Ferric pyrophosphate

Volume 3

Active substance data

Annex B.6: Toxicology and metabolism

Rapporteur Member State: Poland
Co-Rapporteur Member State:

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Level 2: Reasoned statement of the overall conclusions drawn by the Rapporteur Member State

Level 3: Proposed decision with respect to the application

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List(s) of endpoints

List of endpoints

Version history

Date	Reasons for revision
June 2018	Initial DAR
July 2019	DAR version after Comments from Member States, EFSA, applicant
October 2019	DAR version covering EFSA Conclusions Pesticide Peer Review Meeting 11

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B.6 Toxicology and metabolism data

Iron and phosphorus are pervasive in the natural environment. Iron is the second most abundant metal and the fourth most abundant element, which composes about 5% of the Earth's crust. In the natural environment, it is found in the form of minerals such as: hematite, magnetite, siderite or pyrite. The content and distribution of iron in soils varies but typically it is 1–5% (10 – 50 g/kg). Phosphorus resources in soil are scarce and its total concentration ranges between 50 and 3 000 mg of phosphorus/kg (or 275 – 16 500 mg/kg expressed as pyrophosphate P2O73-). Apatite is the main source of phosphorus in soil. Both minerals are indispensable for proper development and functioning of plants, animals and humans. Iron participates in electron transfer, DNA synthesis, oxygen transport and many other cellular functions. Phosphorus, on the other hand, is a component of ATP and ADP, nucleic acids and numerous coenzymes. It also needs to be mentioned that ferric pyrophosphate has been approved as a safe and effective source of iron added to food, even in infants. In accordance with Regulation (EU) No. 609/2013 of the European Parliament and of the Council of 12 June 2013, it was approved for use in baby food for infants and young children, processed cereal-based foods and food for children, food for special medical purposes, and total diet replacement. Also the Food and Drug Administration (FDA) positively assessed ferric pyrophosphate, placing it on the list of substances generally recognized as safe (GRAS).

In face of prevalent natural occurrence of iron and phosphorus, and the potential absorption of ferric pyrophosphate from water or supplements, exposure to this substance will not increase significantly as a result of its use as PPP. Exposure related to absorption of pyrophosphate in other ways is not expected as the substance is non-volatile and the product has a form of non-dusty granules.

Thus, in the present Application, ferric pyrophosphate is considered safe and to prove this thesis selected toxicity tests are presented. Data on exposure cited from acknowledged scientific sources combined with low toxicity evidenced in the studies presented indicate that further studies are not necessary.

1. United States Environmental Protection Agency; Reregistration eligibility Document (RED): Iron Salts US EPA 1993

2. Mineral Information Institute pod red. Draggan S.: Iron. Publication online http://www.eoearth.org/view/article/51cbee407896bb431f696707, 2011

3. Schulte E.E.: Understanding Plant Nutrients: Soil and Applied Iron, 1992

4. Bezak-Mazur E., Stoińska R.: The importance of phosphorus in the environment – review article. Archives of Waste Management and Environmental Protection, 2013, 15, pp. 33-42

5. Fidler M.C., Walczyk T., Davidsson L., Zeder C., Sakaguchi N., Juneja L.R., Hurrell R.F.: A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. British Journal of Nutrition, 2004, 91, pp.107-112

6. U.S. Food and Drug Administration, 1988, SCOGS-Report Number: 35; 21 CFR Section: 184.1304

B.6.1 Absorption, distribution, metabolism and excretion in mammals

B.6.1.1 Absorption, distribution, metabolism and excretion by oral route

Iron absorption plays the major role in maintaining homeostasis in the human body. Only a fraction of daily ingested iron is absorbed. Iron absorption takes place in the entire intestine but mainly in the duodenum. Considerably lower amounts are absorbed in the stomach. Iron absorption depends on several factors such as its content in food, stores in the organism and its form in food. Iron is absorbed in the form of divalent cations, whereas ferric iron is released from food as a result of digestion by gastric acid in the stomach, next it is reduced to ferrous iron and only then it is absorbed. Ferrous iron (Fe^{2+}) comprises about 10% of the daily iron supply in food and it is absorbed in about 20%. Its absorption is decreased by calcium present in food. On the other hand, ferric iron (Fe^{3+}) comprises 80% of the daily iron supply in food and its absorption is low - from 1 to 5% - and depends on other components of food. Iron transport through membranes requires energy and is supported by carriers, thus the process might become saturated and decrease the speed of iron absorption. Ferrous iron is absorbed by the mucosa of the gastrointestinal tract and then converted to the ferric state. In intestinal epithelial cells, it binds to apoferritin, forming ferritin. Transported from the epithelial cells to blood, iron binds to transferrin - protein which transports iron to the bone marrow, where it is used in erythropoiesis. Organisms are protected from the toxic effect of iron mainly by the liver (also the spleen and bone marrow to a lesser extent), where it is stored in the form of soluble complex of ferritin and hemosiderin (its insoluble derivative). The excessive amounts of the stored iron can be released any time from this buffer pool. About 25% of the total amount of iron is stored in the liver (about 2/3 as ferritin and 1/3 as hemosiderin).

Cells absorb iron through receptors binding transferrin to Fe^{3+}, which are transmembrane proteins consisting of two glycoprotein monomers connected with a sulfur bridge. On the inner side of the cell membrane, there are fatty acids linked with the proteins by covalent bonds. Receptor-transferrin-iron complex is absorbed via endocytosis, forming vacuoles in the cytoplasm. Acidic environment of vacuoles causes transferrin to release iron, then the receptor-transferrin complex is transferred back to the cell surface, ready for another round of iron uptake.

The extent of iron absorption demonstrates intra- and inter-subject variability, which is mainly influenced by dietary factors and characteristics of the organism itself (age, sex, health condition etc.) The ingested substances might modify the level of absorption e.g. by chelating and/or change in iron oxidation level, effect on the mucosa and function of intestines, or the competitive mechanism of other minerals in protein transport. Inductors and inhibitors of iron absorption are provided with food. The former include e.g. meat and vitamin C, the latter - calcium, polyphenols, phosphates, carbonates and soy proteins. Induction and inhibition of absorption by these dietary components is closely related to oxidoreduction processes and formation of soluble monomers or insoluble polymers. Anions which form relevant salts with Fe cations also determine the size of the absorbed dose because they differ in the level of solubility (ferric pyrophosphate is an insoluble compound, whereas ferric sulphate is a highly soluble salt). If iron is provided in an assimilable form, the size of the dose provided and current demand in the organism will constitute factors influencing the absorption level.

Human organism has a very limited ability to remove excess iron, thus protection from overload with this mineral consists in limiting its absorption. The only natural ways of daily iron loss are epidermis exfoliation and sweating (0.2-0.3 mg/day), excretion in urine (<0.1 mg/day), gastrointestinal secretion and deposition in hair. The total daily loss of iron in healthy men is about 1 mg. In women, this amount is somewhat higher due to menstruation, pregnancy and lactation.

Due to poor iron absorption from food and food processing, developed countries for years have been fortifying food products with sources of iron. In Great Britain, the obligation to add iron compounds to flour has existed since 1953 - flour needs to contain no less than 1.65 mg of iron/100 g. The European law, on the other hand, states that modified milk for infants based on cow's milk should contain 0.07-0.3 mg of iron/100 kcal. Certain food products such as cereal bars or breakfast cereals are fortified by manufacturers even though there is no binding guideline (content ranges between 70 and 120 mg/kg). What is popular is prophylactic supplementation, where dosing amounts to 7-50 mg/day.

According to WHO/FAO recommendations, the substance that should be used for iron supplementation in food in the first place is ferric sulphate, in the last place - ferric pyrophosphate. Even though ferric pyrophosphate is poorly absorbed, it is used in diet fortification as a compound that causes no organoleptic changes in food.

Ferric pyrophosphate is virtually insoluble in water, which makes it hard to assimilate. Research showed that average absorption of iron from food fortified with pyrophosphate was only 2%.

RMS: A correction factor of 50% for oral bioavailability of ferric pyrophosphate is recommended for setting the acceptable operator exposure level (AOEL) of iron; as previously agreed during the peer review on iron sulfate and ferric phosphate (EFSA Journal 2012;10(1):2521 and EFSA Journal 2015;13(1):3973 respectively).

1. Scientific Advisory Committee on Nutrition (SACN), Iron and Health, SACN, 2010
2. World Health Organization/Food and Agricultural Organization of the United Nations. Guidelines on food fortification with micronutrients. Geneva: WHO, 2006
3. Moretti D., Zimmermann M. B., Wegmüller R., Walczyk T., Zeder C., Hurrell R. F.: Iron status and food matrix strongly affect the relative bioavailability of ferric pyrophosphate in humans, Am J Clin Nutr, 2006; 83, pp.632– 638

B.6.1.2 Absorption, distribution, metabolism and excretion by other routes

Absorption, distribution, metabolism and excretion of ferric pyrophosphate after exposure other than oral are very limited. This is related to the physicochemical properties of the substance - it is insoluble in water, lipids and organic solvents, which makes the transdermal exposure extremely low. The compound has a form of non-volatile powder, which is supposed to be added to PPP as a solid - granules, whose size prevents absorption via inhalation.

RMS comment: Ferric pyrophosphate is characterised by the low bioavailability following oral administration. Due to the poor solubility in water and lipids the absorption in the body is low. Ferric pyrophosphate does not accumulate in the organism and the main resources of iron are stored in liver. A low level of iron excretion is observed under normal physiological conditions.

B.6.2 Acute toxicity

B.6.2.1 Oral

Report: [redacted] 2013, Acute Oral Toxicity Study on Rats – Fixed Dose Method

Study test: PO-2/13

Guidelines: OECD Guideline No. 420 / EU Method B.1.BIS

GLP: Yes

Abstract:

The acute oral toxicity study – fixed dose method was performed in order to obtain information on health hazards likely to arise from a single oral exposure caused by the consumption of the test item ferric pyrophosphate. The experiment commenced with a sighting study in which one animal received the test item at a dose of 300 mg/kg b.w. Since this dose did not cause any toxicity signs or mortality, the test item at a dose of 2000 mg/kg b.w was administered once to the next female. On the grounds of the results of the sighting study, four animals in the main study were given the test item at a dose of 2000 mg/kg b.w. (the animal used in the sighting study was included in the main study). Before the administration, the animals had been fasted for about 18-19 hours. The test item in the form of a suspension in 0.5% carboxymethylcellulose in a volume of 0.5 mL/100 g b.w. was administered using a metal stomach tube. After the administration of the test item, the animals were observed for 14 days. General and detailed clinical observations were conducted daily during the entire experiment. Body weights of the animals were determined on days 0 (directly before the administration of the test item), 7, and 14.

After a 14-day observation period, all animals which survived the experiment were euthanized and subjected to a necropsy and a detailed gross examination. Following a single administration of the test item at a dose of 2000 mg/kg b.w. to the next four animals used in the main study, the animals did not exhibit any clinical signs during the whole observation period. The animals survived the 14-day observation period. In all animals which survived the experiment, body weight gain was stated. The gross examinations of the animals did not reveal any pathological changes.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: cream-colored powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: temperature: 10 - 30ºC, humidity: 30 - 70%

2. Vehicle and/or positive control: 0.5% carboxymethylcellulose

3. Test animals:

Species: rat (female)

Strain: Wistar

Age: 9-11 week

Weight at dosing: average body weight was 197 g

Source: [redacted]

Acclimation period: 5 days

Diet: "Murigran" standard granulated fodder produced by Wytwórnia Koncentratów i Mieszanek Paszowych AGROPOL, Motycz

Water: tap water

Housing:. The animals were housed in plastic cages covered with wire bar lids. The dimensions of the cages were 58 x 37 x 21 cm . In the sighting study, the animals were housed individually. In the main study, there were four animals per cage. UV-sterilized wood shavings were used as bedding.

Environmental conditions:

Temperature: 19 – 24 °C

Humidity: 5 – 60 %

(air humidity was recorded at the level of 5 to 60%. Such a wide range of the parameter was related to the regulation of the air-conditioning device at that time. Analysing the possible influence of humidity on the course of the experiment, changes in this parameter did not affect the course of the experiment and the results of the study. During the observation of animals, no clinical changes were observed and no pathological changes were observed in macroscopic studies. Water was available for the animals all the time)

Air changes: about 16 times / hour

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN AND METHODS:

1. In life dates: 19.02.2013 – 20.03.2013

2.Animal assignment and treatment:

Sighting study: The test item at a dose of 300 mg/kg b.w. was administered to one animal. Since the dose of 300 mg/kg b.w. did not cause any clinical signs or mortality, the next female received the test item at a dose

of 2000 mg/kg b.w.

Main study: On the grounds of the results of the sighting study, four animals in the main study were given the test item at a dose of 2000 mg/kg b.w. (the animal used in the sighting study was included in the main study). The animals survived the 14-day observation period. Body weights of the animals were determined on days 0 (directly before the administration of the test item), 7, and 14. After a 14-day observation period, all animals which survived the experiment were euthanized and subjected to a necropsy and a detailed gross examination.

Table CA B 6.2-1 Ferric pyrophosphate acute oral toxicity to rats

Administered dose of the test item (mg/kg m.c.)	Sighting study	Sighting study	Main study
	300	2000	
Mortality	0/1	0/1	0/4
Clinicalsigns	none	none	none

3. Statistics: The data did not warrant statistical analysis.

II. RESULTS AND DISCUSSION

A. MORTALITY

Details in Table CA B 6.2-1. No mortalities were observed during the experimental period.

B. CLINICAL OBSERVATIONS

Following a single administration of the test item at a dose of 300 mg/kg b.w. to one animal used in the sighting study, the animal did not exhibit any clinical signs during the whole observation period. The animal survived the 14-day observation period.

Following a single administration of the test item at a dose of 2000 mg/kg b.w. to one animal used in the sighting study, the animal did not exhibit any clinical signs during the whole observation period. The animal survived the 14-day observation period.

Following a single administration of the test item at a dose of 2000 mg/kg b.w. to the next four animals used in the main study, the animals did not exhibit any clinical signs during the whole observation period. The animals survived the 14-day observation period.

C. BODY WEIGHT

Sighting study: In both animals, body weight gain was stated during the whole experiment.
Main study: In all animals, body weight gain was stated during the whole experiment.

D. NECROPSY

Sighting study: The gross examination did not reveal any pathological changes.

Main study: The gross examination did not reveal any pathological changes.

E. DEFICIENCIES

During the experiment, the relative air humidity was lower than 30% a few times. These changes did not influence the results of the experiment. No other deviations from the Study Plan were stated.

III. CONCLUSIONS

Following a single administration of the test item at a dose of 2000 mg/kg b.w. to the next four animals used in the main study, the animals did not exhibit any clinical signs during the whole observation period. The animals survived the 14-day observation period. In all animals which survived the experiment, body weight gain was stated. The gross examinations of the animals did not reveal any pathological changes.

On the grounds of the study results, the test item, i.e. Ferric Pyrophosphate can be classified to the following categories:

– category 5/beyond classification – according to the Globally Harmonized System (GHS),

– beyond categorization – according to the Regulation (EC) No. 1272/2008 of the European Parliament and of the Council of December 16th, 2008 on classification, labelling, and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC and amending Regulation (EC) No. 1907/2006.

RMS comment: The study is considered acceptable.

The study was performed according to OECD guideline No. 420 with exception of following deviation: the relative air humidity during the experiment was lower than 30% a few times. These changes did not influence the results of the experiment and the study is considered suitable for evaluation.

An oral limit test was performed in 5 fasted female rats with a single dose of 2000mg/kg bw ferric pyrophosphate (purity: 101.73%). The active ingredient was suspended in 0.5% carboxymethylcellulose in water. No mortalities and no clinical signs were observed in treated animals. All animals gained body weight over the study period. No pathological changes were observed at necropsy.

The acute oral LD_{50}> 2000 mg/kg bw in female rats.

No classification in regard to acute oral toxicity is required for ferric pyrophosphate according to criteria of the Regulation 1272/2008.

B.6.2.2 Dermal

Due to the fact that the substance's acute oral toxicity, LD_{50}, is higher than 2000 mg/kg bw (point 6.2.1, test code PO-2/13), according to Commission Regulation (EU) No. 283/2013 a study of acute dermal toxicity is not necessary.

RMS comment: No acute dermal toxicity study is submitted.

The justification for waiving the acute dermal toxicity study of ferric pyrophosphate is scientifically justified and in accordance to Commission Regulation (EU) No. 283/2013.

B.6.2.3 Inhalation

Report: [redacted] 2013, Acute Inhalation Toxicity Study in Rats with Ferric Pyrophosphate

Study test: 4150

Guidelines: OECD GT. 403: „Acute Inhalation Toxicity"

GLP: Yes

Abstract:

Three male and three female Wistar rats were exposed to Ferric Pyrophosphate for a single 4-hours period at exposure concentration of 2.69 mg/Lair (maximum attainable concentration). The animals were observed daily during the acclimatization period and mortality/viability and clinical signs were recorded. All animals were observed for clinical signs at 1, 2, 3 and 4 hours during exposure (only gross abnormal signs were observed as the animals were restrained), at 30 min post exposure on test day 0 and once daily thereafter during days 1-14. Mortality/viability was recorded at 1, 2, 3 and 4 hours during exposure and once after the exposure (day 0) (in common with the clinical signs) and twice daily during days 1-14. Rectal temperature was (Post exposure – from day 1 to day 14 i.e., twice daily morning and evening) observed. Body weights were recorded on test days 0 (prior to exposure) 1, 3, 7 and 14. At the end of observation all animals were necropsied and examined macroscopically. No mortality was recorded throughout the study. All exposed animals appeared normal throughout the experimental period. Rectal temperature recorded in all the animals was normal throughout the study. All animals had gained body weight by day 1, 3, 7 and day 14 as compared to day 0. No abnormalities were detected in any of the animals on necropsy at the end of observation period. The LC_{50} (4-hours exposure) of FERRIC PYROPHOSPHATE obtained in this study, based on combine gender mortality data, was estimated to be greater than 2.69 mg/L air (maximum attainable concentration).

MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream white powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: Room Temperature (20 to 30ºC)

2. Vehicle and/or positive control: -

3. Test animals:

Species: rat

Strain: Wistar

Age: males: 9-10 weeks, females: 9-10 weeks

Weight at dosing: male: 228,6 – 234,7 grams, female: 201,2 – 204,9 grams

Source: [redacted]

Acclimation period: 7 days

Diet: Nutrilab rodent feed (Batch No. 0001331749) from Provimi Animal Nutrition India Pvt. Ltd., was provided ad libitum, except during treatment period, when they were restrained in the restraining tubes.

Water: Aquaguard filtered tap water was provided ad libitum, except during exposure period, when they were restrained in the restraining tubes.

Housing:. Housed in groups by sex of three animals in Polycarbonate cages (approximate internal dimensions of 365 mm x 202 mm x 180 mm height) with paddy husk bedding. **Environmental conditions:**

Temperature: 21.7 °C - 23.6 °C

Humidity: 60%-70% RH

Air changes: The animal room was air-conditioned with adequate (above 10) air changes per hour.

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN AND METHODS:

1. In life dates: group I 04.09.2013 – 11.09.2013, group II 19.09.2013 – 03.10.2013

2.Animal assignment and treatment:

Range Finding Study: A dust aerosol was generated from the test item using a rotating brush aerosol generator and piston with speed set at a rate of 130/minute. The actual concentration achieved at this speed was 2.80 mg/L and the nominal concentration was 6.60 mg/L. Group – I; One male and one female rat were exposed to this concentration, for 4 hours and observed for 7 days. All animals survived at the above concentrations. Both animals appeared normal throughout the experimental period. Subsequently these animals were euthanized without necropsy.

Limit test: Based on the results of range finding study, Group of rats (Group – II; 3 males and 3 females) was exposed to 2.69 mg/L (actual concentration) and 7.01 mg/L (nominal concentration). To attain this nominal concentration, dust aerosol was generated from the test item using a rotating brush aerosol generator and piston with speed was set at a rate of 130/min.

Table CA B 6.2-2 Doses, mortality / animals treated

Dose (mg/L), actual concentration	Males	Females	Combined
2.80	0/1	0/1	0/2
2.69	0/3	0/3	0/6

3.Generation of the test atmosphere / chamber description:

The flow-past system was constructed in anodised alloy and consists of 16 ports per stage. The flow-past nose-only rodent exposure chamber was entirely modular allowing inhalation systems. The animals were confined individually in restraint tubes which were positioned radially around the flow-past, nose-only exposure chamber . The design of this chamber is based upon the fluid dynamic modeling of the test aerosol flow. It ensures a uniform test item distribution, provides a constant stream of "fresh" test item to each animal, and precludes re-breathing the exhaled air.

A dust aerosol was generated from the test item as such without any preparation using a rotating brush aerosol generator (CR3020) connected to a micronising jet mill. The aerosol generated was then discharged into the exposure chamber.The particle size distribution was measured gravimetrically four times during each exposure using a 7 stage cascade Mercer Impactor.Mass Median Aerodynamic Diameter (MMAD) were within required range of 1 to 4 µm.

4. Statistics: No statistical analysis was performed since the study was terminated using single exposure group only.

II. RESULTS AND DISCUSSION

A.MORTALITY

No mortalities were observed during the experimental period (Table CA B 6.2-2).

B. CLINICAL OBSERVATIONS

All animals appeared normal throughout the experimental period. Rectal temperature was observed twice daily for all the animals from day 1 to day 14 and was found normal. The rectal temperature for male was 37.47°C to 38.00°C and for female 37.77°C to 38.83°C respectively, for 14 days post exposure. The mean temperature values were accepted range.

C. BODY WEIGHT

All animals had gained body weight by day 1, 3, 7 and day 14 as compared to day 0

D. NECROPSY

No abnormalities were detected in any of the animals on necropsy at the end of observation period.

E. DEFICIENCIES: Not reported

III CONCLUSIONS

No mortality was recorded throughout the study. All exposed animals appeared normal throughout the experimental period. Rectal temperature recorded in all the animals was normal throughout the study. All animals had gained body weight by day 1, 3, 7 and day 14 as compared to day 0. No abnormalities were detected in any of the animals on necropsy at the end of observation period.

The LC_{50} (4-hours exposure) of FERRIC PYROPHOSPHATE obtained in this study, based on combine gender mortality data, was estimated to be greater than 2.69 mg/Lair (maximum attainable concentration).

RMS comment: The study is considered acceptable.

The study was performed according to OECD guideline No. 403.

Acute inhalation (4h, nose-only) toxicity testing of ferric pyrophosphate(purity: 101.73%) in three male and three female rats. No mortality was recorded throughout the study to the technically highest attainable concentration of 2.69 mg test substance/l air. All exposed animals appeared normal throughout the experimental period. All animals had gained body weight over the study period. No abnormalities were detected in any of the animals on necropsy at the end of observation period.

The acute inhalation LC_{50} (4 h) of ferric pyrophosphate for male and female rats was >2.69 mg/L air (highest technically attainable concentration).

No classification in regard to acute inhalation toxicity is required for ferric pyrophosphate according to criteria of the Regulation 1272/2008.

B.6.2.4 Skin irritation

Report: [redacted]2013, Ferric Pyrophosphate Acute Toxicity: Dermal Irritation/Corrosion

Study test: Report No:13-154, Study No:95/13/4

Guidelines: Test was performed according to Method B.4 - Acute Toxicity: Dermal Irritation/Corrosion, Council Regulation (EC) No.440/2008, published in O.J. L 142, 2008.

GLP: Yes

Abstract:

The test substance, Ferric Pyrophosphate, was tested in the study for acute dermal irritation/corrosion. Rabbits (New Zealand Albino breed) were used for the test.The rabbits were exposed to 0.5 g of test substance, applied onto clipped skin using a semi-occlusive dressing. At first the test substance was applied on the skin of one rabbit (test animal No. 13). Rabbit No. 13 was investigated at 3 minutes, 1 hour and 4 hours after application of the test substance immediately after the patch was removed. No evidence of a corrosive effect or symptoms of irritation were observed on the skin after application. In confirmatory test, two others rabbits (rabbit No. 14 and No. 15) were used with 4-hour exposition period. Skin reactions were evaluated after patch removal and observations were made at 1, 24, 48 and 72 hours after exposure. The same observations were made also in rabbit No.13. No symptoms of irritation on the skin were observed. No other signs of intoxication were observed. No skin irritation was caused by 4-hour exposure of rabbits to Ferric Pyrophosphate.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream white powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: The test substance was stored in the dark place at laboratory temperature during the study.

2. Vehicle and/or positive control: -

3. Test animals:

Species: rabbit

Strain: New Zealand (albino)

Age: adults albino rabbits

Weight at dosing: 2.60 – 2.80 kg

Source: -

Acclimation period: 5 days

Diet: pelleted standard diet for rabbits and guinea-pigs *ad libitum*

Water: drinking tap water *ad libitum*

Housing: individually in cages without bedding in conventional animal room

Environmental conditions:

Temperature: room temperature 17 - 23ºC, permanently monitored

Humidity: 30 – 70 %, permanently monitored

Air changes: Not reported

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN AND METHODS:

1. In life dates: 29.04.2013- 04.05.2013

2.Animal assignment and treatment:

Code number on inner side of ear of animal, number of study on each cage. Only adult animals with healthy intact skin were used. Approximately 24 hours before application the fur on the dorsal area of the trunk was removed by close clipping (area – 6 x 6 cm).The test substance was applied in delivered form in amount 0.5 g per animal. The test substance was applied on intact skin and then it was covered by gauze patch, foil and cellulose cotton and held in place with non-irritating tape - Spofaplast. At the end of each exposure period the patch was removed and remaining sample was washed with water. The test substance was applied to one animal. Three patches were applied sequentially to one animal. The first patch was removed and skin reaction was evaluated after 3 minutes. The second patch was applied for one hour and then the skin evaluation was performed. The third patch was applied for four hours and skin reaction was evaluated immediately after skin patch removal. Because no corrosive or severe irritant effect was observed after a 4-hour exposure in initial test, the response was verified in confirmatory test using two additional animals, each with one patch for an exposure period of 4 hours. After 4-hour exposure rabbits were evaluated for skin reactions according to standard time schedule at 1, 24, 48 and 72 hours after patch removal.

II. RESULTS AND DISCUSSION

A. FINDINGS

In initial test three patches were applied sequentially to one animal (rabbit no. 13). Because no corrosive or severe irritant effect was observed even after 4-hour exposure, the response was further observed in regular time intervals at 1, 24, 48 and 72 hours after 4-hour exposure. Because during the initial test no corrosive or severe irritating effect was observed, two additional animals (rabbits no. 14 and 15) were used to confirm the negative response. No skin reaction was observed during any of observation periods. There was no evidence of a corrosive effect on the skin. No symptoms of systemic toxicity were observed in the animals during the test period and no mortality occurred. No skin reaction was observed in all rabbits. At 1, 24, 48 and 72 hours after exposure no signs of erythema and oedema were recorded.

B. DEVIATIONS

No deviations from study plan or test guidelines occurred during the study.

III. CONCLUSIONS

The test substance, Ferric Pyrophosphate, was tested for acute dermal irritation/corrosion. Three rabbits were exposed to 0.5 g of test substance, applied onto clipped skin for 4 hours using a semi-occlusive dressing. Skin reactions were evaluated after patch removal and observations were made at the 1, 24, 48 and 72 hours after exposure. At 1, 24, 48 and 72 hours after exposure no sign of erythema and oedema were recorded. No evidence of corrosive effects was observed during all study on the skin of all animals. No skin irritation was caused by 4-hour exposure of rabbits to Ferric Pyrophosphate.

RMS comment: The study is considered acceptable.

The study was performed according to Method B.4, Council Regulation (EC) No.440/2008.

The active substance ferric pyrophosphate, was tested in the study for acute dermal irritation/corrosion in rabbits. One rabbit was investigated at 3 minutes, 1 hour and 4 hours after application of the test substance immediately after the patch was removed. No evidence of a corrosive effect or symptoms of irritation were observed on the skin after application. In confirmatory test, two others rabbits were used with 4-hour exposition period. Skin reactions were evaluated for signs of erythema/eschar and oedema at 1, 24, 48 and 72 hours after exposure in all animals. No symptoms of irritation on the skin were observed. No other signs of intoxication were observed. No skin irritation was caused by 4-hour exposure of rabbits to ferric pyrophosphate.

Ferric pyrophosphate has no skin irritating properties.

No classification in regard to acute dermal irritation/corrosion is required for ferric pyrophosphate according to criteria of the Regulation 1272/2008.

B.6.2.5 Eye irritation

Report: [redacted] 2013, Ferric Pyrophosphate Acute Toxicity: Acute Eye Irritation/Corrosion

Study test: Report No:13-170, Study No:95/13/5

Guidelines: The test was performed according to the Method B.5 Acute Toxicity: Eye Irritation/Corrosion. Council Regulation (EC) No.440/2008, published in O.J. L 142, 2008.

GLP: Yes

Abstract:

The test substance, Ferric Pyrophosphate, was tested for the assessment of eye irritation/corrosion effects using albino rabbit (New Zealand Albino breed).

The test was performed initially using one animal. Because a corrosive or severe irritating effects were not observed in initial test, the response was confirmed using two additional animals. The following changes were observed on eye at 1 hour after application: conjunctivae – diffuse, crimson colour, individual vessels not easily discernible or diffuse beefy red and chemosis – some swelling above normal or obvious swelling with partial eversion of lids were observed in all rabbits. Diffuse, crimson colour, individual vessels not easily discernible of conjunctivae were observed in all rabbits at 24, 48 and 72 hours after application. On the 4th day some blood vessels hyperaemic (injected) of conjunctivae were observed in all rabbits. In one rabbit (No.16) this change persisted also to the 5th day. In two rabbits (No. 17 and 18) eye alterations vanished on the 5th day and in rabbit No.16 eye alterations vanished on the 6th day after application. No clinical signs of systemic intoxication were detected. Examination of eye irritation after single application demonstrated, that the test substance, Ferric Pyrophosphate, is mildly irritating for eye of rabbit but the injury observed was largely not persistent.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream white powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: The test substance was stored in the dark place at laboratory temperature during the study.

2. Vehicle and/or positive control: -

3. Test animals:

Species: rabbit

Strain: New Zealand (albino)

Age: adults albino rabbits

Weight at dosing: 3.10– 3.50 kg

Source: -

Acclimation period: 5 days

Diet:.pelleted standard diet for rabbits and guinea-pigs *ad libitum*

Water: drinking tap water *ad libitum*

Housing:. individually in cages without bedding in conventional animal room

Environmental conditions:

Temperature: room temperature 17 - 23ºC, permanently monitored

Humidity: 30 – 70 %, permanently monitored

Air changes: Not reported

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN AND METHODS:

1. In life dates: 12.05.2013 – 20.05.2013

2.Animal assignment and treatment:

Code number on inner side of ear of animal, number of study on each cage. Only healthy animals without eyes defects were used for testing. The test substance was used in delivered form and was metered in quantities 0.1 g on animal immediately before application. The test substance was placed in the conjunctival sac of one eye of animal after gently pulling the lower lid away from the ball. After application the lids were gently held together for about one second to prevent loss of the test substance. Untreated eye (on the right side) served as a control. At 1 hour after application the eye was irrigated with aqua pro injection. The eyes were examined at 1, 24, 48 and 72 hours after application, then 1x daily. The examinations were performed at first as subjective observation by inspection and then the ophthalmoscopy was performed by hand slit lamp. After recording the observations at 72 hours, the eye of rabbits were examined with the aid of fluorescein – only damage of cornea was observed. The duration of the observation period was sufficient to evaluate fully the magnitude and reversibility of the effects observed. The grades of ocular reaction for single animal (observation of conjunctivae, cornea and iris) were recorded at each examination. Clinical observation of the animals was carried out before application and daily during the study. Observations included changes in fur, skin, visible mucous membranes, behaviour of animals, somatomotor activity and presence of lacrimation, salivation and discharge from nostrils, function of respiratory and digestive system. In the end of observation period the animals were sacrificed by injection of veterinary preparation T61 (1 mL/kg iv).

II. RESULTS AND DISCUSSION

A. FINDINGS

No symptoms of systemic toxicity were observed in the animals during clinical observation in the test period and no mortality occurred. Weight increments were adequate to species, sex and age of animals in experiment.

Table CA B 6.2-3 Examination of Treated Eye

Number hour / day	**Rabbit No. 16**	**Rabbit No. 17**	**Rabbit No. 18**
1 hour	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – some swelling above normal **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse beefy red **Chemosis**– obvious swelling with partial eversion of lids **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse beefy red **Chemosis**– obvious swelling with partial eversion of lids **Cornea** – no alterations **Iris** – no alterations
24 hours	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis**– no alterations **Cornea –** no alterations **Iris** – no alterations
48 hours	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris –** no alterations
72 hours	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations The examination of eye with the aid of fluorescein showed no damage of cornea.	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations The examination of eye with the aid of fluorescein showed no damage of cornea.	**Conjunctivae** – diffuse, crimson colour, individual vessels not easily discernible **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations The examination of eye with the aid of fluorescein showed no damage of cornea.
4th day	**Conjunctivae** – some blood vessels hyperaemic (injected) **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – some blood vessels hyperaemic (injected) **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – some blood vessels hyperaemic (injected) **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations

5th day:	**Conjunctivae** – some blood vessels hyperaemic (injected) **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – no alterations **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – no alterations **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations
6th day	**Conjunctivae** – no alterations **Chemosis** – no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – no alterations **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations	**Conjunctivae** – no alterations **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations
7th day	**Conjunctivae** – no alterations **Chemosis**– no alterations **Cornea** – no alterations **Iris** – no alterations	-	-

Table CA B 6.2-4 Result of reaction of treated eye (grades)

Animal No.	Ocular lesions	Time interval of examination							
		1h	24 h	48h	72h	4th day	5th day	6th day	7th day
16	Cornea	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0
	Conjunctivae	2	**2**	**2**	**2**	1	1	0	0
	Chemosis	1	0	0	0	0	0	0	0
17	Cornea	0	0	0	0	0	0	0	-
	Iris	0	0	0	0	0	0	0	-
	Conjunctivae	3	**2**	**2**	**2**	1	0	0	-
	Chemosis	2	0	0	0	0	0	0	-
18	Cornea	0	0	0	0	0	0	0	-
	Iris	0	0	0	0	0	0	0	-
	Conjunctivae	3	**2**	**2**	**2**	1	0	0	-
	Chemosis	2	0	0	0	0	0	0	-

B. DEVIATIONS

No deviations from study plant or test guidelines occurred during the study.

The deviations of the toxicological studies from the most updated OECD guidelines

- New procedure of use of topical anesthetics and systemic analgesics was added.

No impact on the study results.

- 'Observations should allow for determination of severity and reversibility before proceeding to a confirmatory test in a second animal.'

No impact on the study. The results of initial test indicated the substance not to be corrosive or severely irritating to the eye and further testing was performed.

- Condition of performing confirmatory test was changed. Sentence 'If results from the second animal are sufficient to allow for a hazard classification determination, then no further testing should be conducted.' was added.

No impact on the study results. In the previous version of guideline sequential manner was recommended in case when in the initial test severe irritant effects indicating a possible strong (irreversible) was observed. New guideline recommends sequential manner in any irritant effect is observed. If test was performed according new guideline, only two animals would be used, but results would remain unchanged, because substance caused the same response in second and third animal.

- Procedure of clinical observations and grading of eye reactions was developed.

According to new guideline animals should be evaluated several times daily for the first 3 days. In the study animals were evaluated at 1, 24, 48 and 72 hours after application. Test animals should be routinely evaluated for the entire duration of the study for clinical signs of pain and/or distress at least twice daily, with a minimum of 6 hours between observations or more often if necessary. Clinical observations of animals was carried out before application and daily during the study. Insertion of new schedule had no impact on the study, because no clinical sigs of systemic intoxication were detected. Duration of the observation period was sufficient to evaluate fully the magnitude and reversibility of the effects observed. Updated guideline recommends use of fluorescein staining, which was used in the study, hence no deviations from new guideline occurred. No digital photographs was taken, but according to updated guideline photographs are optional. Description of ocular lesions considered predictive of severe irritant or corrosive injuries and injuries that are not expected to fully reverse by the end of the 21-day observation period was added, but it has no impact on the study results, because tested substance is mildly irritating for eye of rabbit and injury observed was reversible.

- According to updated guideline test report should include information on anaesthetics and analgesics used in study, but in the study no anaesthetics and analgesics were administrated.

Methods OECD 405 and OECD 437 were updated, but no significant changes were made.

III. CONCLUSIONS

The following changes were observed on eye at 1 hour after application: conjunctivae – diffuse, crimson colour, individual vessels not easily discernible or diffuse beefy red and chemosis – some swelling above normal or obvious swelling with partial eversion of lids in all rabbits were observed. Different changes of conjunctivae were observed in all rabbits at 24-hour, 48-hour, 72- hour and at the 4th day after application. In one rabbit change of conjunctivae persisted also to the 5th day.

In two rabbits eye alterations vanished on the 5th day and in one rabbit eye alterations vanished on the 6th day after application. No clinical signs of systemic intoxication were detected. Examination of eye irritation after

single application demonstrated, that the test substance, Ferric Pyrophosphate, is mildly irritating for eye of rabbit but the injury observed was largely not persistent.

RMS comment: The study is considered acceptable.

The study was performed according to Method B.5, Council Regulation (EC) No.440/2008.

The active substance ferric pyrophosphate, was tested in the study for acute eye irritation in rabbits.

The test substance caused effects in the eyes of all animals in form of conjunctival reddening and chemosis. All signs of irritation were completely reversible within 5 days. No clinical signs of systemic toxicity were observed during the study.

The mean scores of conjunctivae redness following grading at 24, 48 and 72 hours after installation of the test material for each of the three test animals were: 2, 2, 2, therefore classification: irritating to eyes (Category 2) is required for ferric pyrophosphate according to criteria of the Regulation 1272/2008.

Ferric pyrophosphate should be classified as Eye Irrit. 2, H319 Causes serious eye irritation.

B.6.2.6 Skin sensitisation

Ferric pyrophosphate is used as a dietary supplement and is added to food for nutritional fortification, thus skin sensitization potential can be excluded.
More data provided separately (Volume 4) - confidential information.

RMS comment: Ferric pyrophosphate is used as a dietary supplement and is added to food for nutritional fortification. A skin sensitising potential of ferric pyrophosphate can be excluded based on the extensive experience with ferric pyrophosphate and the absence of such effects.

B.6.2.7 Phototoxicity

Phototoxicity study is technically not possible to supply (point 1.5 and 5.2.7 of Annex of Commission Regulation (EU) No. 283/2013) due to poor solubility of the active substance in water and organic solvents, which also makes it impossible to determine the UV spectrum and molecular extinction.

B.6.3 Short-term toxicity

B.6.3.1 Oral 28-day study

Report: [redacted] 2013, Repeated Dose 28-Day Oral Toxicity Study in Rodents of iron pyrophosphate
Study test: 0003/0016/T

Guidelines: **OECD TG 407, Annex to Regulation (EC) No 440/2008, Method B7**

GLP: Yes

Abstract:

Ferric (III) pyrophosphate was orally administered using a stomach gavage to three groups of experimental animals (one group – 6 females and 6 males), as a suspension in 0,5% methylcellulose solution in graded doses (**100, 500, 1000 mg/kg body weight) once a day, in the same volume (1 ml), for 28 days**, seven days a week. The control group was run in parallel and administered 0,5% methylcellulose solution in the same volume as the test material. During the dosing period, each day animals were subjected to a general observation twice a day in order to detect signs of toxicity and morbidity. Once a week, all animals were subjected to detailed clinical observations. Measurement of body weight of animals was conducted prior to administration of the test item and once a week during the whole experiment. Measurements of food and water intake were performed once a week. All animals after dosing period were examined macroscopically and microscopically (histopathological examination was carried out only in animals from the control group and the group receiving ferric pyrophosphate at 1000 mg/kg body weight dose) of organs taken during autopsy, also hematological, biochemical analysis and urine tests were performed.

There were no statistically significant changes in terms of all parameters, in comparison with the control group. Additionally, all parameters were in the range of reference standards. No changes in the examined parameters give rise to classify dose 1000 mg/kg body weight as a limit does in Repeated Does 90-Day Oral Toxicity Study in Rodents. The use of higher doses is not justified, due to the lack of information on the use of ferric (III) pyrophosphate at higher doses in the commercial/industrial use.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: temperature: 10 - 30ºC, humidity: 30 - 70%

2. Vehicle and/or positive control: 0.5% carboxymethylcellulose

3. Test animals:

Species: rats

Strain: Wistar

Age: 6 weeks

Weight at dosing: males: 156 – 194 g, females: 109 – 166 g

Source: [redacted]

Acclimation period: 5 days

Diet:. The animals were fed with conventional laboratory diets Labofeed B Plant Feed “Moravian”

Water: filtered tap water

Housing:. Animal cages: 540 x 390 x 210 mm

Environmental conditions:

Temperature: 22ºC ± 3ºC

Humidity: 30-70%

Air changes: Not reported

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN:

1. In life dates: control group – male and female – 14.05.2013 – 10.06.2013, test group – male and female: dose 1000 mg/kg b.w. – 14.05.2013 – 10.06.2013, test group – male and female: dose 500 mg/kg b.w. – 15.05.2013 – 11.06.2013, test group – male and female: dose 100 mg/kg b.w. – 15.05.2013 – 11.06.2013

2. Animal assignment and treatment: Han Wistar rats, female and male in number of 48 subjects (6 females and males in each of the three dosage levels and 6 females and males in the control group) were used in the study. Each animal was individually labeled according to the procedure SPO-O/20. Animals were given individual numbers and have been assigned into appropriate groups.

Table CA 5.3- 1 Study design

Test group	Dose per animal		Animals assigned	
	Male (mg/kg bw/day)	Female mg/kg bw/day)	Male	Female
1 (control)	0	0	6	6
2	100	100	6	6
3	500	500	6	6
4	1000	1000	6	6

3. Diet preparation and analysis: The animals were fed with conventional laboratory diets Labofeed B Plant Feed "Morawski". The animals had free access to water..

4. Statistics: Measured parameters were subjected to statistical analysis. For analysis of the differences between the groups, normality tests and analysis of the variance were used. For variables not compatible with a normal distribution or not having equal variance, Kruskal-Wallis test was used. For variables that had a distribution consistent with the normal and equal variance, analysis of variance test for unrelated samples was used.

C. METHODS:

1. Observations: On the day of acceptance of the animals, a general medical and veterinary examination was performed. During adoption each animal was weighed and its weight, together with the number, was inscribed in the Adoption Card and Animal Quarantine. On the day of animal transfer, a detailed medical and veterinary examination was performed. Each animal was individually labeled according to the procedure SPO-O/20. During the dosing period, each day, animals were subjected to a general observation twice a day for signs of toxicity and morbidity. Once a week, all animals were subjected to detailed clinical observations.

2. Body weight: Animal body weight measurement was carried out before the administration of the test material, and once a week during the entire period of the experiment.

3. Food consumption and compound intake: Measurement of the food and water consumption was performed once a week. Ferric (III) pyrophosphate was orally administered to three groups of experimental animals (one group – 6 females and 6 males), using a stomach gavage as a suspension in 0,5% methylcellulose solution in graded doses (100, 500, 1000 mg/kg body weight) once a day, in the same volume (1 ml), for 28 days, seven days a week. The control group was run in parallel and administered 0,5% methylcellulose solution in the same volume as the test material. The total number of animals used in the experiment was 24 females and 24 males.

4. Opthalmoscopic examination: Not reported

5. Haematology & clinical chemistry: Hematology and blood chemistry and urinalysis were performed by the DNA Research Centre in Poznań. The scope of hematology included: hematocrit, haemoglobin, red blood cell count (also: HGB, HCT, MCV, MCH, MCHC), white blood cell count, platelet count.

Scope of serum biochemistry analysis: sodium, potassium, iron, chlorides, calcium, ferritin, glucose, total cholesterol, triglycerides, urea, creatinine, total protein and albumin, alanin aminotransferase, aspartate aminotransferase, alkaline phosphatas, bile acids, bilirubin.

6. Urinalysis: Analysis of the urine of animals is auxiliary. The analyzed parameters showed no significant differences compared with the control group of animals in the group of dose 1000 mg/kg body weight. On the basis of a single study performed by dipstick testing one cannot define a definitive diagnosis and formulate definite conclusions, the result should be verified by other medical tests. No other biochemical parameters indicate impaired renal function and the presence of other disorders in which an abnormal urinalysis results.

7. Sacrifice and pathology: Macroscopic examination included external surface of the body, all orifices, cranial, thoracic and abdominal cavity together with its contents. All animals participating in the study were subjected to a full detailed autopsy in accordance with the SPT-T/VIVO/05 procedure. All gross pathological changes, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, eyeball with optic nerve, stomach, esophagus, small and gross intestines (including Peyer’s patches), salivary

glands, liver, kidneys with ureters, adrenal glands, spleen, heart, aorta, thymus, thyroid with parathyroid, larynx, trachea and lungs, gonads (testes, ovaries), accessory sex organs (uterus, cervix, epididymis, prostate and seminal vesicles and the coagulation glands), vagina, mammary gland, urinary bladder, lymph nodes, peripheral nerve (sciatic or tibial), along with muscle, some bone marrow, femur with the knee joint, tongue, have been secured in a buffered formalin solution and subjected to histopathological examination.

II. RESULTS AND DISCUSSION

The tables with detailed data obtained in this study provided by applicant were placed in Appendix 1A of this document.

A. OBSERVATIONS:

1. Clinical signs of toxicity: There were no clinical signs of toxicity.
2. Mortality: There were no deaths.

B. BODY WEIGHT AND BODY WEIGHT GAIN

The body weights of all animals were within the reference ranges for Han Wistar rats. There were no statistically significant differences between animals 1000 mg/kg body weight and the control group.

C. FOOD CONSUMPTION AND COMPOUND INTAKE

Fodder consumption in the study did not differ from the reference values ranging 8-20g/day. Water consumption was also within range of reference standards of about 30 ml/day, however in the case of young animals water intake ranges from 5-80 ml/day. Ferric (III) pyrophosphate was orally administered to three groups of experimental animals, every day using a stomach gavage as a suspension in 0,5% methylcellulose solution in graded doses (100, 500, 1000 mg/kg body weight), one dose level to a group of twelve animals (6 females and 6 males) over a period of 28 days, seven days a week. A control group of twelve animals was also differential (6 females and 6 males) and administered 0,5% methylcellulose solution as a carrier during the dosing period.

D. BLOOD ANALYSIS:

1. Hematological findings: Analyzed parameters did not show statistically significant differences as compared with the control group of animals in a dose of 1000 mg/kg body weight, except the MCH parameter in the case of males, however all values were within the range of reference values.
2. Clinical chemistry findings: The analyzed parameters in the group of animals dose 1000 mg/kg body weight were within the range of reference values apart from the concentration of potassium in the control group. Statistically significant differences were observed in the values of alanine aminotransferase, aspartate aminotransferase and potassium concentration between the control group and a group of animals dose 1000 mg/kg body weight, but these values were higher in the control group than in the study group and in addition

were within the reference values. No clinical signs confirmed hepatic dysfunction and diseases that cause elevated potassium level. Patomorphological analysis of liver of animals also did not give rise to apply for a disturbance of its functioning.

E. SACRIFICE AND PATHOLOGY:

1. Organ weight: Organ weights taken as an anatomical specimen in the study group did not differ significantly from the control group animals.

2. Macroscopic examination:

Macroscopic examination included verification of:

- the external body – there were no pathological changes
- all orifices – there were no pathological changes
- cranial cavity – there were no pathological changes
- thoracic and abdominal cavities and their contents – there were no pathological changes. All animals in the study were subjected to a full, detailed post – mortem examination in accordance with the procedure SPT-T/VIVO/05. During animals autopsy following organs were taken for histopathological examination and examined for macroscopic changes:
- all gross lesions – not collected– no macroscopically visible pathological changes
- brain (representative regions including cerebrum, cerebellum and pons) – no macroscopically visible pathological changes
- spinal cord – no macroscopically visible pathological changes
- eye with optic nerve – no macroscopically visible pathological changes
- stomach – no macroscopically visible pathological changes
- oesophagus – no macroscopically visible pathological changes
- small and gross intestines (including Peyer's patches) – no macroscopically visible pathological changes
- salivary glands – no macroscopically visible pathological changes
- liver – no macroscopically visible pathological changes
- kidneys with ureter – no macroscopically visible pathological changes
- adrenals – no macroscopically visible pathological changes
- spleen– no macroscopically visible pathological changes
- heart – no macroscopically visible pathological changes
- aorta – no macroscopically visible pathological changes
- thymus – no macroscopically visible pathological changes
- thyroid with parathyroids – no macroscopically visible pathological changes
- larynx – no macroscopically visible pathological changes
- trachea and lungs – no macroscopically visible pathological changes

- gonads (testis and ovaries) – no macroscopically visible pathological changes
- accessory sex organs (uterus and cervix, epididymides, prostate + seminal vesicles with coagulating glands) – no macroscopically visible pathological changes
- vagina – no macroscopically visible pathological changes
- mammary gland – no macroscopically visible pathological changes
- urinary bladder – no macroscopically visible pathological changes
- lymph nodes (of the thorax and lumbar) – no macroscopically visible pathological changes
- peripheral nerve (sciatic) with muscle – no macroscopically visible pathological changes
- part of bone narrow – no macroscopically visible pathological changes
- femur with knee joint – no macroscopically visible pathological changes
- tongue – no macroscopically visible pathological changes

F. DEVIATIONS

In the study differential leukocyte formula assay and the number of reticulocytes in peripheral blood (peripheral blood image) were not performed. This was done because in the peripheral blood of rat occur almost exclusively differentiated blood cells, with the exception of erythroblasts, which are present in very small proportion, as matamyelocytes. Blood cells counts have different survival time, it is relatively short and ranges from about 40 – 100 days for erythrocytes to 8 – 14 days for granulocytes and 6 – 8 days for platelets. In the same experimental terms, blood exhibits high stability of the composition, what is a consequence of the balance between hematopoietic and blood cell loss processes.

Arrangement of hematopoietic tissues in animals is different. Myelopoiesis in rats (formation and differentiation of granulocytes, erythrocytes and platelets) takes place in the bone marrow, lymphopoiesis (formation and differentiation of lymphocytes) in the lymph nodes; lymphopoiesis takes place mainly in the spleen but also myelopoiesis in the case of adults. Lymphopoiesis takes place in the spleen - in lymphoid follicles and in the red pulp of the organ, where also monocytes develop. Lymphopoiesis also proceed in the wall of the rat gastrointestinal tract and in the liver, where cell cultures corresponding to the hematopoietic cells may be also located.

The blood cells are being constantly replaced, erythrocytes, platelets and lymphocytes undergo phagocytosis in the spleen. To a lesser extent, granulocytes are removed by the spleen. Granulocytes are also captured by the lungs. Also the phagocytic cells of liver (Browicz – Kupffer cells) takes place in the removal of aging cells. Between the hematopoietic and blood cell loss organs there is a functional balance that provides a constant composition of the peripheral blood.

In the process of myeloid synthesis of erythrocytes, young cells getting into the bloodstream contain residues of ribosomes (reticulum), are called reticulocytes. Reticulocytes reflect a myeloid synthesis of red blood cells over the past 48 hours. Increasing number of reticulocytes is being observed in the normo- or macrocytic anemias undergoing regeneration. Usually after acute bleeding, blood transfusion, use of folic acid or

completion of chemotherapy and in hemolytic anemia. In such cases, there is also a disorder of other parameters of complete blood count, such as increased anisocytosis and macrocytosis. A smaller number of reticulocytes appears in macrocytic anemia, not undergoing regeneration usually because of alcoholism with or without hypersplenism or hypothyroidism. Myelogram (bone marrow smear) can identify three main non regenerating macrocytic anemia, namely acute myeloid anemia, Addison – Biermer anemia or refractory anemia. Also in the course of normocytic anemia with not undergoing regeneration, a smaller number of reticulocytes appears and occurs in chronic renal failure, pituitary failure or rheumatoid diseases. Also here, bone marrow smear execution precludes such anemia causes as bone marrow fibrosis, aplasia, acute leukemia or isolated erythroblastopenia or associated with thymoma.

In the study any changes in analyzed hematological or biochemical parameters were registered, including analysis of bone marrow smears and evaluation of pathological organs involved in myelo- and lymphopoiesis. Image of animal bone marrow and the structure of all organs was normal. There was no leukocyte formula assay and the number of reticulocytes in peripheral blood was analysis performed, having regard to theforegoing. The study is the preliminary evaluation of the toxicity of the tested compound, hence at this stage the assessment is sufficient.

III. CONCLUSIONS

Lack of changes in the studied parameters gives rise to classify 1000 mg/kg body weight dose as the dose in the test limit in Repeated Dose 90-Day Oral Toxicity Study in Rodents. The use of higher doses is not justified, due to the lack of information on the use of ferric (III) pyrophosphate at higher doses in the commercial/industrial use.

RMS comment: The study was performed according to OECD guideline 407 with exception of following deviation: differential leukocyte formula assay and the number of reticulocytes in peripheral blood (peripheral blood image) were not performed. The justification of deviations from the method is acceptable and the study is considered suitable for evaluation.

There were no deaths and clinical signs of toxicity following 28 days oral exposure to Iron (III) Pyrophosphate. Hematological parameters did not show statistically significant differences between the exposed groups and the control. Statistically significant differences as higher values of alanine aminotransferase, aspartate aminotransferase and potassium concentration in the control group in comparison to the group exposed to 1000 mg/kg were within the historical reference values. No signs of toxicity related to elevated enzymes and potassium level were noted. Pathomorphological analysis of liver of animals did not reveal hepatic disfunction. Macroscopic examination indicated no pathological changes in the tested organs.

Summing up no findings indicated systemic toxicity of ferric pyrophosphate at the dose of 1000 mg/kg bw/day were found in the Repeated Dose 28-Day Oral Toxicity Study in Rodents, so NOAEL based on results of this study is 1000 mg/kg bw/day.

