# Bioavailability of Fe, Cu, Zn and antioxidant defence in anemic rat supplemented with a mixture of heme/non-heme Fe

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**Summary**

Scarce information is available about the recovery of anemia with different iron (Fe) sources on enzymatic antioxidant defence; therefore the current study was designed to assess its effect on utilization of Fe, Cu and Zn and antioxidant defence in liver, erythrocytes, duodenal mucosa and plasma. Weanling female rat were divided in two groups: a control group ($n$ = 10) receiving a normal Fe diet with $FeSO_4$ (45.73 mg·kg$^{-1}$) and an anemic group ($n$ = 20) receiving a low-Fe diet (6.92 mg·kg$^{-1}$) for 45 days. For the next thirty days, the anemic rats were fed with a normal Fe diet or with a mixture of heme/non-heme Fe (1 mg of Fe of hydrolysed bovine blood, honey and propolis, and 20 mg $FeSO_4$ for 27.92 mg·kg$^{-1}$ of Fe diet). The anemic group supplemented with the mixture of heme/non-heme Fe showed a positive hematological recovery and the same utilization of the minerals as the group supplemented with $FeSO_4$ only. This group also featured a better balance of antioxidant enzymes. The recovery of anemia with the mixture of heme/non-heme could be considered an alternative therapy to palliate the Fe