B.6.3.2 Oral 90-day study

Report: [redacted]2014, Repeated dose 90-day oral toxicity study in rodents

Study test: 0003/0017/T

Guidelines: OECD TG 408, Annex to Regulation (EC) No 440/2008, Method B.26

GLP: Yes

Abstract:

Iron (III) Pyrophosphate was administered to one group of experimental animals per os (limit test - 15 male and 15 female) using a stomach gavage as a suspension in 0.5% methyl **cellulose at a dose of 1000 mg/kg b.w.** once a day for a period of 90 days, seven days a week. Simultaneously, control group (8 males and 8 females) was administered vehicle (0.5 % methyl cellulose solution) at the same volume as the test material, and a satellite group (8 females and eight males) receiving the test material at a dose of 1000 mg/kg b.w. During the administration of the test material the body weight, water intake and feed were measured. General clinical observations and specific clinical observations was performed also during administration of test material. From the 11th week of experiment, behavioural tests were carried out and the last day before the section the animals underwent neurological examination. After completion of the test material, all animals were subjected to a detailed post-mortem during which the material was taken for haematological, biochemical and pathological.

Based on our audit in accordance with the Annex to Regulation (EC) No 440/2008, Method B 26 and a guideline of the OECD TG 408, there is no evidence of toxicity caused by the action of iron pyrophosphate, what was confirm by haematological, biochemical and histopathological test, as also analysis of behavioral and neurological disorders.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: temperature: 10 - 30ºC, humidity: 30 - 70%

2. Vehicle and/or positive control: 0.5% carboxymethylcellulose

3. Test animals:

Species: Rats

Strain: Wistar

Age: 6 weeks males and females

Weight at dosing: males: 202 – 269 g, females: 138 – 196 g

Source: [redacted]

Acclimation period: 5 days

Diet:. The animals were fed with conventional laboratory diets Labofeed B Plant Feed “Morawski”

Water: Filtered tap water

Housing:. Animal cages: 540 x 390 x 210 mm, Number of animals per cage: 3-5

Environmental conditions:

Temperature: 22ºC ± 3ºC

Humidity: 30-70%

Air changes: Not reported

Photoperiod: 12 hours light / 12 hours dark

B. STUDY DESIGN:

1. In life dates: test group – females 1000 mg/kg b.w. - 25.06.2013 – 22.09.2013, test group – males dose: 1000 mg/kg b.w. – 26.06.2013 – 23.09.2013, satellite group – males and females dose: 1000 mg/kg b.w. – 26.06.2013 – 23.09.2013, control group – males and females - 27.06.2013 – 24.09.2013.

2. Animal assignment and treatment: The method involves oral administration of test substance in the form of suspension in 0.5% methylcellulose by means of intragastric gavage in a constant volume (max 1 mL / 100 g bw). The test substance was administered to animals daily through 90 days in 1000 mg / kg bw dose. Simultaneously with the administration group, a non-exposed to the test substance, receiving a 0.5% methylcellulose solution (8 males and 8 females) control group and a satellite group (8 males and 8 females) which was given the same dose as the experimental group were implemented. During the study all animals were observed closely for signs of toxicity. After administration period the animals were killed and necropsied, with tissues and organs transferred for histopathological examination. The satellite group was observed by next 14 days in order to detect delay in, persistence of or recovery from potential toxic effects.

Tabela CA 5.3-2 Study design

Group	Dose per animal		Animals assigned	
	Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1. test	1000	1000	15	15
2. control	0	0	8	8
3. satellite	1000	1000	8	8

3. Diet preparation and analysis: The animals were fed with conventional laboratory diets Labofeed B Plant Feed "Morawski". The animals had free access to water.

4. Statistics: Measured parameters were subjected to statistical analysis. For analysis of the differences between the groups, normality tests and analysis of the variance were used. For variables not compatible with a normal

distribution or not having equal variance, Kruskal-Wallis test was used. For variables that had a distribution consistent with the normal and equal variance, analysis of variance test for unrelated samples was used. The statistical significance of all calculation was established at p=0.05. Results above this value were considered as not arised from random variation of the group and their toxicological significance was analyzed further taking into account the clinical symptoms and reference value of evaluated parameters.

C. METHODS:

1. Observations: On the day of animal transfer, a detailed medical and veterinary examination was made. During specific medical and veterinary examination qualifying animal for experiment were also performed ophthalmoscopic examination in accordance with the procedure SPT-T/VIVO/15.

2. Body weight: Animal body weight measurements were carried out before administration of the test substance and once a week during the entire experiment.

3. Food consumption and compound intake: Measurements of food and water consumption was performed once a week. The test material was administered as a suspension in 0.5% methylcellulose in a volume of 1.5 ml. Doses of the test material was prepared before administration. Iron (III) Pyrophosphate was administered to one group of experimental animals per os (limit test - 15 male and 15 female) using a stomach gavage as a suspension in 0.5% methyl cellulose at a dose of 1000 mg/kg b.w. once a day for a period of 90 days, seven days a week. Simultaneously, control group (8 males and 8 females) was administered vehicle (0.5 % methyl cellulose solution) at the same volume as the test material, and a satellite group (8 females and eight males) receiving the test material at a dose of 1000 mg/kg b.w.

4. Opthalmoscopic examination: The study was performed using an ophthalmoscope. Rated reaction of the pupil to light the lamp ophthalmoscope. Checked whether pupils have the same size and if their edges are regular, was also studied reaction of the pupils to light.

5. Haematology and clinical chemistry: The hematology analysis included: hemoglobin, hematocrit, erythrocyte count and reticulocyte count, mean corpuscular volume - MCV, mean corpuscular hemoglobin - MCH, the average concentration of hemoglobin in red blood cells, anisocytosis of red blood cells - RDW, platelet count, platelet anisocytosis - PDW, the total number of leukocytes and leucogram, smear and microscopic evaluation of peripheral blood and bone marrow, prothrombin time - PT , thrombin time - TT, partial thromboplastin time - APTT. The scope of serum. Biochemistry included: sodium, potassium, calcium, magnesium, chloride, phosphorus, iron, ferritin, total iron binding capacity TIBC, unsaturated iron binding capacity UIBC, glucose, total cholesterol, triglycerides, urea, creatinine, total protein, and albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, bile acids, bilirubin, amylase, lipase, the activity of cholinesterase.

6. Urinalysis: In the last week of experience collected the urine to the overall analysis (collection daily) which included: appearance, volume, sodium, potassium, chloride, os molarity, specific gravity, pH, protein, glucose and blood / blood cells, ketones, bilirubin, nitrite, urobilinogen (qualitatively).

7. Sacrifice and pathology: After completion of the test material, all animals were subjected to a detailed post-mortem during which the material was taken for haematological , biochemical and pathological.

In the research of all the major pathological lesions, brain (representative regions including cerebrum, cerebellum and pons, pituitary gland), spinal cord eyeball with optic nerve, stomach, esophagus, small and large intestines (including Peyer's patches), parotid, liver, kidneys ureters, adrenal glands, spleen, heart, aorta, thymus, pancreas, clipping the skin, thyroid with parathyroid, larynx, trachea and lungs, gonads (testes, ovaries), secondary sex organs (uterus, cervix, epididymis, prostate with seminal vesicles and coagulation glands), vagina, mammary gland, urinary bladder, lymph nodes (mandibular and lumbar), peripheral nerve (sciatic), along with muscle, some bone marrow, femur with the knee joint, tongue, have been secured and subjected to histopathological examination.

Standard post mortem examinations were performed - external examinations and macroscopic assessment of all organs followed by histopathology using the classical hematoxylin and eosin stain (H+E) technique.

II. RESULTS AND DISCUSSION

The tables with detailed data obtained in this study provided by applicant were placed in Appendix 1B of this document.

For quality control is proposed that control data are compared to historical control values. Study was performed according to version of guideline valid on date of dossier submission, hence submission of historical data was not required. All reference values presented in the report come from the literature - handbooks on laboratory animal breeding data, as well as a laboratory that performs animal blood analysis. In statistical calculations, comparison between the values of the test groups and the control group was always used. The reference values presented in the report have an support to interpretation of research results. In the conducted study, all results of the study groups were compared statistically to the parallel control group. Control groups were carried under the same conditions in the same laboratory. Historical data reference values, which has been used the analysis of results are available from laboratory animals breeders and literature (please see attached file). In the presented study, HCD was auxiliary data - a comparative value data. In all cases, statistically significant differences in comparison to the concurrent control did not allow to determine the toxic effect of the tested material because did not exceed the reference values (for the same strain of rats and for the standard breeding condition) and did not correlate with clinical symptoms. Based on these results it cannot be proven a clear thesis on the impact tested material. All observed small deviations in the values were not progressing to the pathology of the organs or studied systems

A. OBSERVATIONS:

1. Clinical signs of toxicity: There were no treatment-related clinical signs at any dose.
2. Mortality: There were no deaths.

B. BODY WEIGHT AND BODY WEIGHT GAIN

The body weights of all animals were within the reference ranges for Wistar rats (by National Laboratory Animal Center - NLAC). There were no statistically significant differences between body weight of animals receiving a dose of 1000 mg/kg and the body weight of control group. During the whole period the experiment the animals gained weight. In the case of the satellite group, the body weight remained at the same level after end of dosage. There were no differences in response of males and females in case of body weight. This study was conducted with one dose level that there is impossible to show dependency of body weight changes on the dose.

C. FOOD CONSUMPTION AND COMPOUND INTAKE

Food consumption by animals participating in the study did not differ from the reference values ranging 8-20 g/day. Water consumption also ranged in reference standards of approximately 30 mL/day, however, in case of juveniles the water intake ranges from 5-80 mL/day. There was no statistically significant difference in food and water consumption of animals receiving iron pyrophosphate in relation to the control group. Iron (III) Pyrophosphate was administered to one group of experimental animals per os (limit test - 15 male and 15 female) using a stomach gavage as a suspension in 0.5% methyl cellulose at a dose of 1000 mg/kg b.w. once a day for a period of 90 days, seven days a week. Simultaneously, control group (8 males and 8 females) was administered vehicle (0.5 % methyl cellulose solution) at the same volume as the test material, and a satellite group (8 females and eight males) receiving the test material at a dose of 1000 mg/kg b.w.

D. REPRODUCTIVE EFFECTS:

Pathologic discharge from reproductive organs – absence.

E. BLOOD ANALYSIS:

1. Haematological findings:

In terms of values analysed statistically significant differences were found:

• WBC (leukocytes) - Statistically significantly higher in females in the study group in comparison to the control group.

• RBC (red blood cell count) - Statistically significantly higher in male and female test group in relation to the control group.

• HCT (haematocrit) - A statistically significantly higher in male and female test group in relation to the control group.

• Reticulocytes - Statistically significantly higher in females in the study group with respect to the control group.

The above- mentioned changes, although it showed a statistically significant difference, however, do not exceed the reference values and did not correlate with other clinical symptoms. Based on the analysis results cannot provide a clear thesis on the impact in this area of iron pyrophosphate.

2. Plasma and serum analysis:

In terms of the parameters shown marked differences statistically significant differences in the groups of females and males receiving ferric pyrophosphate in relation to the control group.

For females demonstrated statistically significant differences in the case of indications:

• unsaturated iron binding capacity UIBC (value higher than in the control group)

• glucose (a value lower than in the control group)

• phosphorus (value higher than in the control group)

• total cholesterol (value lower than in the control group), triglycerides (value higher than in the control group)

• urea (the higher than in the control group)

• total protein (a higher value than in the control group) and albumin (a value higher than in the control group)

For males demonstrated statistically significant differences in the case of indications:

• magnesium (the higher than in the control group)

• phosphorus (less than control)

• iron (value higher than in the control group)

• total iron binding capacity TIBC (value lower than in the control group)

• unsaturated iron binding capacity UIBC (value lower than in the control group)

• urea (the higher than in the control group)

• creatinine (a value higher than in the control group)

• alanine aminotransferase (value higher than in the control group), alkaline phosphatase (value higher than in the control group)

• amylase (a value higher than in the control group)

Statistically significant differences that occurred in the analyzed biochemical parameters do not allow an assumption that they were caused by iron pyrophosphate. In most cases, where the reference values are available, results ranged within the scope. In other cases, where reference values are not available, clinical picture does not allow classification of these lesions as a result of action of iron pyrophosphate, due to slight differences combined with large standard deviations or, as in the case of UIBC, contradictory results in males and females.

The analysis of results cannot provide a clear thesis on the impact in this area of iron pyrophosphate.

All the changes did not exceed the reference values and did not correlate with other clinical symptoms.

The results of the total leukocyte count, erythrocyte count, hemoglobin, MCV, MCH, MCHC, platelet count, hematocrit did not show statistically significant differences as compared to the control group in the animal group of 1000 mg / kg bw., apart from the MCH parameter for males, however, all values were within the ranges of reference values for total leukocyte count, erythrocyte count, hemoglobin concentration, MCV,

MCH, MCHC, platelet count, hematocrit for Wistar Han rats aged 8-16 weeks. In case MCH value, in males it wasn't correlate with clinical signs and it is not enough to sufficient to be considered a dosage effect.

F. SACRIFICE AND PATHOLOGY:

1. Organ weight: Absolute and relative organ weights, were unaffected by treatment.

2. Macroscopic examination:

All animals participating in experiment were subjected to a full, detailed autopsy procedure (SPT-T/VIVO/05). At autopsy, the animals were collected for histopathology and examined for macroscopic lesions:

• all gross lesions – not collected– no macroscopically visible pathological changes

• brain (representative regions including cerebrum, cerebellum and pons) – no macroscopically visible pathological changes

• spinal cord – no macroscopically visible pathological changes

• eye with optic nerve – no macroscopically visible pathological changes

• stomach – no macroscopically visible pathological changes

• oesophagus – no macroscopically visible pathological changes

• small and gross intestines (including Peyer's patches) – no macroscopically visible pathological changes

• salivary glands – no macroscopically visible pathological changes

• liver – no macroscopically visible pathological changes

• kidneys with ureter – no macroscopically visible pathological changes

• adrenals – no macroscopically visible pathological changes

• spleen– no macroscopically visible pathological changes

• heart – no macroscopically visible pathological changes

• aorta – no macroscopically visible pathological changes

• thymus – no macroscopically visible pathological changes

• thyroid with parathyroids – no macroscopically visible pathological changes

• pancreas - no macroscopically visible pathological changes

• larynx – no macroscopically visible pathological changes

• trachea and lungs – no macroscopically visible pathological changes

• gonads (testis and ovaries) – no macroscopically visible pathological changes

• accessory sex organs (uterus and cervix, epididymides, prostate + seminal vesicles with coagulating glands) – no macroscopically visible pathological changes

• vagina – no macroscopically visible pathological changes

• mammary gland – no macroscopically visible pathological changes

• urinary bladder – no macroscopically visible pathological changes

• lymph nodes (of the thorax and lumbar) – no macroscopically visible pathological changes

• peripheral nerve (sciatic) with muscle – no macroscopically visible pathological changes

• part of bone narrow – no macroscopically visible pathological changes
• femur with knee joint – no macroscopically visible pathological changes
• tongue – no macroscopically visible pathological changes

G. DEVIATIONS

According to the OECD, where the 28-day study is conducted as a preliminary study to examine the 90-day, preferably for both studies are animals of the same species and from the same source. In this study the animals from different breeders: Wistar Han and Wistar Rat had been used. Both strains are recommended for toxicological studies. Breeding conducted under standardized conditions in accordance with the recommendations of the International Council for Laboratory Animal Science, what makes the result obtained using laboratory animals is independent of the space and time, and it is possible to repeat, precisely because of the behavior of uniform standards for breeding. Due to the very small amount of test material and execution primarily hematological and biochemical parameters, the parameters of coagulation had not been evaluated for all animals. The results of blood coagulation parameters are auxiliary and sufficient to complete the status of animals health.

Organs (stomach + duodenum, kidneys + ureters + adrenal glands, thyroid gland with parathyroid, larynx, trachea and lungs + heart + aorta + thymus gland, gonads (testes, ovaries) + auxiliary genitals + vagina + bladder; peripheral nerve with muscle + femur with the knee joint) were taken in an anatomical preparation without dissection of individual organs. The organs were prepared with this method for the pathology lab at the request a pathologist, which prevents errors associated with the exact description of the type or organ parts. All the organs were assessed with micro and macroscopic evaluation and there were no deviations from the normal status in all groups of animals.

The occurrence of hemolysis - in the case of animal 103 hemolysis occurred to a low intensity, extent due to excessive animal's response to the anesthetic, and in such cases, often the hemolysis. Cases of haemolysis in the thrombin time study were caused by a small volume of investigated material and hematology apparatus marking attempts as hemolyzed. In this study coagulation, a series of analyzes aimed at comprehensive assessment of the coagulation system. Existing deficiencies in the case of the analyzed group of animals had no impact on results of the evaluation.

<u>The deviations of this toxicological study from the most updated OECD guideline</u>

Repeated dose 90-day oral toxicity study in rodents has been carried out according to OECD TG 408 (1998) valid at the day of dossier submission (2015). After dossier submission the new version of this guidance adopted on 25 June 2018 has been published.

The revised guideline pays attention to identification of chemicals with potential to cause endocrine effects. Therefore, new required endpoints have been added: measurement of thyroxine (T4), triiodothyronine (T3), thyroid stimulating hormone (TSH), thyroid gland weight, serum total cholesterol, low-density lipoproteins

(LDL) and high-density lipoproteins (HDL). Other hormone measures are optional on a case-by-case basis. Evaluation of testis, epididymis, sperm parameters, prostate and seminal vesicles and pituitary gland, ovaries, cervix, vagina, skeletal muscle and bone should be performed. Moreover, detailed description of best practice for sampling collection, handling and analysis and factors which might influence the hormones determinations have been added.

Above parameters were not evaluated in 'Repeated dose 90-day oral toxicity study in rodents' (study code: 0003/0017/T), because were not required in 2014.

However, endocrine disruption properties have been assessed in accordance with Commission Regulation (EU) 2018/605 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties and ECHA, EFSA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, EFSA Journal 2018;16(6):531. Please see attached file 'Report on ED assessment'.

No endocrine disruption properties have been identified. Taking into consideration above and animal welfare, in our opinion obtained results are sufficient and there is no need to reply repeated dose 90-day oral toxicity study in rodents.

Other changes:

- addition of point regarding standards of animal care

No impact on the study. Study was performed according to standard operating/experimental/technical procedures as for example 'Adoption and quarantine of animals' or 'Routine veterinary health control of laboratory animals'.

- requirement of detailed justification for the choice of other rodent species

Not relevant, because rats were used.

- threshold value of dietary levels of phytoestrogenes

Conventional laboratory diet was used. No endocrine disruption properties of tested substance has been identified, therefore impact on the study results can be excluded.

- individually housing of animals is possible only when it is scientifically justified

Not relevant.

- the least invasive method of animals identification must be used.

Each animal was individually labeled according to the procedure SPT-T/VIVO/05.

- for quality control is proposed that control data are compared to historical control values.

Study was performed according to version of guideline valid on date of dossier submission, hence submission of historical data was not required. All reference values presented in the report come from the literature - handbooks on laboratory animal breeding data, as well as a laboratory that performs animal blood analysis. In statistical calculations, comparison between the values of the test groups and the control group was always used. The reference values presented in the report have an support to interpretation of research results. In the

conducted study, all results of the study groups were compared statistically to the parallel control group. Control groups were carried under the same conditions in the same laboratory. Historical data reference values, which has been used the analysis of results are available from laboratory animals breeders and literature (please see attached file). In the presented study, HCD was auxiliary data - a comparative value data. In all cases, statistically significant differences in comparison to the concurrent control did not allow to determine the toxic effect of the tested material because did not exceed the reference values (for the same strain of rats and for the standard breeding condition) and did not correlate with clinical symptoms. Based on these results it cannot be proven a clear thesis on the impact tested material. All observed small deviations in the values were not progressing to the pathology of the organs or studied systems.

III. CONCLUSIONS

The test material was administered daily for 90 days at a dose of 1000 mg/kg bw. During the study, the signs of toxicity among animals were observed. Based on our audit in accordance with the Annex to Regulation (EC) No 440/2008, Method B 26 and a guideline of the OECD TG 408, there is no evidence of toxicity caused by the action of iron pyrophosphate, what was confirm by hematological, biochemical and histopathological test, as also analysis of behavioral and neurological disorders.

RMS comment: The study was performed according to OECD guideline 408 with exception of following deviation: different rat strain was used in this study than in 28 days oral study, the parameters of coagulation had not been evaluated for all animals and some organs were taken in the form of an anatomical preparation without dissection of individual organs . The justification of deviations from the method is acceptable and the study is considered suitable for evaluation.

There were no deaths and clinical signs of toxicity following 90 days oral exposure to Iron (III) Pyrophosphate. Hematological parameters showed statistically significant increase of leukocytes and reticulocytes in exposed females and red blood cell counts and hematocrit in exposed male and female. These changes were within the reference values and did not correlate with other clinical symptoms. The analysis of plasma and serum revealed statistically significant increase of the following parameters measured in exposed females: unsaturated iron binding capacity UIBC, phosphorus, triglycerides, urea, total protein and albumin. The level of glucose and total cholesterol were decreased in exposed females.

In case of males the following parameters were higher in exposed group: level of magnesium, iron, urea, creatinine, alanine aminotransferase, alkaline phosphatase and amylase. Other parameters as phosphorus, total iron binding capacity TIBC and unsaturated iron binding capacity UIBC were lower in comparison to control. These changes were within the reference values if they existed. In other case these alteration could not be associated with the iron pyrophosphate influence as they were slight and the standard deviations were large. The autopsy demonstrated no pathological changes in the tested organs.

Summing up no severe adverse effects indicated systemic toxicity of ferric pyrophosphate at the dose of 1000 mg/kg bw/day were found in the Repeated Dose 90-Day Oral Toxicity Study in Rodents, so NOAEL based on results of this study is 1000 mg/kg bw/day.

The EFSA experts at the Pesticide Peer Review Meeting 11 (05 September 2019) confirmed the NOAEL proposed by the RMS: i.e. 1000 mg/kg bw per day (high dose level tested; 90-day rat study).

B.6.3.3 Other routes

Potential exposure, other than oral, is very limited. This is related to the physicochemical properties of the substance - it is insoluble in water, lipids and organic solvents, which makes the transdermal exposure extremely low. The compound has a form of non-volatile powder, which is supposed to be added to PPP as a solid - granules, whose size prevents absorption via inhalation.

B.6.4 Genotoxicity

B.6.4.1 *In vitro* studies

B.6.4.1.1 *In vitro* studies (Bacterial Reverse Mutation Test)

Report: Nikhil S. Sathe, 2014, Bacterial Reverse Mutation Test of Ferric Pyrophosphate using Salmonella typhimurium Tester Strains

Study test: 1248

Guidelines: OECD TG 471, section 4: "Bacterial Reverse mutation Test"

GLP: Yes

Abstract:

This study was performed to evaluate the ability of Ferric Pyrophosphate (FPP) to induce a mutagenic response in histidine requiring tester strains viz., TA98, TA100, TA1535, TA1537 and TA102. A dose range finding assay was performed using eight doses with 5000 µg/plate as the top dose. The assay was conducted using the plate incorporation method to select doses for the Definitive Assay. Tester strains TA98 and TA100 were used in the Dose Range Finding Assay in the presence and absence of 10% v/v S9 Mix. Ferric Pyrophosphate was not found to induce cytotoxicity in the form of inhibition of background bacterial lawn and inhibition of spontaneous revertants in both TA98 and TA100 over the dose range (1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate) selected. Ferric Pyrophosphate was also found non-mutagenic to both TA98 and TA100 over the dose range (1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate) selected in presence and absence of metabolic activation.

Five concentrations (312.5, 625, 1250, 2500, 5000 µg/plate) were selected for the Definitive Assay. The Definitive Assay was conducted using the plate incorporation method in the presence (10% v/v S9 Mix) and absence of metabolic activation. The results of the dose range finding assay and definitive Assay were

confirmed in the Confirmatory Assay by using all the five tester strains with (10% v/v S9 Mix) and without metabolic activation using Pre-incubation method.

Test item (Ferric Pyrophosphate) was found non mutagenic to TA1535, TA1537 and TA102 over the dose range selected (312.5, 625, 1250, 2500, 5000 µg/plate) in presence (10% v/v S9 Mix) and absence of metabolic activation in definitive assay.

Ferric pyrophosphate was found non mutagenic to TA98, TA100, TA1535, TA1537 and TA102 when tested in presence and absence of metabolic activation over the dose range (312.5, 625, 1250, 2500, 5000 µg/plate) in confirmatory assay.

Revertant frequencies of TA98, TA1537, TA1535, TA100 and TA102 were comparable with controls in dose range finding assay, definitive as well as confirmatory assay.

From the results of this study, it can be concluded that Ferric Pyrophosphate (FPP) is non-mutagenic to all the five tester strains viz. TA98, TA100, TA1535, TA1537 and TA102 when tested at 5000 µg/plate in presence (10% v/v S9 Mix) as well as in absence of metabolic activation.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: 27 ± 9°C, away from light

Solvent used: dimethyl sulphoxide (DMSO)

2. Control Materials:

Solvent/ final concentration: dimethyl sulphoxide (DMSO) at 100 µl/plate

Positive: Positive controls were included in the dose range finding assay, definitive assay and confirmatory assay. The doses used for the definitive assay and confirmatory assay are as enlisted in Table CA 5.4-1.

Table CA 5.4-1 Positive controls with activation and without activation

Strain	Positive Controls (without S9 activation)	Dose (µg/plate)	Positive Controls (with S9 activation)	Dose (µg/plate)
TA98	2-Nitrofluorene (2NF)	5.0	2-Aminoanthracene (2AA)	5.0
TA100	Sodium Azide (SA)	2.5	2-Aminoanthracene (2AA)	5.0
TA1535	Sodium Azide (SA)	2.5	2-Aminoanthracene (2AA)	5.0
TA1537	9-Aminoacridine (9AA)	50.0	2-Aminoanthracene (2AA)	5.0
TA102	Mitomycin C (MMC)	0.5	2-Aminoanthracene (2AA)	5.0

3. Activitation: The S9 liver microsomal fraction was prepared from the rat liver (Sprague Dawley) Sprague Dawley female rats were administered Phenobarbital intraperitoneally and β-Naphthoflavone orally for three consecutive days. The liver was removed aseptically and homogenized with 0.15 M KCl. The homogenate was centrifuged at 9000g at 4 °C for 10 minutes and the supernatant was collected. Aliquots of 1 mL were prepared and stored at – 80°C ± 15°C.

Table 5.4-2 S9 mix composition

	Component	Volume (per 50 mL)
S9 mix composition	Rat liver S9 fraction	5.0 mL
	$MgCl_2$ – KCl	1.0 mL
	1 M glucose-6-phosphate	0.25 mL
	0.1 M NADP	2.0 mL
	0.2 M Phosphate buffer pH 7.4	25.0 mL
	Sterile Milli Q water	16.75 mL

4. Test organisms: Salmonella typhimurium, strain: TA98, TA100, TA1535, TA1537 and TA102, source: Bioreliance, Inc.

5. Test Concentration:

a) preliminary cytotoxicity assay:

Dose Range Finding Assay: A Dose Range Finding Assay was performed with strains TA98 and TA100 with (10% v/v S9 Mix) and without metabolic activation using plate incorporation method, in order to evaluate the cytotoxicity of Ferric Pyrophosphate to the bacteria and to select the range of doses for the „Definitive Assay‟. Eight different doses were tested (1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate). Each dose, vehicle control and positive controls were tested in triplicate. The precipitation of Ferric Pyrophosphate at the doses tested was observed and recorded.

b) Mutation assays:

Definitive Assay: The Definitive Assay was conducted using the „Plate Incorporation Method‟ at the dose levels 312.5, 625, 1250, 2500, 5000 µg/plate on the basis of the results obtained in the „Dose Range Finding Assay‟. TA98 and TA100 were not repeated in the definitive assay. Ferric Pyrophosphate was assessed for its mutagenic potential using three bacterial strains (TA1535, TA1537, and TA102) both in the presence (10% v/v S9 Mix) and in the absence of metabolic activation.

Confirmatory Assay: A confirmatory assay was conducted to confirm the results of the Dose range finding assay and Definitive Assay using the „Pre-Incubation Method‟ with dose levels of 312.5, 625, 1250, 2500, 5000 µg/plate in triplicates.

B. TEST PERFORMANCE:

1. Plate incorporation mutation assay:

Tubes containing 2 mL of molten top agar with 0.5 mM of histidine and biotin maintained at 45 ± 2 °C were used for plating of the Tester Strains. To the molten top agar, 100 µL of test item solution, 100 µL positive control or Vehicle control was added. 0.1 mL of overnight grown bacterial culture was added. Finally, 0.5 mL of S9 mixture was added to the tubes wherever metabolic activation was required or 0.5 mL of 0.2 M phosphate buffer wherever metabolic activation was not required. The mixture was mixed by overtaxing briefly and rapidly poured on labeled Minimal Glucose Agar (MGA) plates. After the contents solidified, the petri dishes were inverted and incubated at 37 ± 2 °C for 48 hrs.

2. Pre-incubation assay

Following ingredients were added to a sterile test tube in the order indicated below:

– 500 µL of S9 Mix if metabolic activation is required or 500 µL of 0.2 M Phosphate Buffer if metabolic activation is not required,

– 100 µL of bacterial suspension,

– Test Item solution (0.1 ml) at each dose level or 0.1 ml vehicle control or positive control solution.

– The Mixture was incubated for approximately 30 minutes in a shaker incubator set to 37 + 2 °C and 70 rpm.

– After the incubation period, 2 ml of molten Top Agar (containing 0.5 mM of histidine and biotin) held at 45 ± 1°C was added to the tubes, the mixture was briefly vortexed and then poured on MGA plates.

– After the contents solidify, the petri plates were inverted and incubated at 37 ± 2 °C for 48 h.

3. Statistics:

Mean, Standard deviation and fold increase was calculated using MS-Excel.

4. Mutagenicity Evaluation Criteria:

After the incubation period, colonies were counted and the test item (FPP) was considered negative (non-mutagenic) for TA98, TA100, TA1535, TA1537 and TA102 based on the following criteria for the test item to be called mutagenic:

a) If the background average is ten colonies or above, the average number of revertants for one test item dose must exceed three times the background average (two times for strains TA100 and TA102), either with or without metabolic activation. If the background average is below ten colonies, the average number of revertants for one test item dose must exceed 20 colonies, either with or without metabolic activation.

b) If there is a dose-related increase that does not meet the first criteria (a). In this case, the highest average number of revertant colonies must be outside the laboratory's spontaneous reversion historical data, either with or without metabolic activation. A positive result indicates that the test article induces mutations in Salmonella typhimurium cells.

II. RESULTS AND DISCUSSION

A. DOSE RANGE FINDING ASSAY

During Dose Range Finding Assay, precipitation was observed at 150, 500, 1500 and 5000 µg/plate when tested in presence and absence of metabolic activation. Test item (FPP) was found non cytotoxic in both TA98

and TA100 at the 5000 µg/plate when tested in presence (10 % v/v S9 Mix) and absence of metabolic activation. Test item (FPP) was found non mutagenic to both TA98 and TA100 when tested at 5000, 1500, 500, 150, 50, 15, 5, 1.5 µg/plate when tested in presence (10 % v/v S9 Mix) and absence of metabolic activation. The average of revertant colonies obtained at all individual dose levels for TA98 and TA100 strains with and without metabolic activation was comparable with their respective vehicle controls. The positive controls induced a strong mutagenic response by inducing a multifold increase in number of colonies over the vehicle control.

B. DEFINITIVE ASSAY

Ferric pyrophosphate was found non cytotoxic at 5000 µg/plate and precipitate formed by Ferric pyrophosphate was not interfering with the scoring and hence five dose levels were selected based on the data from dose range finding assay Viz. 312.5, 625, 1250, 2500, 5000 µg/plate. Definitive assay was performed with strains TA1335, TA1537 and TA102. Test item (FPP) was found non mutagenic to TA1535, TA1537 and TA102 over the dose range viz. 312.5, 625, 1250, 2500, 5000 µg/plate when tested in presence (10 % v/v S9 Mix) and absence of metabolic activation. The average of revertant colonies obtained at all individual dose levels for TA1535, TA1537 and TA102 strains with and without metabolic activation was comparable with their respective vehicle controls. The positive controls induced a strong mutagenic response by inducing a multi-fold increase in the number of revertant colonies over the vehicle controls.

Table 5.4-3 Definitive Assay – Without Metabolic Activation (-S9)

Test Item/VC/PC	Concentration (µg/plate)	His⁺ Revertant Colonies/Plate□								
		TA1535			TA1537			TA102		
		Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠
VC	0	11.67	3.51	NA	7.00	1.00	NA	356.67	29.69	NA
FPP	5000	12.00	3.00	1.03	6.00	1.00	0.86	347.33	31.01	0.97
FPP	2500	12.33	1.53	1.06	5.33	0.58	0.76	344.33	45.21	0.97
FPP	1250	13.67	4.73	1.17	6.67	1.53	0.95	323.67	5.86	0.91
FPP	625	12.00	2.00	1.03	6.67	1.15	0.95	373.33	29.96	1.05
FPP	312.5	14.67	3.21	1.26	6.67	1.53	0.95	323.33	18.15	0.91

Key: NA = Not Applicable, S.D. = Standard deviation, –S9 = without metabolic activation system, +S9 = with 10% v/v metabolic activation system, VC = Vehicle control, FPP = Ferric Pyrophosphate Note: □ = Mean of three replicates, ≠ - Fold Increase

Table 5.4-4 Definitive Assay – With Metabolic Activation (+S9)

Test Item/VC/PC	Concentration (µg/plate)	His⁺ Revertant Colonies/Plate□								
		TA1535			TA1537			TA102		
		Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠
VC	0	10.67	1.15	NA	6.67	0.58	NA	281.00	18.52	NA
FPP	5000	11.67	1.53	1.09	7.33	1.53	1.10	250.00	25.87	0.89
FPP	2500	8.00	1.00	0.75	5.67	0.58	0.85	274.67	5.86	0.98
FPP	1250	8.33	1.53	0.78	7.33	0.58	1.10	272.00	19.08	0.97
FPP	625	11.33	2.08	1.06	6.33	1.15	0.95	275.33	16.77	0.98
FPP	312.5	9.33	3.51	0.88	5.67	0.58	0.85	340.00	15.62	1.21

Key: NA = Not Applicable, S.D. = Standard deviation, –S9 = without metabolic activation system, +S9 = with 10% v/v metabolic activation system, VC = Vehicle control, FPP = Ferric Pyrophosphate Note: ¤ = Mean of three replicates, ≠ - Fold Increase

C. CONFIRMATORY ASSAYS

Confirmatory assay was performed with strains TA98, TA100, TA1535, TA1537 and TA102.The results obtained in the Definitive Assay were confirmed in the Confirmatory assay by using the „Pre-Incubation method". Test item (FPP) was confirmed to be non-mutagenic to all the five tester strains viz. TA98, TA100, TA1535, TA1537 and TA102 when tested in presence (10 % v/v S9 Mix) and in absence of metabolic activation. The average number of revertant colonies obtained at all individual dose levels for TA98, TA100, TA1535, TA1537 and TA102 strains with and without metabolic activation was comparable with their respective vehicle controls.

Table 5.4-5. Confirmatory Assay – Without Metabolic Activation (-S9)

Test Item/ VC/PC	Concentration (µg/plate)	His⁺ Revertant Colonies/Plate														
		TA98			TA100			TA1535			TA1537			TA102		
		Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠
VC	0	21.00	1.00	NA	94.33	11.93	NA	10.33	3.51	NA	8.00	2.65	NA	230.67	13.61	NA
FPP	5000	22.00	1.00	1.05	84.67	4.16	0.90	6.00	1.00	0.58	5.67	0.58	0.71	223.33	16.26	0.97
FPP	2500	20.00	1.00	0.95	84.33	9.45	0.89	7.33	1.53	0.71	7.33	1.15	0.92	224.00	3.00	0.97
FPP	1250	20.00	4.00	0.95	91.67	8.50	0.97	8.67	4.73	0.84	9.33	4.04	1.17	226.33	19.55	0.98
FPP	625	21.00	2.00	1.00	85.33	6.11	0.90	9.33	1.53	0.90	5.33	0.58	0.67	246.67	8.33	1.07
FPP	312.5	18.67	1.53	0.89	89.33	9.29	0.95	9.67	3.06	0.94	6.33	0.58	0.79	223.00	14.42	0.97

Key: NA = Not Applicable, S.D. = Standard deviation, –S9 = without metabolic activation system, +S9 = with 10% v/v metabolic activation system, VC = Vehicle control, FPP = Ferric Pyrophosphate Note: ¤ = Mean of three replicates, ≠ - Fold Increase

Table 5.4-6. Confirmatory Assay – With Metabolic Activation (-S9)

Test Item/VC/PC	Concentration (µg/plate)	His⁺ Revertant Colonies/Plate														
		TA98			TA100			TA1535			TA1537			TA102		
		Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠	Mean	S.D.	≠
VC	0	23.00	3.61	NA	111.33	7.51	NA	8.33	1.53	NA	6.67	2.89	NA	274.33	6.43	274.33
FPP	5000	27.33	3.79	1.19	95.67	12.90	0.86	7.33	1.53	0.88	5.33	0.58	0.80	263.67	13.58	0.96
FPP	2500	25.33	4.04	1.10	118.00	16.82	1.06	8.33	2.08	1.00	6.00	1.00	0.90	247.33	16.92	0.90
FPP	1250	28.33	1.53	1.23	104.67	6.66	0.94	8.33	2.08	1.00	8.33	1.53	1.25	267.00	21.28	0.97
FPP	625	26.33	9.29	1.14	112.33	5.51	1.01	6.67	2.08	0.80	6.00	2.00	0.90	267.67	20.50	0.98
FPP	312.5	24.33	0.58	1.06	115.67	3.06	1.04	7.00	1.73	0.84	7.00	1.00	1.05	303.33	6.66	1.11

Key: NA = Not Applicable, S.D. = Standard deviation, –S9 = without metabolic activation system, +S9 = with 10% v/v metabolic activation system, VC = Vehicle control, FPP = Ferric Pyrophosphate Note: ¤ = Mean of three replicates, ≠ - Fold Increase

Figure 1. Dose Response Curve – Dose Range Finding Assay - without metabolic activation

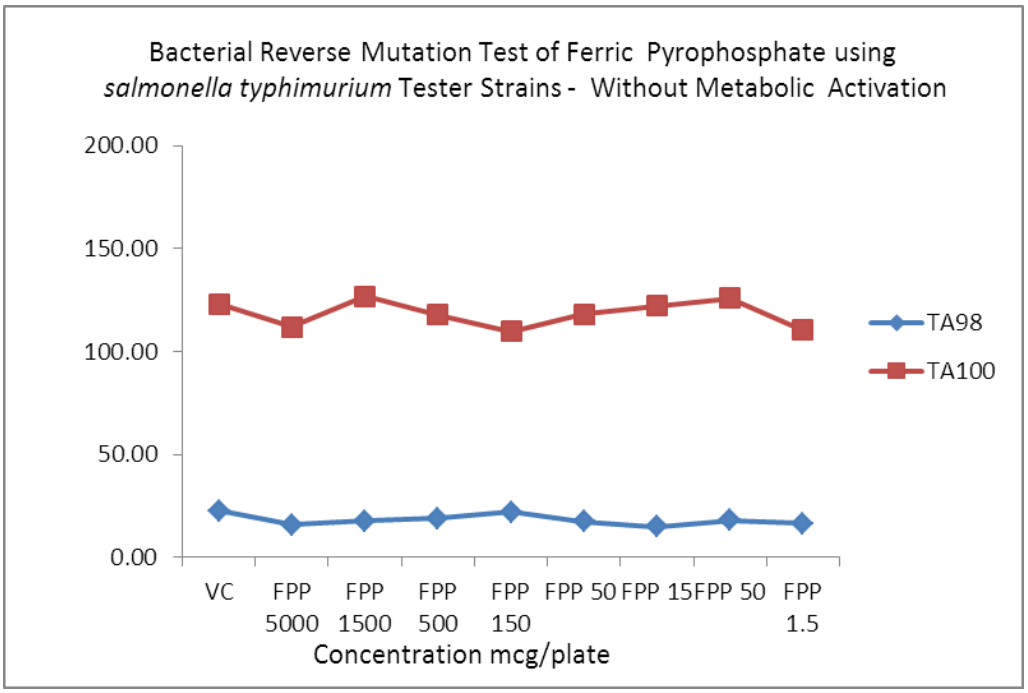


Figure 2. Dose Response Curve – Dose Range Finding Assay - with metabolic activation

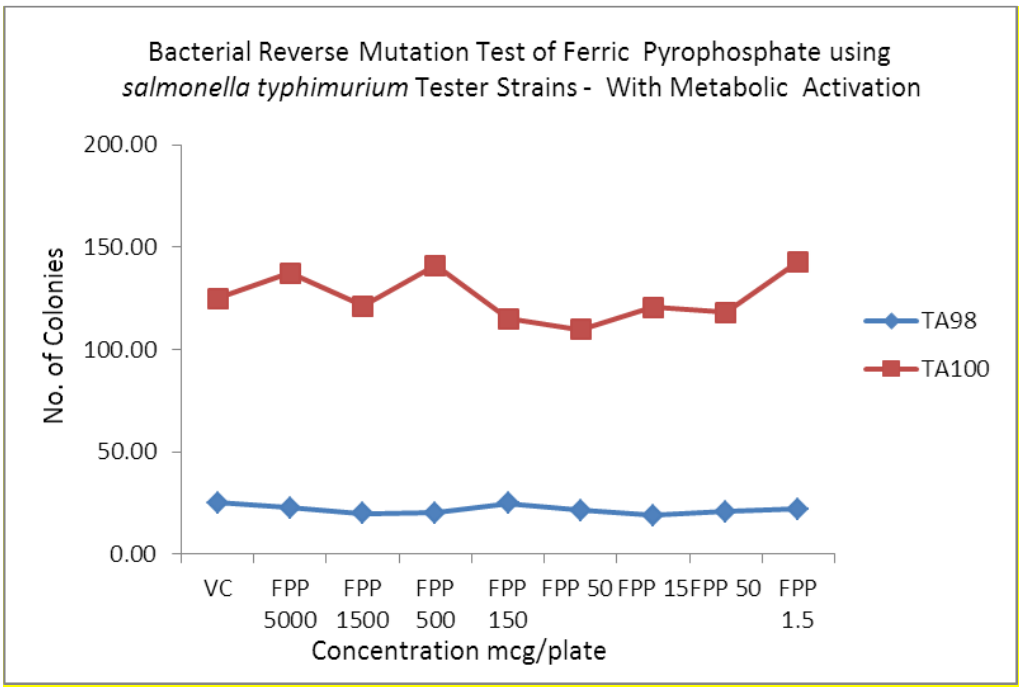


Figure 3. Dose Response Curve – Definitive Assay - without metabolic activation

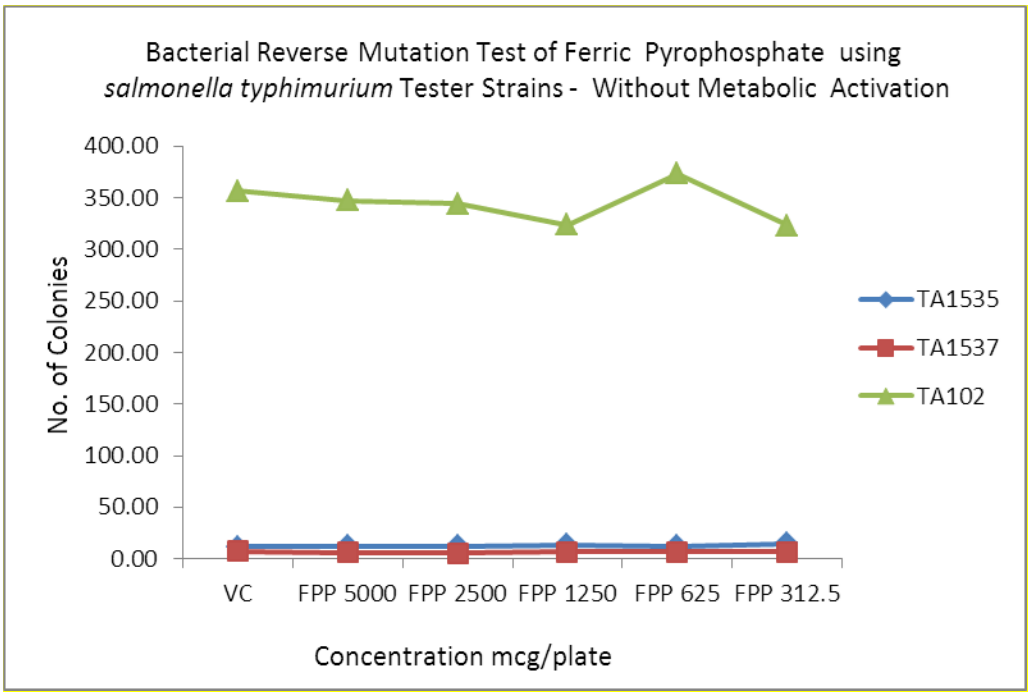


Figure 4. Dose Response Curve – Definitive Assay - with metabolic activation

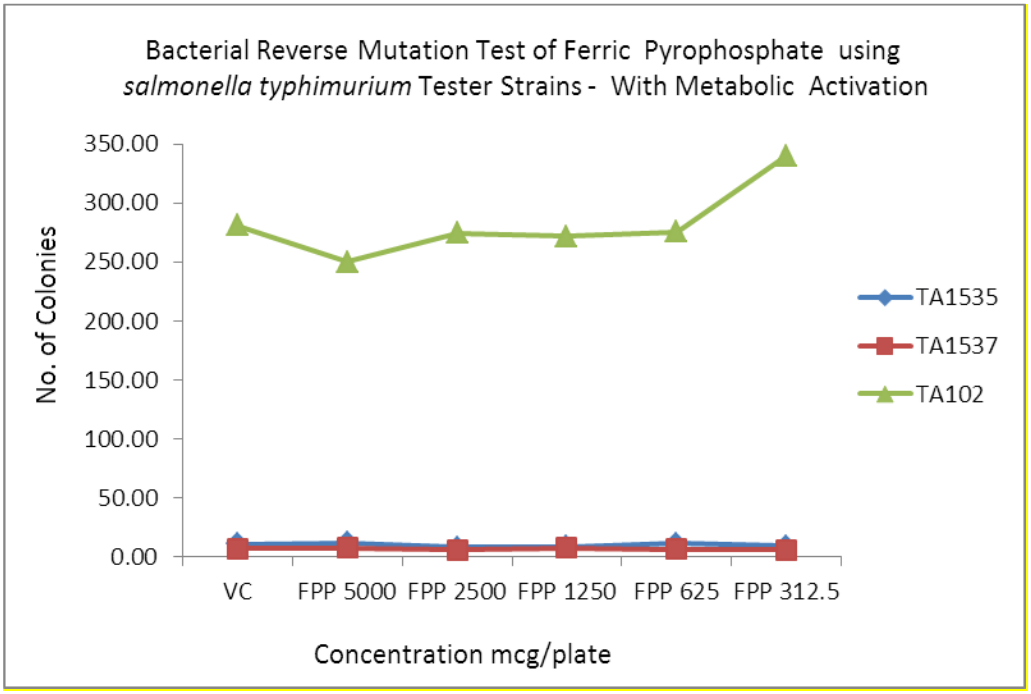


Figure 5. Dose Response Curve – Confirmatory Assay - without metabolic activation

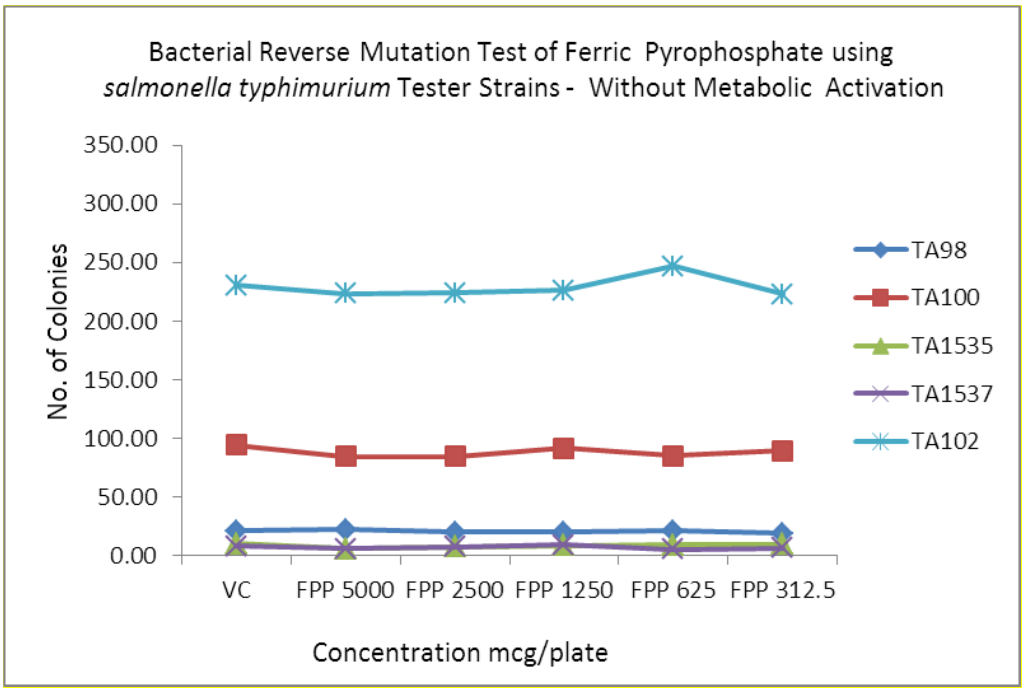
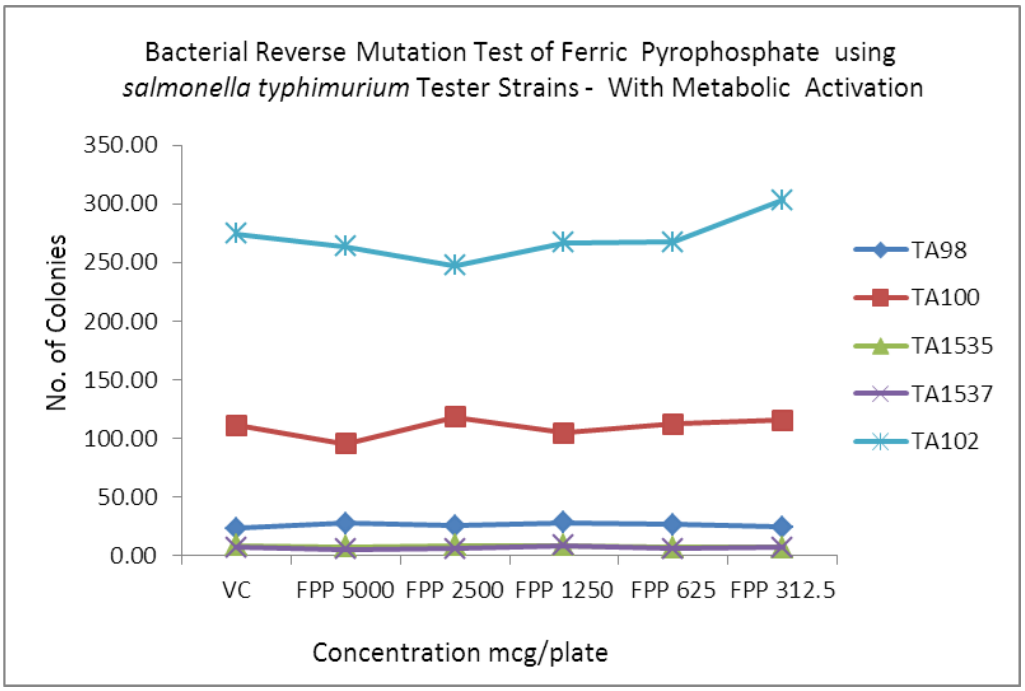


Figure 6. Dose Response Curve – Confirmatory Assay - with metabolic activation



III. CONCLUSIONS

From the results of this study, it can be concluded that Ferric Pyrophosphate is non-mutagenic to all the five tester strains viz. TA98, TA100, TA1535, TA1537 and TA102 when tested at 5000 µg/plate in presence (10% v/v S9 Mix) as well as in absence of metabolic activation.

RMS comment: The study was carried out in compliance with OECD 471 guideline and it is acceptable. Under the condition of this study Ferric Pyrophosphate was not mutagenic when tested in a Salmonella strains with and without metabolic activation.

B.6.4.1.2 *In vitro* studies (Mammalian Cell Gene Mutation Test)

Report: N. Shetty, 2014, In Vitro Mouse Lymphoma Forward Mutation Assay of Ferric Pyrophosphate by using Mouse Lymphoma (L5178Y TK+/-) Cell Line
Study test: VLL/1013/G/T079
Guidelines: OECD TG 476, section 4: "In Vitro Mammalian Cell Gene Mutation Test"
GLP: Yes

Abstract:
The potential mutagenic activity of Ferric Pyrophosphate was tested in the mouse lymphoma assay. The mouse lymphoma L5178Y cell line, heterozygous at the TK locus and designated as clone 3.7.2C, was used to test the mutagenicity of Ferric Pyrophosphate with and without metabolic activation system. The study was conducted in two independent phases – preliminary cytotoxicity assay and mouse lymphoma mutagenicity assay.
Based on the solubility in DMSO at 250 mg/mL and precipitation of test item, Ferric Pyrophosphate was tested up to the highest concentration of 1250 µg/mL. The preliminary dose range finding cytotoxicity experiment with a treatment period of approximately 4 hours was performed with and without S9 activation at the concentrations such as 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250 and 2500 µg/mL. In addition, a non-activation assay with a treatment period of approximately 24 hours was performed at the same test concentrations. The test item was observed to form a 'slight precipitation' and 'heavy precipitation' upon dilution into treatment medium and at the end of the treatment period at 1250 and 2500 µg/mL respectively. Additionally the lower test concentration of 625 µg/mL also showed slight precipitation at the end of the treatment period. No precipitations were observed at all the lower concentrations tested.
Cytotoxicity was determined by measuring the relative cloning efficiency and relative total growth of the cultures after the treatment period. No reduction in % relative cloning efficiency and relative total growth was observed in any of the test item treated groups when compared to vehicle control. On the basis of the results of preliminary cytotoxicity study and item solubility, dispersion characteristics the Ferric Pyrophosphate was tested at 78.1, 156.3, 312.5, 625.0 and 1250 µg/mL in the mouse lymphoma mutagenicity assay.
Mouse lymphoma mutagenicity assay with a treatment period of approximately 4 hours was performed with and without S9 activation at the concentrations such as 78.1, 156.3, 312.5, 625.0 and 1250 µg/mL. In addition, confirmatory non-activation assay with a treatment period of approximately 24 hours was performed at the same test concentrations. Concurrent negative, vehicle and positive and negative controls both with and

without metabolic activation were included in each experiment. Methyl methanesulfonate and Benzo(a)pyrene were used as positive controls for experiments without and with metabolic activation system respectively.

At the end of test item exposure period, cells in suspension were subcultured to determine cytotoxicity and were incubated for further 2-days for phenotypic expression prior to mutantselection. At the end of expression period, mutant frequency was determined by seeding known numbers of cells in medium containing the selective agent (TFT) to detect mutant cells, and in medium without selective agent to determine the cloning efficiency (viability). The results did not indicate evidence of any cytotoxic effect. No dose-related decreases were noted in the % relative cloning efficiency and relative total growth in any of the test item treated groups when compared to vehicle control. In the absence of cytotoxicity, all the test concentrations were analyzed for mutant frequencies. No statistically or biologically significant increases in the frequency of mutants were noted at any of the test concentration levels tested. All the values of treated groups were similar to the negative and vehicle control values. In the presence or absence of metabolic activation, the test item Ferric Pyrophosphate did not induce any increase in the mutant frequency when tested up to 1250 µg/mL using long or short period of treatment.

Positive control (MMS & BAP) treated cultures showed a statistically significant increase in the number of TFT resistant colonies as compared to the vehicle control indicating the assay was sensitive and responsive to mutagens.

From the results of this study and according to the criteria of the test protocol, it is concluded that when tested up to 1250 µg/mL the test item, Ferric Pyrophosphate did not induce forward mutation at the thymidine kinase (TK) locus of L5178Y mouse lymphoma cells either with or without metabolic activation under this test conditions.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: Room temperature, keep away from light, keep tightly closed

Solvent used: dimethyl sulphoxide (DMSO)

2. Control Materials:

Culture medium: RPMI-1640 supplemented with fetal bovine serum (10% by volume), L-glutamine, 25mM HEPES buffer, penicillin and streptomycin.

Positive:

Without activation: Methyl methanesulfonate was used as direct acting mutagen. For the non-activation assay with a 4 hour treatment, Methyl methanesulfonate was treated at two concentrations of 13 and 18 µg/mL. For non-activation studies with a 24 hours treatment period, MMS was used at two concentrations of 6.5 and 9 µg/mL.

With activation: Benzo(a)pyrene was used in the presence of metabolic activation system. This chemical was treated at two concentrations of 3 and 4 µg/mL as a positive control for assay performed with S9 activation.

3. Activation: Aroclor 1254 induced male Sprague Dawley rat liver post mitochondrial fraction was used for the assay with metabolic activation system (S9 Homogenate). Aliquots of the post mitochondrial fraction (S9 fraction), retained frozen at -70°C were thawed immediately before use and added to other components that form the activation system - S9 Mix.

Table 6.4.1.2-1 S9 mix composition

	Components	Volume (mL)
S9 mix composition	Glucose-6-phosphate (180 mg/mL)	mixed in the ratio 1:1:1:2
	NADP (25 mg/mL)	
	150 mM KCl	
	S9 fraction	

The final concentration of the liver homogenate in the test system was used at 2 % v/v. Cultures treated in the absence of S-9 received 1 mL, 150 mM KCl.

4. Test System: The mouse lymphoma L5178Y cell line, heterozygous at the TK locus and designated as clone 3.7.2C was used for this assay. Stock cultures were obtained from ATCC and stored in the liquid nitrogen. All laboratory cultures were maintained in logarithmic growth by serial subculturing for not more than 4 months and were replaced by cells from frozen stock. All cultures were grown in a incubator at 37 ± 1°C in a humidified atmosphere of 5 ± 1.5 % CO_2 in air.

Preparation of cultures

Cells were propagated from stock cultures, seeded in culture media (RPMI 1640 with 10 % FBS) and incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1.5 % CO_2 in air. A continuous cell stock log was kept to record growth, doubling times and subculture operations. The cell doubling time was found to be between 8.6 to 8.7 hours. Prior to use in the mutagenicity assay, the L5178Y cells were cultured in RPMI-1640 (with 10 % FBS) containing HAT (Hypoxanthine, Aminopterin and Thymidine) for 24 hours to select against newly arising $TK^{-/-}$ mutants, and then placed in the RPMI 1640 media (with 10 % FBS) containing HT (Hypoxanthine and Thymidine) for one day prior to use in mutagenicity study.

5. Test for Mycoplasma Contamination: Mycoplasma detection was performed on stock cultures after preparing stocks for freezing. It was performed by using commercially available kit (Universal Mycoplasma Detection Kit - ATCC) employing PCR method. The cell line L5178Y $TK^{+/-}$ Clone (3.7.2C), was found to be free from mycoplasma contamination.

6. Karyotype Stability: Karyotype stability, as measured by mean chromosomal number, was performed prior to discarding the stock cultures to ensure that changes in the culture have not occurred. The results showed that modal chromosome number for the L5178Y $TK^{+/-}$ Clone (3.7.2C) cell line as 40.

B. TEST PERFORMANCE

1. Preliminary Cytotoxicity Assay: The preliminary dose range finding cytotoxicity experiment with a treatment period of approximately 4 hours was performed with and without S9 activation at the concentrations of 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250 and 2500 µg/mL. In addition, a confirmatory non-activation assay with a treatment period of approximately 24 hours was performed at the same test concentrations. In all cases, precipitation was evaluated at the beginning and at the end of the treatment period.

Cytotoxicity was determined by measuring the relative cloning efficiency and relative total growth of the cultures after the treatment period.

2. Initial Mouse Lymphoma Mutagenicity Assay:

a) Initial Non Activation Assay

Logarithmically growing laboratory stock cultures were adjusted approximately to 1 x 10^6 cells/mL and exposed to the test item, Ferric Pyrophosphate at 78.1, 156.3, 312.5, 625.0, and 1250.0 µg/mL in the absence of a metabolic activation system for 4 hours. MMS at 13 and 18 µg/mL was used as positive control substance. Each concentrations of test item together with the concurrent negative, vehicle and positive controls was tested in duplicate.

A standard expression period of 2 days was used to allow for mutant recovery, growth and expression of the $TK^{-/-}$ phenotype.

b) Initial Activation Assay

The activation assays was performed identical to the non-activation assays except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the treatment period of approximately 4 hours. The S9 activation mix (2% v/v) was prepared prior to use and was included with treatment medium. BAP at 3 and 4 µg/mL was used as positive control substance. Each concentrations of test item together with the concurrent negative, vehicle and positive controls was tested in duplicate.

3. Statistic:

The statistical analysis was performed using “SAS® 9.2, Enterprise Guide version 4.2 for Windows (SAS Institute Inc., Cary, NC, USA)”. The averages of negative control and vehicle control were compared using Student’s t test. The average control mutant frequency was compared with the average MF from each treatment dose based on ANOVA and further Dunnett’s test for multiple comparisons. The data was also checked for a linear trend in mutant frequency with treatment dose using weighted regression method.

4. Data Evaluation criteria:

The test item was evaluated as positive, negative, or equivocal in this assay. A positive evaluation indicates that the test item is mutagen in this test system. A negative evaluation indicates the test item is nonmutagenic

in this test system and causes no responses that can be interpreted as positive. However, there are test items for which the results remain questionable; any test item in this category will be evaluated as equivocal in this test system.

The test item is evaluated as positive if all the following criteria are met:

- the assay is valid,
- the mutant frequency at one or more doses is significantly greater than that of the negative/vehicle control ($p < 0.05$),
- there is a significant dose-relationship as indicated by the linear trend analysis ($p < 0.05$).

A test item, for which the results do not meet the above criteria for positive response, is considered nonmutagenic in this system.

II. RESULTS AND DISCUSSION

A. TEST ITEM SOLUBILITY TESTING

Solubility of the test item in DMSO was found to be up to 250 mg/mL. The trial dose formulation preparation was carried out by dissolving 500.22 mg of test item in DMSO, volume made up to 2001 µL and was sonicated for 10 minutes (which is equivalent to 250 mg/mL). The test item, Ferric Pyrophosphate formed an whitish homogenous suspension in the vehicle (dimethylsulfoxide) at 250 mg/mL, the highest concentration prepared for use in the preliminary cytotoxicity assay. Later precipitation of test item if any, was verified by adding 200 µL of the test item formulation from the stock solutions (125 and 250 mg/mL) to 19.8 mL culture medium. The culture medium treated with test item formulation at 125 and 250 mg/mL showed slight precipitation and heavy precipitation respectively. Based on the solubility results, Ferric Pyrophosphate was tested up to 2500 µg/mL in the preliminary cytotoxicity assay.

B. PRELIMINATORY CYTOTOXICITY ASSAY

No signs of cytotoxicity were noted in the concentration range of 19.5 to 2500 µg/mL. No dose-related decreases were noted in the % relative cloning efficiency and relative total growth in any of the test item, Ferric Pyrophosphate treated groups when compared to vehicle control. On the basis of the results of preliminary cytotoxicity study and item solubility, dispersion characteristics the Ferric Pyrophosphate was tested at 78.1, 156.3, 312.5, 625.0 and 1250 µg/mL in mouse lymphoma mutagenicity assay.

C. MUTATION ASSAYS – INITIAL ASSAY

Initial Activation Assay: In the absence of cytotoxicity, all the test concentration levels were included in the analysis of the mutant frequencies. When compared to negative and vehicle control, no statistically significant increases in the frequency of mutants were noted over the range of test concentration levels tested (78.1, 156.3,

312.5, 625.0, and 1250.0 µg/mL). All the values in treated groups were similar to the negative and vehicle values.

Benzo(a)pyrene treated cultures at 3 and 4 µg/mL showed a statistically significant increase in the number of TFT resistant colonies as compared to the vehicle control indicating the assay was sensitive and responsive to mutagens.

Initial Non activation Assay: In the absence of cytotoxicity, all the test concentrations were included in the analysis of the mutant frequencies. When compared to negative and vehicle control, no statistically significant increases in the frequency of mutants were noted over the range of concentrations tested (78.1, 156.3, 312.5, 625.0 and 1250.0 µg/mL). All the values in treated groups were similar to the negative and vehicle values.

Methyl methanesulfonate treated cultures at 13 and 18 µg/mL showed a statistically significant increase in the number of TFT resistant colonies as compared to the vehicle control indicating the assay was sensitive and responsive to mutagens.

Table 6.4.1.2-2 Summary of Cell Treatment Without Metabolic Activation - initial non activation assay

Test Concentrations (µg/mL)	**Without S9 Mix**					
	Number of Cultures	**Treatment Period (Hrs)**	**Expression Period (days)**	**Number of Cells Seeded per well**		**Culture Period (Days)**
				For Cloning Efficiency	**For Mutant Detection**	
0.0 (NC)	2	4	2	1.6	2×10^3	14
0.0 (VC)	2	4	2	1.6	2×10^3	14
78.1	2	4	2	1.6	2×10^3	14
156.3	2	4	2	1.6	2×10^3	14
312.5	2	4	2	1.6	2×10^3	14
625.0	2	4	2	1.6	2×10^3	14
1250.0	2	4	2	1.6	2×10^3	14
13.0 - MMS	2	4	2	1.6	2×10^3	14
18.0 - MMS	2	4	2	1.6	2×10^3	14

Table 6.4.1.2-3. Summary of Cell Treatment With Metabolic Activation - Initial Activation Assay

Test Concentrations (µg/mL)	With S9 Mix					
	Number of Cultures	Treatment Period (Hrs)	Expression Period (days)	Number of Cells Seeded per well		Culture Period (Days)
				For Cloning Efficiency	For Mutant Detection	
0.0 (NC)	2	4	2	1.6	2 x 10^3	14
0.0 (VC)	2	4	2	1.6	2 x 10^3	14
78.1	2	4	2	1.6	2 x 10^3	14
156.3	2	4	2	1.6	2 x 10^3	14
312.5	2	4	2	1.6	2 x 10^3	14
625.0	2	4	2	1.6	2 x 10^3	14
1250.0	2	4	2	1.6	2 x 10^3	14
3.0 - BAP	2	4	2	1.6	2 x 10^3	14
4.0 - BAP	2	4	2	1.6	2 x 10^3	14

D. CONFIRMATORY ASSAY

In the absence of cytotoxicity, all the test concentrations were included in the analysis of the mutant frequencies. When compared to negative and vehicle control, no statistically significant increases in the frequency of mutants were noted over the range of test concentrations tested (78.1, 156.3, 312.5, 625.0 and 1250.0 µg/mL). All the values in treated groups were similar to the negative and vehicle values.

Methyl methanesulfonate treated cultures at 6.5 and 9.0 µg/mL showed a statistically significant increase in the number of TFT resistant colonies as compared to the vehicle control indicating the assay was sensitive and responsive to mutagens.

Table 6.4.1.2-4 Summary of Cell Treatment With Metabolic Activation - Confirmatory Non-activation Mouse Lymphoma Assay

Test Concentrations (µg/mL)	Without S9 Mix					
	Number of Cultures	Treatment Period (Hrs)	Expression Period (days)	Number of Cells Seeded per well		Culture Period (Days)
				For Cloning Efficiency	For Mutant Detection	
0.0 (NC)	2	24	2	1.6	2 x 10^3	15
0.0 (VC)	2	24	2	1.6	2 x 10^3	15
78.1	2	24	2	1.6	2 x 10^3	15
156.3	2	24	2	1.6	2 x 10^3	15
312.5	2	24	2	1.6	2 x 10^3	15
625.0	2	24	2	1.6	2 x 10^3	15
1250.0	2	24	2	1.6	2 x 10^3	15
6.5 - MMS	2	24	2	1.6	2 x 10^3	15
9.0 - MMS	2	24	2	1.6	2 x 10^3	15

E. DEVIATIONS

Dossier was submitted in 2015, hence it should be assessed according to guidelines valid in 2015.

Ferric pyrophoshpate is almost insoluble substance, hence it was tested in limit of solubility (OECD 476 §17). Therefore, ferric pyrophosphate was tested up to 2500 µg/ml in the preliminary toxicity assay. The dose range finding information was then used to select concentration levels for mutation assay. In the absence of cytotoxicity in the entire concentration range tested (19.5 - 2500 µg/ml) the test concentration for mutation assay were limited by solubility and precipitation of test item in the medium. The test item was observed to form a 'slight precipitation' and 'heavy precipitation' upon dilution into treatment medium and at the end of the treatment period at 1250 and 2500 µg/ml respectively. Therefore, on the basis of test item solubility and precipitation in the medium 1250 µg/ml was selected as highest test concentration for mutation assay. Substance was not tested in concentration 2 mg/ml due to two reasons. Firstly, doses was chosen by using separate factor 2 and as the highest tested dose was 2500 µg/ml, next lower concentration was 1250 µg/ml. Secondly, in 2014 valid guideline OECD 476 did not obliged to test substance in concentration of 2 mg/ml.

III. CONCLUSIONS

From the results of this study and according to the criteria of the test protocol, it is concluded that when tested up to 1250 µg/mL the test item, Ferric Pyrophosphate did not induce forward mutation at the thymidine kinase (TK) locus of L5178Y mouse lymphoma cells either with or without metabolic activation under this test conditions.

RMS comment: The study was carried out in compliance with OECD 476 guideline and it is acceptable. Under the condition of this study Ferric Pyrophosphate was not mutagenic when tested in the mouse lymphoma assay with and without metabolic activation.

B.6.4.1.3 *In vitro* studies (Mammalian Cell Micronucleus Test)

Report: Stępnik M. 2013, Assessment of the mutagenic potential of ferric pyrophosphate in micronucleus test in vitro on the human lymphocytes

Study test: ZTM/2013/1/MN

Guidelines: OECD TG 487 "In Vitro Mammalian Cell Micronucleus Test"

GLP: Yes

Abstract:

Ferric pyrophosphate was tested for potential mutagenic properties on human peripheral blood lymphocytes in Micronucleus test in vitro (MN test). The lymphocytes were isolated from two healthy, non-smoking donors. In the preliminary study, lymphocyte cultures were exposed to Ferric pyrophosphate at 5 concentrations. The

concentration range (0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, 0.0005 mg/ml) was prepared using dilution factor of 3.16 (i.e. √10). The cells were exposed for 3 hrs, in the presence or absence of metabolic activation system (+S9 and – S9), and for 24 hrs, without metabolic activation system (-S9). As a result of the study, in lymphocytes from donor 1 and donor 2 exposed to Ferric pyrophosphate at the concentrations of 0.05 - 0.0005 mg/ml for 3 and 24 hours no significant changes in Cytokinesis-Block Proliferation Index (CBPI), Replicative Index (RI) and the percentage of the cytostasis were observed. In the positive control cultures (Mitomycin C, 0.15 µg/ml (3 h – S9) or 0.05 µg/ml (24 h – S9); Vinblastine, 10 ng/ml (3 h – S9) or 0.6 ng/ml (24 h – S9); Cyclophospamide, 2.5 µg/ml (3 h + S9)) an increase in % cytostasis was observed, however, the values were within an acceptable range (i.e. less than 55%). In the main study, Ferric pyrophosphate used at 4 concentrations of 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, did not induce any statistically significant increase in micronuclei frequency in the exposed lymphocyte cultures compared to control cultures (Student's t-test at $p < 0.05$) neither after 3-hr exposure (with or without metabolic activation system) nor after 24-hr exposure (without metabolic activation system). Reference mutagens without S9-mix, i.e. Mitomycin C (0.15 µg/ml (3 h – S9) or 0.05 µg/ml (24 h – S9)) and Vinblastin (10 ng/ml (3 h – S9) or 0.6 ng/ml (24 h – S9)), as well as with S9-mix, i.e. Cyclophosphamide (2.5 µg/ml (3 h +S9)), induced statistically significant increases in MN frequency comparing to the control values. These results met the acceptance criteria of the test. The results obtained indicate that under the experimental conditions used, Ferric pyrophosphate does not induce mutagenic effect in Micronucleus test on human peripheral blood lymphocytes

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: at room temperature 21-23°C, protected from water and moisture

2. Control Materials:

Negative control : culture medium and NaCl

Culture medium: RPMI medium with 10% bovine, L-glutamine (200 mM), antibiotics mix which included penicillin (100 µg/ml) and streptomycin (100 µg/ml).

Positive control without activation: Mitomycin C and Vinblastine

With activation: Cyclophosphamide

3. Activation: Cofactors-supplemented post-mitochondrial S9 fraction was used as metabolic activation system (S9-mix). To the negative and positive (CP) controls as well as test samples (Ferric pyrophosphate), 1 ml of S9-mix was added. Final concentration of S9 fraction in culture medium was 1% (v/v). S9-mix was prepared

directly before use by mixing on ice 9 ml of buffer (0.75 mM NADP, 12 mM glucose-6-phosphate, 5 mM MgCl2) with S9 fraction in ratio of 3:3:3:1 (v/v). During the exposure without metabolic activation, culture medium was used instead of S9-mix. According to the manufacturer, protein content in the S9 fraction was 39.7 mg/ml.

4. Test System: human peripheral blood lymphocytes

5. Concentration test:

a) Preliminary study: In the preliminary study, lymphocyte cultures were exposed to Ferric pyrophosphate at 5 concentrations. The concentration range (0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, 0.0005 mg/ml) was prepared using dilution factor of 3.16 (i.e. √10). The cells were exposed for 3 hrs, in the presence or absence of metabolic activation system (+S9 and –S9), and for 24 hrs, without metabolic activation system (-S9).

b) Main study: In the main study, Ferric pyrophosphate used at 4 concentrations of 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, did not induce any statistically significant increase in micronuclei frequency in the exposed lymphocyte cultures compared to control cultures (Student's t-test at $p < 0.05$) neither after 3-hr exposure (with or without metabolic activation system) nor after 24-hr exposure (without metabolic activation system).

B. TEST PERFORMANCE

1. The main study

According to the OECD TG 487 which recommends using in the main study the highest test concentration not exceeding 55 ± 5% cytostasis, after calculation of CBPI, RI and % cytostasis based on the results of the preliminary study, for the main study the following four Ferric pyrophosphate concentrations were selected: 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml. The cells were exposed for 3 or 24 hours, with or without metabolic activation, respectively.

2. Statistic:

To assess statistical difference of MN frequency between the exposed cell cultures and control cultures, two-tailed Student's t-test was applied at the significance level of $p < 0.05$. All statistical calculations were performed using GraphPad Prism v.6.01 for Windows (GraphPad Prism Software, Inc. USA).

3. Evaluation criteria

In the negative control cultures with diluent/vehicle reproducibly low frequency of micronuclei in binuclears should be observed. The chemicals used as positive controls (MMC, VIN and CP for the exposure without and with metabolic activation system, respectively) should induce statistically significant increase in the MN frequency compared to control values. If the results do not meet the acceptance criteria the test shall be repeated, however in the final interpretation of the data the biological and scientific plausibility of the observed response is taken into consideration.

II. RESULTS AND DISCUSSION

The tables with detailed data obtained in this study provided by applicant were placed in Appendix 1C of this document.

A. THE PRELIMINARY STUDY ON FERRIC PYROPHOSPHATE CYTOTOXICITY

In the preliminary study the lymphocytes from 2 donors were exposed to Ferric pyrophosphate at the concentrations of 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, 0.0005 mg/ml for 3 hours (with or without metabolic activation system) and for 24 hours (without metabolic activation). For each donor CBPI, RI and % cytostasis were calculated (for 500 cells/culture). As a result of the study, in lymphocytes from donor 1 and donor 2 exposed to Ferric pyrophosphate at concentrations 0.05 - 0.0005 mg/ml for 3 or 24 hours no statistically significant changes in Cytokinesis-Block Proliferation Index (CBPI), Replicative Index (RI) or the percentage of the cytostasis were observed. In the positive control cultures (Mitomycin C, 0.15 µg/ml (3 h) or 0.05 µg/ml (24 h); Vinblastine, 10 ng/ml (3 h) or 0.6 ng/ml (24 h); Cyclophospamide, 2.5 µg/ml (24 h)) an increase in % cytostasis was observed however, the values were within acceptable range (i.e. less than 55%).

B. THE MAIN STUDY

In the first stage of the main study the cells were exposed to Ferric pyrophosphate at 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml for 3 hours with or without metabolic activation. At each time point 2000 binucleated lymphocytes were analysed, i.e. 1000 cells from each donor (500 cells from 2 separate cultures). It was demonstrated that neither concentration of Ferric pyrophosphate in the presence or absence of S9-mix induced any significant increase in micronuclei frequency in comparison to control. In contrast, positive control substances without S9-mix, i.e. Mitomycin C (0.15 µg/ml) and Vinblastin (10 ng/ml) significantly increased MN frequency (donor 1:54 and 48 vs. 2‰ in control, respectively and donor 2:57 and 47 vs. 2‰ in control, respectively). Similarly, cyclophosphamide (2.5 µg/ml) in the presence of S9-mix increased MN frequency in the cells (donor 1:30 vs. 1‰ in control and donor 2:30 vs. 1‰ in control). Based on these results it was decided to conduct 24-hrs exposure to Ferric pyrophosphate without S9 activation system. It was demonstrated that neither concentration of Ferric pyrophosphate after 24-hr exposure in the absence of S9-mix induced any significant increase in micronuclei frequency in comparison to control. In contrast, positive control substances without S9-mix, i.e. Mitomycin C and Vinblastin, significantly increased MN frequency (donor 1: 40 and 45 vs. 2‰ in control, respectively and donor 2: 52 and 50 vs. 1‰ in control, respectively).

C. DEVIATIONS

Dossier was submitted in 2015, hence should be assessed according to guidelines valid in 2015.

The wording 'should aim' and 'not exceeding' may make a difference, but in case of this study it is not relevant, because it did not have an impact on chosen doses as the most important factor was a solubility of active substance.

Before the studies, solubility of Ferric pyrophosphate at the test concentrations of 5 mg/ml, 1.58 mg/ml, 0.5 mg/ml, 0.158 mg/ml, 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, and pH and osmolality of hence prepared dilutions in complete RPMI culture medium (with serum), were measured.

The substance was very poorly soluble at all final concentrations tested. In microscopical examination in the culture vessels precipitates of the test material were visible at all concentrations, both after 3 and 24 hrs of incubation at 37oC. The results of measurements of pH value performed after 3 and 24 hours of incubation at 37oC indicate no significant influence of Ferric pyrophosphate on pH.

The osmolality measurements indicated a significant decrease of the values at the Ferric pyrophosphate concentrations of 5 – 0.5 mg/ml. At the concentrations ≤0.158 mg/ml no changes in comparison to control values measured for RPMI culture medium with serum were observed.

Based on the observations of solubility and osmolality of ferric pyrophosphate dilutions, it was decided that in the preliminary study, human lymphocytes will be exposed to the test material at the concentrations of 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml or 0.0005 mg/ml. These concentrations should not have any influence on lymphocyte culture conditions, i.e. stimulation to proliferation or growth, and neither they should hamper further microscopical analysis resulting from enhanced precipitate formation.

<u>The deviations of the toxicological studies from the most updated OECD guidelines</u>

1. Cells:

- new types of cells were added
- the use of synchronised cells during treatment with the test chemical is not recommended by the new guideline

Above mentioned changes did not have an impact on study validity. In the test human peripheral blood lymphocytes were used, which is in line with the new guideline. The lymphocytes originate from healthy non-smoker blood donors, without known recent exposure to genotoxic compounds or radiation. Cells were not synchronised during exposure to the test substance.

2. Media and culture conditions:

- temperature of 37°C and concentration 5% of CO2 was specified

No impact on the study results. Maintained temperature was 37°C and concentration of CO_2 was 5%.

3. Preparation of cultures:

- cells propagated from stock cultures should grow exponentially until harvest time

Not applicable, human lymphocytes were used.

4. Solvents:

- concentration of solvents was specified

Not relevant, the tested substance was dissolved in complete RPMI culture medium.

5. Measuring cell proliferation and cytotoxicity and choosing treatment concentrations

- pH might be adjusted by buffering the final treatment medium in case when the test chemical causes a marked change in the pH of medium

Not applicable, no significant influence of Ferric pyrophosphate on pH was observed.

- Cytotoxicity should be determined in the main experiment.

Not relevant, for each donor CBPI, RI and % of cytostasis were calculated.

6. Treatment schedule

- For lymphocytes, the most efficient approach is to start the treatment with the test chemical at 44 - 48 hours after PHA stimulation, when cells will be dividing asynchronously.

Not relevant, the tested substance was added at 44 hour after PHA stimulation

- Option B has been deleted

Not relevant, option A was used.

Analysis:

- CBPI, RI, RPD or RICC should be assessed concurrently.

Not relevant, CBPI and RI were assessed concurrently.

Proficiency of laboratory and Historical control data

- New sections were added.

Not relevant, laboratory had a historical control data. Reference mutagens without S9-mix, i.e. Mitomycin C and Vinblastin as well as with S9-mix, i.e. Cyclophosphamide, induced statistically significant increases in MN frequency comparing to the control values. These results were within the range of historical values and meet the acceptance criteria of the test.

Presentation of the results

- New section was added.

Not relevant, all required data are presented in tabular form.

Evaluation and interpretation of results

- New criteria for clearly positive and clearly negative results were established.

Not relevant, all new criteria for clearly negative results are met.

III. CONCLUSIONS

Ferric pyrophosphate at the concentrations of 0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml, 0.0005 mg/ml does not induce any statistically significant changes in Cytokinesis-Block Proliferation Index (CBPI), Replicative Index (RI) or the percentage of the cytostasis of human peripheral blood lymphocytes exposed for 3 hours (with or without metabolic activation system) or for 24 hours (without metabolic activation).

Ferric pyrophosphate at the concentrations used (0.05 mg/ml, 0.0158 mg/ml, 0.005 mg/ml, 0.00158 mg/ml), both after 3-hr exposure (with or without metabolic activation system) and after 24-hr exposure (without metabolic activation system) did not induce any statistically significant increase in micronuclei frequency in exposed cell cultures compared to control cultures (Student's t-test at $p < 0.05$).

Reference mutagens without S9-mix, i.e. Mitomycin C and Vinblastin as well as with S9-mix, i.e. Cyclophosphamide, induced statistically significant increases in MN frequency comparing to the control values. These results are within the range of historical values and meet the acceptance criteria of the test.
The results obtained indicate that under the experimental conditions used, Ferric pyrophosphate does not induce mutagenic effect in Micronucleus test on human peripheral blood lymphocytes.

RMS comment: The study was carried out in compliance with OECD 487 guideline and it is acceptable. Under the condition of this study Ferric Pyrophosphate was not mutagenic when tested in *in vitro* micronucleus test on human peripheral blood lymphocytes with and without metabolic activation.

B.6.4.2 *In vivo* studies in somatic cells

B.6.4.2.1 Mammalian erythrocyte micronucleus test

Report: [redacted], 2014, Mammalian erythrocyte micronucleus test with using animals from repeated dose 90-day oral toxicity study in rodents
Study test: 0003/0030/T
Guidelines: OECD Test Guideline 474
GLP: Yes

Abstract:
The aim of the study was detection of cytogenetic damages induced by the iron (III) pyrophosphate to the chromosomes or the mitotic apparatus of erythroblasts by analysis of micronuclei forming in erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals from repeated dose 90-day oral toxicity study in rodents. Ferric (III) pyrophosphate was orally administered using a stomach gavage to one group of experimental animals (one group – 15 females and 15 males), as a suspension in 0,5% methylcellulose solution in dose 1000 mg/kg b.w., once a day, in the volume max. 1 ml/100 g b.w., for 90 days, seven days a week. The control group was run in parallel and administered 0,5 % methylcellulose solution (8 females and 8 males) in the same volume as the test material. Also a positive control group (8 females and 8 males) was introduced that was administerd with ethyl methanesulphonate. All animals after dosing period were sacrificed and an autopsy was performed. During the autopsy bone marrow cells were obtained from the femurs immediately following sacrifice. Peripheral blood was obtained from heart during sacrifice. Then smear preparations from blood cells and bone marrow were made and then stained with Giemsa stain. All smear preparations were evaluated for the presence of the micronuclei.
In this study, 2000 immature erythrocytes and 2000 mature erythrocytes of bone marrow and peripheral blood per animal were scored for the incidence of micronucleated erythrocytes. During analysis of slides the proportion of immature erythrocytes among mature erythrocytes of peripheral blood and bone marrow were scored by evaluation of 2000 cells. In the range of tested parameters there are no statistically significant

differences between all tested groups of animals and control group treated with 0,5 % methylcellulose solution ($p > 0,1$). There are no statistically significant differences in the number of polychromatic erythrocytes containing micronuclei, number of polychromatic erythrocytes to number of normochromatic erythrocytes ratio in comparison with the results of animals treated 0,5 % methylcellulose solution (unlike the positive control group where the differences in compared parameters were statistically significant at the significance level $p < 0,001$). Results of conducted study indicate the test material iron (III) pyrophosphate does not cause cytogenetic damages which effect forming micronuclei in the immature erythrocytes of the test species.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Ferric pyrophosphate

Description: Cream powder

Batch: 120327086

Purity: 101.73%

CAS: 10058-44-3

Stability of test compound: temperature: 10 - 30ºC, humidity: 30 - 70%

2. Test animals:

Species: rats

Strain: Wistar

Age: 6 weeks males and females

Weight at dosing: males: 202 – 269 g, females: 138 – 196 g

Source: [redacted]

Acclimation period: 5 days

Diet:. The animals were fed with conventional laboratory diets Labofeed B Plant Feed “Morawski”

Water: filtered tap water

Housing:. Animal cages: 540 x 390 x 210 mm, Number of animals per cage : 3-5

Environmental conditions:

Temperature: 22ºC ± 3ºC

Humidity: 30-70%

Air changes: Not reported

Photoperiod: 12 hours light / 12 hours dark

3. Test materials: peripheral blood and bone marrow

B. TEST PERFORMANCE

1. Sampling times:

Peripheral blood was obtained from heart during sacrifice. During the autopsy bone marrow cells were obtained from the femurs immediately following sacrifice.

2. Statistic:

Measured parameters were analyzed statistically. The differences between groups were analysed with normality test and variation analysis. For variables without normal distribution or without equal variances test Kruskal Wallis test was used. For variables with normal distribution and equal variances variance test for independent samples (ANOVA for independent samples) was used. For all tests significance levels – p = 0,05. The result of comparison was considered statistically significant below this value. In the range of significance level 0,05-0,1 results demanded additional analysis (there were no equivocal results in the study). In comparisons of groups where significance levels occurred above p=0,1 were considered as showing no statistically significant difference.

3. Evaluation criteria

Micronuclei were characterized by following conditions: stained in the same way as nucleus, regular, globular structure not connected with nucleus, structures comprise less than 1/3 volume of nucleus.

II. RESULTS AND DISCUSSION

The tables with detailed data from this study provided by applicant are shown in Appendix 1D of this document.

A. THE MAIN STUDY

In the range of tested parameters there were no statistically significant differences in all tested groups of animals in comparison with control group treated 0,5 % methylcellulose solution. There were no statistically significant differences in the number of polychromatic erythrocytes containing micronuclei and number of polychromatic erythrocytes to number of normochromatic erythrocytes ratio in comparison with the results of animals treated 0,5 % methylcellulose solution. Such differences were demonstrated in relation to the positive control group, where the values were statistically significant (significance level $p < 0.003$ for all parameters compared) thus confirming the effect of ethyl methanesulfonate. Positive control group was used to demonstrate the formation of micronuclei in the group treated with a substance inducing cytogenetic damages which result forming of micronucleus containing covering chromosome fragments or whole chromosomes. The substance used as a positive control should produce micronuclei after administration in appropriate dose, on the level of detectable increase relative to the background. This conditions were fulfilled with ethyl methanesulfonate. On the base of conducted study the test material iron (III) pyrophosphate does not cause cytogenetic damages which effect forming micronuclei in the immature erythrocytes in vivo in mammals.

B. DEVIATIONS

The deviations of the toxicological studies from the most updated OECD guidelines

Mammalian erythrocyte micronucleus test with using animals from repeated dose 90-day oral toxicity study in rats has been performed in 2014 according to OECD Guideline for the testing of chemicals, Mammalian Erythrocyte Micronucleus Test (474) adopted in 21 July 1997. After ferric pyrophosphate dossier submission a updated version of guideline was adopted in 29 July 2016.

Changes in the new guideline:

- Points 14 - 18: Laboratory should establish historical positive and negative range and distribution.

Historical data on positive and negative data were not required by previous guideline. Using of published data was permitted.

Based on the literature review for the test material and substances of similar structure, genotoxicity of iron pyrophosphate was not expected. In the research, genotoxicity effects were not observed in case of iron saccharate in Ames test, L5178Y TK +/- mouse lymphoma assay, in micronucleus test in mice or chromosome aberration test of human lymphocytes. Iron nitrilotriacetate induced oxidative DNA damage in Chinese hamster V79 cells. Ferric citrate showed no such properties. Iron ions induced the formation of protein-DNA connections in mammalian cells cultures. Many of iron salts were tested to demonstrate the mutagenic properties - strain of Saccharomyces cerevisiae D-4, Salmonella typhimurium strain TA-1535 , TA- 1537 and TA -1538 and without the activation were used. There were plate assays and in suspension. Iron pyrophosphate was inactive in all models and in addition lactate, orthophosphate and ferric sodium pyrophosphate. Iron sulfate was active in the suspension test with activation in particular strain TA -1537. Ferrous gluconate showed mutagenic properties in TA 1538 strain in test with activation but in other tests showed no such properties.

Negative results of mutagenicity tests give rise to determine iron pyrophosphate as no mutagenic.

The dose 350 mg/kg b.w. of ethylmethanesulphonate was determined based on literature review concerning the genotoxicity of ethyl methanesulphonate. Based on the literature, the chosen dose did not cause death, but at the same time caused forming the highest number of micronucleated cells after oral application.

- Point 20 'Rodents should be housed in small groups (no more than five per cage) of the same sex and treatment group if no aggressive behaviour is expected, preferably in solid floor cages with appropriate environmental enrichment.'

Above changes has no impact on the study. Number of animals per cage – females: test group – 5 rats, control group and satellite group – 4 rats per cage; males: test group – 4 and 3 rats per cage, control and satellite group – 4 rats per cage; positive control group: females and males – 4 rats per cage.

- Point 21 Rodents, ideally 6-10 weeks old at start of treatment.

No impact the on study, because in the study 6 weeks old rats were used.

- Point 21 'The individual animals are identified uniquely using a humane, minimally invasive method (e.g. by ringing, lagging, micro-chipping or biometric identification, but not ear or toe clipping).'

No impact the on study, animals were identified by using waterproof marker.

- Point 21 'Cross contamination by the positive control and the test chemical should be avoided. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.'

No impact on the study, because at the start of experiment body weight of each animals in groups did not differ by more than ± 20 % of average body weight. Cross contamination can be excluded, because animals from different groups were separated.

- 'For inhalation exposure, test chemicals can be administrated as a gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties.'

Not applicable, ferric pyrophosphate is not a gas.

- 'Examples of commonly used compatible solvents/vehicles include water, physiological saline, methylcellulose solution, carboxymethyl cellulose sodium salt solution, olive oil and corn oil. In the absence of historical or published control data showing that no micronuclei and other deleterious effects are induced by a chosen atypical solvent / vehicle, an initial study should be conducted in order to establish the acceptability of the solvent/vehicle control.'

No impact on study, because methylcellulose was use as vehicle.

- A group of animals treated with a positive control substance should normally be included with each test. This may be waived when the testing laboratory has demonstrated proficiency in the conduct of the test and has established a historical positive control range. When a concurrent positive control group is not included, scoring controls (fixed and unstained slides or cell suspension samples, as appropriate for the method of scoring) should be included in each experiment. These can be obtained by including within the scoring of the study appropriate reference samples that have been obtained and stored from a separate positive control experiment conducted periodically (e.g. every 6-18 months); for example, during proficiency testing and on a regular basis thereafter, where necessary.'

No impact on the study. Positive control has been included in the study.

- If peripheral blood is used, a pre-treatment sample is acceptable instead of a concurrent negative control for short-term studies when the resulting data are consistent with the historical control database for the testing laboratory. It has been shown for rats that pre-treatment sampling of small volumes (e.g. below 100 µL/day) has minimal impact on micronucleus background frequency.

Not applicable. Pre-treatment sample was not used in the study.

- Points 31 - 33 dose settings criteria.

No impact on the study. Maximum dose of 1000 mg/kg body weight/day was used.

- Point 35 'The volume should not normally exceed 1 mL/100 g body weight (...).'

No impact on the study. Maximum volume 1 mL/100 g body weight has been used.

- Point 36 '2 or more treatments are performed, administrated at 24-hour intervals, especially when integrating this test into other toxicity studies'

No impact on the study. Test was integrating into repeated dose 90-day oral toxicity study in rodents.

- Point 37 a and b – not applicable due to different treatment schedule

- Point 37 c 'If three or more daily treatments are used (e.g. three or more treatments at approximately 24 hour intervals), bone marrow samples should be collected no later than 24 hours after the last treatment and peripheral blood should be collected no later than 40 hours after the last treatment (31). This treatment option accommodates combination of the comet assay (e.g. sampling 2-6 hours after the last treatment) with the micronucleus test, and integration of the micronucleus test with repeated-dose toxicity studies. Accumulated data suggested that micronucleus induction can be observed over these wider timeframes when 3 or more administrations have occurred (15).'

No impact on the study. Blood and bone marrow was collected after 24 hours from last dose of ferric pyrophosphate administration.

- Point 38 'Other dosing or sampling regimens may be used when relevant and scientifically justified, and to facilitate integration with other toxicity tests.'

No impact on the study. Test was integrated with repeated dose 90-day oral toxicity study in rodents.

- Point 39 'General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality. All animals should be weighed at study initiation, at least once a week during repeated dose studies, and at euthanasia. In studies of at least one-week duration, measurements of food consumption should be made at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excessive toxicity should be humanely euthanized prior to completion of the test period (28). Under certain circumstances, animal body temperature could be monitored, since treatment induced hyper- and hypothermia have been implicated in producing spurious results (32) (33) (34).'

No impact on the study. During dosing period, each day animals were under general observation for determination of health condition, toxicity and morbidity signs. The observations were made twice a day on workdays and once a day on weekends. In case when symptoms of disease occurred, animals were clinically observed every day until recovery. Animal body weight measurements were carried out before administration of the test substance and once a week during the entire experiment. Measurements of food and water consumption were performed once a week. Non-lethal indicators of excessive toxicity has not been observed. One animal was euthanized because of the occurrence of malignancy which empeded test material gavage. The histopathological examination demonstrated inflammatory character – abscess not caused by iron pyrophosphate.

- Point 40 'A blood sample should be taken at appropriate time(s) in order to permit investigation of the plasma levels of the test substances for the purposes of demonstrating that exposure of the bone marrow occurred, where warranted and where other exposure data do not exist (see paragraph 48). '

No impact on the study results. Blood samples were taken according to point 38 c.

- Point 40 'Small volumes of peripheral blood can be obtained, according to adequate animal welfare standards, either using a method that permits survival of the test animal, such as bleeding from the tail vein or other appropriate blood vessel, or by cardiac puncture or sampling from a large vessel at animal euthanasia. For both bone marrow or peripheral blood-derived erythrocytes, depending on the method of analysis, cells may be immediately stained supravitally (16) (17) (18), smear preparations are made and then stained for microscopy, or fixed and stained appropriately for flow cytometric analysis.'

No impact on the study results. At the end of the study animals were anesthetized using ketamine with xylazine and then blood was taken from the heart chamber. During the autopsy bone marrow cells were obtained from the femurs immediately following sacrifice. The smear preparations from blood cells and bone marrow were made and then stained with Giemsa stain.

- Point 42 'Where these methods are applicable, anti-kinetochore antibodies (39), FISH with pancentromeric DNA probes (40), or primed in situ labelling with pancentromere-specific primers, together with appropriate DNA counterstaining (41), can be used to identify the nature of the micronuclei (chromosome/chromosomal fragment) in order to determine whether the mechanism of micronucleus induction is due to clastogenic and/or aneugenic activity. Other methods for differentiation between clastogens and aneugens may be used if they have been shown to be effective.'

Not applicable, in the conducted study there were no statistically significant differences in the number of polychromatic erythrocytes containing micronuclei and number of polychromatic erythrocytes to number of normochromatic erythrocytes ratio in comparison with control group, hence identification of the nature of the micronuclei was not necessary.

- Point 43 'The proportion of immature among total (immature + mature) erythrocytes is determined for each animal by counting a total of at least 500 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood.'

Above mentioned changes has not an impact on the study, because 2000 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood was counted.

- Point 43 'At least 4000 immature erythrocytes per animal should be scored for the incidence of micronucleated immature erythrocytes.'

In the study 2000 immature erythrocytes per animal was scored, but it was enough for statistic analysis. In the range of tested parameters there are no statistically significant differences between all tested groups of animals and control group treated with 0.5 % methylcellulose solution (statistically significant at the significance level p p>0,1). There are no statistically significant differences in the number of polychromatic erythrocytes containing micronuclei, number of polychromatic erythrocytes to number of normochromatic erythrocytes ratio in comparison with the results of animals treated 0.5 % methylcellulose solution (unlike the positive control group where the differences in compared parameters were statistically significant at the significance level p<0,001).

- Point 43 'If the historical negative control database indicates the mean background micronucleated immature erythrocyte frequency is <0.1% in the testing laboratory, consideration should be given to scoring additional cells.'

Historical control data were not required by version of guideline valid on day of dossier submission.

- Point 43 'When analysing samples, the proportion of immature erythrocytes to total erythrocytes in treated animals should not be less than 20% of the vehicle/solvent control proportion when scoring by microscopy and not less than approximately 5% of the vehicle/solvent control proportion when scoring CD71+ immature erythrocytes by cytometric methods (see paragraph 31) (29). For example for a bone marrow assay scored by microscopy, if the control proportion of immature erythrocytes in the bone marrow is 50%, the upper limit of toxicity would be 10% immature erythrocytes.'

No impact on the study results. The number of immature erythrocytes among total erythrocytes was not less than 20 % of control group scoring by microscopy.

- Point 44 'Because the rat spleen sequesters and destroys micronucleated erythrocytes, to maintain high assay sensitivity when analysing rat peripheral blood, it is preferable to restrict the analysis of micronucleated immature erythrocytes to the youngest fraction. When using automated analysis methods, these most immature erythrocytes can be identified based on their high RNA content, or the high level of transferrin receptors (CD71+) expressed on their surface (31). However, direct comparison of different staining methods has shown that satisfactory results can be obtained with various methods, including conventional acridine orange staining (3) (4).'

No impact on the study results. In the study bone marrow and peripherial blood has been assessed. Results of microscopic evaluation of bone marrow are not affected by spleen activity. Results of peripherial blood analysis have an auxiliary value, confirming study results carried on bone marrow. In the range of tested parameters there were no statistically significant differences in all tested groups of animals in comparison with control group treated 0.5 % methylcellusose solution. Such differences were demonstrated in relation to the positive control group, where values were statistically significant.

Point 46 The following criteria determine the acceptability of the test:

a) The concurrent negative control data are considered acceptable for addition to the laboratory historical control database (see paragraphs 15-18).

Historical control data were not required by version of guideline valid on day of dossier submission.

b) The concurrent positive controls or scoring controls should induce responses that are compatible with those generated in the historical positive control database and produce a statistically significant increase compared with the concurrent negative control (see paragraphs 24-25).

Historical control data were not required by version of guideline valid on day of dossier submission.

c) The appropriate number of doses and cells has been analysed.

The number of doses and analysed was in line with version of guideline valid on day of dossier submission.

d) The criteria for the selection of highest dose are consistent with those described in paragraphs 30-33.

The tested dose is consistent with recommended dose for administration period of 14 days or more – 1000 mg kg/b.w.

- Point 48 'Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) None of the treatment groups exhibits a statistically significant increase in the frequency of micronucleated immature erythrocytes compared with the concurrent negative control,

In the range of tested parameters there are no statistically significant differences between all tested groups of animals and control group treated with 0.5 % methylcellulose solution (statistically significant at the significance level p p>0,1).

b) There is no dose-related increase at any sampling time when evaluated by an appropriate trend test,

Not relevant, because one dose was tested.

c) All results are inside the distribution of the historical negative control data (e.g. Poisson-based 95% control limits),

Historical control data were not required by version of guideline valid on day of dossier submission.

d) Bone marrow exposure to the test substance(s) occurred.'

See point 40.

- Point 49 'There is no requirement for verification of a clear positive or clear negative response.'

No impact on the study results, as clear negative response was not verified.

- Point 50 'In cases where the response is not clearly negative or positive and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgment and/or further investigations of the existing experiments completed. In some cases, analyzing more cells or performing a repeat experiment using modified experimental conditions could be useful.'

Not relevant, clear negative response was obtained.

III. CONCLUSIONS

In the study 2000 immature erythrocytes and 2000 mature erythrocytes of bone marrow and peripheral blood were evaluated for the incidence of micronucleated erythrocytes. During analysis of slides the proportion of immature erythrocytes among mature erythrocytes of peripheral blood and bone marrow were scored by evaluated of 2000 cells. There were statistically no significant differences in the number of polychromatic erythrocytes to the number of normochromatic erythrocytes. On the base of conducted study the test material iron (III) pyrophosphate does not cause cytogenetic damages which effect forming micronuclei in the immature erythrocytes in vivo in mammals.

RMS comment: The study was carried out in compliance with OECD 474 guideline and it is acceptable. Ferric Pyrophosphate did not cause cytogenetic damages which effect forming micronuclei in the immature

erythrocytes in vivo in mammals. Under the condition of the test the substance is not clastogenic in the rat bone marrow micronucleus test.

B.6.4.3 *In vivo* studies in germ cells

During pregnancy, physiological changes in the organism of a pregnant woman cause a decrease in the level of hemoglobin, which may lead to anemia. To meet iron demand increased by 1000 mg, stored iron is released in the organism, but dietary fortification or even supplementation are also indicated. Ferric pyrophosphate is one of iron sources approved in the European Union for food fortification. No premises suggesting the substance used orally might potentially have a toxic effect on germ cells and reproduction are known of, and the risk related to its use in plant production products can be ruled out.

1. Bothwell T.H.: Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr July 2000, 72, pp. 257s-264s

B.6.5 Long-term toxicity and carcinogenicity

Due to limited exposure, both by oral and non-oral routes, chronic toxicity of ferric pyrophosphate is deemed very unlikely. Chronic toxicity might however be caused by excessive alcohol consumption, especially alcohol rich in iron (such as wine or beer), which additionally increases permeability of the intestinal mucosa to Fe ions. Still, the amount of iron absorbed in such circumstances is moderate. Rarer causes of chronic iron toxicity include genetic diseases such as primary and neonatal hemochromatosis, and thalassemia. The symptoms are mainly manifested as cirrhosis, diabetes, cardiac and endocrine diseases, as well as the characteristic brown skin discoloration.

Given very poor iron absorption and lack of ferric pyrophosphate toxicity demonstrated in acute and repeated dose toxicity tests, the risk of chronic toxicity is deemed negligible.

Evidence for the link between iron exposure and chronic diseases is derived mainly from epidemiological studies, which have their limitations. The most important one is the lack of reliable assessment of iron intake with food and lifestyle of the participants. Most of the available studies are based on small populations, which results in low statistical power of the data obtained.

The experimental studies with multiple intravenous administration of iron in dextran conducted on mice and rats demonstrated that tumours form in the site of injection. Tests on the primates have not confirmed these observations.

Based on population observations, the link between the risk of colorectal and duodenal cancer development and iron intake with food, ferritin serum concentration or heterozygosity in hereditary haemochromatosis was studied. Results of epidemiological studies suggest that there might be a correlation between the increased iron supply (total or heme iron) and increased risk of colorectal and duodenal cancer development, however these differences were not statistically significant. Study results do not provide conclusive evidence that considerable iron overload and increased ferritin concentration might contribute to cancer development. Heterozygosity in

haemochromatosis might be related to this phenomenon but this relation has also proved to be statistically insignificant. Thus, it is not possible to draw definitive conclusions. Results of studies on red meat consumption, which is a source of heme iron, invariably pointed to an increase of risk of colorectal and duodenal cancer development. However, these studies do not exclude the role of confounding variables such as environmental factors or e.g. lifestyle of the patients.

Nor it is not possible to determine the dose-effect relation and the threshold value of the amount of consumed and processed red meat.

The amount of iron that could be ingested as a result of the use of PPP containing ferric pyrophosphate in crops and ornamental plants compared to daily iron consumption with food is negligible.

Comprehensive review of the role of iron in human nutrition and consideration of potential adverse effects was presented in the report 'Iron and Health' of Scientific Advisory Committee on Nutrition. Please see below chapter 'Iron and cancer' published by SACN, which in clear way presents data from publications and review reports (Agget et al., 2010).

'Iron and cancer

7.30 Although it is uncertain whether, or how, iron might be carcinogenic, it has been suggested that oxygen free radical formation catalysed by iron might play a role in this process (Toyokuni, 1996; Okada, 1996). Two main pathways have been suggested: increased DNA damage induced either directly or indirectly by impeding DNA repair, and modulation of nuclear redox sensitive transcriptional regulators through signal transduction mechanisms (Galaris and Evangelou, 2002). Haem iron, but not inorganic iron, also increases production of N-nitroso compounds in the lumen of the gastrointestinal tract (Cross et al, 2003); many N-nitroso compounds have been shown to be human and animal carcinogens (IARC58, 1978). In addition, iron is a limiting nutrient for the growth and replication of cancer cells in the human body (Weinberg, 1984).

7.31 Higher concentrations of body iron might increase cancer risk by increasing oxidative stress to cells and by providing iron for growth and replication of cancer cells. Animal studies have shown that iron is essential for proliferation of neoplastic cells (Siegers et al, 1991). Iron supplementation was found to enhance the rate of tumour growth in mice with chemically induced colonic neoplasia (Siegers et al, 1992); the increase in tumour rate was dependent on the iron concentration in the diet. Seril et al (2002) found that a 2-fold iron enriched diet significantly increased the incidence of ulcerative colitis-associated colorectal tumours in mice. Hann et al (1988) observed that tumour growth in mice inoculated with colonic adenoma-carcinoma cells was decreased after they were fed an iron deficient diet.

7.32 Some epidemiological studies have suggested an association between blood donation (which reduces body iron) and lower cancer risk (Merk et al, 1990; Kato et al, 2007). Zacharski et al (2008) analysed cancer risk in patients with peripheral arterial disease (n=1277; mean age 67 years) participating in a randomised controlled trial on the effect of iron reduction by phlebotomy (at six-month intervals) on vascular outcomes (Zacharski et al, 2007). Mean serum ferritin concentrations between the iron reduction and control groups were similar at baseline but were significantly lower in the iron reduction group after phlebotomy (79.7 µg/L versus 122.5 µg/L; p<0.001). After a mean follow-up of 4.5 years, cancer risk was reported to be lower in the iron reduction group (hazard ratio, 0.65; 95% CI, 0.43–0.97; p=0.036).

7.33 Attention has mainly focused on the relationship between iron and colorectal cancer, based on the hypothesis that high intakes of iron might increase colorectal cancer risk by intraluminal (Graf and Eaton, 1985) or systemic effects (Stevens and Kalkwarf, 1990). Since most dietary iron is not absorbed, luminal exposure to excessive dietary iron may result in direct oxidative damage to the colorectal lumen.

Epidemiological studies of iron and colorectal cancer

7.34 The relationship between dietary iron and cancer was considered by COMA in their report, Nutritional Aspects of the Development of Cancer (DH, 1998), which reviewed studies on iron and cancer published up to 1996. Based on the available evidence at that time, COMA concluded that high iron stores may be related to increased risk of colorectal cancer, but since iron stores may not be related directly to iron intake, further prospective studies were required to confirm whether iron intake has a role in colorectal cancer.

7.35 The World Cancer Research Fund (WCRF) has also reviewed the evidence for an association between iron and colorectal cancer (WCRF, 2007). Four cohort studies on iron intake and colorectal cancer (Wurzelmann et al, 1996; Glynn et al, 1996; Kato et al, 1999; Konings et al, 2002) and 1 cohort study on haem iron intake and colorectal cancer (Lee et al, 2004) were considered. Four studies suggested an increased risk of colorectal cancer among people with the highest iron intake compared to those with the lowest intake, which was statistically significant in 2 studies (Wurzelmann et al, 1996; Lee et al, 2004). The WCRF concluded that there was limited evidence that foods containing iron are a cause of colorectal cancer and commented that the evidence was "sparse, of poor quality, and inconsistent".

7.36 In this report, consideration of the relationship between iron and colorectal cancer is based on prospective studies published since 1996[59] which have examined the relationship between colorectal cancer and dietary iron (including supplements) and serum ferritin concentration (although serum ferritin concentrations do not necessarily represent the amount of iron to which the colorectal mucosa has been exposed). Studies of individuals heterozygous for hereditary haemochromatosis were also considered. Details of these studies, including sample size, duration of follow-up, and allowance made for confounding factors, can be found in Annex 7 (see Tables A14–A17 below).

7.37 Five prospective studies (Annex 7, Table A14) have reported on dietary iron and colorectal cancer; most studies did not include iron intake from dietary supplements. Six out of the 7 relative risks reported were above 1; of these, one was significant. The median relative risk was 1.08.

Table A14: Total dietary iron and colorectal cancer risk

Study/year/country	Age baseline (years)	Mean follow-up (years)	Cases	Non-cases	Cancer site	Comparison (median intake or quantile range)	Adjustments	RR/OR/HR (95% CI)
Wurzelmann *et al*, 1996 USA	25–74	15	52 M+F	8,740 M+F	Proximal colon	Top fourth vs bottom fourth *(intakes in quartiles not specified)*	Age, sex.	1.44 (1.23–1.69) *p trend not given*
As above	As above	As above	57 M+F	As above	Distal colon	As above	As above.	1.03 (0.80–1.32) *p trend not given*
Kato *et al*, 1999 USA	34–65	4.7	105 F	523 F	Colorectum	Top fourth vs bottom fourth (intakes in quartiles not specified)	Age, beer intake, physical activity, family history CRC.	1.17 (0.6–2.3) *p trend=0.44*
Balder *et al*, 2006 NETHERLANDS	55–69	9.3	869 M	2,156 M	Colorectum	Top fifth (17 mg/day) vs bottom fifth (9.5 mg/day)	Age, BMI, family history, smoking, physical activity; intakes of energy, alcohol, vegetables.	1.34 (0.93–1.93) *p trend=0.12*
Balder *et al*, 2006 NETHERLANDS	As above	As above	666 F	2,215 F	As above	Top fifth (15 mg/day) vs bottom fifth (8.5 mg/day)	As above.	1.08 (0.72–1.62) p trend=0.90
Cross *et al*, 2006 FINLAND	50–69	14.2	130 M	260 M (smokers)	Colorectum	Top fourth (25 mg/day) vs bottom fourth (12.2 mg/day)	Age, education, BMI, smoking, physical activity, energy intake, alcohol, aspirin use.	0.4 (0.1–1.1) *p trend=0.06*
Kabat *et al*, 2007 CANADA	40–59	16.4	617 F	49,037 F	Colorectum	Top fifth (≥14.99 mg/day) vs bottom fifth (<11.90 mg/day)	Age, BMI, menopausal status, HRT, smoking, alcohol, education, physical activity; intakes of energy, fat, fibre, folic acid.	1.07 (0.8–1.43) *p trend=0.94*

Abbreviations: BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; HR, hazard ratio; HRT, hormone replacement therapy; OR, odds ratio; RR, relative risk

7.38 Four prospective studies have reported on dietary haem iron and colorectal cancer risk (Annex 7, Table A15). Two of these studies (Lee et al, 2004; Larsson et al, 2005) assumed haem iron content from all types of meat to be 40%, while in the other 2 studies (Balder et al, 2006; Kabat et al, 2007) haem iron content was estimated according to the type of meat. Kabat et al (2007) found similar results using the two different methods. Five of the 6 relative risks were above 1; none were significant. The median relative risk was 1.26.

Table A15: Haem iron and colorectal cancer risk

Study/year/country	Age baseline (years)	Mean follow-up (years)	Cases	Non-cases	Cancer site	Comparison (median intake or quantile range)	Adjustments	RR/OR/HR (95% CI)
Lee *et al*, 2004* USA	55–69	15	438 F	33,967	Proximal colon	Top fifth (≥2.05 mg/day) vs bottom fifth (≤0.76 mg/day)	Age, energy, BMI, physical activity, smoking, alcohol, HRT, diabetes, intake of: saturated fat, calcium, vitamin E, folate, fibre, multivitamins.	1.41 (0.90–2.21) *p trend=0.24*
Lee *et al*, 2004** USA	As above	As above	303 F	As above	Distal colon	As above	As above.	0.65 (0.38–1.11) *p trend=0.09*
Larsson *et al*, 2005*** SWEDEN	40–75	14.8	547 F	60,886 F	Colon	Top fifth (≥2.06 mg/day) vs bottom fifth (<0.67 mg/day)	Age, BMI, education; intakes of energy, saturated fat, folate, calcium, fibre, zinc.	1.31 (0.98–1.75) *p trend=0.03*
Balder *et al*, 2006 NETHERLANDS	55–69	9.3	869 M	2,156 M	Colorectum	Top fifth (1.85 mg/day) vs bottom fifth (0.60 mg/day)	Age, BMI, family history, smoking, physical activity; intakes of energy, alcohol, vegetables.	1.32 (0.96–1.80) *p trend=0.08*
Balder *et al*, 2006 NETHERLANDS	As above	As above	666 F	2,215 F	As above	Top fifth (1.54 mg/day) vs bottom fifth (0.47 mg/day)	As above.	1.20 (0.86–1.69) *p trend=0.24*
Kabat *et al*, 2007 CANADA	40–59	16.4	617 F	48,049	Colorectum	Top fifth (>2.95 mg/day) vs bottom fifth (<1.58 mg/day)	Age, BMI, menopausal status, HRT, smoking, alcohol, education, physical activity; intakes of energy, fat, fibre, folic acid.	1.06 (0.8–1.42) *p trend=0.99*

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio; HRT, hormone replacement therapy; OR, odds ratio; RR, relative risk

* Neither haem iron or zinc intake associated with risk of proximal colon cancer; however, when haem and zinc were mutually adjusted, both positive association of haem iron and inverse association of zinc intake were statistically significantly associated with proximal colon cancer (RR, 2.18 [1.24–3.86], p trend=0.01. Strength of associations of both haem iron intake and Zn intake became stronger with increasing levels of consumption.

** After mutual adjustment for haem iron and Zn, only inverse trend for Zn was statistically significant. Association not affected by alcohol consumption.

*** For women consuming 20 g or more per week alcohol, multivariate RR = –2.29 (1.25–4.21), p trend=0.007.

7.39 Two small prospective studies (Kato et al, 1999; Cross et al, 2006) have reported on serum ferritin concentration and colorectal cancer risk (Annex 7, Table A16). Both observed a significant reduction in risk with increasing serum ferritin concentrations. In the study by Kato et al (1999), the authors suggested that low ferritin concentrations might have been caused by blood loss associated with preclinical colorectal cancer because the average time between blood donation and cancer diagnoses was only 4.7 years. Cross et al (2006) suggested that low serum ferritin concentrations

observed in their study might indicate that less iron was absorbed and therefore more was present in the gut lumen where it could exert direct oxidative damage; that it could be due to increased iron requirement for tumour growth; or, although all cases were diagnosed at least five years after blood collection, that bleeding from a tumour could explain the lower serum ferritin concentrations because of the long induction period (5–10 years) associated with colorectal cancer.

7.40 Most studies assessing the relationship between iron intake or iron stores and colorectal cancer risk were relatively small and, therefore, probably had insufficient statistical power to detect small or moderate associations. Hereditary haemochromatosis and cancer

7.41 Hepatocellular carcinoma, the main form of liver cancer, is very strongly associated with haemochromatosis. Bradbear et al (1985) first quantified the excess risk for hepatocellular carcinoma as 200-fold in patients with genetic haemochromatosis. Subsequent studies have confirmed this strong association (Hsing et al, 1995; Fracanzani et al, 2001) and indicated that the increased risk generally follows the development of cirrhosis.

7.42 The risk for cancers other than hepatocellular carcinoma in patients with hereditary haemochromatosis has also been investigated in several small studies. Bradbear (1985) reported no excess of non-hepatocellular cancers, based on eight cancers, in a cohort (n=208) of patients with hereditary haemochromatosis. Fracanzani et al (2001) reported that the risk for non-hepatic cancers among 230 patients with hereditary haemochromatosis compared to 230 patients with non iron–related chronic liver disease was 1.8 (95% CI, 0.8–4.0). Geier (2002) reported that, among 59 patients with hereditary haemochromatosis, there were 13 non-hepatocellular cancers and a standardised incidence ratio of 1.40 (p<0.04).

Heterozygosity for type 1 hereditary haemochromatosis and colorectal cancer

7.43 Seven studies have assessed the association between heterozygosity for genetic haemochromatosis and colorectal cancer (Annex 7, Table A17).

Table A17: Prospective studies of C282Y heterozygosity and colorectal cancer risk

Study/year	Country	Cases	Non-cases	Odds ratio/Relative risk (95% Confidence interval)
Nelson *et al*, 1995	USA	47 M	26 M	1.28 (1.07–1.53)
As above	USA	45 F	36 F	1.08 (0.87–1.34)
Altes *et al*, 1999	Spain	116 M+F	108 M+F	0.86 (0.25–2.94)
Beckman *et al*, 1999	Sweden	173 M +F	294 M + F	1.02 (0.57–1.82)
Macdonald *et al*, 1999	Australia	229 M+F	228 M+F	0.90 (0.48–1.69)
Shaheen *et al*, 2003	USA	475 M+F	833 M+F	1.27 (0.83–1.95)
Van der A *et al*, 2003	Netherlands	240 F	635 F	1.20 (0.6–2.2)
Robinson *et al*, 2005	UK	327 M+F	322 M+F	1.01 (0.73–1.40)

7.44 Six out of the 8 relative risks reported were greater than 1; one of these was statistically significant (Nelson et al, 1995). The median relative risk was 1.05. Most studies were relatively small and may not have had sufficient power to detect small or moderate associations.

7.45 In the study which reported a significantly increased risk of colorectal cancer associated with C282Y heterozygosity (Nelson et al, 1995), colorectal cancer risk was assessed from postal questionnaires sent to individuals homozygous for genetic haemochromatosis regarding the health histories of their parents (who were assumed to be heterozygotes). Their spouses were asked to

complete questionnaires on the health histories of their parents (who were assumed not to be heterozygotes). Heterozygosity for hereditary haemochromatosis was not confirmed by DNA analysis and colorectal cancer was not confirmed by hospital records.

7.46 Robinson et al (2005) reported that although C282Y or H63D heterozygosity was not associated with colorectal cancer risk, individuals who were compound heterozygotes (C282Y/H63D) were at increased risk of colorectal cancer compared with those with a single mutation (odds ratio, 3.03; 95% CI, 1.06–8.61; p=0.038); however, this finding did not reach statistical significance after adjustment for multiple post hoc testing.

Meat and colorectal cancer

7.47 A number of plausible biological mechanisms have been suggested for an association between meat and colorectal cancer. Consumption of red and processed meat, but not white meat or fish, is associated with increased endogenous production of potentially carcinogenic N-nitroso compounds in the colon (Bingham et al, 2002). One mechanism proposed for the association is that the presence of haem iron, which is found in higher amounts in red and processed meat, enhances nitrosation. It has also been proposed that nitrites or nitrates which are added to some processed meat as preservatives could increase exogenous exposure to N-nitroso compounds or their precursors. Heterocyclic amines and polycyclic aromatic hydrocarbons, which are formed when foods are cooked at very high temperatures, have also been proposed to increase the risk of colon cancer (Sugimura, 2000); however, their production is not specific to the cooking of red and processed meat. Toxicological data examining whether differences in colorectal cancer risk between white, red and processed meat can be explained on the basis of cooked food mutagens, preservatives, N-nitroso compounds or haem iron can be found in Annex 8. There are currently no convincing data that provide an explanation for the observed differences in colorectal cancer risk between high consumers of white meat/fish and high consumers of red and processed meat.

7.48 It has also been suggested that the fat contained in red meat could affect colorectal cancer risk by increasing production of secondary bile acids which have been associated with promoting colon cancer (Narisawa et al, 1978). In addition, people eating diets high in red meat may eat fewer foods, such as fruit and vegetables, which might be protective against cancer risk (WCRF, 2007).

7.49 Several studies have examined the association between red and processed meat intake and colorectal cancer risk. There are a number of difficulties in interpreting the results from these studies including lack of consistency in definitions of red and processed meat, adequacy of dietary assessment methods, and variability in quantification of intakes. These limitations are discussed in further detail in paragraphs 7.62–7.70.

7.50 The relationship between meat intake and colorectal cancer was previously considered by COMA in their report, Nutritional Aspects of the Development of Cancer (DH, 1998), which reviewed epidemiological studies published up to 1996. The report concluded that evidence from cohort studies was: inconsistent for an association between total meat consumption and risk of colorectal cancer; moderately consistent for a positive association between consumption of red or processed meat and risk of colorectal cancer; and moderately consistent that poultry (white meat) and fish were not associated with colorectal cancer risk. COMA recommended that average consumption of red and processed meat (90 g/day cooked weight or 8–10 portions/week which was current at that time) should not increase and that higher consumers (above 140 g/day or 12–14 portions/week) should consider reducing their intakes.

7.51 A meta-analysis of 13 cohort studies published up to 1999 (Sandhu et al, 2001) reported that an increase in intake of 100 g/day of all meat significantly increased risk of colorectal cancer by 12–14%, 100 g/day red meat significantly increased risk by 13–17%, and 25 g/day increase in intake of processed meat was associated with an increased risk of 49%.

7.52 A subsequent meta-analysis included nine cohort studies of red meat and seven cohort studies of processed meat published from 1973 to 1999 (Norat et al, 2002). Compared to lowest level of intake, the highest level of red meat consumption was associated with a significant increase in colorectal cancer risk (relative risk, 1.27; 95% CI, 1.11-1.45). The relative risk for an increase of 120 g/day of red meat was 1.22 (95% CI, 1.05–1.41). Processed meat was also associated with a significant increase in risk: relative risks were 1.39 (95% CI, 1.09–1.76) for highest compared to the lowest level of intake and 1.54 (95% CI, 1.10–2.17) for an increase of 30 g/day of processed meat. No significant association was found between total meat consumption and colorectal cancer risk.

7.53 Another meta-analysis (Larsson and Wolk, 2006), which updated and expanded the previous analyses to include all prospective studies published up to March 2006 (15 studies of red meat; 14 studies of processed meat), included up to six times as many cases of colorectal cancer (7367 cases; n=1,042,824) compared to the two earlier meta-analyses. The relative risk of colorectal cancer for individuals in the highest category of red meat consumption compared with those in the lowest category was 1.28 (95% CI, 1.15–1.42); an increase of 120 g/day of red meat was associated with a relative risk of 1.28 (95% CI, 1.18–1.39). The relative risk of colorectal cancer for individuals with the highest compared to the lowest processed meat intake was 1.20 (95% CI, 1.11–1.31) and an increase of 30 g/day of processed meat intake was associated with a relative risk of 1.09 (95% CI, 1.05–1.13).

7.54 The relationship between red and processed meat intake and colorectal cancer risk was also considered by the WCRF (2007). Sixteen cohort studies (published from 1975 to 2005) on red meat intake and colorectal cancer risk were identified. All showed an increased colorectal cancer risk for highest versus lowest intake, which was significant in 4 studies. Meta-analysis of 7 studies that measured red meat intake in times/week and 3 studies that measured red meat intake in g/day reported relative risks of 1.43 (95% CI, 1.05–1.94) per times/day and 1.29 (95% CI, 1.04–1.6) per 100 g/day. Fourteen cohort studies (published from 1990 to 2005) on processed meat and colorectal cancer risk were considered. Twelve studies showed an increased colorectal cancer risk for highest versus lowest intake which was statistically significant in 3 studies. A meta-analysis of 5 of these studies reported a relative risk of 1.21 (95% CI, 1.04–1.42) for 50 g/day of processed meat intake.
7.55 The WCRF concluded that red and processed meat "is a convincing cause of colorectal cancer" and recommended that the population average consumption of red meat[60] should be no more than 300 g (cooked weight) per week and that very little, if any, should be processed. On an individual basis, the report recommended that consumption of red meat should be less than 500 g (cooked weight) per week, with very little or any processed meat. The report also concluded that there was limited evidence to indicate that eating fish protects against colorectal cancer and that there was insufficient evidence to draw conclusions on the association between poultry (white meat) and colorectal cancer risk.

Prospective studies of red and processed meat intake and colorectal cancer risk

7.56 Prospective studies published after 1996[61] on the association between red meat and processed meat intake and colorectal cancer are considered in this report. Details of these studies are provided in Annex 7 (Tables A18–A19).

Red meat intake and colorectal cancer risk (Annex 7, Table A18)

7.57 Twenty-one prospective studies have been published since 1996 (including some updated analyses from previously published cohorts). Two of these studies (Balder et al, 2006; Sato et al, 2006) considered the risk of colorectal cancer with total meat consumption rather than only red meat. Twenty-one out of the 25 relative risks reported were greater than one, 3 significantly so (Chao et al, 2005; Larsson et al, 2005; Cross et al, 2007). The median relative risk for highest versus lowest red meat intake was 1.17. The increased relative risk was statistically significant in 1 out of the 4 largest studies (Wei et al, 2004; Chao et al, 2005; Norat et al, 2005; Cross et al, 2007); the trend was statistically significant in 2 of the 4 studies and close to significance in 1 study.

7.58 Significantly increased colorectal cancer risk was associated with red meat intakes of ≥ 114 g/day for men, ≥ 80 g/day for women (Chao et al, 2005); ≥ 94 g/day (Larsson et al, 2005); and 62.7 g per 1000 kcal (equivalent to 145 g/day for men and 102 g/ day for women62) (Cross et al, 2007). In all the studies which reported a significantly increased risk of colorectal cancer, processed meat was included under the category of red meat.

Processed meat intake and colorectal cancer risk (Annex 7, Table A19)

7.59 Fourteen prospective studies have been published since 1996 (including some updated analyses from previously published cohorts). Thirteen out of the 18 relative risks for highest compared to lowest processed meat intake were greater than 1; of these, 5 were significant (English et al, 2004; Wei et al, 2004; Norat et al, 2005; Oba et al, 2006; Cross et al, 2007). The median relative risk was 1.16. The relative risk was statistically significant in 3 out of the 4 largest studies (Wei et al, 2004; Chao et al, 2005; Norat et al, 2005; Cross et al, 2007), and the trend was statistically significant in all the studies.

7.60 Increased colorectal cancer risk was significantly associated with processed meat intakes of ≥ 20.3 g/day (Oba et al, 2006); ≥ 29 g/day (English et al, 2004); ≥ 80 g/day (Norat et al, 2005); 22.6 g per 1000 kcal (equivalent to 52.3 g/day for men and 36.9 g/ day for women[63]) (Cross et al, 2007); and 5 times per week or more (Wei et al, 2004).

Colorectal cancer risk in vegetarians

7.61 A pooled analysis (Key et al, 2009a) of individual participant data (n=61,566) from 2 studies in the UK which examined cancer incidence in vegetarians (Sanjoaquin et al, 2004; Key et al, 2009b) observed no significant difference in colorectal cancer incidence between meat eaters and vegetarians (relative risk in vegetarians compared to meat eaters = 1.12; 95% CI, 0.87–1.44) after 12 years of follow-up. However, the analysis did not fully characterise meat consumption patterns and the total meat intake of non-vegetarians was much lower than the average UK intakes of meat consumers reported in the National Diet and Nutrition Survey[64] (Henderson et al, 2002).

Limitations in interpreting the results from prospective studies on red and processed meat intake and colorectal cancer risk

7.62 The majority of prospective studies published after 1996 suggest that high intakes of red and processed meat are associated with an increased risk of colorectal cancer. Although the association was not statistically significant in most studies, the sample sizes in the majority of studies may not have been large enough to ensure adequate power to detect a significant association.

7.63 There are a number of methodological inconsistencies between the different studies which make comparisons difficult. These include adequacy of the dietary assessment methods to obtain reliable estimates of red and processed meat intake; lack of consistency in the categorisation of red and processed meat; and variability in the reporting of quantities of red and processed meat intake. 7.64 In most studies, red meat and processed meat intake is based on a single dietary assessment at the start of the study. This does not take account of changes in dietary patterns over a number of years and could therefore be an unreliable estimate of intake over the specified follow-up period. The relevant period between dietary intake and development of cancer is also uncertain and dietary intakes estimated at baseline may not be the relevant period for dietary assessment in relation to cancer risk.

7.64 In most studies, red meat and processed meat intake is based on a single dietary assessment at the start of the study. This does not take account of changes in dietary patterns over a number of years and could therefore be an unreliable estimate of intake over the specified follow-up period. The relevant period between dietary intake and development of cancer is also uncertain and dietary intakes estimated at baseline may not be the relevant period for dietary assessment in relation to cancer risk.

7.65 There are also considerable inconsistencies between studies in categorisation and definition of red and processed meat: some studies collected detailed information of the foods included under the red and processed meat categories, while others used very broad classifications (e.g., beef, pork, lamb). In addition, some separated red and processed meat categories and only included fresh or untreated red meat in the red meat category, while other studies included processed meat under the red meat category.

7.66 Another difficulty is the variable ways in which quantities of red and processed meat intake were reported. While most studies compared highest versus lowest intake in grams per day/week/month, some reported intake as grams per 1000 kcal, frequency of intake per week/month, or servings per day. There are also large differences in the quantiles of intake between studies so that the amounts in the lowest quantiles in some studies are higher than the top quantiles in others. For red meat, intakes in the highest quantiles ranged from more than 40 to 158 g/day and in the lowest quantile from 10 to 61 g/day; processed meat intakes ranged from 16.3 to 80 g/day or more in the top quantiles and from 0 to 12 g/day in the lowest quantiles.

7.67 Some studies also analysed the association between colorectal cancer risk and red/processed meat intake for men and women combined, although intake in the highest and lowest quantiles differed by sex (Brink et al, 2005; Chao et al, 2005). In the study by Chao et al (2005), a significant association was found for men and women combined in the highest compared to the lowest quintile of red meat intake; however, intakes in the highest quintile were above 114 g/day for men and above 80 g/day for women. [111]

7.68 All these inconsistencies make it difficult to quantify a level of red or processed meat intake that may be associated with colorectal cancer risk and to make recommendations on levels of consumption. The potential effect of a recommendation to reduce the consumption of red meat and processed meat intake on iron and zinc intakes in the UK is considered in section 10.

7.69 Although results from prospective studies of dietary fibre and colorectal cancer are inconsistent, it has been suggested that higher intakes of foods containing fibre may protect against colorectal cancer risk (WCRF, 2007; Bingham et al, 2003). The fibre content of the diet might be a surrogate

marker for another plant component such as phytate, which has been shown to reduce the incidence of experimentally induced large intestinal tumours in animal studies (Shamsuddin and Ullah, 1989); however, only eight of the 21 studies on red meat intake and colorectal cancer risk and five of the 14 studies on processed meat intake and colorectal cancer risk adjusted for fibre intake.

7.70 There are also a number of other factors that have been associated with colorectal cancer risk. These include genetic predisposition, high total fat intake, low fruit and vegetable intake, low physical activity, and meat preparation and cooking methods. Although most of the newer larger studies have controlled for confounding factors, other studies have varied in the adjustments made. Additionally the effects of residual confounding cannot be excluded.'

RMS: The existing epidemiological data do not provide any evidence that the intake of ferric pyrophosphate (ferric ions) creates a risk of colorectal and duodenal cancer development in humans. In case of intake of heme iron associated with increased consumption of red and processed meat there is limited evidence suggesting association with increased risk of colorectal cancer, however this does not seem to be related with assessment of carcinogenicity of ferric pyrophosphate (ferric ions)

REFERENCES:

1. Agget et al., Scientific Advisory Committee on Nutrition (SACN), Iron and Health, SACN, 2010

2. Joint FAO/WHO Expert Committee on Food Additives. Toxicological evaluation of certain food additives and food contaminants. WHO Food Additives Series, No. 18, 571, Iron; 1983

B.6.6 Reproductive toxicity

During pregnancy, physiological changes in the organism of a pregnant woman cause a decrease in the level of hemoglobin, which may lead to anemia. To meet iron demand increased by 1000 mg, stored iron is released in the organism, but dietary fortification or even supplementation are also indicated. Ferric pyrophosphate is one of iron sources approved in the European Union for food fortification. No premises suggesting the substance used orally might potentially have a toxic effect on germ cells and reproduction are known of, and the risk related to its use in plant production products can be ruled out. WHO report cites the results of studies on the influence of iron and its compounds on reproduction, which show that no maternal toxicity or teratogenic effects were observed for doses up to 160 mg/kg bw in mice and rats (ferric sodium pyrophosphate).

Most essential nutrients exhibit a U-shaped dose-response relationship, which means that both their too low and too high levels in organism may be associated with health risks. This property is also probable for iron and especially relevant in reference to reproductive toxicity [1].

Functional impairment in iron deficiency is well investigated. Iron is particularly important in pregnancy and infancy to meet the high demands for hematopoiesis, growth and development. During pregnancy, physiological changes in the organism of a pregnant woman cause a decrease in the level of hemoglobin, which may lead to anemia. To meet iron demand increased by 1000 mg, stored iron

is released in the organism, but dietary fortification or even supplementation are also indicated. Ferric pyrophosphate is one of iron sources approved in the European Union for food fortification. WHO report cites the results of studies on the influence of iron and its compounds on reproduction, which show that no maternal toxicity or teratogenic effects were observed for doses up to 160 mg/kg bw in mice and rats (ferric sodium pyrophosphate) [2].

State of the art in reference to iron deficiency was comprehensively assessed in a Cochrane review. Much attention has been given to conditions of iron deficiency (ID) and iron deficient anemia (IDA) because of their high global prevalence estimated in vulnerable life stages. Globally, over 40% of pregnant women and 47% of preschool children are anemic from all causes. The World Health Organization (WHO) estimates that 50% of these anemias are due to ID and reflect IDA. The WHO recommends universal iron supplementation for pregnant women and young children 6 to 24 months because of this high prevalence of IDA. The prevalence of IDA in European countries varies from a low of 3% in Switzerland to 15% in Belgium in European pregnant women and below 5% in Northern and Western Europe in European young children. It appears that the majority of populations in developed countries are likely to be iron-replete, however it is notable that there is lack of established cut points for iron-replete status [3].

Overall, women taking iron supplements are less likely to have low birthweight newborns compared with controls and mean birthweight is significantly greater for those infants whose mothers received iron during pregnancy. Preventive iron supplementation reduces the risk of maternal anaemia at term by 70% and iron deficiency at term [3]. Moreover, evidence obtained from well-designed cohort study established that iron supplement use or greater intake of total, heme and nonheme iron is associated with lower risk of ovulatory infertility [4].

Emerging studies describe potential correlation of iron overload with birth and infant adverse health outcomes including growth retardation, fetal malformations or preterm birth. Their level of evidence is however considerably low, as they are biased by presence of cofounders (e.g. coexisting inflammation, genetic and ethnic factors), measurement uncertainties, lack of control or population sample of insufficient size [1]. Fetal loss and malformations were studied in a series of patients with iron overdose in pregnancy, however the study was limited by number of enrolled patients and lack of control group. Authors stated that outcomes were considered as not appropriate for a causal relationship. Another study concerned patients with beta-thalassemia, who experience iron overload and impaired fertility. In these patients sexual maturation is delayed and patients have hypogonadism and sperm DNA damage [5]. Several metals, including iron, copper, nickel, lead, and cadmium, may increase ROS production, decrease glutathione and other antioxidant levels, enhance the lipid peroxidation of the cell membrane, cause apoptosis, and contribute to the oxidative damage of DNA. Damage to the sperm membrane reduces sperm's motility and ability to fuse with the oocyte, whereas damage to sperm DNA compromises paternal genomic contribution to the embryo and increases the risk of infertility, miscarriage, or serious disease in the offspring [6]. Reactive chemical nature of the iron molecule, particularly its redox chemistry and interaction with oxygen, underlie both its essential functions and cytotoxic actions. Its ability to form iron polymers through hydroxide complexes is also important for its storage complexed to the protein, ferritin. In physiologic concentrations, iron functions in both oxygen transport and energy production through its redox potential. In excess, however, iron is a pro-oxidant and produces reactive hydroxyl radicals and other reactive oxygen species that damage DNA, proteins, lipids, other cellular molecules and stem cells. Thus, ensuring adequate availability, but not excess, drives iron homeostasis. Both iron deficiency and iron overload can affect redox state, and these conditions can be restored to physiological conditions using iron supplementation and iron chelation, respectively [1].

In conclusion, it is also essential to address the potential exposure of humans to toxic levels of iron. The risk of systemic iron overload from dietary sources is negligible with normal intestinal function. Chronic iron overload may occur as a result of specific clinical conditions and genetic mutations, but there is no evidence that heterozygotes for haemochromatosis are at increased risk of iron overload [7]. Exposure to iron in ferric pyrophosphate in a form of plant protection product is considered to be distinctly lower and as such its impact on reproductive toxicity is regarded as not significant.

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1. Brannon P, Taylor C, Iron Supplementation during Pregnancy and Infancy: Uncertainties and Implications for Research and Policy, Nutrients 2017, 9, 1327
2. Joint FAO/WHO Expert Committee on Food Additives. Toxicological evaluation of certain food additives and food contaminants. WHO Food Additives Series, No. 18, 571. Iron; 1983
3. Peña-Rosas JP, De-Regil LM, Garcia-Casal MN, Dowswell T. Daily oral iron supplementation during pregnancy. Cochrane Database of Systematic Reviews 2015, Issue 7. Art. No.: CD004736
4. Chavarro JE, Rich-Edward SJW, Rosner BA, Willett WC: Iron intake and risk of ovulatory infertility. Obstet Gynecol 2006;108
5. Golub MS (ed) (2006) Metals, fertility, and reproductive toxicity. Taylor and Francis, Boca Raton
6. Pizent A, et al. Reproductive toxicity of metals in men, Arh Hig Rada Toksikol 2012;63
7. EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2015. Scientific Opinion on Dietary Reference Values for iron. EFSA Journal 2015;13(10):4254

B.6.6.1 Generational studies

Based on the information presented in point 6.6. and the rule of limiting testing on vertebrates, it has been established that a study is not required.

B.6.6.2 Developmental toxicity studies

Based on the information presented in point 6.6. and the rule of limiting testing on vertebrates, it has been established that a study is not required.

RMS: Based on the reviewed epidemiological, clinical and animal data it can be conclude that ferric phosphate does not pose reproductive hazard for humans or mammals, thus the exposure linked with its application as an active substance in plant protection product does not create an unacceptable risk.

B.6.7 Neurotoxicity

Ferric pyrophosphate has been used as food additive for many years, even in small children. In accordance with Regulation (EU) No. 609/2013 of the European Parliament and of the Council of 12 June 2013, it was approved for use in baby food for infants and young children, processed cereal-based foods and food for children, food for special medical purposes, and total diet replacement. Also the Food and Drug Administration (FDA) positively assessed ferric pyrophosphate, placing it on the list of substances generally recognized as safe (GRAS). The substance is very poorly soluble in water, poorly absorbed in the organism and it does not

bioaccumulate in organisms. The above data indicates that the risk of neurotoxicity related to ferric pyrophosphate exposure does not exist.

1. U.S. Food and Drug Administration, 1988 SCOGS-Report Number: 35; 21 CFR Section: 184.1304

B.6.7.1 Neurotoxicity studies in rodents

Based on the information presented in point 6.7 and the rule of limiting testing on vertebrates, it has been established that a study is not required.

B.6.7.2 Delayed polyneuropathy studies

Based on the information presented in point 6.7 and the rule of limiting testing on vertebrates, it has been established that a study is not required.

B.6.8 Other toxicological studies

There is no reason to perform additional studies of ferric pyrophosphate.

B.6.8.1 Toxicity studies of metabolites and relevant impurities

There is no reason to perform additional studies of ferric pyrophosphate.

B.6.8.2 Supplementary studies on the active substance

There is no reason to perform additional studies of ferric pyrophosphate.

Immunotoxicity studies

A specific immunotoxicity test are not necessary. Immune system related endpoints as WBC, globulin, lymph nodes, spleen weight and histopathology, thymus weight and histopathology, Payer's patches, bone marrow and survival have been assessed in the repeated dose 28-day and 90-day oral toxicity in rodents. The histopathological examination show no pathological changes observed in all the examined organs (normal structure of the organ). Statistically significantly higher WBC in females in the study group with respect to the control was observed, however changes did not exceed the reference/historical control values and did not correlate with other clinical symptoms. An image of the bone marrow of animals in the group of 1000 mg / kg b.w. did not deviate from the norm – no registered differences in relation to the control group. In the sub-chronic 90 days rodent toxicity no changes that might suggest a immunotoxic effect of iron pyrophosphate have been indicated.

Moreover, ferric pyrophosphate is used as food additive and diet supplement.

RMS: The justification for not carrying out immunotoxicity study is acceptable, noting that iron intake is essential for development and survival.

B.6.8.3 Studies on endocrine disruption

Ferric pyrophosphate is a substance commonly considered safe and approved for use as a dietary supplement and food additive, thus it does not interfere with the endocrine system. There is no reason to perform additional studies of ferric pyrophosphate.

The data necessary for assessment ED properties of ferric pyrophosphate in mammals and humans may be retrieved from the short term toxicity studies – the Repeated Dose 28-Day Oral Toxicity Study in Rodents and the Repeated Dose 90-Day Oral Toxicity Study in Rodents (Majka, 2018, Updated assessment of the endocrine disruption (ED) potential of Ferric pyrophosphate).

According to OECD (2018) Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, an assessment of endocrine disrupting activity in repeated dose 28-day and 90- day oral toxicity studies in rodents may be performed on the basis of results of macro- and microscopic examination and of the weight of following organs: adrenals, testes, epididymis, uterus/cervix, ovaries, prostate + seminal vesicles with coagulating glans, vagina, thyroid with parathyroid, mammary glands.

In both repeated dose studies, the 28-Day Repeated Dose Study and the 90-Day Repeated Dose Study, no results were found that could indicate the endocrine disrupting properties of ferric pyrophosphate. Although the studies, run in 2014, could not be conducted according to revised OECD TG 408 (published in June 2018), in which more emphasis has been placed on endocrine disrupting potential of test chemical by adding, among others, serum/plasma evaluation of thyroxine (T4), T3 and TSH. The clear lack in 28-Day and 90-day Repeated Dose Studies of any histopathological changes in examined organs indicate with very high plausibility that Ferric pyrophosphate does not possess the endocrine disrupting properties.

The lack of endocrine disrupting properties of Ferric pyrophosphate for mammals is supported by human data. In accordance with the Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013, the compound was approved for use in baby food for infants and young children, processed cereal-based foods and food for children, food for special medical purposes, and total diet replacement. Also the Food and Drug Administration (FDA) positively assessed Ferric pyrophosphate, placing it on the list of substances generally recognized as safe (GRAS). In the publication of Expert Group on Vitamins and Minerals: “Safe Upper Levels for Vitamins and Minerals” UK EVM (2003) it is stated that analyses of a large number of studies involving oral iron supplementation shows that, apart from observed sometimes gastrointestinal side effects, such studies have generally not addressed issues of potential adverse effects associated with oral iron supplementation. What’s more, the gastrointestinal effects were noted primarily in subjects given Ferrous sulphate rather, than Ferric compounds.

Results of both repeated dose studies on rats and the lack of systemic adverse effects in human populations supplemented with iron compounds indicate that the absence of concern for endocrine disrupting potential of

Ferric pyrophosphate should be concluded and further studies on endocrine disrupting potential of that compound are not necessary.

According to Commission Regulation (EU) 2018/605 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties, Ferric pyrophosphate, considering mammals, should be considered as not having endocrine disrupting properties.

RMS: According to the ED criteria[1], a substance shall be considered as having ED properties if it meets all of the following criteria:
a) it shows an adverse effect in non-target organisms, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub) that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;
b) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;
c) the adverse effect is a consequence of the endocrine mode of action.

In the 28-day and 90-day repeated dose toxicity studies ([redacted], 2013; [redacted]2014) (2015) no adverse effects, that could be related to endocrine disruption, were not seen in rats exposed to Ferric Pyrophosphate by gavage at the dose of 1000 mg/kg bw/day. The studies has been performed according to OECD TG 407 and OECD TG 408 capable to detect such ED modalities as (anti)estrogen, (anti)androgen, thyroid, steroidogenesis, which are relevant for identification of ED properties of the tested substance. In both studies the following endpoints, essential according to OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals for detection of ED properties, were not affected: weight of thyroid gland, adrenals, testes, epididymides, uterus, ovaries, prostate + seminal vesicles with coagulating glands. There were no histopathologic changes in pituitary, thyroid gland, gonads, uterus, accessory sex organs, male and female mammary gland, testes and adrenals.

The 28-day and 90-day repeated dose toxicity studies (OECD TG 407 and 408) are methods belonging to level 3-5 of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals, revised 2017, including In vivo assays providing data on adverse effects on endocrine-relevant endpoints. The lack of significant effect on weight of thyroid gland, adrenals, testes, epididymides, uterus, ovaries, prostate + seminal vesicles with coagulating glands and lack of histopathologic changes in pituitary, thyroid gland, gonads, uterus, accessory sex organs, male and female mammary gland, testes and adrenals, show that the first criterion, i.e. the adverse effect in exposed mammals, was not fulfilled and that ferric pyrophosphate should not be considered as having endocrine disrupting properties in mammals. Studies on the mode of action are not necessary.

The EFSA experts at the Pesticide Peer Review Meeting 11 (05 September 2019) agreed to waive the requirement for an ED assessment for ferric pyrophosphate based on scientific rationale as provided by the RMS in the RAR.

[1] Commission Regulation (EU) 2018/605 of 19 April 2018 setting out scientific criteria for the determination of endocrine disrupting and amending Annex II to Regulation (EC) 1107/2009. OJ L 101, 20.4.2018, p. 33–36.
Available online: http://data.europa.eu/eli/reg/2018/605/oj

B.6.9 Medical data and information

B.6.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

No cases of chronic and sub-chronic poisoning with ferric pyrophosphate among production plant employees are known (identification data of the manufacturer included in document J), even though the substance has been produced as a food additive for many years. The substance is poorly soluble in water, non-volatile, poorly absorbed by the organism and does not accumulate in it.

B.6.9.2 Data collected on humans

No cases of poisoning symptoms occurring after ferric pyrophosphate exposure are known of, even though the substance has been used as a food additive for many years. The substance is poorly soluble in water, non-volatile, poorly absorbed by the organism and does not accumulate in it.

B.6.9.3 Direct observations

No cases of poisoning symptoms occurring after ferric pyrophosphate exposure are known of, even though the substance has been used as a food additive for many years. The substance is poorly soluble in water, non-volatile, poorly absorbed by the organism and does not accumulate in it.

B.6.9.4 Epidemiological studies

No cases of poisoning symptoms occurring after ferric pyrophosphate exposure are known of, even though the substance has been used as a food additive for many years. The substance is poorly soluble in water, non-volatile, poorly absorbed by the organism and does not accumulate in it.

B.6.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

Ferric pyrophosphate has been used as food additive for many years, even in small children. In accordance with Regulation (EU) No. 609/2013 of the European Parliament and of the Council of 12 June 2013, it was approved for use in baby food for infants and young children, processed cereal-based foods and food for children, food for special medical purposes, and total diet replacement. Also the Food and Drug Administration (FDA) positively assessed ferric pyrophosphate, placing it on the list of substances generally recognized as safe (GRAS). The substance is very poorly soluble in water, poorly absorbed in the organism and it does not bioaccumulate in organisms. Poisoning with this substance is very unlikely. The knowledge on acute poisonings with iron salts has been mainly derived from long experience related to the use of fortified food and dietary supplements replenishing iron deficiencies in the organism. Poisonings result mainly form

accidental ingestion of large amounts of tablets with high iron salt content (very often in colourful sweet coatings, which makes them attractive to children). Ferric pyrophosphate preparations available in the European market contain about 10-30 mg of elemental iron, whereas the findings of a British expert group indicate that systemic symptoms of acute iron poisoning in children might appear after ingestion of about 60 mg of iron/kg bw. Lethal dose for an adult is about 1400 mg/kg bw. Ferric pyrophosphate contains only about 25% of iron, therefore poisoning with this substance is very unlikely. Potential iron poisoning might be manifested by gastrointestinal symptoms such as constipation, nausea, diarrhoea, vomiting.

1. Fidler M.C., Walczyk T., Davidsson L., Zeder C., Sakaguchi N., Juneja L.R., Hurrell R.F.: A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. British Journal of Nutrition, 2004, 91, pp.107-112
2. U.S. Food and Drug Administration, 1988, SCOGS-Report Number: 35; 21 CFR Section: 184.1304
3. UK EVM (Expert Group on Vitamins and Minerals). 2003. Safe Upper Levels for Vitamins and Minerals, 2003, Publikacja online: http://cot.food.gov.uk/pdfs/vitmin2003.pdf

B.6.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

Based on information presented in points 6.9.1-6.9.5, iron poisoning by oral route is deemed very unlikely. If, however, iron poisoning occurs, current guidelines for management of a poisoned patient, based on experience and observation, include:

1) 6-hour observation without hospitalisation if less than 20 mg of iron/kg bw have been ingested and there are no symptoms

2) colon cleansing if more than 20 mg of iron/kg bw have been ingested, shortly after ingestion of a product containing iron salts or if the patient displays symptoms of mild poisoning (procedure: irrigation with an electrolyte solution of polyethylene glycol administered at 1.5-2 l/h in adults and 20-40 ml/kg bw/h in children until clear rectal effluent is obtained); due to the time needed to reach maximum blood concentration, dependent on the form of the ingested iron, the concentration should be monitored for up to about 8 h from ingestion.

3) administration of deferoxamine when maximum measured concentration exceeds 500 µg/dL or when systemic toxicity symptoms appear in the patient (continuous intravenous infusion with the starting dose of 15 mg/kg bw/h or intramuscular injection)

In case of contact with eyes, rinse them with plenty of water. In case of inhalation exposure, leave the area where dustiness occurs. Toxic effects are not expected.

1. Balmadrid C., Bono M.: Recognizing and Managing Iron Toxicity. Emergency Medicine, 2009; pp. 36-41

B.6.9.7 Expected effects of poisoning

Based on information presented in points 6.9.1-6.9.5, poisoning is deemed very unlikely. If, however, acute iron poisoning by oral route occurs, its stages are as follow:

Stage 1) GI symptoms (from 30 minutes to 6 hours): vomiting, abdominal pain, diarrhoea, hematemesis, melaena, drowsiness and fatigue

Stage 2) latent (from 6 hours to 24 hours): iron is distributed in the mononuclear phagocyte system and does not cause GI symptoms; hyperventilation might occur as a result of metabolic acidosis and oliguria as a result of hypovolaemia

Stage 3) shock and metabolic acidosis (from 7 hours to 72 hours): different types of cardiac failure might occur - hypovelemic, cardiogenic and/or distributive. At this stage, gastrointestinal bleeding, intestinal perforation, coagulopathy, and hepatic and renal dysfunction might occur.

Stage 4) hepatotoxicity, liver cell necrosis (from 12 hours to 96 hours): acute iron poisoning might result in the so called fulminant hepatic failure. This is a rare phenomenon occurring after iron ingestion but it can be lethal.

Stage 5) intestinal obstruction (2 weeks to 8 weeks): the mucosa of the gastrointestinal tract scars in the process of regeneration, which might cause intestinal obstruction.

1. Balmadrid C., Bono M.: Recognizing and Managing Iron Toxicity. Emergency Medicine, 2009; pp. 36-41

B.6.11 References relied on

Reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y / N	Owner
B.6.1.1 *also:* *B.6.5*	Aggett P. et al.	2010	*Iron and Health,* Scientific Advisory Committee on Nutrition (SACN) nGLP / Published	N	Public
B.6 *also:* *B.8.1* *B.7.2*	Anonymous	1993	*United States Environmental Protection Agency; Reregistration eligibility Document (RED): Iron Salts US EPA 1993* nGLP / Published	N	Public
B.6 *also:* *B.6.9.5* *B.6.7*	Anonymous	1988	*U.S. Food and Drug Administration, SCOGS-Report Number: 35; 21 CFR Section: 184.1304* nGLP / Published	N	Public
B.6.1.1	Anonymous	2006	*World Health Organization/Food and Agricultural Organization of the United Nations. Guidelines on food fortification with micronutrients.* nGLP / Published	N	Public
B.6.5 *also:* *B.6.6* *B.6.9*	Anonymous	1983	*Joint FAO/WHO Expert Committee on Food Additives. Toxicological evaluation of certain food additives and food contaminants. WHO Food Additives Series, No. 18, 571, Iron* nGLP / Published	N	Public
B.6.7 *also:* *B.6* *B.6.9.5*	Anonymous	1988	*U.S. Food and Drug Administration, SCOGS-Report Number: 35; 21 CFR Section: 184.1304* nGLP / Published	N	Public
B.6.9.5 *also:* *B.6* *B.6.7*	Anonymous	1988	*U.S. Food and Drug Administration, SCOGS-Report Number: 35; 21 CFR Section: 184.1304* nGLP / Published	N	Public
B.6.9.5	Anonymous	2003	*UK EVM (Expert Group on Vitamins and Minerals). 2003. Safe Upper Levels for Vitamins and Minerals, Publication online: http://cot.food.gov.uk/sites/default/files/cot/vitmin2003.pdf* nGLP / Published	N	Public
B.6.9.6 *also:* *B.6.9.7*	Balmadrid C. et al.	2009	*Recognizing and Managing Iron Toxicity, Emergency Medicine; pp. 36-41* nGLP / Published	N	Public
B.6 *also:* *B.8.1* *B.7.2* *B.8.2*	Bezak-Mazur E., Stoińska R.	2013	*The importance of phosphorus in the environment – review article. Archives of Waste Management and Environmental Protection 15, 33-42.* nGLP / Published	N	Public
B.6.4.3	Bothwell T.H.	2000	*Iron requirements in pregnancy and strategies to meet them. Am J Clin Nutr,72, pp. 257s-264s*	N	Public

Reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y / N	Owner
B.6.2.6	Davidsson L.	2000	*Iron bioavailability in infants from an infant cereal fortified with ferric pyrophosphate or ferrous fumarate.* Am J Clin Nutr. 2000 Jun; 71(6):1597-602 nGLP / Opublikowane	N	Public
B.6 *also:* *B.8.1* *B.7.2*	Draggan S. et al.	2011	*Mineral Information Institute: Iron.* Published online http://www.eoearth.org/view/article/51cbee407896bb431f696707 nGLP / Published	N	Public
B.6.2.6	Fidler M.C. et al.	2004	*Effect of ascorbic acid and particle size on iron absorption from ferric pyrophosphate in adult women.* Int J Vitam Nutr Res. 2004, 74: 294-300 nGLP / Opublikowane	N	Public
B.6.9.5 *also:* *B.6* *B.6.7* *B.6.2*	Fidler M.C. et al.	2004	*A micronised, dispersible ferric pyrophosphate with high relative bioavailability in man. British Journal of Nutrition 91, pp. 107-112* nGLP / Published	N	Public
B.6.3.1	[redacted]	2013	*Repeated Dose 28-Day Oral Toxicity Study in Rodents of iron pyrophosphate;* [redacted] Study code: 0003/0016/T GLP / Unpublished	Y	BROS
B.6.3.2	[redacted]	2014a	*Repeated dose 90-day oral toxicity study in rodents;* [redacted] Study code: 0003/0017/T GLP / Unpublished	Y	BROS
B.6.4.2	[redacted]	2014b	*Mammalian erythrocyte micronucleus test with using animals from repeated dose 90-day oral toxicity study in rodents;* [redacted] Study code: 0003/0030/T GLP / Unpublished	Y	BROS
B.6.2.3	[redacted]	2013	*Acute Inhalation Toxicity Study in Rats with Ferric pyrophosphate;* [redacted] Study code: 4150 GLP / Unpublished	Y	BROS
B.6.8.3	Majka J	2018	*Updated assessment of the endocrine disruption (ED) potential of Ferric pyrophosphate* nGLP / Unpublished	Y	BROS
B.6.1.1	Moretti D. et al.	2006	*Iron status and food matrix strongly affect the relative bioavailability of ferric pyrophosphate in human, Am J Clin Nutr 2006;83:632– 638* nGLP / Published	N	Public
B.6.2.1 *also:* *B.9.1.2.1*	[redacted]	2013	*Ferric pyrophosphate* *Acute Oral Toxicity Study on Rats – Fixed Dose Method;* [redacted] Study code: PO-2/13 GLP / Unpublished	Y	BROS

Reference number	Author(s)	Year	Title Source (where different from company) Company, Report No GLP or GEP status (where relevant) Published or not	Data Protection Claimed Y / N	Owner
B.6.4.1.1	Nikhil S. Sathe	2014	*Bacterial Reverse Mutation Test of Ferric Pyrophosphate using Salmonella typhimurium Tester Strains;* Laboratory Animal Research Services, Study code: 1248 GLP / Unpublished	Y	BROS
B.6.3.2	Provencher Bolliger A.	2014	*Reference Intervals for Selected Hematology, Coagulation and Clinical Chemistry Parameters in Charles River CD [Crl:CD®(SD)] and Wistar Hannover [Crl:WI(Han)] Rats* nGLP / Published	N	Public
B.6.2.4	[redacted]	2013a	*Ferric pyrophosphate – Acute Toxicity: Dermal Irritation / Corrosion;* [redacted] Report: 13-154, Study No.: 95/13/4, GLP / Unpublished	Y	BROS
B.6.2.5	[redacted]	2013b	*Ferric pyrophosphate – Acute Eye Irritation / Corrosion*; Research Institute of Organic Syntheses Inc. Report: 13-170, Study code: 95/13/5 GLP / Unpublished	Y	BROS
B.6 *also:* *B.8.1* *B.7.2*	Schulte E.E.	1992	*Understanding Plant Nutrients: Soil and Applied Iron* nGLP / Published	N	Public
B.6.4.1.2	Shetty N.	2014	*In Vitro Mouse Lymphoma Forward Mutation Assay of Ferric Pyrophosphate by using Mouse Lymphoma (L5178Y TK$^{+/-}$) Cell Line;* Vimta Study code: VLL/1013/G/T079 GLP / Unpublished	Y	BROS
B.6.4.1.3	Stępnik M.	2013	*Assessment of the mutagenic potential of ferric pyrophosphate in micronucleus test in vitro on human lymphocytes (OECD TG 487);* Nofer Institute of Occupational Medicine, Study code: ZTM/2013/1/MN GLP / Unpublished	Y	BROS

APPENDIX 1A.

The numerical data from the study [redacted] 2013, Repeated Dose 28-Day Oral Toxicity Study in Rodents of iron pyrophosphate (Vol. 3 CA, B.6.3, Short-term toxicity

Table 1. Weight of organs taken from animals of all test groups [g].

Animal No.	Sex	Dose [mg/kg bw]	All Gross lesions	Brain	Spinal cored	Eye with optic nerve	Stomach + duodenum	small and gross intestines	Liver	salivary glands	kidneys with ureter + adrenals	Spleen	thyroid with parathyroids + larynx + trachea and lungs + heart + aorta + thymus	gonads (testis and ovaries) + auxiliary genitalia + vagina + bladder	Mammary gland	lymph nodes (number)	peripheral nerve along with muscle + femur with the knee joint)	Bone narrow (smear – 1, pathomorfology – 2) **	Tounge
7/13/1	♀	1000	abs.	1,84	0,9	0,13	2,35	0,92	7,42	0,10	0,97+0,98	0,53	2,92	2,30	0,27	2	8,20	1+2	0,41
8/13/1	♀	1000	abs.	1,78	1,12	0,15	2,11	0,97	6,23	0,00	1,04+0,87	0,53	3,27	1,26	0,13	2	6,49	2	0,38
9/13/1	♀	1000	abs.	1,97	0,66	0,13	2,36	1,11	6,19	0,06	0,99+1,18	0,47	3,37	2,02	0,31	2	7,34	1+2	0,45
10/13/1	♀	1000	abs.	1,74	0,81	0,19	1,79	0,57	5,16	0,06	1,00+0,92	0,48	2,86	1,80	*	2	5,63	1+2	0,50
11/13/1	♀	1000	abs.	1,76	1,04	0,12	2,39	0,74	5,45	0,06	1,10+0,93	0,52	3,27	2,20	0,07	2	6,49	1+2	0,58
12/13/1	♀	1000	abs.	1,85	0,81	0,13	2,45	0,64	5,18	0,06	1,15+1,07	0,51	2,99	1,55	0,10	2	5,72	1+2	0,55
15/13/1	♂	1000	abs.	1,95	1,11	0,15	3,03	0,87	7,76	0,08	1,41+1,56	0,68	3,43	11,07	-	2	9,11	1+2	0,71
16/13/1	♂	1000	abs.	1,97	1,05	0,15	3,18	0,62	8,04	0,08	1,45+1,44	0,75	3,77	10,38	-	2	9,30	1+2	0,55
17/13/1	♂	1000	abs.	1,86	0,94	0,15	1,95	0,94	7,09	0,13	1,23+1,25	0,52	3,44	7,57	-	2	8,27	1+2	0,43
18/13/1	♂	1000	abs.	1,89	1,48	0,13	2,35	1,34	7,67	0,14	1,36+1,37	0,58	3,96	9,52	-	2	9,17	1+2	0,63
19/13/1	♂	1000	abs.	1,98	1,48	0,12	1,72	1,27	7,63	0,15	1,39+1,36	0,58	3,52	10,02	-	2	8,05	1+2	0,83
20/13/1	♂	1000	abs.	2,02	1,40	0,16	2,95	0,74	8,67	0,13	1,52+1,46	0,95	4,13	11,47	-	2	7,90	2	0,76
24/13/1	♀	500	abs.	1,66	1,06	0,13	3,30	0,58	6,03	0,08	1,01+1,14	0,52	3,17	2,35	0,13	2	8,12	2	0,64
25/13/1	♀	500	abs.	1,63	1,23	0,11	2,44	0,86	5,13	0,06	0,88+0,94	0,43	3,38	1,78	0,09	2	6,94	2	0,67
26/13/1	♀	500	abs.	2,11	0,99	0,15	3,77	0,88	6,27	0,08	0,86+0,97	0,51	4,11	1,75	0,13	2	8,38	1+2	0,65
27/13/1	♀	500	abs.	1,91	0,65	0,17	2,63	0,78	6,16	0,08	1,07+1,27	0,54	3,34	2,49	0,04	2	7,37	1+2	0,86
28/13/1	♀	500	abs.	1,86	0,91	0,13	2,10	1,10	5,89	0,07	1,08+1,20	0,60	3,14	2,35	0,06	2	7,99	2	0,65
31/13/1	♂	500	abs.	1,93	1,04	0,15	2,83	0,87	8,47	0,09	1,32+1,47	0,62	4,33	9,01	-	2	8,42	2	0,50
32/13/1	♂	500	abs.	1,80	0,74	0,17	2,48	0,63	7,70	0,09	1,76+1,44	0,67	3,76	9,60	-	2	9,64	1+2	0,80

Animal No.	Sex	Dose [mg/kg bw]	All Gross lesions	Brain	Spinal cored	Eye with optic nerve	Stomach + duodenum	small and gross intestines	Liver	salivary glands	kidneys with ureter + adrenals	Spleen	thyroid with parathyroids + larynx + trachea and lungs + heart + aorta + thymus	gonads (testis and ovaries) + auxiliary genitalia + vagina + bladder	Mammary gland	lymph nodes (number)	peripheral nerve along with muscle + femur with the knee joint)	Bone narrow (smear – 1, pathomorfology – 2) **	Tounge
33/13/1	♂	**500**	abs.	1,91	1,15	0,14	2,29	0,75	6,86	0,06	1,27+1,41	0,51	4,09	9,64	-	2	8,38	2	0,59
34/13/1	♂	**500**	abs.	1,91	1,52	0,16	2,29	1,19	8,76	0,09	1,71+1,65	0,96	3,91	10,47	-	2	12,70	2	0,88
35/13/1	♂	**500**	abs.	1,94	1,08	0,17	2,64	0,66	8,09	0,08	1,26+1,47	0,66	3,45	8,60	-	2	9,99	1+2	0,55
36/13/1	♂	**500**	abs.	1,97	1,78	0,16	3,12	0,77	8,85	0,10	1,46+1,40	0,66	3,82	10,99	-	2	11,28	1+2	0,85
40/13/1	♀	**100**	abs.	1,71	0,94	0,15	3,63	0,52	6,20	0,07	1,13+1,18	0,64	3,45	1,62	0,14	2	8,38	2	0,44
41/13/1	♀	**100**	abs.	1,93	0,93	0,13	2,22	0,65	7,14	0,07	0,95+1,08	0,55	3,12	2,13	0,08	2	6,65	1+2	0,74
42/13/1	♀	**100**	abs.	1,86	1,29	0,15	2,21	0,57	6,04	0,09	1,02+0,97	0,52	3,10	2,83	0,04	2	9,30	1+2	0,46
43/13/1	♀	**100**	abs.	1,79	1,16	0,15	2,07	0,47	5,49	0,08	0,93+0,96	0,52	3,01	1,70	0,17	2	7,77	1+2	0,51
44/13/1	♀	**100**	abs.	1,78	0,88	0,15	2,10	0,48	5,12	0,09	1,01+0,77	0,45	2,85	2,08	0,05	2	7,13	2	0,61
47/13/1	♂	**100**	abs.	2,02	1,42	0,14	2,48	0,55	9,61	0,11	1,49+1,45	0,56	3,90	11,91	-	2	9,49	1+2	0,64
48/13/1	♂	**100**	abs.	1,91	1,15	0,16	2,23	0,72	10,30	0,09	1,47+1,49	0,69	4,30	11,59	-	2	11,67	1+2	0,70
49/13/1	♂	**100**	abs.	1,86	1,17	0,15	2,86	0,64	8,44	0,08	1,49+1,48	0,65	3,63	10,76	-	2	9,12	1+2	0,63
50/13/1	♂	**100**	abs.	1,99	1,42	0,16	3,25	0,82	11,44	0,12	1,66+1,68	0,80	4,61	10,81	-	2	11,91	2	0,78
51/13/1	♂	**100**	abs.	1,98	1,17	0,17	2,86	0,53	9,60	0,09	1,73+1,54	0,67	4,05	12,41	-	2	12,72	2	0,66
52/13/1	♂	**100**	abs.	2,10	1,07	0,13	2,86	0,67	11,12	0,07	1,87+1,88	0,67	4,31	13,27	-	2	13,41	1+2	0,61
13/13/1	♀	**control**	abs.	1,76	1,05	0,21	2,22	0,09+0,31	5,97	0,06	0,92+0,98	0,35	3,24	1,62	0,04	2	6,71	2	0,81
14/13/1	♀	**control**	abs.	1,87	0,76	0,12	3,48	0,29+0,52	6,18	0,07	0,96+0,96	0,48	3,34	1,35	0,27	2	7,23	1+2	0,56
29/13/1	♀	**control**	abs.	1,89	0,79	0,14	2,43	0,39+0,55	5,64	0,05	0,91+0,90	0,55	3,13	1,50	0,04	2	6,20	1+2	0,84
30/13/1	♀	**control**	abs.	1,65	0,94	0,16	2,40	0,17+0,27	5,35	0,02	0,83+0,83	0,44	2,65	1,92	0,11	2	6,60	1+2	0,64
45/13/1	♀	**control**	abs.	1,78	1,17	0,08	2,14	0,28+0,14	5,40	0,04	1,06+0,97	0,50	3,00	2,12	0,1	2	5,46	1+2	0,50
46/13/1	♀	**control**	abs.	1,72	0,87	0,11	2,60	0,30+0,45	6,94	0,07	1,25+1,20	0,78	3,43	2,40	0,28	2	7,38	1+2	0,70
21/13/1	♂	**control**	abs.	1,96	1,03	0,21	2,13	0,40+1,10	11,65	0,09	1,20+1,20	0,84	3,43	7,26	-	2	9,53	1+2	0,67
22/13/1	♂	**control**	abs.	1,80	0,91	0,16	3,89	0,9 (total)	11,44	0,12	1,37+1,42	0,62	3,74	9,54	-	2	10,69	1+2	0,46
37/13/1	♂	**control**	abs.	1,55	0,82	0,15	3,51	0,35+0,73	10,54	0,01	1,31+1,26	0,69	3,27	9,84	-	2	8,40	2	0,58

Animal No.	Sex	Dose [mg/kg bw]	All Gross lesions	Brain	Spinal cored	Eye with optic nerve	Stomach + duodenum	small and gross intestines	Liver	salivary glands	kidneys with ureter + adrenals	Spleen	thyroid with parathyroids + larynx + trachea and lungs + heart + aorta + thymus	gonads (testis and ovaries) + auxiliary genitalia + vagina + bladder	Mammary gland	lymph nodes (number)	peripheral nerve along with muscle + femur with the knee joint)	Bone narrow (smear – 1, pathomorfology – 2) **	Tounge
38/13/1	♂	**control**	abs.	1,88	1,33	0,15	4,57	0,39+0,47	9,87	0,09	1,16+1,12	0,75	3,43	9,01	-	2	8,43	1+2	0,45
53/13/1	♂	**control**	abs.	1,89	1,06	0,19	3,73	0,74+0,79	9,52	0,12	1,69+1,89	0,62	4,15	10,09	-	2	9,15	1+2	0,94
54/13/1	♂	**control**	abs.	1,78	1,12	0,16	3,31	0,36+0,64	7,87	0,17	1,44+1,35	0,64	3,72	9,84	-	2	9,65	1+2	0,48

abs. - absence

* not collected. The remaining number of animals in the study is the minimum number prescribed by the OECD 407 guideline.
** bone marrow – smear – not collected for the total number of animals. The remaining number of animals in the study is the minimum number recommended by the OECD 407 guideline.

Histopathology examination

Due to the lack of pathological changes in the tested organs of animals, from group receiving dose 1000 mg/kg and control group, there were no examinations in 500 an 100 mg/kg body weight group according to OECD 407 guideline: Repeated Dose 28-Day Oral Toxicity Study in Rodents.

Table 2. Pathological analysis of individual organs - results

Organ	Noticed pathological lesions
1. All gross pathological changes	Not collected – non gross pathomorphologic lesions.
2. Brain	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
3. Spinal cord	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
4. Eyeball with optic nerve	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
5. Stomach	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
6. Esophagus	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
7. Small and gross intestines (including Peyer's patches)	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
8. Salivary glands	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
9. Liver	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
10. Kidneys with ureters	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
11. Adrenal glands	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
12. Spleen	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
13. Heart	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
14. Aorta	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
15. Thymus	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
16. Thyroid with parathyroid	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
17. Larynx, trachea and lungs	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
18. Gonads (testes, ovaries)	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
19. Accessory sex organs (uterus, cervix, epididymis, prostate and seminal vesicles and the coagulation glands)	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
20. Vagina	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
21. Mammary gland	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
22. Urinary bladder	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
23. Lymph nodes (mandibulary and lumbag)	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
24. Peripheral nerve (sciatic or tibial), along with muscle	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
25. Bone marrow	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group

26. Femur with the knee joint	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group
27. Tongue	lack of pathomorphologic lesions in 1000 mg/kg bw group and control group

Hematology and biochemistry analysis

Hematology and biochemical blood analyses, and urinalysis included:

Hematological analysis

- Hematocrit
- Haemoglobin
- Red blood cell count (also: HGB, HCT, MCV, MCH, MCHC)
- White blood cell
- Platelet Count
- Blood clotting time

Results of the total number of leukocytes, erythrocytes, hemoglobin, MCV, MCH, MCHC, number of platelets and hematocrit are shown in the Table 3. Analyzed parameters did not show statistically significant differences as compared with the control group of animals in a dose of 1000 mg/kg body weight, except the MCH parameter in the case of males, however all values were within the range of reference values. The reference values of the total number of leukocytes, erythrocytes, hemoglobin, MCV, MCH, MCHC, number of platelets and hematocrit for Han Wistar rats at the age of 8 – 16 weeks are shown in the Table 4.

Table 3. Results hematological analysis the total number of leukocytes, erythrocytes, hemoglobin, MCV, MCH, MCHC, platelet count and hematocrit.

Animal No.	Sex	Dose [mg/kg]	WBC [thous./µl]	RBC [mln/µl]	HGB [g/dl]	HCT [%]	MCV [fl]	MCH [pg]	MCHC [g/dl]	PLT [thous./µl]
7/13/1	♀	**1000**	5,3	9,67	18,5	55,3	57	19,1	33,5	611
8/13/1	♀	**1000**	5,7	8,44	16,9	47,7	57	20	35,4	712
9/13/1	♀	**1000**	5,7	7,95	15,5	44,9	56	19,6	34,6	725
10/13/1	♀	**1000**	3,3	7,63	15,2	42,6	56	19,9	35,6	723
11/13/1	♀	**1000**	3,5	7,89	15,2	43,8	56	19,2	34,6	665
12/13/1	♀	**1000**	4	7,65	15,3	43,2	56	19,9	35,3	633
15/13/1	♂	**1000**	5,1	8,51	16,9	48,2	57	19,9	35,1	601
16/13/1	♂	**1000**	6,7	8,08	15,9	44,7	55	19,6	35,5	608
17/13/1	♂	**1000**	6,7	8,51	16,4	47,7	56	19,3	34,4	639
18/13/1	♂	**1000**	5,9	8,61	17	50,4	59	19,7	33,7	681
19/13/1	♂	**1000**	5,4	8,14	16,6	47,2	58	20,4	35,2	730
20/13/1	♂	**1000**	5,3	8,03	16,3	46,8	58	20,3	34,8	658
24/13/1	♀	**500**	4,5	7,72	15,4	44	57	20	35	557
25/13/1	♀	**500**	7	7,71	14,9	42,9	56	19,3	34,7	654
26/13/1	♀	**500**	4,5	7,89	14,7	41,7	53	18,6	35,2	672
27/13/1	♀	**500**	4,2	7,31	14,7	40,8	56	20,1	36	753
28/13/1	♀	**500**	3,4	7,33	14,6	40,1	55	19,9	36,4	795
31/13/1	♂	**500**	2	3,28	7,4	18,9	58	22,4	38,8	72

32/13/1	♂	**500**	5,6	8,39	16,2	47,9	57	19,3	33,8	638
33/13/1	♂	**500**	5,8	9,42	17,6	52,9	56	18,7	33,3	633
34/13/1	♂	**500**	6,9	7,79	15,1	42,6	55	19,4	35,5	538
35/13/1	♂	**500**	7,8	8,67	17,2	50,1	58	19,8	34,4	675
36/13/1	♂	**500**	4,4	7,86	15,4	43,8	56	19,6	35,1	568
40/13/1	♀	**100**	5,1	7,47	14,4	42,6	57	19,2	33,7	557
41/13/1	♀	**100**	3,7	7,28	14,7	42,1	58	20,2	34,9	684
42/13/1	♀	**100**	2,8	7,2	14,5	41,4	58	20,1	34,9	557
43/13/1	♀	**100**	4,2	7,18	14,4	40,7	57	20,1	35,3	629
44/13/1	♀	**100**	2,6	7,08	14,2	39,8	56	20,1	35,7	564
47/13/1	♂	**100**	6,9	7,58	16,1	44	58	21,3	36,7	723
48/13/1	♂	**100**	5,7	7,33	15,6	43,5	59	21,3	35,9	615
49/13/1	♂	**100**	6,1	8,11	16,1	46	57	19,8	34,9	680
50/13/1	♂	**100**	5,3	7,41	14,8	42,1	57	20	35,1	680
51/13/1	♂	**100**	6,6	7,9	15,4	44,4	56	19,5	34,7	768
52/13/1	♂	**100**	4,9	7,78	15,3	42,4	55	19,7	36,1	761
13/13/1	♀	**control**	5,2	6,91	13,9	39,2	57	20,1	35,4	659
14/13/1	♀	**control**	4,8	7,04	14,3	40,2	57	20,3	35,4	688
29/13/1	♀	**control**	4	6,29	13	36,7	58	20,7	35,4	555
30/13/1	♀	**control**	2,9	7,32	14,6	40,7	56	19,9	35,8	548
45/13/1	♀	**control**	3,2	8,36	16,5	47,2	56	19,8	35	709
46/13/1	♀	**control**	4,7	7,47	14,5	42,4	57	19,5	34,3	594
21/13/1	♂	**control**	15,3	9,67	18,7	55,8	58	19,3	33,4	627
22/13/1	♂	**control**	5,9	10,49	20,1	62,8	60	19,2	32,1	536
37/13/1	♂	**control**	6,2	9,79	19,1	57,5	59	19,5	33,2	361
38/13/1	♂	**control**	5,4	8,41	16,9	49,9	57	19,3	33,9	614
53/13/1	♂	**control**	5,6	7,69	14,8	42,6	55	19,3	34,8	812
54/13/1	♂	**control**	7,2	8,06	16	45,6	57	19,8	35,1	674

Table 4. Reference values of the total number of leukocytes, erythrocytes, hemoglobin, MCV, MCH, MCHC, platelet count and hematocrit.

Sex	**WBC [thous./µl]**	**RBC [mln/µl]**	**HGB [g/dl]**	**HCT [%]**	**MCV [fl]**	**MCH [pg]**	**MCHC g/dl]**	**PLT [thous./µl]**
♂	1,96-8,25	7,27-9,65	13,7-17,6	39,6-52,5	48,9-57,9	17,1-20,4	32,9-37,5	638-1177
♀	1,3-7,49	7,07-9,03	13,7-16,8	37,9-49,9	49,9-58,3	17,8-20,9	33,2-37,9	680-1200

Smear and microscopic evaluation of bone marrow

Smear and microscopic evaluation of bone marrow was made in animals, where it was possible due to the small amount of material available for examination and the need to provide samples of bone primarily for histopathological examination. In all analyzed cases, animals of the control and the dose of 1000 mg/kg body weight group, cellularity of marrow preparation was very rich. Red blood cells system percentage in the standard range– about 38% ± 3, Normoblastic erythropoiesis. Granulocytic system percentage in the standard - about 50% ± 10, lymphocytes in the standard - about 13% ± 4, other: mast cells and naked nuclei. An image of the bone marrow in the group of animals 1000 mg/kg body weight did not deviate from the

norm – no differences in the control group were observed. Detailed results of the analysis of bone marrow smears are shown in Table 5.

Table 5. Detailed results of microscope assessment of bone narrow for animals from 1000 mg/kg bw group and control group

Animal No.	Sex	Dose [mg/kg]	Bone narrow smear [1 – not performed, 2 – performed and evaluated]	Result
7/13/1	♀	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 37%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 53%. Lymphocytes 9%. Other: mast cells and naked nuclei.
8/13/1	♀	1000	1	-
9/13/1	♀	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 35%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 49%. Lymphocytes 9%. Other: mast cells and naked nuclei.
10/13/1	♀	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 40%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 47%. Lymphocytes 11%. Other: mast cells and naked nuclei.
11/13/1	♀	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 34%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 54%. Lymphocytes 10%. Other: mast cells and naked nuclei.
12/13/1	♀	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 37%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 52%. Lymphocytes 9%. Other: mast cells and naked nuclei.
15/13/1	♂	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 36%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 50%. Lymphocytes 10%. Other: mast cells and naked nuclei.

Animal No.	Sex	Dose [mg/kg]	Bone narrow smear [1 – not performed, 2 – performed and evaluated]	Result
16/13/1	♂	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 36%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 52%. Lymphocytes 10%.
17/13/1	♂	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 35%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 53%. Lymphocytes 9%. Other: mast cells and naked nuclei.
18/13/1	♂	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 40%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 43%. Lymphocytes 11%. Other: mast cells and naked nuclei.
19/13/1	♂	1000	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 32%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 45%. Lymphocytes 11%. Other: mast cells and naked nuclei.
20/13/1	♂	1000	1	-
13/13/1	♀	control	1	-
14/13/1	♀	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 34%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 51%. Lymphocytes focyty 9%. Other: mast cells and naked nuclei.
29/13/1	♀	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 37%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 49%. Lymphocytes 10%. Other: mast cells and naked nuclei.
30/13/1	♀	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 38%. Normoblastic erythropoiesis Granulocytic system percentage in the standard 46%. Lymphocytes 11%. Other: mast cells and naked nuclei.

Animal No.	Sex	Dose [mg/kg]	Bone narrow smear [1 – not performed, 2 – performed and evaluated]	Result
45/13/1	♀	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 34%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 50%. Lymphocytes 9%. Other: mast cells and naked nuclei.
46/13/1	♀	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 36%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 48%. Lymphocytes 14%. Other: mast cells and naked nuclei.
21/13/1	♂	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 39%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 50%. Lymphocytes 8%. Other: mast cells and naked nuclei
22/13/1	♂	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 41%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 47%. Lymphocytes 9%. Other: mast cells and naked nuclei
37/13/1	♂	control	1	-
38/13/1	♂	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 35%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 45%. Lymphocytes 15%. Other: mast cells and naked nuclei
53/13/1	♂	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 38%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 43%. Lymphocytes 10%. Other: mast cells and naked nuclei
54/13/1	♂	control	2	Rich bone marrow cellularity. Red blood cells system percentage in the standard 38%. Normoblastic erythropoiesis. Granulocytic system percentage in the standard 47%. Lymphocytes 11%. Other: mast cells and naked nuclei

Clotting time determination

Results of coagulation time in tested animals are shown in Table 6. Clotting times showed no statistically significant differences in comparison with the control group of animals in a dose of 1000 mg/kg body weight and were within the ranges of reference values. The clothing time reference values in rat are 2 – 4 minutes.

Table 6. Clotting time results

Animal No.	Sex	Dose [mg/kg bw]	Clotting time [min]
13/13/1	♀	control	02:45
14/13/1	♀	control	02:52
29/13/1	♀	control	02:46
30/13/1	♀	control	03:02
45/13/1	♀	control	02:52
46/13/1	♀	control	02:41
21/13/1	♂	control	02:34
22/13/1	♂	control	02:02
37/13/1	♂	control	02:15
38/13/1	♂	control	02:23
53/13/1	♂	control	02:27
54/13/1	♂	control	02:17
7/13/1	♀	1000	02:25
8/13/1	♀	1000	02:30
9/13/1	♀	1000	02:22
10/13/1	♀	1000	03:03
11/13/1	♀	1000	03:04
12/13/1	♀	1000	03:06
15/13/1	♂	1000	02:55
16/13/1	♂	1000	02:48
17/13/1	♂	1000	02:51
18/13/1	♂	1000	02:45
19/13/1	♂	1000	02:32
20/13/1	♂	1000	02:41
40/13/1	♀	100	02:47
41/13/1	♀	100	02:40
42/13/1	♀	100	03:33
43/13/1	♀	100	02:43
44/13/1	♀	100	02:50
47/13/1	♂	100	03:00
48/13/1	♂	100	02:24
49/13/1	♂	100	02:39
50/13/1	♂	100	02:42
51/13/1	♂	100	02:39
52/13/1	♂	100	02:37
24/13/1	♀	500	02:51
25/13/1	♀	500	02:41
26/13/1	♀	500	03:05
27/13/1	♀	500	02:54
28/13/1	♀	500	02:47
31/13/1	♂	500	Lack of blood *
32/13/1	♂	500	02:16
33/13/1	♂	500	02:55
34/13/1	♂	500	02:16
35/13/1	♂	500	02:59
36/13/1	♂	500	02:46

Due to the small amount of collected blood, this study was abandoned for hematology and biochemistry analysis. The remaining number of animals in the study is the minimum number recommended by OECD 407 guideline.

Biochemistry determination of serum from all animals:

- Potassium
- Sodium
- Iron
- Chlorides
- Calcium
- Ferritin
- Glucose
- Total cholesterol
- Triglycerides
- Urea
- Creatinin
- Total protein and albumin
- Alanin aminotransferase, aspartate aminotransferase, alkaline phosphatase
- Bile acids
- Bilirubin

Results of potassium, sodium, iron, ferritin, glucose, total cholesterol, triglycerides, urea, creatinine, total protein and albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin and bile acids are shown in Table 7.

Reference values of potassium, sodium, iron, ferritin, glucose, total cholesterol, triglycerides, urea, creatinine, total protein and albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bile acids and bilirubin for Han Wistar rats at the age of 8 -16 weeks are shown in Table 8.

Table 7. Determination results of potasium, sodium, iron, ferritin, glucose, total cholesterol, triglicerides, urea, creatinin, total protein and albumin, alanin aminotransferase, aspartate aminotransferase, alkaline phosphatase, bile acids and bilirubin.

Ani. No.	Sex	Dose [mg/kg]	Sodium [mmol/l]	Potassium [mmol/l]	Cholesterol [mg/dl]	Triglicerides [mmol/l]	Creatinin [mg/dl]	Urea [mg/dl]	Aspat [U/l]	Alat [U/l]	Total bilirubin [mg/dl]	Albumin [g/dl]	Total protein [g/dl]	Glucose [mg/dl]	alkaline phosphatase [U/L]	Calcium [mmol/l]	Iron [µg/dl]	Chlorides [mmol/l]	Ferritin [ng/ml]	Bile acids [umol/l]
7/13/1	♀	1000	146	6,80	38	0,55	0,57	45	93	26	0,28	3,6	5,39	97	50	2,98	339	103	176,81	21,1
8/13/1	♀	1000	141	5,60	59	2,00	0,50	49	68	20	0,28	3,6	5,47	66	78	2,93	201	102	162,40	17,4
9/13/1	♀	1000	141	5,40	33	0,66	0,48	44	93	26	0,26	3,6	5,38	87	92	2,89	276	100	206,60	22,7
10/13/1	♀	1000	142	4,60	61	0,69	0,52	43	95	22	0,29	6,5	5,25	27	139	2,80	229	102	195,43	17,3
11/13/1	♀	1000	142	4,80	51	0,49	0,47	51	75	18	0,30	3,5	5,34	115	96	2,92	184	101	168,39	10,6
12/13/1	♀	1000	141	5,00	71	0,62	0,44	49	82	20	0,2 6	3,4	5,18	111	103	2,86	246	103	110,98	15,4
15/13/1	♂	1000	140	5,30	49	0,72	0,43	50	72	31	0,29	3,4	5,37	184	247	2,87	147	99	209,57	19,7
16/13/1	♂	1000	142	5,70	47	0,52	0,42	48	81	41	0,29	3,2	5,08	181	177	2,87	136	99	166,82	28,3
17/13/1	♂	1000	141	5,70	35	0,43	0,53	46	113	26	0,26	3,2	4,93	230	187	2,73	146	101	194,73	12,7
18/13/1	♂	1000	142	5,60	42	0,70	0,49	56	92	40	0,28	3,3	5,21	158	248	2,80	191	101	241,02	27,6
19/13/1	♂	1000	140	5,60	62	0,70	0,52	55	72	27	0,29	3,4	5,26	232	155	2,80	173	99	177,32	11,4
20/13/1	♂	1000	143	5,20	54	0,68	0,49	59	65	26	0,24	3,2	4,93	244	164	2,87	196	101	191,14	35,2
24/13/1	♀	500	139	4,40	57	0,64	0,44	39	100	34	0,29	3,2	6,65	228	138	2,92	442	98	171,38	28,2
25/13/1	♀	500	14	6,90	51	0,52	0,55	38	87	28	0,25	3,4	6,86	344	176	3,26	435	98	144,32	10,4
26/13/1	♀	500	143	4,60	36	0,26	0,53	57	86	34	0,25	3,3	6,79	202	123	2,80	375	103	131,19	39,1
27/13/1	♀	500	140	4,60	44	0,65	0,43	32	71	29	0,29	3,2	6,75	212	112	2,71	299	103	136,33	8,5
28/13/1	♀	500	140	5,20	46	0,81	0,47	37	72	25	0,27	3,2	6,39	178	63	2,96	286	101	213,99	14,7
31/13/1	♂	500	138	4,80	56	0,71	0,39	36	114	41	0,26	3,0	6,36	235	123	2,61	196	102	250,89	6,9
32/13/1	♂	500	141	5,50	47	0,68	0,37	41	87	39	0,28	3,1	6,50	232	196	2,77	331	101	191,79	19,8
33/13/1	♂	500	141	6,20	56	0,74	0,48	49	91	25	0,29	3,4	6,91	299	187	2,89	215	101	249,84	8,4
34/13/1	♂	500	139	6,40	50	0,77	0,45	31	82	30	0,29	3,1	6,38	240	209	2,84	163	100	153,79	12,7
35/13/1	♂	500	142	6,10	58	0,42	0,54	43	93	37	0,27	3,0	6,66	402	199	3,17	217	101	201,72	7,0
36/13/1	♂	500	143	5,60	61	0,46	0,36	32	81	35	0,27	3,0	6,44	174	159	2,78	184	104	199,93	10,4
40/13/1	♀	100	140	4,50	40	0,46	0,38	44	103	32	0,35	3,3	6,75	80	90	2,78	408	100	206,68	85,4

Ani. No.	Sex	Dose [mg/kg]	Sodium [mmol/l]	Potassium [mmol/l]	Cholesterol [mg/dl]	Triglicerides [mmol/l]	Creatinin [mg/dl]	Urea [mg/dl]	Aspat [U/l]	Alat [U/l]	Total bilirubin [mg/dl]	Albumin [g/dl]	Total protein [g/dl]	Glucose [mg/dl]	alkaline phosphatase [U/L]	Calcium [mmol/l]	Iron [µg/dl]	Chlorides [mmol/l]	Ferritin [ng/ml]	Bile acids [umol/l]
41/13/1	♀	100	140	5,50	84	0,65	0,49	47	86	32	0,31	2,8	6,40	100	108	2,82	348	100	157,57	39,5
42/13/1	♀	100	142	4,40	56	0,84	0,49	51	96	32	0,27	3,5	7,10	178	113	2,84	408	103	146,85	33,0
43/13/1	♀	100	141	5,10	53	1,01	0,47	56	100	37	0,34	3,3	6,50	142	111	2,78	362	104	177,28	72,5
44/13/1	♀	100	139	4,80	42	0,58	0,52	54	98	43	0,30	3,2	6,22	142	113	2,64	317	103	125,05	27,3
47/13/1	♂	100	141	5,10	72	1,08	0,46	53	87	49	0,29	3,5	6,91	247	196	2,77	191	98	169,96	28,9
48/13/1	♂	100	139	5,10	60	0,80	0,45	46	75	34	0,28	3,2	6,61	209	170	2,78	290	97	153,17	20,1
49/13/	♂	100	141	5,30	53	0,70	0,46	50	77	42	0,33	3,2	6,73	228	172	2,79	260	102	189,24	15,4
50/13/1	♂	100	142	5,00	40	1,23	0,43	42	84	39	0,30	3,2	6,66	216	123	2,80	184	100	195,51	8,2
51/13/1	♂	100	140	5,20	40	1,08	0,42	51	76	52	0,31	3,3	6,76	185	144	2,96	407	99	220,57	38,6
52/13/1	♂	100	140	5,20	53	1,80	0,35	40	68	35	0,27	3,2	6,71	206	119	2,89	168	100	206,43	11,8
13/13/1	♀	100	141	4,90	55	1,00	0,45	45	110	25	0,27	3,3	5,11	90	96	2,86	342	101	149,14	25,8
14/13/1	♀	control	143	5,20	44	1,00	0,51	53	106	24	0,29	3,7	5,50	100	98	2,91	331	103	145,58	51,4
29/13/1	♀	control	140	4,60	23	0,35	0,46	38	82	27	0,26	2,8	4,29	112	119	2,56	279	102	111,92	8,5
30/13/1	♀	control	142	4,40	40	1,00	0,43	40	110	37	0,25	3,3	4,80	96	98	2,61	273	102	115,46	13,3
45/13/1	♀	control	144	4,10	62	1,00	0,46	53	107	26	0,28	3,7	5,68	137	112	3,01	237	102	180,27	15,3
46/13/1	♀	control	142	6,00	47	0,40	0,51	56	97	36	0,29	3,4	5,12	113	78	2,83	389	102	159,62	38,3
21/13/1	♂	control	142	8,20	62	0,60	0,57	52	121	46	0,27	3,1	5,32	98	222	2,90	88	100	266,15	15,1
22/13/1	♂	control	143	7,40	42	0,70	0,56	40	112	37	0,32	3,2	4,82	31	217	2,70	145	99	207,61	30,8
37/13/1	♂	control	142	7,50	37	0,27	0,66	58	192	51	0,29	3,2	4,74	163	197	2,77	206	100	228,08	51,2
38/13/1	♂	control	143	7,70	38	0,34	0,62	60	97	63	0,30	3,1	4,92	146	195	2,99	135	101	200,51	124,5
53/13/1	♂	control	142	5,80	49	0,65	0,45	54	94	45	0,28	3,5	5,46	174	126	2,98	150	98	206,43	16,5
54/13/1	♂	control	140	5,50	56	0,60	0,49	63	94	43	0,28	3,4	5,18	232	202	2,80	115	101	188,88	14,2

Table 8. Reference value of potasium, sodium, iron, ferritin, glucose, total cholesterol, triglicerides, urea, creatinin, total protein and albumin, alanin aminotransferase, aspartate aminotransferase, alkaline phosphatase, bile acids and bilirubin determination for Wistar Han rats in 8-16 week old.

Sex	Sodium [mmol/l]	Potassium [mmol/l]	Cholesterol [mg/dl]	Triglicerides [mmol/l]	Creatinin [mg/dl]	Urea [mg/dl]	Aspat [U/l]	Alat [U/l]	Total bilirubin [mg/dl]	Albumin [g/dl]	Total protein [g/dl]	Glucose [mg/dl]	alkaline phosphatase [U/L]	Calcium [mmol/l]	Iron [µg/dl]	Chlorides [mmol/l]	Ferritin [ng/ml]	Bile acids [umol/l]
♂	142-151	3,82-5,55	37-85	20-114	0,2-0,5	12,3-	74-	18-	0,05-0,15	3,4-4,8	5,2-7,1	70-208	62-230	9,5-11,5	n/d	100-106	n/d	n/d
♀	140-150	3,31-4,9	24-73	14-46	0,2-0,6	13,2-	65-	16-	0,05-0,18	3,6-5,5	5,5-7,7	76-175	26-147	9,7-11,2	n/d	100-107	n/d	n/d

n/d – no data

Scope of urine analysis (urine was collected during the last week of experiment):

- Color
- Volume
- Osmolarity
- Specific gravity
- pH
- Protein
- Glucose
- Blood/blood cells

Table 9. Result of urine test.

Ani. No.	Sex	Dose [mg/kg]	Urine volume [ml]	Colour	lucidity	pH	Urine specific gravity	Protein [mg/dl]	Glucose [mg/dl]	Urobilinogen [mg/dl]	Bilirubin [mg/dl]	Ketones [mg/dl]	Nitrite [mg/dl]	Erythrocytes [ery/µl]	Leukocytes [leu/µl]	Urea – Urine * [mg/dl]	Osmolality [mOsm]
7/13/1	♀	1000	9	straw	clear	6	1,01	30	absent	normal	absent	absent	POS	10	absent	2468	241,0922
8/13/1	♀	1000	11	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	1611	226,8232
9/13/1	♀	1000	8,5	straw	clear	5	1,015	30	absent	2	2	absent	POS	10	absent	5844	297,3027
10/13/1	♀	1000	12	straw	clear	6,5	1,005	absent	absent	normal	absent	absent	POS	absent	absent	1670	227,8055
11/13/1	♀	1000	11	straw	clear	6	1,01	absent	absent	normal	1	absent	POS	absent	absent	2704	245,0216
12/13/1	♀	1000	12,5	straw	clear	6	1,01	30	absent	normal	absent	absent	absent	10	25	2407	240,0766
15/13/1	♂	1000	14	yellow	slightly	6,5	1,01	30	absent	normal	1	absent	absent	10	absent	2942	248,9843
16/13/1	♂	1000	11,5	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	1112	218,5148
17/13/1	♂	1000	9,5	straw	slightly cloudy	7	1,005	absent	absent	normal	absent	absent	absent	10	absent	1104	218,3816
18/13/1	♂	1000	14	straw	slightly cloudy	7	1,005	30	absent	normal	absent	absent	absent	absent	absent	1134	218,8811
19/13/1	♂	1000	17,5	straw	slightly cloudy	8	1,01	absent	absent	normal	absent	absent	absent	10	absent	1004	216,7166
20/13/1	♂	1000	10,5	straw	clear	6,5	1,01	30	absent	normal	absent	absent	absent	absent	absent	1586	226,4069
24/13/1	♀	500	13,5	straw	clear	8	1,01	absent	absent	normal	absent	absent	absent	10	absent	1284,6	221,3886
25/13/1	♀	500	16	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	50	absent	1829,1	230,4545
26/13/1	♀	500	13,5	straw	clear	7	1,005	absent	absent	normal	absent	absent	POS*	absent	absent	1192,3	219,8518
27/13/1	♀	500	11,5	straw	clear	7	1	absent	absent	normal	absent	absent	absent	10	absent	1047,6	217,4426
28/13/1	♀	500	10	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	10	absent	1300	221,645
31/13/1	♂	500	12	straw	clear	9	1,01	30	absent	normal	absent	absent	absent	absent	absent	1746,7	229,0826
32/13/1	♂	500	16,5	straw	clear	7	1,005	30	absent	normal	absent	absent	POS	10	absent	2132,5	235,5062
33/13/1	♂	500	10	straw	clear	8	1,01	30	absent	normal	absent	absent	absent	absent	absent	2138,7	235,6094
34/13/1	♂	500	18	straw	clear	7	1,01	30	absent	normal	1	absent	absent	absent	absent	4144,1	268,9993
35/13/1	♂	500	11,5	straw	clear	8	1,01	absent	absent	normal	absent	absent	absent	absent	absent	1551,3	225,8292
36/13/1	♂	500	15,5	straw	clear	7	1,005	Absent	absent	normal	Absent	Absent	Absent	Absent	Absent	1032,8	217,1961

Ani. No.	Sex	Dose [mg/kg]	Urine volume [ml]	Colour	lucidity	pH	Urine specific gravity	Protein [mg/dl]	Glucose [mg/dl]	Urobilinogen [mg/dl]	Bilirubin [mg/dl]	Ketones [mg/dl]	Nitrite [mg/dl]	Erythrocytes [ery/µl]	Leukocytes [leu/µl]	Urea – Urine * [mg/dl]	Osmolality [mOsm]
40/13/1	♀	100	7,5	straw	slightly cloudy	7	1	absent	absent	normal	absent	absent	absent	10	absent	656	210,9224
41/13/1	♀	100	8,5	straw	slightly cloudy	6,5	1,01	100	absent	normal	absent	absent	absent	absent	500	908	215,1182
42/13/1	♀	100	15,5	straw	clear	6,5	1	absent	absent	normal	absent	absent	absent	absent	absent	775	212,9038
43/13/1	♀	100	10	straw	clear	7	1	absent	absent	normal	absent	absent	absent	absent	absent	815	213,5698
44/13/1	♀	100	8,5	straw	clear	8	1,005	absent	absent	normal	absent	absent	absent	absent	absent	637	210,6061
47/13/1	♂	100	18	straw	slightly cloudy	8	1,01	30	absent	normal	absent	absent	absent	10	absent	1154	219,2141
48/13/1	♂	100	11,5	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	1610	226,8065
49/13/	♂	100	9	straw	clear	8	1,01	absent	absent	normal	absent	absent	absent	absent	absent	1236,3	220,5844
50/13/1	♂	100	16,5	straw	clear	8	1,01	30	absent	normal	absent	absent	absent	absent	absent	1221,2	220,333
51/13/1	♂	100	11,5	straw	clear	8	1,005	absent	absent	normal	absent	absent	absent	10	absent	740,8	212,3343
52/13/1	♂	100	14	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	1321,9	222,0097
13/13/1	♀	control	8,5	yellow	clear	6,5	1,015	30	absent	normal	1	absent	absent	10	absent	4776	279,5205
14/13/1	♀	control	10,8	yellow	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	1606	226,7399
29/13/1	♀	control	12	yellow	slightly cloudy	6,5	1,01	30	absent	normal	1	absent	absent	absent	absent	3285	254,6953
30/13/1	♀	control	10	straw	clear	7	1	absent	absent	normal	absent	absent	absent	absent	absent	884	214,7186
45/13/1	♀	control	9,5	yellow	slightly cloudy	6	1,02	30	absent	normal	2	25	absent	absent	absent	6641	310,5728
46/13/1	♀	control	12	dark yellow	slightly cloudy	8	1,02	100	absent	normal	4	25	absent	absent	25	7059	317,5325
21/13/1	♂	control	9,5	dark yellow	slightly cloudy	7	1,01	30	absent	2	2	absent	absent	absent	25	3733	262,1545
22/13/1	♂	control	10,5	yellow	slightly cloudy	7	1,01	30	absent	normal	1	absent	absent	absent	absent	2342	238,9943
37/13/1	♂	control	16	dark yellow	slightly cloudy	6,5	1,015	30	absent	2	2	absent	absent	absent	25	5610	293,4066

38/13/1	♂	control	14,5	yellow	slightly cloudy	8	1,01	30	absent	normal	absent	absent	absent	10	absent	1618	226,9397
53/13/1	♂	control	9	straw	clear	9	1,005	absent	absent	normal	absent	absent	absent	absent	absent	752	212,5208
54/13/1	♂	control	12,5	straw	slightly cloudy	7	1,005	Absent	absent	normal	Absent	Absent	Absent	Absent	Absent	986	216,4169

- parametr indicates to determine urine osmolality – in addition, it may be a parameter estimates capacity of the kidneys.

Table 10. Reference values of urine test.

Urine volume [ml]	Colour	lucidity	pH	Urine specific gravity	Protein [mg/dl]	Glucose [mg/dl]	Urobilinogen [mg/dl]	Bilirubin [mg/dl]	Ketones [mg/dl]	Nitrite [mg/dl]	Erythrocytes [ery/µl]	Leukocytes [leu/µl]	Urea – Urine * [mg/dl]	Osmolality [mOsm]
15-30 ml/24h[6]	n/d	n/d	n/d	n/d	Trace amount[6]	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	10 - 2000[6]

n/d – no data

APPENDIX 1B.
The detailed data from the study [redacted] 2014, Repeated dose 90-day oral toxicity study of iron pyrophosphate in rodents (Vol. 3 CA, B.6.3.2, Oral 90-day study)

Clinical observations

During the dosing period, each day, animals were subjected to a general observation twice a day in order to detect signs of toxicity and morbidity. Results of general clinical observations are shown in Table 1. During the entire dosing period was not observed disturbing symptoms illness or toxicity symptoms.

Table 1. Results of general clinical observation.

Order no.	Animal no.	Sex	Dose [mg/kg bw]/Group	Occurrence of signs of toxicity or morbidity in observation period (descriptively if present)
1	55/13/1	♀	1000	Absence
2	56/13/1	♀	1000	Absence
3	57/13/1	♀	1000	Absence
4	58/13/1	♀	1000	Absence
5	59/13/1	♀	1000	Animal was euthanized 25.08.2013 because of the occurrence of malignancy which impeded test material gavage. The malignancy was noted 27.07.2013. The histopathological examination demonstrated inflammatory character - abscess – not caused by iron pyrophosphate
6	60/13/1	♀	1000	Absence
7	61/13/1	♀	1000	Absence
8	62/13/1	♀	1000	Absence
9	63/13/1	♀	1000	Absence
10	64/13/1	♀	1000	Absence
11	65/13/1	♀	1000	Absence
12	66/13/1	♀	1000	Absence
13	67/13/1	♀	1000	Absence
14	68/13/1	♀	1000	Absence
15	69/13/1	♀	1000	Absence
16	70/13/1	♂	1000	The animal died due to dose administration to the pleural cavity 28.07.2013 17
17	71/13/1	♂	1000	Absence
18	72/13/1	♂	1000	Absence
19	73/13/1	♂	1000	Absence
20	74/13/1	♂	1000	Absence
21	75/13/1	♂	1000	Absence
22	76/13/1	♂	1000	Absence
23	77/13/1	♂	1000	The animal died due to dose administration to the pleural cavity 8.08.2013
24	78/13/1	♂	1000	Absence
25	79/13/1	♂	1000	Absence
26	80/13/1	♂	1000	Absence
27	81/13/1	♂	1000	Absence

Order no.	Animal no.	Sex	Dose [mg/kg bw]/Group	Occurrence of signs of toxicity or morbidity in observation period (descriptively if present)
28	82/13/1	♂	1000	Absence
29	83/13/1	♂	1000	Absence
30	84/13/1	♂	1000	Absence
31	85/13/1	♀	satellite group	Absence
32	86/13/1	♀	satellite group	Absence
33	87/13/1	♀	satellite group	Absence
34	88/13/1	♀	satellite group	Absence
35	89/13/1	♀	satellite group	The animal was excluded from the group because of the occurring throughout the experimental period, starting from the third week of the study, disorder behaviour: increased response to sound and an unusual reaction to the capture - difficult to catch, and poor response to light.
36	90/13/1	♀	satellite group	Absence
37	91/13/1	♀	satellite group	Absence
38	92/13/1	♀	satellite group	Absence
39	93/13/1	♂	satellite group	Absence
40	94/13/1	♂	satellite group	Absence
41	95/13/1	♂	satellite group	Absence
42	96/13/1	♂	satellite group	Absence
43	97/13/1	♂	satellite group	Absence
44	98/13/1	♂	satellite group	Absence
45	99/13/1	♂	satellite group	Absence
46	100/13/1	♂	satellite group	Absence
47	101/13/1	♀	control group	Absence
48	102/13/1	♀	control group	Absence
49	103/13/1	♀	control group	Absence
50	104/13/1	♀	control group	Absence
51	105/13/1	♀	control group	Absence
52	106/13/1	♀	control group	Absence
53	107/13/1	♀	control group	Absence
54	108/13/1	♀	control group	Absence
55	109/13/1	♂	control group	Absence
56	110/13/1	♂	control group	Absence
57	111/13/1	♂	control group	Absence
58	112/13/1	♂	control group	Absence
59	113/13/1	♂	control group	Absence
60	114/13/1	♂	control group	Absence
61	115/13/1	♂	control group	Absence
62	116/13/1	♂	control group	Absence

Once a week, all animals were subjected to detailed clinical observations in the form of a thorough check of changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, respiratory pattern. Attention was paid to changes in gait, posture, responses

to touch and to auditory stimuli, as well as the presence of involuntary movements - tonic and clonic, stereotypes (e.g. excessive grooming, repetitive circling) and strange behaviour (e.g. self-mutilation, walking backwards) and any other. Detailed observations were carried outside home cage. Any observed abnormal symptoms were recorded in the detailed observations protocols.

As a result of detailed clinical observations, there were no changes in the health status of animals without the animals No. 59/13/1 and 77/13/1 70/13/1 for the reasons described above..

Table 2. Results of detailed clinical observations

Order no.	Animal no.	Sex	Dose [mg/kg bw]/Group	Occurrence of signs of toxicity or morbidity (descriptively if present)
1	55/13/1	♀	1000	absence
2	56/13/1	♀	1000	absence
3	57/13/1	♀	1000	absence
4	58/13/1	♀	1000	absence
5	60/13/1	♀	1000	absence
6	61/13/1	♀	1000	absence
7	62/13/1	♀	1000	absence
8	63/13/1	♀	1000	absence
9	64/13/1	♀	1000	absence
10	65/13/1	♀	1000	absence
11	66/13/1	♀	1000	absence
12	67/13/1	♀	1000	absence
13	68/13/1	♀	1000	absence
14	69/13/1	♀	1000	absence
15	71/13/1	♂	1000	absence
16	72/13/1	♂	1000	absence
17	73/13/1	♂	1000	absence
18	74/13/1	♂	1000	absence
19	75/13/1	♂	1000	absence
20	76/13/1	♂	1000	absence
21	78/13/1	♂	1000	absence
22	79/13/1	♂	1000	absence
23	80/13/1	♂	1000	absence
24	81/13/1	♂	1000	absence
25	82/13/1	♂	1000	absence
26	83/13/1	♂	1000	absence
27	84/13/1	♂	1000	absence
28	85/13/1	♀	satellite group	absence
29	86/13/1	♀	satellite group	absence
30	87/13/1	♀	satellite group	absence
31	88/13/1	♀	satellite group	absence
33	90/13/1	♀	satellite group	absence
34	91/13/1	♀	satellite group	absence
35	92/13/1	♀	satellite group	absence
36	93/13/1	♂	satellite group	absence
37	94/13/1	♂	satellite group	absence
38	95/13/1	♂	satellite group	absence
39	96/13/1	♂	satellite group	absence
40	97/13/1	♂	satellite group	absence
41	98/13/1	♂	satellite group	absence
42	99/13/1	♂	satellite group	absence
43	100/13/1	♂	satellite group	absence
44	101/13/1	♀	control group	absence
45	102/13/1	♀	control group	absence

46	103/13/1	♀	control group	absence
47	104/13/1	♀	control group	absence
48	105/13/1	♀	control group	absence
49	106/13/1	♀	control group	absence
50	107/13/1	♀	control group	absence
51	108/13/1	♀	control group	absence
52	109/13/1	♂	control group	absence
53	110/13/1	♂	control group	absence
54	111/13/1	♂	control group	absence
55	112/13/1	♂	control group	absence
56	113/13/1	♂	control group	absence
57	114/13/1	♂	control group	absence
58	115/13/1	♂	control group	absence
59	116/13/1	♂	control group	absence

Body weight

Animal body weight measurement was carried out before administration of the test material, and once a week during the entire period of the experiment. At the start of the experiment body weight of the animals in groups did not differ by more than ± 20% of the average body weight. Measurement of body weight for each animal in the subsequent weeks of the experiment is presented in Table 3. The body weights of all animals were within the reference ranges for Wistar rats (by National Lanoratory Animal Center - NLAC). There were no statistically significant differences between animals of 1000 mg/kg body weight group and the control group. During the whole period the experiment the animals gained weight. In the case of the satellite group, the body weight remained at the same level after end of dosage. There were no differences in response of males and females in case of body weight. This study was conducted with one dose level that there is impossible to show dependency of body weight changes on the dose.

Table 3. Measurement of animals body weight in the following weeks of the experiment [g].

Animal no	Sex	Dose [mg/kg bw] / Group	Day of experiment																body weight gain (day of experiment)			
			0	7	14	21	28	35	42	49	56	63	70	77	84	90	97	114	0-28	28-63	63-90	0-90
55/13/1	♀	1000	163	189	209	225	236	247	250	256	253	261	267	264	265	256	-	-	73	25	4	93
56/13/1	♀	1000	158	175	191	200	220	212	222	227	223	225	233	229	236	226	-	-	62	5	11	68
57/13/1	♀	1000	149	160	176	189	194	198	204	212	210	214	219	214	219	204	-	-	45	20	5	55
58/13/1	♀	1000	163	175	183	195	205	214	217	220	222	229	229	234	237	219	-	-	42	24	8	56
59/13/1	♀	1000	154	172	182	165	190	200	205	212	215	-	-	-	-	-	-	-	36	-	-	-
60/13/1	♀	1000	153	173	188	201	208	210	224	228	229	232	235	240	243	232	-	-	55	24	11	79
61/13/1	♀	1000	171	189	201	214	223	226	253	238	241	246	251	246	253	244	-	-	52	23	7	73
62/13/1	♀	1000	179	196	216	229	237	236	254	258	263	264	272	268	269	263	-	-	58	27	5	84
63/13/1	♀	1000	185	207	220	234	243	247	255	257	256	262	263	264	266	253	-	-	58	19	4	68
64/13/1	♀	1000	152	174	184	198	208	215	221	230	231	239	262	237	239	230	-	-	56	31	0	78
65/13/1	♀	1000	143	163	177	187	200	209	212	218	220	230	230	230	232	218	-	-	57	30	2	75
66/13/1	♀	1000	151	173	188	198	202	210	212	218	215	221	221	222	229	212	-	-	51	19	8	61
67/13/1	♀	1000	148	162	177	193	197	200	207	213	209	215	220	220	226	210	-	-	49	18	11	62
68/13/1	♀	1000	191	208	216	225	230	240	243	248	247	254	264	255	268	251	-	-	39	24	14	60
69/13/1	♀	1000	158	176	187	204	208	218	216	225	227	234	232	228	236	222	-	-	50	26	2	64
70/13/1	♂	1000	241	270	294	325	331	-	-	-	-	-	-	-	-	-	-	-	90	-	-	-
71/13/1	♂	1000	204	225	253	276	284	305	312	321	328	337	342	339	351	335	-	-	80	53	14	131
72/13/1	♂	1000	269	301	331	357	376	392	406	421	429	441	444	446	469	447	-	-	107	65	28	178
73/13/1	♂	1000	202	220	240	269	281	297	302	318	316	323	327	326	343	340	-	-	79	42	20	138
74/13/1	♂	1000	238	268	300	335	349	366	378	397	406	422	428	426	441	423	-	-	111	73	19	185
75/13/1	♂	1000	258	288	313	344	364	390	394	414	420	433	441	451	458	448	-	-	106	69	25	190
76/13/1	♂	1000	251	274	309	334	354	369	382	400	401	417	427	437	445	427	-	-	103	63	28	176

Animal no	Sex	Dose [mg/kg bw] / Group	Day of experiment																body weight gain (day of experiment)			
			0	7	14	21	28	35	42	49	56	63	70	77	84	90	97	114	0-28	28-63	63-90	0-90
77/13/1	♂	1000	249	274	299	322	342	303	275	-	-	-	-	-	-	-	-	-	93	-	-	-
78/13/1	♂	1000	268	300	330	366	385	404	407	423	436	450	459	461	473	453	-	-	117	65	23	185
79/13/1	♂	1000	212	227	254	284	299	316	320	327	331	344	345	342	359	338	-	-	87	45	15	126
80/13/1	♂	1000	237	265	289	313	326	339	349	364	371	379	383	383	394	375	-	-	89	53	15	138
81/13/1	♂	1000	221	257	287	327	350	367	380	401	402	414	430	426	447	420	-	-	129	64	33	199
82/13/1	♂	1000	242	278	312	345	365	364	384	407	414	426	432	444	455	420	-	-	123	61	29	178
83/13/1	♂	1000	266	300	330	358	377	383	391	406	410	426	436	437	446	418	-	-	111	49	20	152
84/13/1	♂	1000	251	283	310	340	361	378	387	402	409	421	425	433	440	408	-	-	110	60	19	157
85/13/1	♀	sat.	154	168	182	195	200	215	213	220	220	226	229	231	230	232	238	225	46	26	4	71
86/13/1	♀	sat.	184	208	219	232	240	246	251	261	254	257	258	264	264	262	269	260	56	17	7	76
87/13/1	♀	sat.	140	160	176	186	192	200	197	209	208	212	210	217	217	219	221	209	52	20	5	69
88/13/1	♀	sat.	149	162	173	178	184	189	191	199	199	205	206	207	210	267	271	202	35	21	5	53
89/13/1	♀	sat.	144	161	169	175	184	198	191	205	208	210	209	209	210	214	216	210	40	26	0	66
90/13/1	♀	sat.	169	191	196	211	219	227	232	240	240	245	248	246	254	240	255	240	50	26	9	71
91/13/1	♀	sat.	177	195	209	212	229	236	236	248	246	247	251	253	253	248	258	248	52	18	6	71
92/13/1	♀	sat.	196	218	234	257	259	264	265	278	281	285	287	285	296	280	296	280	63	26	11	84
93/13/1	♂	sat.	230	261	290	320	339	355	370	385	393	402	407	410	423	429	431	410	109	63	21	180
94/13/1	♂	sat.	252	282	314	344	360	384	394	406	416	425	433	444	459	433	440	443	108	65	34	191
95/13/1	♂	sat.	250	282	313	350	370	391	405	418	431	442	443	461	468	457	466	454	120	72	26	204
96/13/1	♂	sat.	239	269	297	322	340	356	367	387	393	405	414	428	431	470	483	421	101	65	26	182
97/13/1	♂	sat.	245	286	317	346	369	384	395	412	418	430	437	443	451	458	458	436	124	61	21	191
98/13/1	♂	sat.	253	299	328	364	386	409	421	438	444	456	458	466	468	472	480	457	133	70	12	204
99/13/1	♂	sat.	213	257	290	313	325	341	356	369	375	388	393	401	397	411	422	402	112	63	9	189

Animal no	Sex	Dose [mg/kg bw] / Group	Day of experiment																body weight gain (day of experiment)			
			0	7	14	21	28	35	42	49	56	63	70	77	84	90	97	114	0-28	28-63	63-90	0-90
100/13/1	♂	sat.	220	251	280	307	322	343	354	372	381	395	399	407	414	416	425	396	102	73	19	176
101/13/1	♀	control	141	161	171	187	191	198	199	206	203	214	214	216	216	206	-	-	50	23	2	65
102/13/1	♀	control	149	168	176	190	200	210	215	217	221	223	222	227	227	216	-	-	51	23	4	67
103/13/1	♀	control	138	158	168	180	183	192	198	207	207	212	215	217	224	209	-	-	45	29	12	71
104/13/1	♀	control	175	197	212	228	230	245	244	256	259	259	269	272	228	257	-	-	55	29	-31	82
105/13/1	♀	control	155	172	185	195	201	209	209	216	219	221	220	224	228	211	-	-	46	20	7	56
106/13/1	♀	control	162	180	193	205	213	221	222	228	230	236	238	245	246	226	-	-	51	23	10	64
107/13/1	♀	control	168	182	192	208	216	225	230	236	240	243	238	249	255	235	-	-	48	27	12	67
108/13/1	♀	control	156	173	179	192	195	200	205	211	212	213	216	218	220	202	-	-	39	18	7	46
109/13/1	♂	control	251	279	303	334	349	369	376	390	398	408	415	417	429	407	-	-	98	59	21	156
110/13/1	♂	control	240	278	308	341	364	382	383	401	407	422	432	429	448	422	-	-	124	58	26	182
111/13/1	♂	control	243	282	316	353	381	401	409	431	444	460	465	469	470	460	-	-	138	79	10	217
112/13/1	♂	control	269	305	336	372	394	416	425	441	444	452	458	460	463	454	-	-	125	58	11	185
113/13/1	♂	control	211	236	267	304	323	342	359	376	381	396	402	411	421	397	-	-	112	73	25	186

Animal no	Sex	Dose [mg/kg bw] / Group	Day of experiment																body weight gain (day of experiment)			
			0	7	14	21	28	35	42	49	56	63	70	77	84	90	97	114	0-28	28-63	63-90	0-90
114/13/1	♂	control	229	262	286	315	330	344	357	370	380	383	393	393	401	390	-	-	101	53	18	161
115/13/1	♂	control	236	268	299	330	345	371	389	403	413	423	430	439	453	428	-	-	109	78	30	192
116/13/1	♂	control	242	275	306	344	364	386	402	416	427	434	442	447	460	435	-	-	122	70	26	193

sat. - satellite group

Water and feed consumption

Measurements of food and water consumption was performed once a week. Food consumption by animals participating in the study did not differ from the reference values in the range of 8-20 g / day. Water consumption also ranged in reference standards of approximately 30 mL / day, however, in case of juveniles the water intake ranges from 5-80 mL / day. There was no statistically significant difference in food and water consumption of animals receiving iron pyrophosphate in relation to the control group. Measurement of water and food consumption in individual cages in the following weeks of the experiment are shown in Table 4.

Table 4. Measurement of water and feed consumption in animals cages in the following weeks of the experiment

Feed consumption per one animal Week																
Cage no	Dose [mg/kg bw] / Group	sex	Week													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1000 Satellite group	♀	95	94	84	101	93	93	93	96	115	102	105	95	-	-
2			106	103	106	114	104	111	106	113	100	113	114	106	-	-
3			99	94	96	101	95	93	93	96	99	99	100	100	-	-
8			86	95	102	97	94	91	93	97	97	84	99	91	85	75
9			90	96	96	111	96	99	101	105	127	108	116	100	86	76
12	Control group		94	91	100	101	98	97	100	105	106	107	108	110	-	-
13			96	89	98	101	98	91	96	99	102	99	97	104	-	-
4	1000 Satellite group	♂	125	139	144	143	119	110	138	146	150	145	163	142	-	-
5			157	145	150	152	127	125	155	157	130	164	154	159	-	-
6			126	146	148	156	148	143	170	155	162	151	156	149	-	-
7			139	148	158	163	139	150	154	159	110	157	154	154	-	-
10			131	147	150	158	145	148	149	157	159	168	163	160	137	119
11			136	154	155	158	153	151	156	157	159	176	193	152	137	113
14	Control group		159	153	165	167	153	152	162	164	170	163	161	171	-	-
15			145	144	150	158	150	153	156	158	162	160	161	164	-	-

Water consumption per one animal [cm³/week]																
Cage no.	Dose [mg/kg bw] / Group	sex	Week													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1000 Satellite group	♀	196	194	173	198	187	202	192	169	208	185	181	192	-	-
2			195	188	168	172	179	180	153	152	152	157	156	151	-	-
3			198	190	162	174	132	184	178	170	152	172	152	131	-	-
8			169	199	200	205	205	208	199	183	160	167	214	189	194	185
9			153	195	188	198	203	218	210	165	180	147	207	165	151	163
12	Control group		163	177	163	180	175	185	176	165	183	184	182	195	-	-
13			181	213	188	208	191	208	194	200	222	191	198	199	-	-
4	1000 Satellite group	♂	194	238	194	205	261	183	187	183	185	196	175	180	-	-
5			213	267	238	253	238	230	257	233	240	266	246	236	-	-
6			187	240	220	236	258	270	229	213	163	218	189	207	-	-
7			249	299	251	287	313	277	250	233	183	218	240	236	-	-
10			215	213	218	228	228	243	223	213	213	210	243	199	244	210
11			227	292	251	256	315	280	275	260	235	210	238	190	193	180

14	Control group		251	263	233	250	248	228	223	210	204	238	222	212		
15			188	277	225	280	262	230	263	243	224	246	220	217		

Behaviour tests

The gripping force test

The first part of the study includes a test of measuring how long the animal placed on the bar continues, gripping his forelegs on the rod. For this purpose, suspended animal forelegs on the stick and from the moment of catching up rat rod is measured by a stopwatch to time the moment you release the lever by the animal. This procedure was repeated twice at an interval of about one minute. The second part of the study includes a measure of how hard a rat holding a stick. For this purpose, suspended animal forelegs on the stick connected to a calibrated dynamometer. 150 recorded consecutive measurements at intervals of 0.1 s, during which the animal's tail pulled until the rod is released by the rat was recorded maximum force at which the rod let go wild. The measurement results forelimb grip strength of animals are shown in Tables 5 and 6.

Table 5. Result of animals gripping time measurement

Order no	Animal no	Sex	Dose [mg/kg bw] / Group	Measurement I [s]	Measurement II [s]	Average of two measurements ± SD
1	55/13/1	♀	1000	4	6	7,93 ± 3,44
2	56/13/1	♀	1000	12	4	
3	57/13/1	♀	1000	5	9	
4	58/13/1	♀	1000	6	7	
5	60/13/1	♀	1000	5	3	
6	61/13/1	♀	1000	8	10	
7	62/13/1	♀	1000	12	15	
8	63/13/1	♀	1000	6	4	
9	64/13/1	♀	1000	12	19	
10	65/13/1	♀	1000	5	16	
11	66/13/1	♀	1000	3	5	
12	67/13/1	♀	1000	10	5	
13	68/13/1	♀	1000	10	9	
14	69/13/1	♀	1000	7	5	
15	71/13/1	♂	1000	9	17	6,69 ± 3,74
16	72/13/1	♂	1000	2	3	
17	73/13/1	♂	1000	7	9	
18	74/13/1	♂	1000	4	14	
19	75/13/1	♂	1000	3	10	
20	76/13/1	♂	1000	2	3	
21	78/13/1	♂	1000	5	19	
22	79/13/1	♂	1000	7	5	
23	80/13/1	♂	1000	3	3	
24	81/13/1	♂	1000	6	16	
25	82/13/1	♂	1000	4	4	
26	83/13/1	♂	1000	4	10	
27	84/13/1	♂	1000	3	2	

Order no	Animal no	Sex	Dose [mg/kg bw] / Group	Measurement I [s]	Measurement II [s]	Average of two measurements ± SD
28	85/13/1	♀	1000/sat.	9	10	
29	86/13/1	♀	1000/sat.	19	9	
30	87/13/1	♀	1000/sat.	4	6	
31	88/13/1	♀	1000/sat.	26	8	
33	90/13/1	♀	1000/sat.	3	4	
34	91/13/1	♀	1000/sat.	8	15	
35	92/13/1	♀	1000/sat.	7	6	9,57 ± 4,93
36	93/13/1	♂	1000/sat.	16	14	
37	94/13/1	♂	1000/sat.	4	2	
38	95/13/1	♂	1000/sat.	4	19	
39	96/13/1	♂	1000/sat.	3	1	8,75 ± 6,53
40	97/13/1	♂	1000/sat.	4	8	
41	98/13/1	♂	1000/sat.	9	8	
42	99/13/1	♂	1000/sat.	3	4	
43	100/13/1	♂	1000/sat.	21	20	
44	101/13/1	♀	control	18	18	
45	102/13/1	♀	control	3	4	
46	103/13/1	♀	control	24	33	
47	104/13/1	♀	control	7	15	14,38 ± 9,52
48	105/13/1	♀	control	7	5	
49	106/13/1	♀	control	14	15	
50	107/13/1	♀	control	35	19	
51	108/13/1	♀	control	4	9	
52	109/13/1	♂	control	17	11	
53	110/13/1	♂	control	2	4	
54	111/13/1	♂	control	8	3	
55	112/13/1	♂	control	8	2	8,31 ± 4,28
56	113/13/1	♂	control	19	7	
57	114/13/1	♂	control	19	2	
58	115/13/1	♂	control	4	5	
59	116/13/1	♂	control	12	10	

SD – standard deviation

Table 6. Measurement of gripping force of animal's forelimb by using dynamometer

Animal no	Sex	Dose [mg/kg bw] / Group	Force [N]	Average ± SD
55/13/1	♀	1000	8,31	
56/13/1	♀	1000	7,10	
57/13/1	♀	1000	8,40	
58/13/1	♀	1000	8,05	
60/13/1	♀	1000	9,08	8,64 ± 1,07
61/13/1	♀	1000	9,91	
62/13/1	♀	1000	10,62	
63/13/1	♀	1000	8,34	
64/13/1	♀	1000	9,46	

Animal no	Sex	Dose [mg/kg bw] / Group	Force [N]	Average ± SD
65/13/1	♀	1000	9,15	
66/13/1	♀	1000	6,37	
67/13/1	♀	1000	8,80	
68/13/1	♀	1000	8,78	
69/13/1	♀	1000	8,55	
71/13/1	♂	1000	11,77	
72/13/1	♂	1000	11,38	
73/13/1	♂	1000	9,89	
74/13/1	♂	1000	12,80	
75/13/1	♂	1000	10,85	
76/13/1	♂	1000	9,33	
78/13/1	♂	1000	13,63	11,58 ± 1,32
79/13/1	♂	1000	12,75	
80/13/1	♂	1000	12,96	
81/13/1	♂	1000	11,35	
82/13/1	♂	1000	10,13	
83/13/1	♂	1000	12,57	
84/13/1	♂	1000	11,12	
85/13/1	♀	Satellite group	7,78	
86/13/1	♀	Satellite group	8,97	
87/13/1	♀	Satellite group	8,02	
88/13/1	♀	Satellite group	8,40	8,54 ± 0,81
90/13/1	♀	Satellite group	7,66	
91/13/1	♀	Satellite group	9,92	
92/13/1	♀	Satellite group	9,01	
93/13/1	♂	Satellite group	10,63	
94/13/1	♂	Satellite group	11,12	
95/13/1	♂	Satellite group	13,15	
96/13/1	♂	Satellite group.	11,66	11,69 ± 1,39
97/13/1	♂	Satellite group	11,67	
98/13/1	♂	Satellite group	13,17	
99/13/1	♂	Satellite group	9,20	
100/13/1	♂	Satellite group	12,92	
101/13/1	♀	Control group	5,85	
102/13/1	♀	Control group	13,61	
103/13/1	♀	Control group	6,79	
104/13/1	♀	Control group	6,91	7,35 ± 2,68
105/13/1	♀	Control group	5,88	
106/13/1	♀	Control group	6,73	
107/13/1	♀	Control group	5,01	
108/13/1	♀	Control group	8,01	
109/13/1	♂	Control group	13,61	
110/13/1	♂	Control group	13,42	
111/13/1	♂	Control group	13,17	12,16 ± 1,26
112/13/1	♂	Control group	11,35	
113/13/1	♂	Control group	11,42	

Animal no	Sex	Dose [mg/kg bw] / Group	Force [N]	Average ± SD
114/13/1	♂	Control group	12,92	
115/13/1	♂	Control group	11,13	
116/13/1	♂	Control group	10,25	
85/13/1	♀	Satellite group	9,97	10,65 ± 0,92
86/13/1	♀	Satellite group	9,08	
87/13/1	♀	Satellite group	11,05	
88/13/1	♀	Satellite group	11,41	
89/13/1	♀	Satellite group	9,75	
90/13/1	♀	Satellite group	11,03	
91/13/1	♀	Satellite group	11,30	
92/13/1	♀	Satellite group	11,61	
93/13/1	♂	Satellite group	11,00	10,16 ± 2,66
94/13/1	♂	Satellite group	11,66	
95/13/1	♂	Satellite group	11,59	
96/13/1	♂	Satellite group	6,45	
97/13/1	♂	Satellite group	6,78	
98/13/1	♂	Satellite group	9,34	
99/13/1	♂	Satellite group	9,93	
100/13/1	♂	Satellite group	14,49	

SD – standard deviation

As a result of statistical analysis, there was no statistically significant differences between treatment groups and satellite group in terms of grip strength measurement forelimb time, whereas animals in the case of force statistically significant differences were found in the group of females compared to the control group and the group of females of the satellite with the control group. However, these results do not allow for a clear evaluation of the impact of the test substance on the strength of grip because of the standard deviation value, which is high and in addition the measurement results show higher strength values for the group receiving iron pyrophosphate than in the control group.

Animal's mobility test.

Animal's mobility is evaluated by placing it in the restricted opened area, in the corner at the wall and recording of the animal's behavior with a video-camera for 10 min. For observation, a limited area of 1 m2 divided into fields of 20 cm side length is used. The test results include the number of crossed lines, animal's moving track (number of fields in which the animal was present in the external, internal and central zone), number of stops (halting and lifting up of the front limbs), the presence of involuntary movements and stereotype behaviours. Additionally, number of stool units and excretion of urine by the animals during the study was reported. The results of the animals' motility test are presented in Table 7.

Table 7. Results of locomotor activity test

Animal no	Dose [mg/kg bw] / Group	Sex	Outer zone	Inner zone	Center	Stand	Grooming	Faeces	Urine
55/13/1	1000	♀	70	0	0	7	1	0	+
56/13/1	1000	♀	50	1	0	4	0	0	-
57/13/1	1000	♀	86	8	1	12	1	0	+

Animal no	Dose [mg/kg bw] / Group	Sex	Outer zone	Inner zone	Center	Stand	Grooming	Faeces	Urine
58/13/1	1000	♀	99	7	2	20	2	4	-
60/13/1	1000	♀	95	5	1	15	1	1	-
61/13/1	1000	♀	54	1	0	9	1	0	+
62/13/1	1000	♀	92	10	3	20	1	4	+
63/13/1	1000	♀	128	6	0	20	3	0	-
64/13/1	1000	♀	112	22	5	20	3	4	+
65/13/1	1000	♀	74	3	0	7	2	0	+
66/13/1	1000	♀	86	4	0	11	2	0	-
67/13/1	1000	♀	120	33	5	23	1	2	+
68/13/1	1000	♀	148	27	4	25	1	0	-
69/13/1	1000	♀	126	28	3	29	2	7	+
71/13/1	1000	♂	68	0	0	14	2	0	-
72/13/1	1000	♂	118	10	1	18	1	5	+
73/13/1	1000	♂	28	0	0	6	2	1	-
74/13/1	1000	♂	40	0	0	7	0	3	-
75/13/1	1000	♂	109	22	2	12	1	5	-
76/13/1	1000	♂	51	13	1	16	0	9	-
78/13/1	1000	♂	41	0	0	8	1	4	-
79/13/1	1000	♂	86	0	0	13	2	8	+
80/13/1	1000	♂	58	1	0	8	0	4	-
81/13/1	1000	♂	36	0	0	7	0	8	-
82/13/1	1000	♂	6	0	0	2	1	0	-
83/13/1	1000	♂	8	0	0	3	1	4	-
84/13/1	1000	♂	29	4	1	6	1	3	-
101/13/1	Control group	♀	38	0	0	5	2	0	-
102/13/1	Control group	♀	134	24	6	14	1	8	-
103/13/1	Control group	♀	107	40	5	21	3	4	-
104/13/1	Control group	♀	84	8	2	9	1	5	+
105/13/1	Control group	♀	51	4	0	11	1	4	-
106/13/1	Control group	♀	159	29	3	20	3	0	-
107/13/1	Control group	♀	62	3	0	14	0	5	+
108/13/1	Control group	♀	90	5	2	19	1	0	-
109/13/1	Control group	♂	60	8	1	10	1	1	-
110/13/1	Control group	♂	138	20	4	16	0	7	-
111/13/1	Control group	♂	90	7	1	7	1	4	-
112/13/1	Control group	♂	117	9	1	15	1	5	-
113/13/1	Control group	♂	35	1	0	4	0	2	-
114/13/1	Control group	♂	72	1	0	18	1	8	+
115/13/1	Control group	♂	107	37	4	21	1	0	-
116/13/1	Control group	♂	6	0	0	0	0	5	-
SATELLITE GROUP – MEASUREMENT I									
85/13/1	Satellite group	♀	6	0	0	2	0	7	+
86/13/1	Satellite group	♀	91	18	4	13	1	9	-
87/13/1	Satellite group	♀	47	1	0	13	0	9	+
88/13/1	Satellite group	♀	18	1	0	2	2	5	+
90/13/1	Satellite group	♀	92	17	1	19	1	4	-
91/13/1	Satellite group.	♀	0	0	0	5	0	2	-
92/13/1	Satellite group	♀	77	26	5	15	0	4	+
93/13/1	Satellite group	♂	4	0	0	4	1	0	-
94/13/1	Satellite group	♂	63	2	0	8	1	4	-
95/13/1	Satellite group.	♂	20	0	0	5	1	11	-
96/13/1	Satellite group.	♂	87	18	1	24	0	1	-
97/13/1	Satellite group	♂	0	0	0	3	1	3	-

Animal no	Dose [mg/kg bw] / Group	Sex	Outer zone	Inner zone	Center	Stand	Grooming	Faeces	Urine
98/13/1	Satellite group	♂	18	0	0	5	1	1	-
99/13/1	Satellite group	♂	67	3	0	8	3	8	-
100/13/1	Satellite group	♂	48	5	1	7	2	0	-
SATELLITE GROUP – MEASUREMENT II – 14 DAYS AFTER END OF DOSE ADMINISTRATION PERIOD									
85/13/1	Satellite group .	♀	114	13	1	11	2	0	-
86/13/1	Satellite group	♀	74	18	3	10	2	6	-
87/13/1	Satellite group	♀	56	0	0	14	1	0	-
88/13/1	Satellite group	♀	40	6	1	4	1	2	-
90/13/1	Satellite group	♀	169	33	4	20	2	4	-
91/13/1	Satellite group	♀	69	5	0	11	2	0	-
92/13/1	Satellite group	♀	98	18	3	17	2	0	-
93/13/1	Satellite group	♂	38	0	0	4	1	0	-
94/13/1	Satellite group	♂	71	3	1	7	2	1	-
95/13/1	Satellite group	♂	38	2	1	5	1	0	-
96/13/1	Satellite group	♂	95	6	0	9	1	0	+
97/13/1	Satellite group	♂	0	0	0	1	0	0	-
98/13/1	Satellite group	♂	72	0	0	11	1	9	-
99/13/1	Satellite group	♂	20	0	0	1	2	2	-
100/13/1	Satellite group	♂	70	3	0	11	2	1	-

+ Animal urinated

- Animal did not urinated

In the studied parameters - number of fields in which the animal was present in the external, internal and central zone, the number of stops (halting and lifting up of the front limbs), the number of stool units and urinary excretion by the animals during the study, there was no statistically significant difference in groups of males and females, as well as the satellite group of males and females in relation to the control group. Conclusion was based on the observation of the animals during the measurement and statistical analysis parameters. In addition, there was no difference in response in male and female at a dose of 1000 mg/g ferric pyrophosphate in the range recorded parameters.

Animal's motor coordination test.

An animal was placed on an illuminated end of a 165 cm long, double-layered beam, on the height of 80 cm. On the other end of the beam there was a dark box with a small amount of food. During the test, time of crossing over the beam by the animal was measured with a stopper and its gait was observed from both sides of the track (e.g. sliding of the rodent on one side - number of slips). It was also reported if the animal entered the box or not. The rat's behaviour on the beam was recorded with a video-camera. The results of the study are given in Table 8.

Table 8. Results of locomotor coordination test of animals.

Ordinal no	Number of animal	Sex	Dose [mg/kg bw]	Crossing time [s]	Slip		Entrance to the box
					Left	Right	
1	55/13/1	♀	1000	7	0	0	x
2	56/13/1	♀	1000	15	1	0	x
3	57/13/1	♀	1000	9	1	0	x
4	58/13/1	♀	1000	5	0	1	x
5	60/13/1	♀	1000	15	0	0	x

Ordinal no	Number of animal	Sex	Dose [mg/kg bw]	Crossing time [s]	Slip		Entrance to the box
					Left	Right	
6	61/13/1	♀	1000	7	0	0	x
7	62/13/1	♀	1000	10	0	0	x
8	63/13/1	♀	1000	5	1	1	x
9	64/13/1	♀	1000	12	0	0	x
10	65/13/1	♀	1000	29	0	0	x
11	66/13/1	♀	1000	34	0	0	x
12	67/13/1	♀	1000	9	0	0	x
13	68/13/1	♀	1000	27	0	0	x
14	69/13/1	♀	1000	25	0	1	x
15	71/13/1	♂	1000	14	0	0	x
16	72/13/1	♂	1000	23	1	1	x
17	73/13/1	♂	1000	49	0	0	x
18	74/13/1	♂	1000	28	0	0	x
19	75/13/1	♂	1000	39	0	0	x
20	76/13/1	♂	1000	24	0	0	x
21	78/13/1	♂	1000	6	1	2	x
22	79/13/1	♂	1000	6	0	0	x
23	80/13/1	♂	1000	10	0	0	x
24	81/13/1	♂	1000	22	1	0	x
25	82/13/1	♂	1000	3	1	1	x
26	83/13/1	♂	1000	31	1	0	x
27	84/13/1	♂	1000	3	0	0	x
28	85/13/1	♀	Satellite group	3	0	1	x
29	86/13/1	♀	Satellite group	54	0	0	x
30	87/13/1	♀	Satellite group	28	0	0	x
31	88/13/1	♀	Satellite group	100	1	0	x
33	90/13/1	♀	Satellite group	3	0	0	x
34	91/13/1	♀	Satellite group	60	0	0	x
35	92/13/1	♀	Satellite group	48	0	0	x
36	93/13/1	♂	Satellite group	10	0	0	x
37	94/13/1	♂	Satellite group	37	0	0	x
38	95/13/1	♂	Satellite group	9	0	0	x
39	96/13/1	♂	Satellite group	4	1	1	x
40	97/13/1	♂	Satellite group	5	0	0	x
41	98/13/1	♂	Satellite group	5	0	2	x
42	99/13/1	♂	Satellite group	8	2	2	x
43	100/13/1	♂	Satellite group	9	0	0	x
44	101/13/1	♀	Control group	23	0	0	x
45	102/13/1	♀	Control group	19	0	0	x
46	103/13/1	♀	Control group	7	0	0	x
47	104/13/1	♀	Control group	25	0	0	x
48	105/13/1	♀	Control group	9	2	1	x
49	106/13/1	♀	Control group	3	1	0	x
50	107/13/1	♀	Control group	95	0	0	x
51	108/13/1	♀	Control group	6	1	0	x
52	109/13/1	♂	Control group	4	0	0	x
53	110/13/1	♂	Control group	5	0	0	x
54	111/13/1	♂	Control group	5	0	0	x
55	112/13/1	♂	Control group	27	0	0	x
56	113/13/1	♂	Control group	20	0	0	x
57	114/13/1	♂	Control group	10	0	0	x
58	115/13/1	♂	Control group	10	0	0	x
59	116/13/1	♂	Control group	20	0	0	x

Ordinal no	Number of animal	Sex	Dose [mg/kg bw]	Crossing time [s]	Slip		Entrance to the box
					Left	Right	
60	85/13/1	♀	Satellite group after 2 w.	22	0	0	x
61	86/13/1	♀	Satellite group after 2 w.	34	0	0	x
62	87/13/1	♀	Satellite group after 2 w.	27	0	0	x
63	88/13/1	♀	Satellite group after 2 w.	36	0	0	x
64	89/13/1	♀	Satellite group after 2 w.	10	1	1	x
65	90/13/1	♀	Satellite group after 2 w.	3	0	2	x
66	91/13/1	♀	Satellite group after 2 w.	20	0	0	x
67	92/13/1	♀	Satellite group after 2 w.	5	0	0	x
68	93/13/1	♂	Satellite group after 2 w.	22	1	1	x
69	94/13/1	♂	Satellite group after 2 w.	14	0	0	x
70	95/13/1	♂	Satellite group after 2 w.	20	0	0	x
71	96/13/1	♂	Satellite group after 2 w.	8	1	0	x
72	97/13/1	♂	Satellite group after 2 w.	15	0	0	x
73	98/13/1	♂	Satellite group after 2 w.	30	1	1	x
74	99/13/1	♂	Satellite group after 2 w.	8	0	0	x
75	100/13/1	♂	Satellite group after 2 w.	13	0	1	x

X – animal enters to the box
w - weeks

The measured crossing time, gait - number of slips, and the fact that the animal entered the box or not, were not significantly different in the experimental groups of females and males as well as in the satellite group of males and females in relation to the control group. Moreover, there were no differences in responces of males and females at dose 1000 mg/kg b.w. of iron pyrophosphate in the range of registered parameters.

Response to pain:

Pain in laboratory animals can occur in every experiment. The pain can be caused by experimental procedures, inflammatory reactions, due to unexpected toxicity or accident. Acute pain is signaled, among others, by licking painful area, assuming position that protects sore spot, scratching or biting aching body parts, abnormal posture, vocalization, anxiety or lying position. Chronic pain, on the other hand, leads to a reduction in activity, licking painful part, oliguria or dysuria (urine output was not evaluated) and often reduced food intake and body weight gain. The study was conducted by squeezing the tip of the tail with forceps and observation of vocalization, defensive moves or escape of the animal. Rapid escape reflex or

vocalization after crimping forceps at the tip of the tail (Kocher forceps clamped on the first security notch) were recognized as correct responses. Excessive reaction (hypersensitivity) manifested in violent defensive reaction under light crimping of the forceps. The results of the study are presented in Table 9.

Table 9. Response to pain

Number of animal	Response to stimuli
55/13/1	Correct reaction
56/13/1	Correct reaction
57/13/1	Correct reaction
58/13/1	Correct reaction
60/13/1	Correct reaction
61/13/1	Correct reaction
62/13/1	Correct reaction
63/13/1	Correct reaction
64/13/1	Correct reaction
65/13/1	Correct reaction
66/13/1	Correct reaction
67/13/1	Correct reaction
68/13/1	Correct reaction
69/13/1	Small oversensitivity
71/13/1	Correct reaction
72/13/1	Correct reaction
73/13/1	Correct reaction
74/13/1	Correct reaction
75/13/1	Correct reaction
76/13/1	Correct reaction
78/13/1	Correct reaction
79/13/1	Correct reaction
80/13/1	Correct reaction
81/13/1	Correct reaction
82/13/1	Correct reaction
83/13/1	Correct reaction
84/13/1	Correct reaction
85/13/1	Correct reaction
86/13/1	Correct reaction
87/13/1	Correct reaction
88/13/1	Correct reaction
90/13/1	Correct reaction
91/13/1	Correct reaction
92/13/1	Correct reaction
93/13/1	Correct reaction
94/13/1	Correct reaction
95/13/1	Correct reaction
96/13/1	Correct reaction
97/13/1	Correct reaction
98/13/1	Correct reaction
99/13/1	Correct reaction
100/13/1	Correct reaction
101/13/1	Correct reaction
102/13/1	Correct reaction
103/13/1	Correct reaction
104/13/1	Correct reaction
105/13/1	Correct reaction

Number of animal	Response to stimuli
106/13/1	Correct reaction
107/13/1	Correct reaction
108/13/1	Correct reaction
109/13/1	Correct reaction
110/13/1	Correct reaction
111/13/1	Correct reaction
112/13/1	Correct reaction
113/13/1	Correct reaction
114/13/1	Correct reaction
115/13/1	Correct reaction
116/13/1	Correct reaction

All examined animals react correctly, while animal 69/13/1 showed only slight signs of stimulation, hence based on a small response in one individual can not be demonstrated that the symptom was caused by the action of iron pyrophosphate.

Response to touch:

Rats in response to touch of the skin usually react with a movement directed toward the spot where the body was affected. The test was carried out using a wooden rod with a blunt end of 1 cm diameter. Animal's move toward the touched spot or stopping during the movement with observation of the situation were recognized as correct responses. Escape of the animal away from the wooden tool after touching was treated as a slight oversensitivity (pace of accelerated gait). Additionally, reactions to touching of animal's abdominal parts with the abovementioned tool was assayed, with absence of pain or irritation in a defensive reaction of the animal recognized as a normal reaction. The results of the study are presented in Table 10.

Table 10. Response to touch

Number of animal	Response to stimuli
55/13/1	Correct reaction
56/13/1	Correct reaction
57/13/1	Correct reaction
58/13/1	Correct reaction
60/13/1	Correct reaction
61/13/1	Correct reaction
62/13/1	Correct reaction
63/13/1	Correct reaction
64/13/1	Correct reaction
65/13/1	Correct reaction
66/13/1	Correct reaction
67/13/1	Correct reaction
68/13/1	Correct reaction
69/13/1	Small oversensitivity
71/13/1	Correct reaction
72/13/1	Correct reaction
73/13/1	Correct reaction
74/13/1	Correct reaction
75/13/1	Correct reaction
76/13/1	Correct reaction
78/13/1	Correct reaction

Number of animal	Response to stimuli
79/13/1	Correct reaction
80/13/1	Correct reaction
81/13/1	Correct reaction
82/13/1	Correct reaction
83/13/1	Correct reaction
84/13/1	Correct reaction
85/13/1	Correct reaction
86/13/1	Correct reaction
87/13/1	Correct reaction
88/13/1	Correct reaction
90/13/1	Correct reaction
91/13/1	Correct reaction
92/13/1	Correct reaction
93/13/1	Correct reaction
94/13/1	Correct reaction
95/13/1	Correct reaction
96/13/1	Correct reaction
97/13/1	Correct reaction
98/13/1	Correct reaction
99/13/1	Correct reaction
100/13/1	Correct reaction
101/13/1	Correct reaction
102/13/1	Correct reaction
103/13/1	Correct reaction
104/13/1	Correct reaction
105/13/1	Correct reaction
106/13/1	Correct reaction
107/13/1	Correct reaction
108/13/1	Correct reaction
109/13/1	Correct reaction
110/13/1	Correct reaction
111/13/1	Correct reaction
112/13/1	Correct reaction
113/13/1	Correct reaction
114/13/1	Correct reaction
115/13/1	Correct reaction
116/13/1	Correct reaction

All examined animals react correctly, while animal 69/13/1 showed only slight signs of stimulation, hence based on a small response in one individual can not be demonstrated that the symptom was caused by the action of iron pyrophosphate.

The novel object recognition test:

The next test was to put the new object in a cage in the form of a coloured plastic ball. After examining the animal a new object and the loss of his interests were placed in a cage next new object and observed recurrence of interest in the subject. The correct response is to show interest in the animal new subject, the study of the subject over a period of several seconds. The results of the novel object recognition test are presented in Table 11.

Table 11. The novel object recognition test

Number of animal	Response to stimuli
55/13/1	Correct reaction
56/13/1	Correct reaction
57/13/1	Correct reaction
58/13/1	Correct reaction
60/13/1	Correct reaction
61/13/1	Correct reaction
62/13/1	Correct reaction
63/13/1	Correct reaction
64/13/1	Correct reaction
65/13/1	Correct reaction
66/13/1	Correct reaction
67/13/1	Correct reaction
68/13/1	Correct reaction
69/13/1	Correct reaction
71/13/1	Correct reaction
72/13/1	Correct reaction
73/13/1	Correct reaction
74/13/1	Correct reaction
75/13/1	Correct reaction
76/13/1	Correct reaction
78/13/1	Correct reaction
79/13/1	Correct reaction
80/13/1	Correct reaction
81/13/1	Correct reaction
82/13/1	Correct reaction
83/13/1	Correct reaction
84/13/1	Correct reaction
85/13/1	Correct reaction
86/13/1	Correct reaction
87/13/1	Correct reaction
88/13/1	Correct reaction
90/13/1	Correct reaction
91/13/1	Correct reaction
92/13/1	Correct reaction
93/13/1	Correct reaction
94/13/1	Correct reaction
95/13/1	Correct reaction
96/13/1	Correct reaction
97/13/1	Correct reaction
98/13/1	Correct reaction
99/13/1	Correct reaction
100/13/1	Correct reaction
101/13/1	Correct reaction
102/13/1	Correct reaction
103/13/1	Correct reaction
104/13/1	Correct reaction

Number of animal	Response to stimuli
105/13/1	Correct reaction
106/13/1	Correct reaction
107/13/1	Correct reaction
108/13/1	Correct reaction
109/13/1	Correct reaction
110/13/1	Correct reaction
111/13/1	Correct reaction
112/13/1	Correct reaction
113/13/1	Correct reaction
114/13/1	Correct reaction
115/13/1	Correct reaction
116/13/1	Correct reaction

All examined animals react correctly in the novel object recognition test.

Response to sound:

The test was part of the daily observation of the animals. The test consists of hitting a finger / object in the cage and observing animal behavior. Animals should respond clearly to hitting the cage through the motions ear pinnae. The results of the study are presented in Table 12.

Table 12. Response to sound

Number of animal	Response to stimuli
55/13/1	Correct reaction
56/13/1	Correct reaction
57/13/1	Correct reaction
58/13/1	Correct reaction
60/13/1	Correct reaction
61/13/1	Correct reaction
62/13/1	Correct reaction
63/13/1	Correct reaction
64/13/1	Correct reaction
65/13/1	Correct reaction
66/13/1	Correct reaction
67/13/1	Correct reaction
68/13/1	Correct reaction
69/13/1	Correct reaction
71/13/1	Correct reaction
72/13/1	Correct reaction
73/13/1	Correct reaction
74/13/1	Correct reaction
75/13/1	Correct reaction
76/13/1	Correct reaction
78/13/1	Correct reaction
79/13/1	Correct reaction
80/13/1	Correct reaction
81/13/1	Correct reaction
82/13/1	Correct reaction
83/13/1	Correct reaction
84/13/1	Correct reaction

Number of animal	Response to stimuli
85/13/1	Correct reaction
86/13/1	Correct reaction
87/13/1	Correct reaction
88/13/1	Correct reaction
90/13/1	Correct reaction
91/13/1	Correct reaction
92/13/1	Correct reaction
93/13/1	Correct reaction
94/13/1	Correct reaction
95/13/1	Correct reaction
96/13/1	Correct reaction
97/13/1	Correct reaction
98/13/1	Correct reaction
99/13/1	Correct reaction
100/13/1	Correct reaction
101/13/1	Correct reaction
102/13/1	Correct reaction
103/13/1	Correct reaction
104/13/1	Correct reaction
105/13/1	Correct reaction
106/13/1	Correct reaction
107/13/1	Correct reaction
108/13/1	Correct reaction
109/13/1	Correct reaction
110/13/1	Correct reaction
111/13/1	Correct reaction
112/13/1	Correct reaction
113/13/1	Correct reaction
114/13/1	Correct reaction
115/13/1	Correct reaction
116/13/1	Correct reaction

All examined animals react correctly.

Response to light

The study was performed using an ophthalmoscope. Rated reaction of the pupilla to light the lamp ophthalmoscope. Checked whether pupills have the same size and if their edges are regular, was also studied reaction of the pupills to light. The proper pupilla was equal, round and equally reactive to light in the form of constriction diameter. Results of the reaction to light eyes of animals show Table 13.

Table 13. Response to light

Number of animal	Response to stimuli
55/13/1	Correct reaction
56/13/1	Correct reaction
57/13/1	Correct reaction
58/13/1	Correct reaction
60/13/1	Correct reaction
61/13/1	Correct reaction

Number of animal	Response to stimuli
62/13/1	Correct reaction
63/13/1	Correct reaction
64/13/1	Correct reaction
65/13/1	Correct reaction
66/13/1	Correct reaction
67/13/1	Correct reaction
68/13/1	Correct reaction
69/13/1	Correct reaction
71/13/1	Correct reaction
72/13/1	Correct reaction
73/13/1	Correct reaction
74/13/1	Correct reaction
75/13/1	Correct reaction
76/13/1	Correct reaction
78/13/1	Correct reaction
79/13/1	Correct reaction
80/13/1	Correct reaction
81/13/1	Correct reaction
82/13/1	Correct reaction
83/13/1	Correct reaction
84/13/1	Correct reaction
85/13/1	Correct reaction
86/13/1	Correct reaction
87/13/1	Correct reaction
88/13/1	Correct reaction
90/13/1	Correct reaction
91/13/1	Correct reaction
92/13/1	Correct reaction
93/13/1	Correct reaction
94/13/1	Correct reaction
95/13/1	Correct reaction
96/13/1	Correct reaction
97/13/1	Correct reaction
98/13/1	Correct reaction
99/13/1	Correct reaction
100/13/1	Correct reaction
101/13/1	Correct reaction
102/13/1	Correct reaction
103/13/1	Correct reaction
104/13/1	Correct reaction
105/13/1	Correct reaction
106/13/1	Correct reaction
107/13/1	Correct reaction
108/13/1	Correct reaction
109/13/1	Correct reaction
110/13/1	Correct reaction
111/13/1	Correct reaction
112/13/1	Correct reaction
113/13/1	Correct reaction

Number of animal	Response to stimuli
114/13/1	Correct reaction
115/13/1	Correct reaction
116/13/1	Correct reaction

The eyes of all animals react correctly to light.

Macroscopic examination

Macroscopic examination included verification of outer surface of the body, all natural body orifice, skull cavity, thoracic and abdominal cavity with content Results of macroscopic examination presents Table 14.

Table 14. Results of macroscopic examination

Animal No	Sex	Dose [mg/kg bw]/ Group	Outer surface of the body	Natural body orifice	Skull cavity	Thoracic and abdominal cavity with content
55/13/1	♀	1000	No changes	No changes	No changes	No changes
56/13/1	♀	1000	No changes	No changes	No changes	No changes
57/13/1	♀	1000	No changes	No changes	No changes	No changes
58/13/1	♀	1000	No changes	No changes	No changes	No changes
59/13/1	♀	1000	No changes	No changes	No changes	Animal was euthanized 25.08.2013 because of the occurance of malignancy which impeded test material gavage. The malignancy was noted 27.07.2013. The histopathological examination demonstrated inflammatory character - abscess – not caused by iron pyrophosphate
60/13/1	♀	1000	No changes	No changes	No changes	No changes
61/13/1	♀	1000	No changes	No changes	No changes	No changes
62/13/1	♀	1000	No changes	No changes	No changes	No changes
63/13/1	♀	1000	No changes	No changes	No changes	No changes
64/13/1	♀	1000	No changes	No changes	No changes	No changes
65/13/1	♀	1000	No changes	No changes	No changes	No changes
66/13/1	♀	1000	No changes	No changes	No changes	No changes
67/13/1	♀	1000	No changes	No changes	No changes	No changes
68/13/1	♀	1000	No changes	No changes	No changes	No changes
69/13/1	♀	1000	No changes	No changes	No changes	No changes
70/13/1	♂	1000	No changes	No changes	No changes	No changes
71/13/1	♂	1000	No changes	No changes	No changes	No changes
72/13/1	♂	1000	No changes	No changes	No changes	No changes
73/13/1	♂	1000	No changes	No changes	No changes	No changes
74/13/1	♂	1000	No changes	No changes	No changes	No changes
75/13/1	♂	1000	No changes	No changes	No changes	No changes
76/13/1	♂	1000	No changes	No changes	No changes	No changes
77/13/1	♂	1000	No changes	No changes	No changes	No changes
78/13/1	♂	1000	No changes	No changes	No changes	No changes
79/13/1	♂	1000	No changes	No changes	No changes	No changes
80/13/1	♂	1000	No changes	No changes	No changes	No changes
81/13/1	♂	1000	No changes	No changes	No changes	No changes

Animal No	Sex	Dose [mg/kg bw]/ Group	Outer surface of the body	Natural body orifice	Skull cavity	Thoracic and abdominal cavity with content
82/13/1	♂	1000	No changes	No changes	No changes	No changes
83/13/1	♂	1000	No changes	No changes	No changes	No changes
84/13/1	♂	1000	No changes	No changes	No changes	No changes
85/13/1	♀	satellit	No changes	No changes	No changes	No changes
86/13/1	♀	satellit	No changes	No changes	No changes	No changes
87/13/1	♀	satellit	No changes	No changes	No changes	No changes
88/13/1	♀	satellit	No changes	No changes	No changes	No changes
89/13/1	♀	satellit	No changes	No changes	No changes	No changes
90/13/1	♀	satellit	No changes	No changes	No changes	No changes
91/13/1	♀	satellit	No changes	No changes	No changes	No changes
92/13/1	♀	satellit	No changes	No changes	No changes	No changes
93/13/1	♂	satellit	No changes	No changes	No changes	No changes
94/13/1	♂	satellit	No changes	No changes	No changes	No changes
95/13/1	♂	satellit	No changes	No changes	No changes	No changes
96/13/1	♂	satellit	No changes	No changes	No changes	No changes
97/13/1	♂	satellit	No changes	No changes	No changes	No changes
98/13/1	♂	satellit	No changes	No changes	No changes	No changes
99/13/1	♂	satellit	No changes	No changes	No changes	No changes
100/13/	♂	satellit	No changes	No changes	No changes	No changes
101/13/	♀	Contro	No changes	No changes	No changes	No changes
102/13/	♀	Contro	No changes	No changes	No changes	No changes
103/13/	♀	Contro	No changes	No changes	No changes	No changes
104/13/	♀	Contro	No changes	No changes	No changes	No changes
105/13/	♀	Contro	No changes	No changes	No changes	No changes
106/13/	♀	Contro	No changes	No changes	No changes	No changes
107/13/	♀	Contro	No changes	No changes	No changes	No changes
108/13/	♀	Contro	No changes	No changes	No changes	No changes
109/13/	♂	Contro	No changes	No changes	No changes	No changes
110/13/	♂	Contro	No changes	No changes	No changes	No changes
111/13/	♂	Contro	No changes	No changes	No changes	No changes
112/13/	♂	Contro	No changes	No changes	No changes	No changes
113/13/	♂	Contro	No changes	No changes	No changes	No changes
114/13/	♂	Contro	No changes	No changes	No changes	No changes
115/13/	♂	Contro	No changes	No changes	No changes	No changes
116/13/	♂	Contro	No changes	No changes	No changes	No changes

Table 15. Weights of organs collected from animals from all test groups [g].

Animal No.	Sex	Dose [mg/kg bw]	All Gross lesions	Brain	Spinal cored	Eye with optic nerve	Stomach + duodenum	small and gross intestines	Liver	Pancreas	salivary glands	kidneys with ureter + adrenals	Spleen	thyroid with parathyroids + larynx + trachea and lungs + heart + aorta + thymus	gonads (testis and ovaries) + auxiliary genitalia + vagina + bladder	Mammary gland	lymph nodes (number)	peripheral nerve along with muscle femur with the knee joint Animal	Tongue	Body weight in Gross necropsy day [g] 55
55/13/1	♀	**1000**	absence	1,93	1,44	0,14	2,84	1,06	6,30	0,79	0,04	1,76	0,47	3,40	2,54	0,40	2	8,74	0,84	256
56/13/1	♀	**1000**	absence	1,80	1,16	0,15	3,09	0,71	6,33	0,50	0,05	1,69	0,47	2,95	2,39	0,28	2	10,02	0,55	226
57/13/1	♀	**1000**	absence	1,79	1,53	0,18	2,46	0,82	5,54	0,75	0,05	1,64	0,41	2,99	2,48	0,48	2	8,87	0,79	204
58/13/1	♀	**1000**	absence	1,73	1,60	0,17	3,26	1,10	5,16	0,61	0,04	1,73	0,37	2,92	1,81	0,63	2	9,68	1,07	219
60/13/1	♀	**1000**	absence	1,84	1,40	0,18	2,10	1,04	6,49	0,60	0,06	1,75	0,47	3,00	2,53	0,59	2	6,62	0,65	232
61/13/1	♀	**1000**	absence	1,88	1,46	0,15	1,96	0,98	5,79	0,49	0,08	1,80	0,41	3,16	2,77	0,35	2	9,66	0,81	244
62/13/1	♀	**1000**	absence	1,83	1,49	0,16	2,25	0,85	6,70	0,83	0,07	2,14	0,62	3,41	2,73	0,20	2	6,25	0,60	263
63/13/1	♀	**1000**	absence	2,17	1,54	0,21	3,33	1,35	5,88	0,61	0,09	2,12	0,55	3,33	3,51	0,25	2	10,02	0,80	253
64/13/1	♀	**1000**	absence	1,70	1,13	0,19	3,13	0,67	5,85	0,50	0,06	1,84	0,56	3,27	2,23	0,69	2	10,14	0,81	230
65/13/1	♀	**1000**	absence	1,89	1,77	0,18	3,51	0,85	5,47	0,75	0,05	1,58	0,46	3,32	2,60	0,44	2	6,22	0,53	218
66/13/1	♀	**1000**	absence	1,84	1,55	0,17	2,50	1,25	5,73	0,71	0,07	1,74	0,43	2,95	2,12	0,45	2	7,69	0,65	212
67/13/1	♀	**1000**	absence	1,90	1,42	0,19	1,90	1,56	5,23	0,67	0,05	1,76	0,43	2,92	3,87	0,53	2	6,73	0,72	210
68/13/1	♀	**1000**	absence	2,03	1,44	0,21	3,75	0,95	5,77	0,69	0,07	1,79	0,51	3,27	2,07	0,51	2	9,36	0,98	251
69/13/1	♀	**1000**	absence	1,84	1,67	0,16	3,98	0,79	5,49	0,73	0,05	2,03	0,42	2,93	3,29	0,55	2	8,24	0,70	222
71/13/1	♂	**1000**	absence	2,05	1,60	0,21	3,99	1,20	8,87	0,76	0,07	2,47	0,70	3,99	12,64	-	2	13,65	0,93	335
72/13/1	♂	**1000**	absence	1,93	1,88	0,23	3,67	1,43	11,78	1,28	0,08	3,81	0,70	5,22	15,65	-	2	15,52	0,98	447
73/13/1	♂	**1000**	absence	2,09	1,96	0,18	6,35	1,59	9,86	0,92	0,05	2,57	0,60	3,84	10,55	-	2	13,89	0,61	340

74/13/1	♂	**1000**	absence	2,12	2,33	0,21	3,11	2,18	12,82	1,11	0,11	3,68	0,70	4,89	15,59	-	2	14,32	0,57	423
75/13/1	♂	**1000**	absence	2,02	1,65	0,22	4,04	1,01	12,80	1,34	0,06	3,76	0,74	4,72	15,70	-	2	12,81	1,06	448
76/13/1	♂	**1000**	absence	1,99	1,80	0,20	6,42	1,57	10,77	1,08	0,08	3,53	0,74	4,73	17,42	-	2	16,79	1,15	427
78/13/1	♂	**1000**	absence	2,08	1,90	0,21	3,32	1,96	12,74	0,99	0,11	4,13	0,87	5,24	16,71	-	2	16,43	1,02	453
79/13/1	♂	**1000**	absence	1,97	1,67	0,23	3,70	1,06	9,16	0,84	0,15	2,85	0,57	3,61	13,06	-	2	12,16	0,56	338
80/13/1	♂	**1000**	absence	1,96	1,48	0,21	3,60	2,09	10,18	1,21	0,09	3,,14	0,67	4,17	14,71	-	2	12,25	0,99	375
81/13/1	♂	**1000**	absence	2,18	1,58	0,22	5,21	1,27	10,54	1,07	0,13	3,65	0,68	4,50	16,81	-	2	16,29	0,99	420
82/13/1	♂	**1000**	absence	2,15	2,10	0,25	2,97	1,87	10,86	0,91	0,13	3,66	0,69	4,53	15,10	-	2	16,56	1,22	420
83/13/1	♂	**1000**	absence	2,03	2,23	0,24	2,74	1,87	11,32	1,22	0,10	3,18	0,78	4,93	14,20	-	2	14,70	1,01	418
84/13/1	♂	**1000**	absence	2,17	1,86	0,22	2,62	1,44	11,17	1,06	0,11	3,23	0,74	4,87	14,30	-	2	16,59	1,08	408
85/13/1	♀	**sat.**	absence	1,78	1,70	0,16	3,26	1,48	5,63	0,66	0,04	1,97	0,54	2,93	3,02	0,40	2	9,58	0,65	225
86/13/1	♀	**sat.**	absence	1,96	1,52	0,23	3,02	1,14	7,01	0,70	0,05	2,58	0,55	3,45	3,08	0,40	2	9,63	0,92	260
87/13/1	♀	**sat.**	absence	1,78	1,87	0,21	2,75	1,22	5,15	0,65	0,05	1,59	0,50	3,19	2,89	0,52	2	8,78	0,68	209
88/13/1	♀	**sat.**	absence	1,83	1,27	0,19	3,86	0,87	5,56	0,55	0,08	1,70	0,43	2,90	2,71	0,27	2	8,66	0,66	202
89/13/1	♀	**sat.**	absence	1,83	1,08	0,15	2,51	0,98	5,50	0,52	0,08	1,94	0,32	2,66	3,13	0,46	2	8,98	0,70	210
90/13/1	♀	**sat.**	absence	1,93	1,46	0,18	2,19	1,63	7,20	0,78	0,09	1,91	0,55	3,32	2,97	1,48	2	12,30	0,99	240
91/13/1	♀	**sat.**	absence	1,95	1,31	0,21	2,62	1,72	6,49	0,79	0,08	2,19	0,46	3,28	3,39	0,49	2	13,52	0,89	248
92/13/1	♀	**sat.**	absence	1,96	1,84	0,17	4,00	1,33	7,11	0,79	0,06	2,33	0,55	3,51	2,50	0,62	2	13,55	1,12	280
93/13/1	♂	**sat.**	absence	2,14	1,51	0,23	3,54	2,70	11,68	0,95	0,10	3,47	0,73	4,44	17,38	-	2	17,38	1,55	410
94/13/1	♂	**sat.**	absence	2,09	1,97	0,21	3,25	2,15	13,14	1,13	0,17	3,99	0,83	5,04	17,57	-	2	16,15	1,03	443
95/13/1	♂	**sat.**	absence	2,01	2,14	0,21	3,10	2,09	13,66	0,10	0,12	3,99	0,78	4,93	18,24	-	2	19,47	1,09	454
96/13/1	♂	**sat.**	absence	2,01	2,24	0,27	3,06	1,73	10,73	1,05	0,13	3,47	0,75	4,79	15,85	-	2	19,97	1,03	421
97/13/1	♂	**sat.**	absence	2,19	1,60	0,21	2,84	1,14	12,78	1,18	0,11	4,11	0,69	5,00	18,95	-	2	16,64	0,91	436
98/13/1	♂	**sat.**	absence	1,89	1,89	0,21	3,72	1,91	12,82	1,00	0,14	4,75	0,80	5,37	23,96	-	2	14,12	0,93	457
99/13/1	♂	**sat.**	absence	1,91	1,43	0,21	2,85	2,11	10,93	1,13	0,19	3,26	0,86	5,25	11,57	-	2	16,48	0,97	402
100/13/1	♂	**sat.**	absence	2,04	1,67	0,17	2,31	1,88	11,00	1,52	0,16	3,22	0,64	4,62	15,98	-	2	15,27	1,02	396

101/13/1	♀	**cont**	absence	1,83	1,17	0,20	2,64	0,95	5,81	0,69	0,05	1,53	0,51	3,03	1,94	-	2	8,97	0,93	206
102/13/1	♀	**cont**	absence	1,87	1,11	0,18	2,58	1,24	7,11	0,70	0,04	1,75	0,50	3,38	2,16	0,47	2	5,71	0,97	216
103/13/1	♀	**cont**	absence	1,87	1,07	0,19	2,66	0,95	5,74	0,56	0,06	1,91	0,52	3,65	2,23	0,47	2	7,66	0,79	209
104/13/1	♀	**cont**	absence	1,90	1,07	0,16	2,52	0,96	6,02	0,74	0,07	1,97	0,45	3,33	3,27	0,41	2	12,52	0,73	257
105/13/1	♀	**cont**	absence	1,81	1,14	0,15	2,14	1,43	4,83	0,77	0,07	1,78	0,45	2,99	2,42	0,43	2	9,59	0,71	211
106/13/1	♀	**cont**	absence	1,78	1,48	0,14	2,57	1,12	5,71	0,78	0,05	1,82	0,51	3,00	2,10	0,42	2	9,90	0,72	226
107/13/1	♀	**cont**	absence	1,82	1,28	0,12	2,26	1,09	6,17	0,83	0,08	1,92	0,50	3,45	2,08	0,35	2	11,55	0,87	235
108/13/1	♀	**cont**	absence	1,79	1,28	0,17	2,92	1,78	4,83	0,61	0,08	1,31	0,47	3,16	1,92	0,41	2	9,15	0,68	202
109/13/1	♂	**cont**	absence	2,37	2,07	0,20	2,58	2,40	10,81	1,23	0,10	3,50	0,68	4,75	16,22	-	2	17,28	0,88	407
110/13/1	♂	**cont**	absence	2,13	1,59	0,20	3,62	2,49	12,85	0,75	0,15	3,61	0,74	5,50	17,10	-	2	16,64	1,22	422
111/13/1	♂	**cont**	absence	2,13	2,02	0,18	2,84	2,52	13,57	1,47	0,21	3,92	0,86	4,75	16,57	-	2	17,01	1,12	460
112/13/1	♂	**cont**	absence	1,98	2,38	0,24	3,46	2,00	13,99	0,99	0,20	4,18	0,85	4,96	19,62	-	2	15,28	0,93	454
113/13/1	♂	**cont**	absence	2,01	2,37	0,20	2,85	1,78	10,28	1,01	0,16	2,97	0,69	4,36	13,71	-	2	17,28	1,42	397
114/13/1	♂	**cont**	absence	2,13	2,28	0,26	2,93	2,82	12,19	0,82	0,14	3,42	0,73	4,49	14,33	-	2	12,62	1,07	390
115/13/1	♂	**cont**	absence	2,07	1,50	0,23	3,40	3,50	11,48	0,86	0,09	3,65	0,70	4,66	18,12	-	2	17,67	1,10	428
116/13/1	♂	**cont**	absence	2,13	1,74	0,14	2,84	2,59	11,82	0,97	0,12	3,68	0,70	4,68	17,41	-	2	17,15	1,08	435

sat - satellite group

cont - control group

Table 16. Average weights of all animals organs [g].

Sex	Dose [mg/kg]	Brain	Spinal cored	Eye with optic nerve	Stomach + duodenum	small and gross intestines	Liver	Pancreas	salivary glands	kidneys with ureter + adrenals	Spleen	thyroid with parathyroids + larynx + trachea and lungs + heart + aorta + thymus	gonads (testis and ovaries) + auxiliary genitalia + vagina + bladder	Mammary gland	lymph nodes (number)	peripheral nerve along with muscle Animal	Tongue
♀	**1000**	1,87	1,47	0,17	2,86	1,00	5,84	0,66	0,06	1,81	0,47	3,13	2,64	0,45	8,45	0,75	231,43
♂	**1000**	2,06	1,85	0,22	3,98	1,58	10,99	1,06	0,10	3,38	0,71	4,56	14,80		14,77	0,94	404,00
♀	**Satellite group**	1,88	1,51	0,19	3,03	1,30	6,21	0,68	0,07	2,03	0,49	3,16	2,96	0,58	10,63	0,83	234,25
♂	**Satellite group**	2,04	1,81	0,22	3,08	1,96	12,09	1,01	0,14	3,78	0,76	4,93	17,44		16,94	1,07	427,38
♀	**Control group**	1,83	1,20	0,16	2,54	1,19	5,78	0,71	0,06	1,75	0,49	3,25	2,27	0,42	9,38	0,80	220,25
♂	**Control group**	2,12	1,99	0,21	3,07	2,51	12,12	1,01	0,15	3,62	0,74	4,77	16,64		16,37	1,10	424,13
ratio of organ weight to body weight of animals																	
♀	**1000**	8,08E-03	6,35E-03	7,35E-04	1,24E-02	4,32E-03	2,52E-02	2,85E-03	2,59E-04	7,82E-03	2,03E-03	1,35E-02	1,14E-02	1,94E-03	3,65E-02	3,24E-03	231,43
♂	**1000**	5,10E-03	4,58E-03	5,45E-04	9,85E-03	3,91E-03	2,72E-02	2,62E-03	2,48E-04	8,37E-03	1,76E-03	1,13E-02	3,66E-02	0,00E+00	3,66E-02	2,33E-03	404,00
♀	**gr. sat.Satellite group**	8,03E-03	6,45E-03	8,11E-04	1,29E-02	5,55E-03	2,65E-02	2,90E-03	2,99E-04	8,67E-03	2,09E-03	1,35E-02	1,26E-02	2,48E-03	4,54E-02	3,54E-03	234,25

♂	**Satellite group**	4,77E-03	4,24E-03	5,15E-04	7,21E-03	4,59E-03	2,83E-02	2,36E-03	3,28E-04	8,84E-03	1,78E-03	1,15E-02	4,08E-02	0,00E+00	3,96E-02	2,50E-03	427,38
♀	**Control group**	8,31E-03	5,45E-03	7,26E-04	1,15E-02	5,40E-03	2,62E-02	3,22E-03	2,72E-04	7,95E-03	2,22E-03	1,48E-02	1,03E-02	1,91E-03	4,26E-02	3,63E-03	220,25
♂	**Control group**	5,00E-03	4,69E-03	4,95E-04	7,24E-03	5,92E-03	2,86E-02	2,38E-03	3,54E-04	8,54E-03	1,74E-03	1,12E-02	3,92E-02	0,00E+00	3,86E-02	2,59E-03	424,13

Histopathological examination

Table 17. Results of histopathological examination of organs

Organ	Observed histopathological changes
1. All gross pathological changes	Not collected – non gross pathomorphologic lesions.
2. Brain	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
3. Spinal cord	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
4. Eyeball with optic nerve	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
5. Stomach	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
6. Esophagus	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
7. Small and gross intestines (including Peyer's patches)	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
8. Salivary glands	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
9. Liver	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
10. Pancreas	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
11. Kidneys with ureters	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
12. Adrenal glands	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
13. Spleen	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
14. Heart	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
15. Aorta	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
16. Thymus	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
17. Thyroid with parathyroid	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
18. Larynx, trachea and lungs	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
19. Gonads (testes, ovaries)	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
20. Accessory sex organs (uterus, cervix, epididymis, prostate and seminal vesicles and the coagulation glands)	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.

Organ	Observed histopathological changes
21. Vagina	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
22. Mammary gland	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
23. Urinary bladder	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
24. Lymph nodes (mandibulary and lumbag)	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
25. Peripheral nerve (sciatic or tibial), along with muscle	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
26. Bone marrow	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
27. Femur with the knee joint	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.
28. Tongue	Lack of pathomorphologic lesions in 1000 mg/kg bw group and control group; Correct structure of the organ.

The histopatological examination show no pathological changes observed in all the examined organs (normal structure of the organ). Due to correct the results of the study in the group receiving ferric pyrophosphate and control group, no histopathological examination was performed in a satellite group of animals, which was used reversibility observation changes induced toxicity.

Haematology and clinical biochemistry

The results of hematological determinations are shown in Table 18 (individual values) and Tables 18A (fenales) and 18 B (males) (mean values ±SD). Table 19 show the available reference values for the studied parameters. In terms of values analyzed statistically significant differences were found:

• WBC (leukocytes) - Statistically significantly higher in females in the study group in comparison to the control group.

• RBC (red blood cell count) - Statistically significantly higher in male and female test group in relation to the control group .

• HCT (hematocrit) - A statistically significantly higher in male and female test group in relation to the control group.

• Reticulocytes - Statistically significantly higher in females in the study group with respect to the control group.

The above- mentioned changes, although it showed a statistically significant difference, however, do not exceed the reference values and did not correlate with other clinical symptoms. Based on the analysis results can not provide a clear thesis on the impact in this area of iron pyrophosphate.

Table 18. Results of haematology (individual values for each animal)

Animal No	Sex	Dose [mg/kg]	Morphology									Coagulogy				
			WBC [thous./µl]	RBC [mln/µl]	HGB [g/dl]	HCT [%]	MCV [fl]	MCH [pg]	MCHC [g/dl]	PLT [tys./µl]	Retikulocytes [part-per-thousand]	APTT	INR	PT	WSK. PT	TT
55/13/1	♀	1000	5,9	7,54	14,5	40,1	53	19,2	36,2	861	31	18,4	0,84	11	120,4	hemolysis
56/13/1	♀	1000	14,2	7,82	15,3	44,3	57	19,6	34,5	829	53	18,2	0,83	11	121,4	hemolysis
57/13/1	♀	1000	8,2	7,56	15	43,1	57	19,9	35	821	30	19	0,81	11	125,9	hemolysis
58/13/1	♀	1000	5,3	8,2	15,3	44,9	55	18,7	34,1	822	b.m.	16,9	0,84	11	120,4	b.m.
60/13/1	♀	1000	4,9	7,25	14,3	39,9	55	19,8	35,9	805	51	22,3	0,79	11	129,5	b.m.
61/13/1	♀	1000	5,4	7,44	15,3	42,3	57	20,5	36,1	874	47	19,8	0,86	12	117,2	b.m.
62/13/1	♀	1000	9,3	9,71	19,7	56	58	20,3	35,2	823	37	17,2	0,83	11	122,5	b.m.
63/13/1	♀	1000	26,8	9,81	20,4	53,6	55	20,7	38	958	34	18,8	0,83	11	122,5	33,3
64/13/1	♀	1000	4,8	7,42	14,6	39,7	54	19,6	36,7	696	35	16,4	0,81	11	127,1	b.m.
65/13/1	♀	1000	10,4	7,64	15,6	41,9	55	20,4	37,3	817	36	16,3	0,83	11	122,5	b.m.
66/13/1	♀	1000	9,9	9,9	19,6	54,9	55	19,6	35,7	804	31	17,8	0,83	11,1	122,5	hemolysis
67/13/1	♀	1000	4,9	7,71	15,2	41,6	54	19,7	36,5	868	40	16,4	0,81	11	124,8	b.m.
68/13/1	♀	1000	6,6	7,57	15,1	41,5	55	20	36,5	832	49	18,8	0,81	11	127,1	hemolysis
69/13/1	♀	1000	5,0	7,78	15,4	42,5	55	19,8	36,3	829	34	16,5	0,83	11	122,5	b.m.
71/13/1	♂	1000	6,8	8,21	14,9	42,1	51	18,1	35,3	934	14	11,6	0,89	12	114,3	24,8
72/13/1	♂	1000	6,6	8,13	15,8	43,6	54	19,4	36,2	886	29	18,8	0,91	12	110,6	21,8
73/13/1	♂	1000	5,9	9,14	17,1	47,8	52	18,7	35,9	740	17	19,9	0,9	12	112,4	b.m.
74/13/1	♂	1000	6,2	8,39	15,6	44,6	53	18,6	35	901	32	17,4	0,89	12	114,3	25,7
75/13/1	♂	1000	6,8	8,7	16,1	46,4	53	18,5	34,7	945	31	18,4	0,9	12	112,4	23,8

76/13/1	♂	1000	4,9	7,93	15,3	42,2	53	19,3	36,3	790	25	18,1	0,89	12	113,3	24,6
78/13/1	♂	1000	5,6	8,02	15,5	42,9	53	19,4	36,2	864	30	16,4	0,86	12	118,3	b.m.
79/13/1	♂	1000	4,8	8,61	16,4	46,4	54	19	35,3	755	28	16,4	0,85	11	119,3	29,1
80/13/1	♂	1000	4,5	8,4	16,1	44,7	53	19	35,7	792	14	16,4	0,86	12	118,3	27,4
81/13/1	♂	1000	4,7	7,79	15	41,4	53	19,3	36,4	801	15	16,2	0,87	12	116,2	27,8
82/13/1	♂	1000	5,2	8,59	16,4	45	52	19,1	36,5	833	21	b.m.	b.m.	b.m.	b.m.	b.m.
83/13/1	♂	1000	4,3	8,22	15,6	42,3	51	18,9	36,8	771	31	16,9	0,85	11	119,3	32,1
84/13/1	♂	1000	5,7	8,53	16,6	45,7	54	19,4	36,3	836	14	16,8	0,88	12	115,3	b.m.
85/13/1	♀	satellite group	6,7	7,21	15	40,9	57	20,8	36,8	832	15	19,8	b.m.	10	b.m.	23
86/13/1	♀	satellite group	3,3	5,41	11,3	30,2	56	20,9	37,5	649	23	18	b.m.	10	b.m.	24,1
87/13/1	♀	satellite group	5	8,72	17,8	47,6	55	20,4	37,4	596	56	18,3	b.m.	10	b.m.	22,6
88/13/1	♀	satellite group	5,7	6,73	14,2	38,1	57	21,1	37,3	674	42	17,8	b.m.	10	b.m.	21,1
89/13/1	♀	satellite group	2,3	8,88	16,8	48,6	55	18,9	34,5	401	57	18,8	b.m.	10	b.m.	26,7
90/13/1	♀	satellite group	5,1	9,31	16,9	49,4	53	18,2	34,2	591	44	16,4	b.m.	10	b.m.	25,7
91/13/1	♀	satellite group	3,2	8,18	15,9	46,3	57	19,5	34,4	881	45	16,6	b.m.	10	b.m.	22,1
92/13/1	♀	satellite group	5	7,95	15,2	43,9	55	19,1	34,6	844	76	20,5	b.m.	10	b.m.	23,5
93/13/1	♂	satellite group	4	8,21	16,1	44,1	54	19,6	36,5	900	62	16,9	b.m.	10	b.m.	24,6
94/13/1	♂	satellite group	6,4	7,62	15	40,8	53	19,7	36,8	733	82	16,5	b.m.	10	b.m.	23,6
95/13/1	♂	satellite group	4,8	6,43	12,4	34,4	53	19,3	36	655	42	18	b.m.	10	b.m.	27,1
96/13/1	♂	satellite group	3,3	10,6	20	57,6	55	19	34,7	193	29	17,7	b.m.	11	b.m.	24,5

97/13/1	♂	satellite group	4,4	8,25	15,2	42,9	52	18,4	35,4	688	24	16,9	b.m.	10	b.m.	28,8
98/13/1	♂	satellite group	6,1	7,47	14,6	40,8	55	19,6	35,9	784	41	21,3	b.m.	10	b.m.	29,4
99/13/1	♂	satellite group	6	9,41	17,3	48,8	52	18,4	35,6	593	62	20,8	b.m.	10	b.m.	26,9
100/13/1	♂	satellite group	4,2	7,8	14,8	40,2	52	19	36,8	464	43	18,8	b.m.	10	b.m.	27,7
101/13/1	♀	control group	7,6	7,59	14,7	40,8	54	19,3	36	824	15	17,3	0,82	11	123,6	20,6
102/13/1	♀	control group	4	7,04	14,9	39,8	57	21,1	37,3	741	30	19	0,83	11	122,5	b.m.
103/13/1	♀	control group	5,8	7,42	16,3	42,6	57	21,9	38,2	862	36	hemolysis	hemolysis	hemolysis	hemolysis	hemolysis
104/13/1	♀	control group	4,3	7,17	56,6	42,3	59	21,7	36,9	718	11	18,3	0,82	11	123,6	b.m.
105/13/1	♀	control group	6,8	7,46	14,9	40,2	54	20	37,1	835	12	18	0,81	11	124,8	b.m.
106/13/1	♀	control group	4,5	5,69	12,3	32,4	57	21,5	37,8	815	25	17,6	0,81	11	125,9	hemolysis
107/13/1	♀	control group	3,8	7,58	15,2	41,7	55	20	36,5	865	12	19,9	0,81	11	125,9	b.m.
108/13/1	♀	control group	4	7,48	14,2	39,9	53	19	35,7	794	16	34,4	0,87	12	116,2	b.m.
109/13/1	♂	control group	7,7	8,09	15,9	42,9	53	19,7	37,1	827	17	17,2	0,86	12	117,2	b.m.
110/13/1	♂	control group	5,8	7,54	15,4	40,3	53	20,4	38,2	810	29	18,8	0,86	12	117,2	b.m.
111/13/1	♂	control group	6,5	8,3	15,9	42,8	52	19,2	37,1	898	72	17,4	0,87	12	116,2	32,6
112/13/1	♂	control group	6,5	7,88	15,6	42,5	54	19,9	36,8	803	26	17,1	0,83	11	122,5	22,1
113/13/1	♂	control group	5,9	8,05	15,2	40,7	51	18,9	37,3	890	19	18,4	0,86	12	118,3	29,5
114/13/1	♂	control group	5,3	8,51	15,8	44,3	52	80,6	35,6	669	54	16,6	0,87	12	116,2	29,3

115/13/1	♂	control group	6,3	7,9	16,3	43,5	55	20,7	37,5	797	29	18,8	0,84	11	120,4	30
116/13/1	♂	control group	6,1	7,83	15	40,7	52	19,1	36,8	905	33	17,4	0,86	12	118,3	29,9

b.m. – lack of material – tests were performed sequentially, so in some cases due to the small blood volume of the rat failed perform all coagulation tests haemolysis –. animal 103/13/1 - hemolysis occurred slightly due to animal's excessive response to anaesthetic, often reason of the haemolysis. The cases of haemolysis in the thrombin time test were caused by an insufficient amount of the material for test and an analyser automatically determined assays as haemolyzed

Table 18 A. The results (mean ± SD) of haematology examinations of female rats exposed to ferric pyrophosphate for 90 days at dose of 1000 mg/kg bw/d

	Control group	1000 mg/kg bw/day for 90 days	1000mg/kg bw/day for 90 days with 14 days recovery period (satellite group)
Parameter	Females	Females	Females
	Morphology		
WBC [thous./µl]	5,1 ± 1,5 N=8	8,9 ± 6,1 N=13[a]	4,5 ± 1,5 N=8
RBC [mln/µl	7,2 ± 0,6 N=8	8,1 ± 1,0 N=13[a]	7,8 ± 1,3 N=8
HGB [g/dl]	19,9 ± 14,9 N=8	16,2 ± 2,1 N=13	15,4 ± 2,0 N=8
HCT [%]	40,0 ± 3,2 N=8	45,1 ± 5,8 N=13[a]	43,1 ± 6,5 N=8
MCV [fl]	55,8 ± 2,1 N=8	55,5 ± 1,3 N=13	55,6 ± 1,4 N=8
MCH [pg]	20,6 ± 1,1 N=8	19,9 ± 0,5 N=13	19,9 ± 1,1 N=8
MCHC [g/dl]	36,9 ± 0,9 N=8	35,3 ± 1,1 N=13	35,8 ± 1,5 N=8
PLT [thous./µl	806,8 ± 53,4 N=8	829,1 ± 57,3 N=13	683,5 ± 162,2 N=8
Reticulocytes [part-per-thousand]	19,6 ± 9,5 N=8	39,6 ± 7,8 N=13[a]	44,8 ± 19,3 N=8

	Coagulation parameters											
APTT	20,6 ± 6,1 N=7				18,1± 1,7 N=14				18,3 ± 1,4 N=8			
INR	0,8 ± 0,0 N=7				0,8 ± 0,0 N=14				n/d			
PT	11,1 ± 0,4 N=7				11,1± 0,3 N=14				10,0 ± 0,0 N=8			
WSK. PT (PR)	123,2 ± 3,3 N=7				123,3 ± 3,2 N=14				n/d			
TT	20,6 N=1				33,3 N=1				23,6 ± 1,9 N=8			
	Microscopic examination of bone marrow											
Red blood cells system [%]	32,0 ± 3,4 N=8				31,4 ± 3,6 N=14				33,8 ± 3,2 N=8			
Granulocytic system [%]	56,8 ± 3,9 N=8				58,1 ± 5,3 N=14				55,3 ± 3,5 N=8			
Lymphocytes [%]	11,3 ± 1,2 N=8				10,6 ± 1,7 N=14				11,0 ± 0,9 N=8			
	Microscopic examination of peripheral blood Percentage of white blood cell types											
Banded Neutrophils [%]	1,9 ± 1,1 N=8				1,5 ± 0,7 N=14				1,8 ± 0,7 N=8			
Segmented Neutrophils [%]	24,4 ± 2,9 N=8				24,2 ± 4,1 N=14				25,3 ± 3,0 N=8			
Eosinophils [%	1,1 ± 0,4 N=8				1,1 ± 0,3 N=14				1,1 ± 0,4 N=8			
Basophils [%]	0,1 ± 0,4 N=8				0,1 ± 0,4 N=14				0,1 ± 0,4 N=8			
Lymphocytes [%]	70,8 ± 2,3 N=8				70,9 ± 4,7 N=14				69,8 ± 3,2 N=8			
Monocytes [%]	1,8 ± 0,5 N=8				2,1 ± 0,9 N=14				2,1 ± 0,8 N=8			

[a]– statistically significant
WBC (leukocytes) – white blood cells count
RBC – red blood cells count
HGB - haemoglobin
HCT – haematocrit
MCV – mean corpuscular volume
MCH – mean corpuscular haemoglobin

MCHC – mean corpuscular haemoglobin concentration
PLT – platelet count
APTT – activated partial thromboplastin time
INR – international normalized ratio
PT – prothrombin time
WSK. PT – prothrombin ratio
TT – thrombin time

Table 18 B. The results (mean ± SD) of haematology examinations of male rats exposed to ferric pyrophosphate for 90 days at dose of 1000 mg/kg bw/d

	Control group	1000 mg/kg bw/day for 90 days	1000mg/kg bw/day for 90 days with 14 days recovery period (satellite group)
Parameter	Males	Males	Males
	Morphology		
WBC [thous./µl]	6,3 ± 0,7 N=8	5,5 ± 0,9 N=13	4,9 ± 1,1 N=8
RBC [mln/µl	8,0 ± 0,3 N=8	8,4 ± 0,4 N=13[a]	8,2 ± 1,3 N=8
HGB [g/dl]	15,6 ± 0,4 N=8	15,9 ± 0,6 N=13	15,7 ± 2,2 N=8
HCT [%]	42,2 ± 1,5 N=8	44,2 ± 2,0 N=13[a]	43,7 ± 6,9 N=8
MCV [fl]	52,8 ± 1,3 N=8	52,8 ± 1,0 N=13	53,3 ± 1,3 N=8
MCH [pg]	27,3 ± 21,5 N=8	19,0 ± 0,4 N=13	19,1 ± 0,5 N=8
MCHC [g/dl]	37,1 ± 0,7 N=8	35,9 ± 0,6 N=13	36,0 ± 0,7 N=8
PLT [thous./µl	824.9 ± 77,3 N=8	834,5 ± 67,2 N=13	626,3 ± 217,5 N=8
Reticulocytes [part-per-thousand]	34,9 ± 18,8 N=8	23,2 ± 7,5 N=13	48,1 ± 19,3 N=8

	Coagulation parameters		
APTT	17,7 ± 0,8 N=8	16,9 ± 2,0 N=12	18,4 ± 1,8 N=8
INR	0,9 ± 0,0 N=8	0,9 ± 0,0 N=12	n/d
PT	11,8 ± 0,5 N=8	11,8 ± 0,4 N=12	10,1 ± 0,4 N=8
WSK. PT (PR)	118,3 ± 2,2 N=8	115,3 ± 2,9 N=12	n/d
TT	28,9 ± 3,5 N=6	26,3 ± 3,1 N=9	26,6 ± 2,1 N=8
	Microscopic examination of bone marrow		
Red blood cells system [%]	34,3 ± 3,6 N=8	31,4 ± 2,1 N=13	32,8 ± 2,7 N=8
Granulocytic system [%]	54,6 ± 4,5 N=8	57,9 ± 3,3 N=13	56,3 ± 3,5 N=8
Lymphocytes [%]	11,1 ± 1,1 N=8	10,7 ± 1,3 N=13	11,0 ± 1,2 N=8
	Microscopic examination of peripheral blood Percentage of white blood cell types		
Banded Neutrophils [%]	0,9 ± 0,4 N=8	1,8 ± 0,4 N=13	1,5 ± 0,5 N=8
Segmented Neutrophils [%]	26,8 ± 4,0 N=8	24,2 ± 2,7 N=13	27,3 ± 3,2 N=8
Eosinophils [%	1,1 ± 0,4 N=8	1,1 ± 0,3 N=13	1,4 ± 0,5 N=8
Basophils [%]	0,3 ± 0,5 N=8	0,1 ± 0,3 N=13	0,1 ± 0,4 N=8
Lymphocytes [%]	69,4 ± 4,4 N=8	70,8 ± 2,8 N=13	67,8 ± 2,7 N=8
Monocytes [%]	1,6 ± 0,7 N=8	2,1 ± 0,8 N=13	2,0 ± 0,5 N=8

WBC (leukocytes) – white blood cells count

RBC – red blood cells count
HGB - haemoglobin
HCT – haematocrit
MCV – mean corpuscular volume
MCH – mean corpuscular haemoglobin
MCHC – mean corpuscular haemoglobin concentration
PLT – platelet count
APTT – activated partial thromboplastin time
INR – international normalized ratio
PT – prothrombin time
WSK. PT – prothrombin ratio
TT – thrombin time

Table 19. Reference values for tested parameters

WBC [thous/µl]	RBC [mln/µl]	HGB [g/dl]	HCT [%]	MCV [fl]	MCH [pg]	MCHC g/dl]	PLT [thous./µl]	Reticulocytes [part-perthousand]	APTT	INR	PT	WSK. PT	TT
4,8-29,8	5,5-11	11,8-18,6	39,3-52	60	17-19,8	32,9-37,5	500-1200	4-77	14,8-19,8	b.d.	11,5-13,8	b.d.	b.d.

b.d. - no data

Smear and microscopic evaluation of bone marrow and peripheral blood

Smear and microscopic evaluation of bone marrow was made in animals. In all analysed cases, animals of the control and the dose of 1000 mg/kg body weight group, cellularity of marrow preparation was rich or very rich. Red blood cells system percentage in the standard – about 32% ± 3, normoblast erythropoiesis. Granulocytic system percentage in the standard - about 56% ± 4, lymphocytes in the standard - about 10% ± 1, other: mast cells and naked nuclei. An image of the bone marrow in the group of animals 1000 mg/kg body weight did not deviate from the norm – no differences in the control group were observed. Hemogram animals treated and control groups showed no differences between the groups. Detailed results of the analysis of bone marrow and peripheral blood smears are shown in Table 20.

Table 20. Detailed results of microscope assessment of bone narrow and peripheral blood smears

Animal No.	Sex	Dose [mg/kg]/ group	Bone marrow					Peripheral blood					
			Cellularity	Red blood cells system [%]	Granulocytic system [%]	Lymphocytes [%]	Others	banded neutrophils[%]	segmented neutrophils [%]	Eosino-phils [%]	Basophils [%]	Lymphocytes [%]	Monocytes [%]
55/13/1	♀	1000	rich	31	59	10	numerous mast cells	1	24	1	0	72	2
56/13/1	♀	1000	rich	29	61	10	quite numerous mast cells naked nuclei rarely	1	21	2	0	73	3
57/13/1	♀	1000	rich	31	58	11	numerous mast cells single naked nuclei	1	24	1	1	72	1
58/13/1	♀	1000	rich	28	63	9	numerous mast cells	1	21	1	0	76	1
60/13/1	♀	1000	rich	32	57	11	quite numerous mast cells naked nuclei rarely	2	25	1	0	71	1
61/13/1	♀	1000	rich	27	65	8	quite numerous mast cells naked nuclei rarely	2	19	1	0	76	2
62/13/1	♀	1000	rich	31	58	11	numerous mast cells	1	24	1	0	73	1
63/13/1	♀	1000	rich	29	62	9	numerous mast cells	2	21	1	0	74	2
64/13/1	♀	1000	rich	37	51	12	numerous mast cells	1	31	1	1	64	2
65/13/1	♀	1000	rich	31	59	10	numerous mast cells	2	23	1	0	72	2
66/13/1	♀	1000	rich	34	54	12	lnumerous mast cells	1	27	1	0	67	4
67/13/1	♀	1000	rich	40	45	15	quite numerous mast cells naked nuclei rarely	3	34	1	0	59	3
68/13/1	♀	1000	rich	28	63	9	numerous mast cells	2	21	1	0	73	3
69/13/1	♀	1000	rich	31	58	11	quite numerous mast cells naked nuclei rarely	1	24	1	0	71	3
71/13/1	♂	1000	rich	33	55	12	numerous mast cells	1	28	1	0	68	2
72/13/1	♂	1000	rich	30	60	10	numerous mast cells	1	26	1	0	71	1
73/13/1	♂	1000	rich	32	57	11	numerous mast cells	2	22	1	0	72	3

74/13/1	♂	1000	rich	36	51	13	numerous mast cells single naked nuclei	2	25	2	0	68	3
75/13/1	♂	1000	rich	30	60	10	numerous mast cells	2	29	1	0	65	3
76/13/1	♂	1000	rich	30	60	10	numerous mast cells	2	23	1	0	72	2
78/13/1	♂	1000	rich	29	62	9	numerous mast cells	2	22	1	0	72	3
79/13/1	♂	1000	rich	31	59	10	numerous mast cells	2	21	1	1	73	2
80/13/1	♂	1000	rich	33	55	12	numerous mast cells, single naked nuclei	2	23	1	0	72	2
81/13/1	♂	1000	rich	33	55	12	quite numerous mast cells naked nuclei rarely	2	26	1	0	69	2
82/13/1	♂	1000	rich	28	63	9	numerous mast cells	2	26	1	0	70	1
83/13/1	♂	1000	rich	31	59	10	numerous mast cells	1	20	1	0	76	2
84/13/1	♂	1000	rich	32	57	11	quite numerous mast cells naked nuclei rarely	2	23	1	0	73	1
85/13/1	♀	satellite group	rich	31	59	10	quite numerous mast cells naked nuclei rarely	2	24	1	0	70	3
86/13/1	♀	satellite group	rich	31	58	11	numerous mast cells, single naked nuclei	2	23	1	0	71	3
87/13/1	♀	satellite group	rich	36	52	12	quite numerous mast cells naked nuclei rarely	1	24	1	0	71	3
88/13/1	♀	satellite group	rich	34	56	10	quite numerous mast cells naked nuclei rarely	3	26	1	0	68	2
89/13/1	♀	satellite group	rich	31	57	12	numerous mast cells	1	28	1	0	69	2
90/13/1	♀	satellite group	rich	31	58	11	numerous mast cells single naked nuclei	2	22	1	1	73	1
91/13/1	♀	satellite group	rich	37	53	10	quite numerous mast cells naked nuclei rarely	2	31	2	0	63	2
92/13/1	♀	satellite group	rich	39	49	12	numerous mast cells	1	24	1	0	73	1
93/13/1	♂	satellite group	rich	35	53	12	quite numerous mast cells naked nuclei rarely	1	26	2	0	69	2
94/13/1	♂	satellite group	rich	34	55	11	numerous mast cells	2	22	2	0	72	2

95/13/1	♂	satellite group	rich	31	57	12	numerous mast cells	1	30	1	0	66	2
96/13/1	♂	satellite group	rich	33	55	12	numerous mast cells single naked nuclei	1	30	1	1	65	2
97/13/1	♂	satellite group .	rich	35	53	12	quite numerous mast cells naked nuclei rarely	2	25	2	0	69	2
98/13/1	♂	satellite group	rich	27	64	9	quite numerous mast cells naked nuclei rarely	2	26	1	0	70	1
99/13/1	♂	satellite group	rich	33	57	10	numerous mast cells single naked nuclei	2	27	1	0	67	3
100/13/1	♂	satellite group	rich	34	56	10	numerous mast cells	1	32	1	0	64	2
101/13/1	♀	control group	rich	34	53	13	quite numerous mast cells naked nuclei rarely	3	20	1	0	74	2
102/13/1	♀	control group	rich	37	51	12	numerous mast cells single naked nuclei	2	24	1	0	71	2
103/13/1	♀	control group	rich	28	62	10	quite numerous mast cells naked nuclei rarely	4	22	1	0	72	1
104/13/1	♀	control group	rich	31	58	11	numerous mast cells	2	26	1	0	69	2
105/13/1	♀	control group	rich	29	61	10	numerous mast cells	1	29	2	0	67	1
106/13/1	♀	control group	rich	36	54	10	quite numerous mast cells naked nuclei rarely	1	27	1	0	69	2
107/13/1	♀	control group	rich	29	59	12	numerous mast cells	1	23	1	1	72	2
108/13/1	♀	control group	rich	32	56	12	quite numerous mast cells naked nuclei rarely	1	24	1	0	72	2
109/13/1	♂	control group	rich	31	58	11	numerous mast cells	1	30	1	0	65	3
110/13/1	♂	control group	rich	36	52	12	numerous mast cells	1	32	1	0	64	2
111/13/1	♂	control group	rich	41	47	12	quite numerous mast cells naked nuclei rarely	1	28	2	1	66	2
112/13/1	♂	control group	rich	29	62	9	quite numerous mast cells naked nuclei rarely	1	27	1	0	69	2

113/13/1	♂	control group	rich	34	55	11	numerous mast cells single naked nuclei	1	24	1	0	73	1
114/13/1	♂	control group	rich	36	52	12	numerous mast cells	1	26	1	0	71	1
115/13/1	♂	control group	rich	34	54	12	numerous mast cells	0	28	1	0	70	1
116/13/1	♂	control group	rich	33	57	10	quite numerous mast cells naked nuclei rarely	1	19	1	1	77	1

Plasma and serum analysis

Serum biochemical analysis included:

- Sodium, potasium, calcium, magnesium, chlorides o phosphorus o iron o ferritin o total iron binding capacity - TIBC
- unsaturated iron binding capacity - UIBC
- glucose
- Total cholesterol, triglicerides
- urea
- creatinin
- total protein and albumin
- globulin
- alanin aminotransferase, aspartate aminotransferase, alkaline phosphatase, GammaGlutamyltransferase (GGT)
- bile acids
- bilirubin
- amylase, lipase
- activity of cholinesterase

Test results are shown in Table 21. Mean values and standard deviations values of all aparameters for each groups are presented in Table 22. Available reference values are shown in Table 23.

In the range of determined paratemters, differences statistically significant were shown in groups of females and males receiving ferric pyrophosphate in relation to the control group.

For females, statistically significant differences were demonstrated in case of following parameters:

• unsaturated iron binding capacity UIBC (value higher than in the control group)
• glucose (a value lower than in the control group)
• phosphorus (value higher than in the control group)
• total cholesterol (value lower than in the control group), triglycerides (value higher than in the control group)
• urea (the higher than in the control group)
• total protein (a higher value than in the control group) and albumin (a value higher than in the control group)

For males demonstrated statistically significant differences in the case of parameters:

• magnesium (the higher than in the control group)
• phosphorus (less than control)
• iron (value higher than in the control group)
• total iron binding capacity TIBC (value lower than in the control group)
• unsaturated iron binding capacity UIBC (value lower than in the control group)

• urea (the higher than in the control group) • creatinine (a value higher than in the control group)
• alanine aminotransferase (value higher than in the control group), alkaline phosphatase (value higher than in the control group)
• amylase (a value higher than in the control group)

Statistically significant differences that occurred in the analyzed biochemical parameters do not allow an assumption that they were caused by iron pyrophosphate. In most cases, where the reference values are available, results ranged within the scope. In other cases, where reference values are not available, clinical picture does not allow classification of these lesions as a result of action of iron pyrophosphate, due to slight differences combined with large standard deviations or, as in the case of UIBC, contradictory results in males and females. In liver damage a very high increase of liver enzymes occurs, with their varying relationship, depending on degree of damage. In the performed study values of liver enzymes range within the reference values, what in combination with lack of clinical signs and normal result of pathomorphological test gives a basis for adopting a lack of toxic effects on the liver function of iron pyrophosphate. Also in case of kidneys, analysis of biochemical results does not confirm the picture of clinical and pathomorphological studies and therefore no neurotoxicity of iron pyrophosphate was stated.

Table 21. Results of determination of biochemistry parameters in rats

Animal no.	Sex	Dose [mg/kg] / group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
			Sodium[mmol/l]	Potassium [mmol/l]	Total cholesterol[mg/dl]	Trójglicerydes [mmol/l]	Creatynin[mg/dl]	Urea [mg/dl]	Aspat[U/l]	Alat[U/l]	Bilirubin [mg/dl]	Albumin[g/dl]	Amylase [U/l]	Total protein [g/dl]	Glukose [mg/dl]	alkaline phosphatase [U/L]	GGTP [U/l]	Lipase [U/l]	Magnesium [mmol/l]	TIBC [µmol/l]	Calcium [mmol/l]	Iron [ug/dl]	Phosphorous [mmol/l]	Chlorides [mmol/l]	Ferritin [ng/ml]	Bile acids [umol/l]	UIBC [µmol/l]	AcCh [µmol/dm^3]
55/13/1	♀	1000	142,00	5,40	69,00	1,34	0,77	71,00	96,00	65,00	0,26	3,40	1150,00	6,15	164,00	49,00	6,00	5,00	1,36	468,00	12,10	256,00	2,57	102,00	295,07	50,40	220,70	0,494949
56/13/1	♀	1000	143,00	5,70	57,00	1,28	0,68	74,00	103,00	50,00	0,23	3,50	978,00	6,19	199,00	40,00	6,00	5,00	1,25	527,00	11,46	189,00	1,96	102,00	293,93	109,50	343,40	0,389171
57/13/1	♀	1000	143,00	5,40	75,00	1,81	0,67	59,00	121,00	55,00	0,19	3,50	965,00	6,27	130,00	40,00	6,00	5,00	1,28	538,00	11,70	447,00	2,17	105,00	232,33	76,00	94,90	0,32
58/13/1	♀	1000	141,00	7,10	70,00	0,80	0,75	54,00	102,00	35,00	0,22	3,60	784,00	6,40	80,00	41,00	6,00	5,00	1,35	484,00	12,10	301,00	266,00	106,00	200,89	89,50	187,30	0,493151
60/13/1	♀	1000	143,00	5,60	73,00	1,24	0,67	48,00	116,00	51,00	0,20	3,40	933,00	6,12	113,00	43,00	6,00	5,00	1,28	520,00	11,74	239,00	2,33	104,00	226,42	50,90	398,50	0,407883
61/13/1	♀	1000	142,00	5,50	79,00	1,25	0,64	51,00	100,00	54,00	0,20	3,30	1016,00	6,01	163,00	49,00	6,00	5,00	1,24	488,00	11,66	344,00	2,27	103,00	271,55	60,90	150,70	0,279001
62/13/1	♀	1000	142,00	5,70	61,00	0,70	0,64	53,00	91,00	45,00	0,24	3,10	783,00	5,79	161,00	54,00	6,00	5,00	1,20	509,00	11,42	196,00	2,38	102,00	289,62	44,00	305,50	0,240892
63/13/1	♀	1000	145,00	4,90	58,00	61,00	67,00	58,00	89,00	40,00	0,22	3,50	730,00	5,95	125,00	50,00	6,00	5,00	1,23	493,00	11,58	268,00	2,74	101,00	234,06	121,10	226,40	0,329738
64/13/1	♀	1000	144,00	6,20	67,00	0,74	0,67	61,00	84,00	51,00	23,00	3,20	804,00	5,64	110,00	66,00	6,00	5,00	1,19	413,00	11,42	255,00	1,83	107,00	241,81	139,80	168,20	0,513288
65/13/1	♀	1000	142,00	5,10	65,00	0,59	0,67	57,00	86,00	56,00	0,24	3,40	889,00	6,06	161,00	45,00	6,00	5,00	1,25	483,00	11,70	341,00	2,36	103,00	285,37	116,30	144,40	0,6
66/13/1	♀	1000	144,00	5,20	63,00	1,03	0,62	47,00	85,00	66,00	0,18	3,40	1176,00	5,99	191,00	50,00	6,00	5,00	1,06	525,00	7,37	303,00	2,13	103,00	207,99	53,70	231,30	0,515528
67/13/1	♀	1000	144,00	4,90	57,00	0,59	0,61	41,00	90,00	38,00	0,21	3,30	994,00	5,61	154,00	44,00	6,00	5,00	1,18	474,00	10,78	342,00	2,25	104,00	227,36	27,60	127,90	0,142424
68/13/1	♀	1000	144,00	4,70	62,00	0,42	0,70	64,00	84,00	63,00	0,22	3,30	695,00	5,85	149,00	50,00	6,00	5,00	1,20	469,00	11,02	250,00	1,86	104,00	293,01	167,00	213,80	0,412214
69/13/1	♀	1000	144,00	4,70	70,00	0,63	0,65	54,00	74,00	51,00	0,26	3,50	612,00	6,06	213,00	59,00	6,00	5,00	1,30	485,00	11,42	285,00	2,04	102,00	254,50	128,30	212,30	0,398148
71/13/1	♂	1000	145,00	5,10	69,00	0,51	0,63	49,00	117,00	46,00	0,20	3,50	1694,00	6,31	156,00	79,00	6,00	5,00	1,27	537,00	11,70	171,00	2,61	106,00	313,92	85,70	353,70	0,363325
72/13/1	♂	1000	144,00	6,10	87,00	0,65	0,60	58,00	126,00	42,00	0,19	3,30	1565,00	6,19	185,00	74,00	6,00	5,00	1,22	504,00	11,42	150,00	2,87	101,00	331,18	53,00	344,10	0,353039
73/13/1	♂	1000	142,00	5,80	60,00	0,42	0,61	69,00	117,00	87,00	0,19	3,50	2000,00	6,08	182,00	92,00	6,00	5,00	1,17	569,00	11,90	208,00	2,26	101,00	255,13	42,10	377,70	0,384424

74/13/1	♂	1000	140,00	6,30	87,00	0,69	0,60	56,00	95,00	49,00	0,19	3,20	2000,00	5,99	202,00	87,00	6,00	5,00	1,14	501,00	11,86	203,00	2,49	100,00	267,26	43,30	327,00	0,464432
75/13/1	♂	1000	142,00	6,20	81,00	0,60	0,65	47,00	116,00	55,00	0,25	3,40	1739,00	6,37	188,00	89,00	6,00	5,00	1,21	534,00	11,46	173,00	2,62	101,00	336,15	48,10	375,10	0,404938
76/13/1	♂	1000	143,00	5,60	85,00	0,57	0,65	46,00	97,00	42,00	0,18	3,30	1442,00	6,17	148,00	69,00	6,00	5,00	1,25	481,00	11,26	183,00	2,63	101,00	267,86	50,60	302,70	0,543319
78/13/1	♂	1000	140,00	5,70	83,00	0,70	0,62	35,00	69,00	33,00	0,17	3,10	1796,00	6,83	226,00	66,00	6,00	5,00	1,35	442,00	11,14	118,00	2,74	100,00	324,44	16,70	341,00	0,454135
79/13/1	♂	1000	143,00	5,80	69,00	0,47	0,67	52,00	109,00	58,00	0,17	3,10	1796,00	5,97	190,00	68,00	6,00	5,00	1,23	449,00	10,50	184,00	2,52	104,00	266,30	27,40	285,50	0,548027
80/13/1	♂	1000	145,00	5,60	69,00	0,66	0,57	45,00	83,00	38,00	0,17	3,20	1987,00	6,14	185,00	64,00	6,00	5,00	1,13	472,00	11,22	204,00	2,24	105,00	190,74	39,10	280,60	0,358896
81/13/1	♂	1000	146,00	5,70	70,00	0,53	0,64	50,00	97,00	46,00	0,18	3,10	1711,00	6,11	164,00	68,00	6,00	5,00	1,19	445,00	11,14	130,00	2,14	104,00	325,80	56,50	302,20	0,446415
82/13/1	♂	1000	148,00	5,60	75,00	0,41	0,59	46,00	92,00	45,00	0,17	3,20	1870,00	6,39	180,00	73,00	6,00	5,00	1,19	455,00	11,26	158,00	2,49	104,00	378,21	8,60	312,30	0,252442
83/13/1	♂	1000	149,00	5,50	93,00	0,61	0,64	38,00	86,00	31,00	0,20	3,30	1931,00	6,27	171,00	64,00	6,00	5,00	1,20	507,00	11,30	120,00	2,61	107,00	268,16	21,80	371,40	0,273764
84/13/1	♂	1000	144,00	6,10	69,00	0,46	0,59	38,00	78,00	27,00	0,17	3,20	1621,00	6,16	246,00	58,00	6,00	5,00	1,14	462,00	11,42	131,00	2,38	105,00	255,86	9,10	343,20	0,17552
85/13/1	♀	sat.	144,00	5,50	71,00	0,56	0,62	45,00	99,00	50,00	0,25	3,20	980,00	5,54	236,00	55,00	6,00	5,13	1,12	451,00	11,46	351,00	2,45	107,00	202,01	87,10	84,40	0,601052
86/13/1	♀	sat.	144,00	5,60	94,00	0,62	0,62	53,00	90,00	40,00	0,25	3,30	1155,00	5,82	190,00	58,00	6,00	5,00	1,15	479,00	11,74	333,00	1,61	109,00	217,72	32,00	136,90	0,545455
87/13/1	♀	sat.	142,00	5,90	84,00	0,46	0,65	46,00	102,00	47,00	0,30	3,20	900,00	5,61	208,00	41,00	6,00	7,30	1,40	440,00	11,38	282,00	2,10	107,00	159,84	80,20	149,90	0,472012
88/13/1	♀	sat.	144,00	5,50	83,00	0,54	0,55	48,00	86,00	54,00	0,27	3,10	1027,00	5,47	217,00	71,00	6,00	5,59	1,09	459,00	11,58	379,00	2,30	108,00	176,11	50,50	71,10	0,430303
89/13/1	♀	sat.	140,00	6,70	62,00	0,64	0,66	29,00	147,00	34,00	0,24	3,50	1083,00	6,24	85,00	51,00	6,00	5,00	1,42	499,00	11,38	367,00	2,21	104,00	180,42	119,70	125,10	0,459600
90/13/1	♀	sat.	147,00	5,30	87,00	0,73	0,63	33,00	222,00	114,00	0,24	3,30	1257,00	5,98	163,00	50,00	6,00	5,00	1,28	522,00	11,38	292,00	2,57	111,00	500,00	35,00	222,70	0,209581
91/13/1	♀	sat.	143,00	5,90	99,00	0,71	0,68	28,00	108,00	36,00	0,23	3,30	984,00	6,00	162,00	38,00	6,00	5,00	1,43	477,00	11,34	341,00	2,28	105,00	244,48	37,50	134,70	0,330140
92/13/1	♀	sat.	144,00	5,70	96,00	0,62	0,63	33,00	86,00	37,00	0,19	3,30	921,00	5,83	156,00	33,00	6,00	5,00	1,09	462,00	11,66	268,00	1,93	105,00	268,01	40,60	191,20	0,390171
93/13/1	♂	sat.	144,00	5,60	69,00	0,41	0,51	36,00	104,00	24,00	0,20	3,20	1379,00	5,84	178,00	47,00	6,00	5,00	1,02	508,00	11,10	122,00	3,30	102,00	208,29	6,30	386,20	0,316716
94/13/1	♂	sat.	142,00	6,60	92,00	0,45	0,51	41,00	107,00	25,00	0,19	3,10	1109,00	5,86	156,00	61,00	6,00	5,22	1,00	496,00	11,78	115,00	3,77	102,00	308,23	12,40	385,30	0,351648
95/13/1	♂	sat.	143,00	6,10	69,00	0,72	0,56	38,00	102,00	23,00	0,20	3,20	1611,00	6,04	208,00	48,00	6,00	5,00	1,08	540,00	11,82	101,00	2,73	101,00	322,44	9,50	435,70	0,160595
96/13/1	♂	sat.	144,00	6,20	78,00	0,31	0,52	36,00	131,00	28,00	0,20	3,20	1116,00	5,95	171,00	58,00	6,00	5,00	1,11	449,00	11,94	118,00	2,98	103,00	313,29	17,00	338,50	0,233184
97/13/1	♂	sat.	140,00	5,80	79,00	0,83	0,56	34,00	111,00	21,00	0,23	3,20	1417,00	6,04	240,00	34,00	6,00	5,00	1,08	506,00	11,42	127,00	3,68	100,00	318,94	15,00	375,70	0,283419
98/13/1	♂	sat.	141,00	5,50	80,00	0,67	0,56	31,00	97,00	25,00	0,21	3,20	1454,00	6,05	211,00	43,00	6,00	5,00	0,96	470,00	11,90	93,00	2,71	102,00	276,04	14,50	379,50	0,342693
99/13/1	♂	sat.	142,00	5,70	66,00	0,29	0,54	35,00	102,00	28,00	0,22	3,40	1221,00	6,01	141,00	51,00	6,00	5,00	1,09	520,00	11,74	124,00	3,03	103,00	337,00	57,70	395,30	0,094256

100/13/1	♂	sat.	143,00	5,00	62,00	0,35	0,52	31,00	105,00	24,00	0,23	3,30	1157,00	6,11	178,00	53,00	6,00	5,00	1,01	497,00	11,82	126,00	2,69	101,00	238,38	48,40	370,60	0,117907
101/13/1	♀	contr.	142,00	5,80	83,00	0,54	0,66	39,00	110,00	61,00	0,22	3,20	1038,00	5,81	214,00	59,00	6,00	5,00	1,43	470,00	11,62	284,00	2,68	107,00	223,39	104,90	211,30	0,450142
102/13/1	♀	contr.	142,00	5,00	103,00	0,62	0,67	49,00	84,00	44,00	0,23	3,30	1318,00	5,87	223,00	50,00	6,00	5,19	1,29	491,00	11,34	267,00	2,43	104,00	237,45	89,20	251,70	0,518047
103/13/1	♀	contr.	141,00	5,10	80,00	0,56	0,67	45,00	88,00	67,00	0,22	3,10	916,00	5,47	221,00	61,00	6,00	6,52	1,45	429,00	11,02	334,00	2,38	106,00	259,32	63,50	117,30	0,429728
104/13/1	♀	contr.	147,00	5,00	93,00	0,55	0,78	45,00	99,00	38,00	0,20	3,30	965,00	5,73	205,00	40,00	6,00	5,00	1,21	399,00	10,94	313,00	1,80	108,00	320,33	27,50	108,30	0,329206
105/13/1	♀	contr.	143,00	5,70	73,00	0,53	0,69	38,00	99,00	39,00	0,24	3,30	919,00	5,64	173,00	42,00	6,00	5,00	1,55	394,00	11,50	311,00	2,70	106,00	240,74	86,00	112,90	0,605375
106/13/1	♀	contr.	142,00	5,20	89,00	0,50	0,61	35,00	89,00	33,00	0,18	3,20	907,00	5,68	182,00	34,00	6,00	5,05	1,25	419,00	11,22	293,00	2,16	105,00	167,93	29,10	146,10	0,318665
107/13/1	♀	contr.	141,00	5,00	96,00	1,00	0,69	39,00	123,00	35,00	0,18	3,20	993,00	5,82	184,00	42,00	6,00	5,00	1,32	398,00	11,18	233,00	2,17	105,00	247,50	32,60	184,30	0,403053
108/13/1	♀	contr.	143,00	4,70	83,00	0,47	0,68	44,00	142,00	46,00	0,20	3,20	901,00	5,68	167,00	46,00	6,00	5,00	1,34	447,00	10,94	283,00	2,09	105,00	131,30	123,00	182,40	
109/13/1	♂	contr.	144,00	5,90	93,00	0,53	0,55	31,00	100,00	22,00	0,19	3,10	1237,00	5,94	167,00	65,00	6,00	5,00	1,04	484,00	11,26	109,00	3,38	103,00	261,39	9,70	370,10	0,206735
110/13/1	♂	contr.	144,00	5,80	100,00	0,64	0,58	38,00	113,00	26,00	0,19	3,10	1399,00	5,86	173,00	47,00	6,00	5,00	1,15	497,00	11,34	94,00	2,82	103,00	285,95	61,70	399,30	0,461314
111/13/1	♂	contr.	148,00	8,50	78,00	0,51	0,64	40,00	95,00	29,00	0,17	3,20	1299,00	6,39	277,00	43,00	6,00	5,92	1,48	513,00	12,95	115,00	4,03	106,00	304,25	10,00	405,00	0,461197
112/13/1	♂	contr.	146,00	6,30	98,00	1,14	0,54	35,00	105,00	24,00	0,17	3,10	1606,00	5,98	212,00	45,00	6,00	5,00	1,07	478,00	11,62	111,00	2,73	104,00	237,41	10,80	370,80	0,55313
113/13/1	♂	contr.	146,00	5,90	65,00	0,38	0,53	37,00	93,00	31,00	0,18	3,30	1109,00	6,20	144,00	53,00	6,00	5,00	1,11	532,00	11,90	119,00	2,85	107,00	321,04	18,50	421,30	0,521994
114/13/1	♂	contr.	145,00	5,90	90,00	0,74	0,54	34,00	110,00	44,00	0,17	3,30	1414,00	6,08	163,00	38,00	6,00	5,00	1,10	515,00	11,66	100,00	2,97	104,00	276,56	7,40	418,40	0,55814
115/13/1	♂	contr.	145,00	5,90	75,00	0,63	0,56	40,00	143,00	24,00	0,17	3,30	1292,00	6,23	160,00	64,00	6,00	6,80	1,20	509,00	11,78	128,00	3,17	103,00	377,86	27,40	383,10	0,190618
116/13/1	♂	contr.	144,00	5,80	83,00	0,59	0,59	34,00	89,00	21,00	0,19	3,30	1321,00	6,31	197,00	46,00	6,00	6,51	1,06	481,00	11,98	98,00	2,80	104,00	299,27	29,00	391,20	0,199697

sat. - satellite group

contr. - control group

Table 22. Mean values and standard deviations values of all parameters for each groups

Number of animals in groups	Dose/ Group	Sex	Mean ±SD	Sodium[mmol/l]	Potassium [mmol/l]	Total cholesterol[mg	Trójglicerydes [mmol/l]	Creatynin[mg/dl]	Urea [mg/dl]	Aspat[U/l]	Alat[U/l]	Bilirubin [mg/dl]	Albumin[g/dl]	Amylase [U/l]	Total protein [g/dl]	Glukose [mg/dl]	alkaline [U/L]	GGTP [U/l]	Lipase [U/l]	Magnesium [mmol/l]	TIBC [µmol/l]	Calcium [mmol/l]	Iron [ug/dl]	Phosphorous [mmol/l]	Sodium[mmol/l]	Potassium [mmol/l]	Total cholesterol[mg	Trójglicerydes [mmol/l]	Creatynin[mg/dl]
14	1000	♀	Mean	143,07	5,44	66,14	5,24	5,41	56,57	94,36	51,43	1,85	3,30	893,50	6,01	150,93	48,57	6,00	5,00	1,24	491,14	11,25	286,86	21,06	103,43	253,85	88,21	216,00	0,40
		♀	SD	1,14	0,64	6,89	16,05	17,73	9,03	12,98	9,59	6,09	0,14	166,90	0,23	36,75	7,43	0,00	0,00	0,08	32,07	1,17	67,45	70,50	1,70	33,73	42,37	84,51	0,12
13	1000	♂	Mean	143,92	5,78	76,69	0,56	0,62	48,38	98,62	46,08	0,19	3,26	1780,92	6,23	186,38	73,15	6,00	5,00	1,21	489,08	11,35	164,08	2,51	103,00	290,85	38,62	332,04	0,39
		♂	SD	2,72	0,33	9,87	0,10	0,03	9,18	17,33	15,18	0,02	0,14	175,75	0,22	26,74	10,61	0,00	0,00	0,06	40,21	0,36	32,25	0,21	2,42	49,09	21,74	33,27	0,11
8	satellite group	♀	Mean	143,50	5,76	84,50	0,61	0,63	39,38	117,50	51,50	0,25	3,28	1038,38	5,81	177,13	49,63	6,00	5,38	1,25	473,63	11,40	326,63	2,18	107,00	243,57	60,33	139,50	0,43
		♀	SD	2,00	0,43	12,71	0,09	0,04	9,66	46,60	26,25	0,03	0,12	121,19	0,26	47,11	12,21	0,00	0,80	0,15	26,77	0,15	41,13	0,30	2,33	109,74	31,82	50,22	0,12
8	satellite group	♂	Mean	142,38	5,81	74,38	0,50	0,54	35,25	107,38	24,75	0,21	3,23	1308,00	5,90	185,30	49,30	6,00	5,03	1,04	498,25	11,60	115,75	3,11	101,75	290,33	22,60	383,35	0,24
		♂	SD	1,41	0,49	9,69	0,21	0,02	3,37	10,38	2,38	0,02	0,09	183,96	0,10	32,29	8,53	0,00	0,08	0,05	28,29	0,29	12,42	0,43	1,04	45,53	19,25	27,10	0,10
8	control group	♀	Mean	142,63	5,19	87,50	0,60	0,68	41,75	104,25	45,38	0,21	3,23	994,63	5,71	196,13	46,75	6,00	5,23	1,36	430,88	11,23	289,75	2,30	105,75	228,50	69,48	164,20	0,41
		♀	SD	1,92	0,38	9,65	0,17	0,05	4,68	19,90	12,36	0,02	0,07	139,22	0,13	22,26	9,39	0,00	0,53	0,11	35,87	0,25	31,12	0,31	1,28	57,47	36,97	51,95	0,10
8	control group	♂	Mean	145,25	6,25	85,25	0,65	0,57	36,13	106,00	27,63	0,18	3,21	1334,63	6,13	186,63	50,13	6,00	5,53	1,15	501,13	11,81	109,25	3,09	104,25	295,47	21,81	394,90	0,39
		♂	SD	1,39	0,92	12,16	0,23	0,04	3,18	17,11	7,42	0,01	0,10	145,31	0,19	42,38	9,80	0,00	0,77	0,14	19,27	0,52	11,51	0,44	1,49	42,28	18,13	19,71	0,17

Table 23. Reference values of determination of biochemistry parameters of rats

Sodium[mmol/l]	Potassium [mmol/l]	Total cholesterol[mg/dl]	Trójglicerydes [mmol/l]	Creatynin[mg/dl]	Urea [mg/dl]	Aspat[U/l]	Alat[U/l]	Bilirubin [mg/dl]	Albumin[g/dl]	Amylase [U/l]	Total protein [g/dl]	Glukose [mg/dl]	alkaline phosphatase [U/L]	GGTP [U/l]	Lipase [U/l]	Magnesium [mmol/l]	TIBC [µmol/l]	Calcium [mmol/l]	Iron [ug/dl]	Phosphorous [mmol/l]	Sodium[mmol/l]	Potassium [mmol/l]	Total cholesterol[mg/dl]	Trójglicerydes [mmol/l]	AcCh
142-151	3,82-5,55	37-85	20-114	0,2-0,5	12,3-24,6	74-143	18-45	0,05-0,15	3,4-4,8	b.d.	5,2-7,1	70-208	62-230	b.d.	b.d.	b.d.	b.d.	10,6-12,3	135-314	b.d.	100-106	b.d.	b.d.	b.d.	b.d.
140-150	3,31-4,9	24-73	14-46	0,2-0,6	13,2-27,1	65-203	16-48	0,05-0,18	3,6-5,5	b.d.	5,5-7,7	76-175	26-147	b.d.	b.d.	b.d.	b.d.			b.d.	100-107	b.d.	b.d.	b.d.	b.d.

b.d.- no data
1st row - male
2nd row - female

Urine analysis

General urine analysis results are shown in Table 24. Available reference values for total urine analysis are shown in Table 25. No significant variations in the overall analysis of urine in the test groups of animals in relation to the control group. Animals did not show clinical signs in the range of disorders of the urinary tract and irregularities in histopathological examination, hence no evidence of toxic effects of iron pyrophosphate in the area of the urinary tract.

Table 24. Results of urine test.

Animal no	Sex	Dose [mg/kg] Group	Volume [ml]	Color	Lucidity	pH	Urine specific gravity	Protein [mg/dl]	Glucose [mg/dl]	Urobilinogen [mg/dl]	Bilirubin [mg/dl]	Ketone bodies [mg/dl	Nitrites [mg/dl]	Erytrocytes [ery/µl]	Leucotytes [leu/µl]	Potassium [mmol/l]	Sodium [mmol/l	Urea * [mg/dl	Chlorides [mmol/l]	Osmolality [mOsm/kg]]
55/13/1	♀	1000	30	straw	clear	8	1,010	100	absent	normal	absent	absent	absent	absent	500	47,93	< 20	1453,6	37	242
56/13/1	♀	1000	44	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	31,80	< 20	891,3	23	148
57/13/1	♀	1000	35	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	21,33	< 20	896,5	< 20	149
58/13/1	♀	1000	37	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	18,27	< 20	769,6	< 20	128
60/13/1	♀	1000	33	straw	clear	8	1,010	absent	absent	normal	absent	absent	absent	absent	absent	42,69	< 20	1191,3	29	198
61/13/1	♀	1000	50	straw	clear	8	1,010	absent	absent	normal	absent	absent	absent	absent	absent	28,86	< 20	815,5	23	136
62/13/1	♀	1000	20	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	36,31	< 20	1248,7	30	208
63/13/1	♀	1000	26	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	43,60	< 20	1659,4	28	276
64/13/1	♀	1000	21	straw	slightly cloudy	8	1,010	absent	absent	normal	absent	absent	absent	absent	absent	57,07	< 20	1723,1	33	287
65/13/1	♀	1000	37	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	30,06	< 20	929,9	25	155
66/13/1	♀	1000	19	straw	clear	8	1,010	absent	absent	normal	absent	absent	absent	absent	absent	60,42	< 20	1443,6	46	240
67/13/1	♀	1000	21	straw	clear	6,5	1,010	absent	absent	normal	absent	absent	absent	absent	absent	45,09	< 20	1659,2	37	276
68/13/1	♀	1000	13	straw	clear	7	1,010	30	absent	normal	1	absent	absent	absent	absent	108,67	22	2741,3	64	478
69/13/1	♀	1000	39	straw	clear	7	1,010	absent	absent	normal	absent	absent	absent	absent	absent	30,58	< 20	954,0	26	159
71/13/1	♂	1000	24	straw	clear	9	1,005	30	absent	normal	absent	absent	POS	absent	absent	48,94	< 20	1732,3	59	288
72/13/1	♂	1000	28	straw	slightly cloudy	9	1,005	30	absent	normal	absent	absent	absent	absent	25	58,02	< 20	1620,4	47	270
73/13/1	♂	1000	33	straw	slightly cloudy	9	1,010	30	absent	normal	absent	absent	POS	absent	absent	45,74	< 20	1147,0	33	191
74/13/1	♂	1000	38	straw	slightly cloudy	9	1,010	30	absent	normal	absent	absent	absent	absent	500	44,38	< 20	1417,2	40	236
75/13/1	♂	1000	35	straw	clear	9	1,005	30	absent	normal	absent	absent	absent	absent	25	51,64	< 20	1371,2	34	228
76/13/1	♂	1000	25	straw	slightly cloudy	9	1,010	30	absent	normal	absent	absent	POS	absent	25	61,22	< 20	1932,5	67	322
78/13/1	♂	1000	16	straw	cloudy	8	1,010	30	absent	normal	1	absent	absent	absent	75	112,30	< 20	3103,8	85	517
79/13/1	♂	1000	25	straw	slightly cloudy	9	1,010	absent	absent	normal	absent	absent	POS	absent	25	44,46	< 20	1317,2	36	219
80/13/1	♂	1000	30	straw	clear	9	1,005	absent	absent	normal	absent	absent	absent	absent	absent	39,80	< 20	1291,0	26	215
81/13/1	♂	1000	35	straw	clear	9	1,010	absent	absent	normal	absent	absent	POS	absent	absent	39,41	< 20	1184,3	33	197
82/13/1	♂	1000	25	straw	slightly cloudy	9	1,005	30	absent	normal	absent	absent	POS	absent	absent	72,45	< 20	1820,3	50	303
83/13/1	♂	1000	28	straw	slightly cloudy	9	1,005	30	absent	normal	1	absent	POS	absent	25	56,00	< 20	1549,2	56	258
84/13/1	♂	1000	43	straw	clear	9	1,005	30	absent	normal	absent	absent	POS	absent	absent	41,13	< 20	1071,5	23	178
85/13/1	♀	1000/sat.	37	straw	clear	7	1,005	absent	absent	normal	absent	absent	nieobecne	absent	absent	14,67	< 20	736,5	< 20	123
86/13/1	♀	1000/sat.	20	straw	slightly cloudy	9	1,005	absent	absent	normal	1	absent	POS	absent	absent	74,01	26	1783,9	66	323

87/13/1	♀	1000/sat.	20	straw	clear	7	1,010	absent	absent	normal	1	absent	POS	absent	absent	69,72	< 20	1974,2	63	329
88/13/1	♀	1000/sat.	16	straw	clear	7	1,010	absent	absent	normal	1	absent	POS	absent	absent	62,39	32	1909,1	43	350
89/13/1	♀	1000/sat.	26	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	10	absent	32,29	< 20	1111,4	21	185
90/13/1	♀	1000/sat.	16	straw	clear	7	1,010	absent	absent	normal	1	absent	absent	absent	absent	58,88	< 20	1970,1	33	328
91/13/1	♀	1000/sat.	14	straw	clear	8	1,015	30	absent	normal	1	absent	POS	absent	absent	110,72	33	2602,0	102	466
92/13/1	♀	1000/sat.	47	straw	clear	7	1,005	absent	absent	normal	absent	absent	absent	absent	absent	24,54	< 20	836,8	< 20	139
93/13/1	♂	1000/sat.	12	straw	clear	8	1,015	30	absent	normal	1	absent	absent	50	25	3024,80	121,43	30,0	118	126
94/13/1	♂	1000/sat.	17	straw	clear	9	1,010	30	absent	normal	2	absent	POS	absent	75	2725,30	88,7	14,0	64	91
95/13/1	♂	1000/sat.	17	straw	clear	8	1,015	30	absent	normal	1	absent	absent	absent	25	2672,10	113,4	17,0	91	116
96/13/1	♂	1000/sat.	15	straw	clear	8	1,015	30	absent	normal	2	absent	absent	absent	25	2813,00	85,03	18,0	56	88
97/13/1	♂	1000/sat.	16	straw	clear	9	1,010	30	absent	normal	1	absent	absent	absent	500	2530,70	95,34	21,0	95	99
98/13/1	♂	1000/sat.	10	straw	clear	9	1,015	100	absent	normal	1	absent	absent	absent	500	2097,20	110,11	< 20	94	110
99/13/1	♂	1000/sat.	38	straw	slightly cloudy	8	1,015	absent	absent	normal	absent	absent	POS	absent	absent	1361,70	48,72	< 20	33	49
100/13/1	♂	1000/sat.	36	straw	clear	8	1,010	absent	absent	normal	absent	absent	POS	absent	absent	1105,50	44,42	< 20	23	44
101/13/1	♀	control	9	straw	cloudy	8	1,015	30	absent	normal	1	absent	POS	absent	absent	171,00	33	3566,8	125	627
102/13/1	♀	control	19	straw	cloudy	9	1,010	30	absent	normal	absent	absent	POS	absent	absent	53,33	33	1553,7	48	292
103/13/1	♀	control	10	straw	cloudy	7	1,010	30	absent	normal	1	absent	absent	absent	absent	128,53	40	3570,5	85	634
104/13/1	♀	control	17	straw	clear	7	1,010	absent	absent	normal	1	absent	POS	absent	absent	73,28	27	1996,2	60	359
105/13/1	♀	control	28	straw	slightly cloudy	8	1,010	absent	absent	normal	absent	absent	absent	absent	absent	31,64	20	1084,7	35	201
106/13/1	♀	control	40	straw	clear	9	1,010	absent	absent	normal	absent	absent	POS	absent	absent	23,47	< 20	835,2	21	139
107/13/1	♀	control	22	straw	clear	7	1,010	absent	absent	normal	1	absent	POS	absent	absent	58,24	24	1662,7	40	301
108/13/1	♀	control	34	straw	clear	8	1,015	absent	absent	normal	absent	absent	POS	absent	absent	38,57	< 20	1187,0	24	198
109/13/1	♂	control	16	straw	cloudy	8	1,015	30	absent	normal	1	absent	POS	absent	absent	120,37	21	3263,7	139	564
110/13/1	♂	control	19	straw	cloudy	8	1,015	30	absent	normal	1	absent	POS	absent	25	112,98	25	2763,7	76	485
111/13/1	♂	control	12	straw	slightly cloudy	9	1,015	30	absent	normal	2	absent	POS	absent	25	127,8	22	3121,5	102	542
112/13/1	♂	control	19	straw	cloudy	8	1,015	500	absent	normal	1	absent	POS	absent	500	122,26	18	2888,5	171	499
113/13/1	♂	control	19	straw	cloudy	8	1,015	30	absent	normal	absent	absent	absent	absent	25	87,87	20	2697,2	119	469

114/13/1	♂	control	16	yellow	cloudy	9	1,005	30	absent	normal	1	absent	POS	absent	25	88,31	27	2691,4	108	475
115/13/1	♂	control	34	yellow	cloudy	9	1,010	30	absent	normal	absent	absent	POS	absent	absent	58,02	< 20	1480,7	58	247
116/13/1	♂	control	13	yellow	cloudy	9	1,010	30	absent	normal	2	absent	POS	absent	25	165,40	46	4138,9	275	735

* parametr indicates to determine urine osmolality – in addition, it may be a parameter estimates capacity of the kidneys

Table 25. Reference values of urine test.

Urine volume [ml]	Colour	lucidity	pH	Urine specific gravity	Protein [mg/dl]	Glucose [mg/dl]	Urobilinogen [mg/dl]	Bilirubin [mg/dl]	Ketones [mg/dl]	Nitrite [mg/dl]	Erythrocytes [ery/µl]	Leukocytes [leu/µl]	Urea – Urine * [mg/dl]	Osmolality [mOsm]
15-30 ml/24h[4]	n.d.	n.d.	n.d.	n.d.	Trace amount	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10 – 2000

n.d. - no data

Appendix 1C. The detailed data from the study Stępnik M. 2013, Assessment of the mutagenic potential of ferric pyrophosphate in micronucleus test in vitro on the human lymphocytes (Vol. 3 CA, B.6.4.1.3)

The analysis of Cytokinesis Block Proliferation Index (CBPI), Replication Index (RI) and % cytostasis of peripheral blood lymphocytes from donor 1 (female, 24 years of age) and donor 2 (male, 33 years of age) exposed to Ferric pyrophosphate at selected concentrations.

Table 1. The preliminary study.

Ferric pyrophosphate concentration [mg/ml]		DONOR 1							DONOR 2						
		Code No	Number of cells			CBPI	RI	% Cytostasis	Code No	Number of cells			CBPI	RI	% Cytostasis
			mononuclear	binuclear	multinuclear					mononuclear	binuclear	multinuclear			
3h - S9	K0	P0	medium without macroscopic changes												
	K1	P21	187	205	108	1.84	100	0	P31	150	257	93	1.89	100	0
	K2	P22	151	237	112	1.92	100	0	P32	164	238	98	1.87	98	2
	K3 (NaCl)	P23	175	208	117	1.88	100	0	P33	157	243	100	1.89	100	0
	0.05	P24	132	256	112	1.96	100	0	P34	190	206	104	1.83	93	7
	0.0158	P25	130	262	108	1.96	100	0	P35	179	236	85	1.81	92	8
	0.005	P26	157	235	108	1.90	100	0	P36	143	264	93	1.90	100	0
	0.00158	P27	139	253	108	1.94	100	0	P37	164	240	96	1.86	98	2
	0.0005	P28	126	260	114	1.98	100	0	P38	158	264	78	1.84	95	5
	K^+ (MMC)	P29	142	262	96	1.91	100	0	P39	175	234	91	1.83	94	6
	K^+ (VIN)	P30	154	217	129	1.95	100	0	P40	156	252	92	1.87	98	2
3h + S9	K1	P41	153	231	116	1.93	100	0	P50	157	243	100	1.89	100	0
	K2	P42	149	242	109	1.92	99	1	P51	151	247	102	1.90	100	0
	K3 (NaCl)	P43	144	240	116	1.94	100	0	P52	149	251	100	1.90	100	0
	0.05	P44	140	247	113	1.95	100	0	P53	155	244	101	1.89	100	0
	0.0158	P45	176	217	107	1.86	93	7	P54	153	250	97	1.89	100	0
	0.005	P46	165	217	118	1.91	98	2	P55	153	246	101	1.90	100	0
	0.00158	P47	145	237	118	1.95	100	0	P56	148	238	114	1.93	100	0
	0.0005	P48	153	240	107	1.91	98	2	P57	150	249	101	1.90	100	0
	K^+ (CP)	P49	119	274	107	1.98	105	0	P58	179	227	94	1.83	94	6

24h - S9	K1	P1	119	242	139	2.04	100	0	P11	183	229	88	1.81	100	0
	K2	P2	123	242	135	2.02	98	2	P12	177	208	115	1.88	100	0
	K3 (NaCl)	P3	130	234	144	2.03	99	1	P13	190	212	98	1.82	100	0
	0.05	P4	119	218	163	2.09	100	0	P14	201	207	92	1.78	97	3
	0.0158	P5	128	231	141	2.03	99	1	P15	212	182	106	1.79	97	3
	0.005	P6	126	240	134	2.02	98	2	P16	188	212	100	1.82	100	0
	0.00158	P7	126	229	145	2.04	100	0	P17	190	207	103	1.83	100	0
	0.0005	P8	123	245	132	2.02	98	2	P18	156	252	92	1.87	100	0
	K^+ (MMC)	P9	183	234	83	1.80	77	23	P19	174	242	84	1.82	100	0
	K^+ (VIN)	P10	175	233	92	1.83	80	20	P20	189	238	73	1.77	95	5

Negative controls:
K0 – quality control of the culture medium (RPMI culture medium without blood): any potential macroscopic changes due to, *e.g.* bacterial contamination, *etc.* are monitored.
K1 – RPMI culture medium with blood and with or without S9-mix
K2 – RPMI culture medium with blood (and with or without S9-mix) and deionized sterile water (the Ferric pyrophosphate vehicle)
K3 – RPMI culture medium with blood (and with or without S9-mix) and negative control (10 mM NaCl, Sigma-Aldrich #S5886). Concentration of the negative control substance was selected according to OECD TG 487.

Positive controls (K^+):
- Mitomycin C (MMC; Sigma-Aldrich #M4287):
  - 0.15 µg/ml, 3-hr exposure without S9-mix
- Vinblastine (VIN; Sigma-Aldrich #V1377):
  - 10 ng/ml, 3-hr exposure without S9-mix
- Cyclophosphamide (CP; Sigma-Aldrich #C0768):
  - 2.5 µg/ml, 3-hr exposure with S9-mix.

Table 2. The main study - micronuclei frequency (MN) in binucleated lymphocytes (BN) from **donor 1 (female, 33 years of age)** after **3 hours** of exposure to Ferric pyrophosphate with or without metabolic activation **(±S9)**.

Ferric pyrophosphate concentration [mg/ml]		Sample code No	BN with MN	MN/500BN	Number of BN with 1, 2 or ≥3 MN, respectively			Number of cells			CBPI	Avg CBPI	RI	% Cytostasis
					1	2	3	mononuclear	binuclear	multinuclear				
3h –S9	K0	M0	medium without macroscopic changes											
	K1	M1	1	1	1	0	0	155	255	90	1.87	1.80	100	0
		M2	0	0	0	0	0	161	242	97	1.87		100	0
	K2	M3	2	2	2	0	0	159	257	84	1.85	1.86	98	2
		M4	0	0	0	0	0	158	247	95	1.87		100	0
	K3 (NaCl)	M5	1	1	1	0	0	148	250	102	1.91	1.90	100	0
		M6	0	0	0	0	0	156	246	98	1.88		100	0
	0.05	M7	0	0	0	0	0	141	266	93	1.90	1.89	100	0
		M8	1	1	1	0	0	160	247	93	1.87		99	1
	0.0158	M9	1	1	1	0	0	145	263	92	1.89	1.88	100	0
		M10	1	1	1	0	0	164	240	96	1.86		99	1
	0.005	M11	0	0	0	0	0	156	244	100	1.89	1.88	100	0
		M12	0	0	0	0	0	164	236	100	1.87		100	0
	0.00158	M13	0	0	0	0	0	137	263	100	1.93	1.91	100	0
		M14	1	1	1	0	0	154	242	104	1.90		100	0
	K^+ (MMC)	M15	20	25	16	3	1	199	215	86	1.77	1.79	89	11
		M16	23	29	19	2	2	189	218	93	1.81		93	7
	K^+ (VIN)	M17	22	26	19	2	1	151	256	93	1.88	1.89	100	0
		M18	20	22	18	2	0	149	253	98	1.90		100	0
3h +S9	K1	M37	1	1	1	0	0	154	252	94	1.88	1.88	100	0
		M38	0	0	0	0	0	162	239	99	1.87		100	0
	K2	M39	0	0	0	0	0	158	246	96	1.88	1.88	100	0
		M40	1	1	1	0	0	153	252	95	1.88		100	0
	K3 (NaCl)	M41	1	1	1	0	0	153	259	88	1.87	1.85	99	1
		M42	0	0	0	0	0	182	220	98	1.83		95	5
	0.05	M43	2	2	2	0	0	173	242	85	1.82	1.83	94	6
		M44	0	0	0	0	0	178	230	92	1.83		95	5

	0.0158	M45	1	1	1	0	0	171	234	95	1.85	1.83	96	4
		M46	0	0	0	0	0	191	208	101	1.82		94	6
	0.005	M47	1	1	1	0	0	190	216	94	1.81	1.81	92	8
		M48	1	1	1	0	0	195	206	99	1.81		92	8
	0.00158	M49	0	0	0	0	0	191	206	103	1.82	1.82	94	6
		M50	1	1	1	0	0	194	208	98	1.81		92	8
	K^+ (CP)	M51	9	14	6	1	2	186	241	73	1.77	1.77	88	12
		M52	12	16	9	2	1	180	242	63	1.76		87	13

Negative controls:
K0 – quality control of the culture medium (RPMI culture medium without blood). Any potential macroscopic changes due to, *e.g.* bacterial contamination, *etc*. are monitored.
K1 – RPMI culture medium with blood and without S9-mix
K2 – RPMI culture medium with blood without S9-mix and deionized sterile water (the Ferric pyrophosphate vehicle)
K2 – RPMI culture medium with blood without S9-mix and negative control (10 mM NaCl, Sigma-Aldrich #S5886). Concentration of the negative substance was selected according to OECD TG 487.

Positive controls (K^+):
- Mitomycin C (MMC; Sigma-Aldrich #M4287):
  - 0.05 µg/ml, 24-hr exposure without S9
- Vinblastine (VIN; Sigma-Aldrich #V1377):
  - 0.6 ng/ml, 24-hr exposure without S9

Table 3. The main study - micronuclei frequency (MN) in binucleated lymphocytes (BN) from donor 2 (female, 23 years of age) after 3 hours of exposure Ferric pyrophosphate with or without metabolic activation (±S9)..

Ferric pyrophosphate concentration [mg/ml]		Sample code No	BN with MN	MN/500BN	Number of BN with 1, 2 or ≥3 MN, respectively			Number of cells			CBPI	Avg CBPI	RI	% Cytostasis
					1	2	3	mononuclear	binuclear	multinuclear				
3h –S9	K0	M0	medium without macroscopic changes											
	K1	M19	1	1	1	0	0	157	244	99	1.88	1.90	100	0
		M20	1	1	1	0	0	152	241	107	1.91		100	0
	K2	M21	0	0	0	0	0	158	236	106	1.90	1.88	100	0
		M22	1	1	1	0	0	169	234	97	1.86		94	6
	K3 (NaCl)	M23	0	0	0	0	0	150	252	98	1.90	1.87	100	0
		M24	1	1	1	0	0	181	212	107	1.85		94	6
	0.05	M25	0	0	0	0	0	180	219	101	1.84	1.86	95	5
		M26	1	1	1	0	0	160	241	99	1.88		96	4
	0.0158	M27	1	1	1	0	0	175	232	93	1.84	1.87	95	5
		M28	0	0	0	0	0	158	235	107	1.90		99	1
	0.005	M29	2	2	2	0	0	175	228	97	1.84	1.86	95	5
		M30	0	0	0	0	0	161	237	102	1.88		97	3
	0.00158	M31	1	1	1	0	0	166	229	105	1.88	1.86	99	1
		M32	1	1	1	0	0	178	223	99	1.84		93	7
	K^+ (MMC)	M33	23	29	19	2	2	178	241	81	1.81	1.83	91	9
		M34	24	28	21	2	1	169	240	91	1.84		93	7
	K^+ (VIN)	M35	20	23	18	1	1	183	224	93	1.82	1.79	93	7
		M36	18	24	14	2	2	208	205	87	1.76		83	17
3h +S9	K1	M53	0	0	0	0	0	185	205	110	1.85	1.83	100	0
		M54	1	1	1	0	0	200	191	109	1.82		100	0
	K2	M55	1	1	1	0	0	193	216	91	1.80	1.81	94	6
		M56	0	0	0	0	0	191	203	106	1.83		100	0
	K3 (NaCl)	M57	0	0	0	0	0	195	200	105	1.82	1.81	96	4
		M58	1	1	1	0	0	194	213	93	1.80		98	2
	0.05	M59	0	0	0	0	0	185	224	92	1.81	1.81	96	4
		M60	1	1	1	0	0	191	217	92	1.80		98	2
	0.0158	M61	0	0	0	0	0	182	219	99	1.83	1.83	98	2

		M62	0	0	0	0	0	188	214	98	1.82		100	0
	0.005	M63	1	1	1	0	0	192	212	96	1.81	1.81	95	5
		M64	0	0	0	0	0	188	217	95	1.81		100	0
	0.00158	M65	1	1	1	0	0	188	220	92	1.81	1.82	95	5
		M66	1	1	1	0	0	181	225	94	1.83		100	0
	K^+ (CP)	M67	13	17	10	2	1	201	210	89	1.78	1.77	91	9
		M68	10	13	8	1	1	202	211	87	1.77		94	6

Negative controls:
K0 – quality control of the culture medium (RPMI culture medium without blood). Any potential macroscopic changes due to, *e.g.* bacterial contamination, *etc.* are monitored.
K1 – RPMI culture medium with blood and without S9-mix
K2 – RPMI culture medium with blood without S9-mix and deionized sterile water (the Ferric pyrophosphate vehicle)
K2 – RPMI culture medium with blood without S9-mix and negative control (10 mM NaCl, Sigma-Aldrich #S5886). Concentration of the negative substance was selected according to OECD TG 487.

Positive controls (K^+):
- Mitomycin C (MMC; Sigma-Aldrich #M4287):
  - 0.05 µg/ml, 24-hr exposure without S9
- Vinblastine (VIN; Sigma-Aldrich #V1377):
  - 0.6 ng/ml, 24-hr exposure without S9

Table 4. The main study - micronuclei frequency (MN) in binucleated lymphocytes (BN) from donor 1 (male, 31 years of age) after 24 hours of exposure to Ferric pyrophosphate without metabolic activation (-S9).

Ferric pyrophosphate concentration [mg/ml]		Sample code No	BN with MN	MN/500BN	Number of BN with 1, 2 or ≥3 MN, respectively			Number of cells			CBPI	Avg CBPI	RI	% Cytostasis
					1	2	3	mononuclear	binuclear	multinuclear				
24 h -S9	K0	S0	medium without macroscopic changes											
	K1	S1	1	1	1	0	0	170	232	98	1.86	1.84	100	0
		S2	1	1	1	0	0	189	210	101	1.82		100	0
	K2	S3	1	1	1	0	0	180	223	97	1.83	1.83	97	3
		S4	0	0	0	0	0	188	213	99	1.82		100	0
	K3 (NaCl)	S5	1	1	1	0	0	183	207	110	1.85	1.84	100	0
		S6	1	1	1	0	0	188	213	99	1.82		100	0
	0.05	S7	1	1	1	0	0	179	224	97	1.84	1.83	98	2
		S8	1	1	1	0	0	190	211	99	1.82		99	1
	0.0158	S9	1	1	1	0	0	188	213	99	1.82	1.83	96	4
		S10	1	1	1	0	0	182	219	99	1.83		100	0
	0.005	S11	1	1	1	0	0	192	207	101	1.82	1.85	96	4
		S12	1	1	1	0	0	156	245	99	1.89		100	0
	0.00158	S13	0	0	0	0	0	165	228	107	1.88	1.87	100	0
		S14	1	1	1	0	0	172	225	103	1.86		100	0
	K^+ (MMC)	S15	19	22	17	1	1	176	217	107	1.86	1.85	100	0
		S16	17	18	16	1	0	190	198	112	1.84		100	0
	K^+ (VIN)	S17	18	22	15	2	1	179	225	98	1.84	1.84	98	2
		S18	21	23	19	2	0	183	211	106	1.85		100	0

Negative controls:
K0 – quality control of the culture medium (RPMI culture medium without blood). Any potential macroscopic changes due to, *e.g.* bacterial contamination, *etc.* are monitored.
K1 – RPMI culture medium with blood and without S9-mix
K2 – RPMI culture medium with blood without S9-mix and deionized sterile water (the Ferric pyrophosphate vehicle)
K2 – RPMI culture medium with blood without S9-mix and negative control (10 mM NaCl, Sigma-Aldrich #S5886). Concentration of the negative substance was selected according to OECD TG 487.
Positive controls (K^+):
- Mitomycin C (MMC; Sigma-Aldrich #M4287):
  - 0.05 µg/ml, 24-hr exposure without S9
- Vinblastine (VIN; Sigma-Aldrich #V1377):
  - 0.6 ng/ml, 24-hr exposure without S9

Table 5. The main study - micronuclei frequency (MN) in binucleated lymphocytes (BN) from **donor 2 (male, 35 years of age)** after **24 hours** of exposure to Ferric pyrophosphate without metabolic activation **(-S9)**.

Ferric pyrophosphate concentration [mg/ml]		Sample code No	BN with MN	MN/500BN	Number of BN with 1, 2 or ≥3 MN, respectively			Number of cells			CBPI	Avg CBPI	RI	% Cytostasis
					1	2	3	mononuclear	binuclear	multinuclear				
24 h –S9	K0	S0	medium without macroscopic changes											
	K1	S19	1	1	1	0	0	171	222	108	1.87	1.87	100	0
		S20	0	0	0	0	0	182	208	110	1.86		100	0
	K2	S21	1	1	1	0	0	172	217	111	1.88	1.87	100	0
		S22	1	1	1	0	0	173	220	107	1.87		100	0
	K3 (NaCl)	S23	0	0	0	0	0	150	241	109	1.92	1.91	100	0
		S24	0	0	0	0	0	157	234	109	1.90		100	0
	0.05	S25	1	1	1	0	0	178	215	107	1.86	1.87	98	2
		S26	1	1	1	0	0	176	210	114	1.88		100	0
	0.0158	S27	0	0	0	0	0	167	217	106	1.88	1.86	100	0
		S28	1	1	1	0	0	185	207	108	1.85		99	1
	0.005	S29	1	1	1	0	0	168	222	108	1.88	1.88	100	0
		S30	2	2	2	0	0	173	229	108	1.87		100	0
	0.00158	S31	1	1	1	0	0	183	211	106	1.85	1.84	97	3
		S32	0	0	0	0	0	180	218	101	1.84		98	2
	K^+ (MMC)	S33	21	27	17	2	2	149	237	114	1.93	1.90	100	0
		S34	21	25	18	2	1	171	222	107	1.87		100	0
	K^+ (VIN)	S35	19	24	15	3	1	166	218	116	1.90	1.90	100	0
		S36	20	26	16	2	2	163	229	108	1.89		100	0

Negative controls:

K0 – quality control of the culture medium (RPMI culture medium without blood). Any potential macroscopic changes due to, *e.g.* bacterial contamination, *etc.* are monitored.
K1 – RPMI culture medium with blood and without S9-mix
K2 – RPMI culture medium with blood without S9-mix and deionized sterile water (the Ferric pyrophosphate vehicle)
K2 – RPMI culture medium with blood without S9-mix and negative control (10 mM NaCl, Sigma-Aldrich #S5886). Concentration of the negative substance was selected according to OECD TG 487.
Positive controls (K^+):
- Mitomycin C (MMC; Sigma-Aldrich #M4287):
  - 0.05 µg/ml, 24-hr exposure without S9
- Vinblastine (VIN; Sigma-Aldrich #V1377):
  - 0.6 ng/ml, 24-hr exposure without S9

Appendix 1D: The detailed data from the study [redacted] Mammalian erythrocyte micronucleus test with using animals from repeated dose 90-day oral toxicity study in rodents (Vol. 3 CA, B.6.4.2.1)

Table 1. Evaluation results of microscopic preparations of peripheral blood and bone marrow for individual groups

Animal no.	Sex/Group	Peripheral blood evaluation						Bone marrow evaluation					
		PCE/2000 ery	NCE/ 2000ery	MNPCE/ 2000ery	MNNCE/ 2000ery	PCE/NCE	PCE/Total	PCE/ 2000ery	NCE/ 2000ery	MNPCE/ 2000ery	MNNCE/ 2000ery	PCE/NC E	PCE/Total
55 /13/1	F/1000	44	1956	1	0	0,022	0,022	1013	987	1	0	1,03	0,51
56 /13/1	F/1000	37	1963	1	0	0,019	0,019	1144	856	1	0	1,34	0,57
57 /13/1	F/1000	40	1960	1	0	0,020	0,020	902	1099	0	0	0,82	0,45
58 /13/1	F/1000	52	1948	1	0	0,027	0,026	1155	845	1	0	1,37	0,58
60 /13/1	F/1000	58	1942	1	0	0,030	0,029	1049	951	1	0	1,1	0,52
61 /13/1	F/1000	48	1952	0	0	0,025	0,024	1085	915	0	0	1,19	0,54
62 /13/1	F/1000	29	1971	1	0	0,015	0,015	972	1028	1	0	0,95	0,49
63 /13/1	F/1000	36	1964	0	0	0,018	0,018	1106	894	0	0	1,24	0,55
64 /13/1	F/1000	44	1956	1	0	0,022	0,022	1022	978	1	0	1,05	0,51
65 /13/1	F/1000	40	1960	1	0	0,020	0,020	952	1048	2	0	0,91	0,48
66 /13/1	F/1000	40	1960	1	0	0,020	0,020	963	1037	1	0	0,93	0,48
67 /13/1	F/1000	62	1938	0	0	0,032	0,031	1304	696	0	0	1,87	0,65
68 /13/1	F/1000	42	1958	0	0	0,021	0,021	995	1005	0	0	0,99	0,50
69 /13/1	F/1000	50	1950	0	0	0,026	0,025	1122	878	0	0	1,28	0,56
71 /13/1	M/1000	33	1967	0	0	0,017	0,017	1050	950	0	0	1,11	0,53
72 /13/1	M/1000	38	1962	2	0	0,019	0,019	1152	848	1	0	1,36	0,58
73 /13/1	M/1000	68	1932	0	0	0,035	0,034	1051	949	0	0	1,11	0,53
74 /13/1	M/1000	48	1952	1	0	0,025	0,024	1080	920	1	0	1,17	0,54
75 /13/1	M/1000	46	1954	0	0	0,024	0,023	1314	686	0	0	1,92	0,66
76 /13/1	M/1000	54	1946	1	0	0,028	0,027	1178	822	1	0	1,43	0,59
78 /13/1	M/1000	47	1953	1	0	0,024	0,024	1067	933	1	0	1,14	0,53

Animal no.	Sex/Group	Peripheral blood evaluation						Bone marrow evaluation					
		PCE/2000ery	NCE/2000ery	MNPCE/2000ery	MNNCE/2000ery	PCE/NCE	PCE/Total	PCE/2000ery	NCE/2000ery	MNPCE/2000ery	MNNCE/2000ery	PCE/NCE	PCE/Total
79 /13/1	M/1000	20	1980	0	0	0,010	0,010	800	1200	0	0	0,67	0,40
80 /13/1	M/1000	38	1962	0	0	0,019	0,019	1152	848	0	0	1,36	0,58
81 /13/1	M/1000	35	1965	0	0	0,018	0,018	1092	908	0	0	1,2	0,55
82 /13/1	M/1000	24	1976	1	0	0,012	0,012	966	1034	1	0	0,93	0,48
83 /13/1	M/1000	23	1977	0	0	0,012	0,012	937	1063	0	0	0,88	0,47
84 /13/1	M/1000	35	1965	0	0	0,018	0,018	1100	900	1	0	1,22	0,55
101 /13/1	F/ctrl	72	1928	0	0	0,037	0,036	1195	805	0	0	1,49	0,60
102 /13/1	F/ctrl	38	1962	1	0	0,019	0,019	980	1020	1	0	0,96	0,49
103 /13/1	F/ctrl	51	1949	0	0	0,026	0,026	1062	938	0	0	1,13	0,53
104 /13/1	F/ctrl	40	1960	0	0	0,020	0,020	1018	982	1	0	1,04	0,51
105 /13/1	F/ctrl	40	1960	0	0	0,020	0,020	1018	982	0	0	1,04	0,51
106 /13/1	F/ctrl	55	1945	0	0	0,028	0,028	1114	886	0	0	1,26	0,56
107 /13/1	F/ctrl	39	1961	1	0	0,020	0,020	1004	996	1	0	1,01	0,50
108 /13/1	F/ctrl	40	1960	1	0	0,020	0,020	1010	990	1	0	1,02	0,51
109 /13/1	M/ctrl	37	1963	0	0	0,019	0,019	1128	872	1	0	1,29	0,56
110 /13/1	M/ctrl	45	1955	1	0	0,023	0,023	1286	714	1	0	1,8	0,64
111 /13/1	M/ctrl	25	1975	2	0	0,013	0,013	890	1110	2	0	0,8	0,45
112 /13/1	M/ctrl	38	1962	0	0	0,019	0,019	1156	844	0	0	1,37	0,58
113 /13/1	M/ctrl	38	1962	0	0	0,019	0,019	1040	960	1	0	1,08	0,52
114 /13/1	M/ctrl	32	1968	0	0	0,016	0,016	1026	974	0	0	1,05	0,51
115 /13/1	M/ctrl	33	1967	1	0	0,017	0,017	1058	942	1	0	1,12	0,53
116 /13/1	M/ctrl	38	1962	0	0	0,019	0,019	1162	838	0	0	1,39	0,58
117 /13/1	F/pos.ctrl	28	1972	8	0	0,014	0,014	874	1126	25	0	0,77	0,44
118 /13/1	F/pos.ctrl	12	1988	8	1	0,006	0,006	859	1141	22	0	0,75	0,43

Animal no.	Sex/Group	Peripheral blood evaluation						Bone marrow evaluation					
		PCE/2000 ery	NCE/ 2000ery	MNPCE/ 2000ery	MNNCE/ 2000ery	PCE/NCE	PCE/Total	PCE/ 2000ery	NCE/ 2000ery	MNPCE/ 2000ery	MNNCE/ 2000ery	PCE/NC E	PCE/Total
119 /13/1	F/pos.ctrl	19	1981	9	0	0,010	0,010	867	1133	30	0	0,76	0,43
120 /13/1	F/pos.ctrl	43	1957	5	0	0,022	0,022	914	1086	64	0	0,84	0,46
122 /13/1	F/pos.ctrl	22	1978	3	0	0,011	0,011	848	1152	27	0	0,73	0,42
123 /13/1	F/pos.ctrl	21	1979	4	1	0,011	0,011	852	1148	41	0	0,74	0,43
124 /13/1	F/pos.ctrl	13	1987	7	0	0,007	0,007	900	1100	57	1	0,81	0,45
125 /13/1	F/pos.ctrl	29	1971	6	0	0,015	0,015	805	1195	46	0	0,67	0,40
128 /13/1	M/pos.ctrl	40	1960	11	0	0,020	0,020	779	1221	51	1	0,64	0,39
129 /13/1	M/pos.ctrl	12	1988	6	0	0,006	0,006	849	1151	39	0	0,74	0,42
130 /13/1	M/pos.ctrl	13	1987	7	1	0,007	0,007	879	1121	31	0	0,78	0,44
131 /13/1	M/pos.ctrl	26	1974	4	0	0,013	0,013	868	1132	13	0	0,77	0,43
132 /13/1	M/pos.ctrl	40	1960	6	0	0,020	0,020	817	1183	36	0	0,69	0,41
133 /13/1	M/pos.ctrl	42	1958	5	0	0,021	0,021	867	1133	60	2	0,77	0,43
135 /13/1	M/pos.ctrl	27	1973	5	0	0,014	0,014	880	1120	14	0	0,79	0,44
136 /13/1	M/pos.ctrl	60	1940	13	0	0,031	0,030	922	1078	53	0	0,85	0,46

F – female
M – male
1000 – Dose 1000 mg/kg b.w. of iron (III) pyrophosphate
ctrl – control group treated 0,5 % methylcellulose solution
pos.ctrl – positive control group treated Ethyl methanesulphonate (dose 350 mg/kg b.w.)
PCE/2000ery – number of polichromatic erythrocytes / 2000 erythrocytes
NCE/2000ery – number of normochromatic erythrocytes / 2000 erythrocytes
MNPCE/2000ery – number of polichromatic erythrocytes containing micronuclei / 2000 erythrocytes
MNNCE/2000ery – number of normochromatic erythrocytes containing micronuclei / 2000 erythrocytes
PCE/Total - ratio of polychromatic erythrocytes / 2000 erythrocytes

Table 2. Mean values ± standard deviation of the assessment in each group.

Sex / Group		Assesment of peripherial blood						Assesment of bone narrow					
		PCE /2000	NCE /2000	MNPCE /2000	MNNCE /2000	PCE/NCE	PCE /Total	PCE /2000	NCE /2000	MNPCE /2000	MNNCE /2000	PCE/NCE	PCE /Total
F/1000	Śr.	44	1956	0,64	0,000	0,023	0,023	1056	944	0,64	0,00	1,15	0,528
	SD	9	9	0,50	0,000	0,005	0,005	105	105	0,63	0,00	0,27	0,053
M/1000	Śr.	39	1961	0,46	0,000	0,020	0,020	1072	928	0,46	0,00	1,19	0,536
	SD	13	13	0,66	0,000	0,007	0,007	125	125	0,52	0,00	0,30	0,063
F/ctrl	Śr.	47	1953	0,38	0,000	0,024	0,024	1050	950	0,50	0,00	1,12	0,525
	SD	12	12	0,52	0,000	0,006	0,006	72	72	0,53	0,00	0,18	0,036
M/ctrl	Śr.	36	1965	0,50	0,000	0,018	0,018	1093	907	0,75	0,00	1,24	0,547
	SD	6	6	0,76	0,000	0,003	0,003	118	118	0,71	0,00	0,30	0,059
F/pos.ctrl	Śr.	23	1977	6,25	0,250	0,012	0,012	865	1135	39,00	0,13	0,76	0,432
	SD	10	10	2,12	0,463	0,005	0,005	33	33	15,64	0,35	0,05	0,017
M/pos.ctrl	Śr.	33	1968	7,13	0,125	0,017	0,017	858	1142	37,13	0,38	0,75	0,429
	SD	16	16	3,18	0,354	0,008	0,008	43	43	17,43	0,74	0,06	0,022

F – female
M – male
1000 – Dose 1000 mg/kg b.w. of iron (III) pyrophosphate
ctrl – control group treated with 0,5 % methylcellulose solution
pos.ctrl – positive control group treated with Ethyl methanesulphonate (dose 350 mg/kg b.w.)
M – mean value of the group
SD – standard deviation of the group
PCE/2000 – number of polichromatic erythrocytes / 2000 erythrocytes
NCE/2000 – number of normochromatic erythrocytes / 2000 erythrocytes
MNPCE/2000 – number of polichromatic erythrocytes containing micronuclei / 2000 erythrocytes
MNNCE/2000 – number of normochromatic erythrocytes containing micronuclei / 2000 erythrocytes
PCE/Total – number of polichromatic erythrocytes / total number of erythrocytes

Appendix 2

Literature search on Ferric pyrophosphate Prepared by [redacted] on behalf of Bros Spółka z o.o. March 2019

1. Introduction

This report summarises the results of a scientific peer-review open literature search conducted in order to support the authorization of the active substance ferric pyrophosphate under Regulation (EU) No 1107/2009.

Ferric pyrophosphate acts as a molluscicide and is intended for the control of slugs and snails in all edible and inedible plants. It has not yet been approved as an active substance in plant protection products and is to be regarded as a new active substance according to the regulation.

Article 8(5) of Regulation (EU) No 1107/2009 requires that a scientific peer-reviewed open literature search must be conducted on the active substance, dealing with side-effects on health, the environment and non-target species and published within the last 10 years before the date of submission of the dossier.

The literature search was conducted in accordance with the EFSA guidance on *Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009* (EFSA Journal 2011;9(2):2092). In order to capture all data requirements of interest, a single-concept search strategy was adopted, using search terms for the active substance and its synonyms only, without additional concepts.

The main categories of data requirements on chemical active substances are:

- Toxicological and toxicokinetic studies
- Metabolism and residue data
- Fate and behaviour in the environment
- Ecotoxicological studies
- Other data requirements for which information may have a direct or indirect effect on overall risk assessment.

The literature review should also address endocrine disruption.

2. Criteria for relevance

Publications were deemed relevant if they provided information on ferric pyrophosphate that addressed the data requirements on chemical active substances.

The relevance criteria are presented in Table 1 below.

Table 1: Relevance criteria

Data requirement indicated by the corresponding OECD data point number	Criteria for relevance
Toxicological and metabolism studies (OECD IIA 5.1 to 5.7)	1. Well-defined test material 2. Test species relevant to the mammalian toxicological assessment 3. Number of animals per group sufficient to establish statistical significance 4. Several dose levels tested (at least 3), preferably including a negative control, to establish a dose-response 5. Well-described test methodology 6. Description of the observations, mortality etc., and chemical analysis performed
Residues (OECD IIA 6)	1. Well-defined test material 2. Any information on residues in crops relevant to the EU 3. Well-described test methodology and results
Fate and behaviour in the environment (OECD IIA 7)	1. Any information that could affect the environmental parameters, degradation profile or endpoints used in the risk assessment 2. Any environmental monitoring information relevant to the usage pattern 3. Well-defined test material 4. Well-described test methodology
Ecotoxicological studies (OECD IIA 8)	1. Well-defined test material 2. Relevant test species 3. Number of test organisms per group sufficient to establish statistical significance 4. Several dose levels tested, preferably including a negative control 5. Well-described test methodology 6. Description of the observations, mortality etc., and chemical analysis performed
Other data requirements for which information may have a direct or indirect on the overall risk assessment, including endocrine disruption	1. Well-defined test material 2. Any information that could affect the risk assessment other than under the OECD data points already addressed

3. Search methods and initial results

The scientific literature was searched using the following resources:

- Scopus
- Web of Science
- Embase
- Reaxys
- Agricola

The justification for the choice of each database is provided in Table 2.

The EFSA guidance states that the search strategy should capture literature published during the 10 years prior to the dossier submission date, as required by Article 8(5) of Regulation (EC) No. 1107/2009. The date span of the search was 2005 to 2015, as the dossier for ferric pyrophosphate was submitted for approval in December 2015.

A single-concept search strategy was adopted, using search terms for the active substance and its synonyms only, without additional concepts. The search terms chosen were “ferric pyrophosphate” and “iron pyrophosphate” as well as the CAS number 10058-44-3. Given the range of the data requirements to be addressed, the alternative strategy of multiple focused searches would have required an excessive number of search terms to cover all possibilities. It was assumed that the single-concept strategy would produce a large number of irrelevant documents, but it was decided that this approach would be less time-consuming as well as highly sensitive.

Table 2: Reporting of the search process for scientific peer-reviewed open literature in bibliographic databases

Data requirement(s) captured in the search	**Details of the searches**				
Ferric pyrophosphate – covers all data requirements	**Scopus**	**Web of Science Core Collection**	**Embase**	**Reaxys**	**Agricola**
	Justification for choosing the source: Large multi-disciplinary database of research literature, with over 16,000 titles and over 69 million records.	**Justification for choosing the source:** Large multi-disciplinary database, with over 20,000 journals and 1.4 billion cited references.	**Justification for choosing the source:** Covers the most important international biomedical literature. Over 8,500 journals and >32 million records. Includes Medline.	**Justification for choosing the source:** Covers over 16,000 chemistry-related periodicals.	**Justification for choosing the source:** Publications and resources encompassing all aspects of agriculture and related disciplines.
	Date of the search: 13/03/2019	**Date of the search:** 13/03/2019	**Date of the search:** 13/03/2019	**Date of the search:** 13/03/2019	**Date of the search:** 13/03/2019
	Date span of the search: 2005-2015	**Date span of the search:** 2005-2015	**Date span of the search:** 2005-2015	**Date span of the search:** 2005-2015	**Date span of the search:** 2005-2015
	Date of the latest database update included in the search:				
	Updated daily.	Updated daily.	Updated daily.	Updated weekly.	Updated daily.
	Search strategies used for this data requirement:				
	(ALL ("ferric pyrophosphate") OR ALL ("iron pyrophosphate") OR ALL ("10058-44-3")) AND (PUBYEAR	ALL=("ferric pyrophosphate" OR "iron pyrophosphate" OR "10058-44-3") Timespan: 2005-2015.	('ferric pyrophosphate' OR 'iron pyrophosphate' OR '10058-44-3') AND [2005-2015]/py	(Chemical Name is 'Ferric pyrophosphate' OR Chemical Name is 'Iron pyrophosphate' OR CAS Registry Number is 10058-44-3)	Keyword Anywhere(ferric pyrophosphate) OR Keyword Anywhere(iron pyrophosphate) OR

	> 2004 AND PUBYEAR < 2016)			AND (Publication Year >=2005 AND Publication Year <=2015)	Keyword Anywhere(10058-44-3)(DATE=2005-2015)
	Total number of summary records retrieved:				
	742	179	115	61	44
	Total number of summary records retrieved after removing duplicates: 802				

CA 3 4. Results of study selection process

Following the initial search, a total of 802 summary records (excluding duplicates) were identified. These were screened for their likely relevance by rapid assessment of titles and, where available, abstracts.

A total of 799 summary records were found to be obviously irrelevant and were excluded from further consideration. It must be noted, however, that although these records did not directly address the listed data points or fulfil the relevance criteria, a substantial number were found by assessment of abstracts to provide information on the use of ferric pyrophosphate as a supplement in food to address iron deficiency, indicating that the substance is safe in the context of human health.

The 3 remaining summary records that were potentially relevant were assessed in detail by reading the full-text documents. Following this assessment, all 3 records were excluded from further consideration.

The results of the different stages of the study selection process are presented in Table 3. The full-text documents that were deemed irrelevant and excluded from further consideration are listed in Table 4.

Table 3: Results of the study selection process, for each data requirement or group of data requirements searched

Data requirement(s) captured in the search (as indicated in Table 2)	**n**
Total number of summary records retrieved after all searches of peer-reviewed literature (excluding duplicates)	802
Number of summary records excluded from the search results after rapid assessment for relevance	799
Total number of full-text documents assessed in detail	3
Number of studies excluded from further consideration after detailed assessment for relevance	3
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0

Table 6: Report of the studies excluded after detailed assessment of full-text documents

Author(s)	Year	Title	Source	Reason(s) for not including this study in the dossier
Li, Bo; Brett, Michael T.	2013	The influence of dissolved phosphorus molecular form on recalcitrance and bioavailability	Environmental Pollution 2013;182:37-44	Does not cover any OECD data point and/or does not fulfil the relevance criteria
Lobo A.R., Cocato M.L., de Sá L.R.M., Colli C.	2014	Dietary iron overload: Short- and long-term effects on cecal morphometry in growing rats	Journal of Nutritional Science and Vitaminology 2014;60(6):397-402	Does not cover any OECD data point and/or does not fulfil the relevance criteria
Sturm B., Ternes N., Goldenberg H., Scheiber-Mojdehkar B.	2009	Ferric pyrophosphate: Biochemical characterisation and metabolic fate in HepG2 cells	American Journal of Hematology 2009;84(8):E287	Does not cover any OECD data point and/or does not fulfil the relevance criteria

CA 4 5. Conclusions

A literature search was conducted to identify scientific peer-reviewed open literature relating to ferric pyrophosphate to support the authorization of this active substance under Regulation (EU) No 1107/2009. Several bibliographic databases were searched, and the date span of the search was 2005 to 2015.

A total of 802 unique summary records were identified, and a rapid assessment of titles and, where available, abstracts was conducted. On assessment, 799 of these records were deemed to be irrelevant and excluded from further consideration. The full text of the remaining 3 records was assessed to determine both relevance and reliability, after which all records were excluded. Therefore no literature of direct relevance to the required data points was identified, although a considerable amount of literature was identified describing the use of ferric pyrophosphate as an iron supplement in the human diet, implying that the substance is safe in the context of human health.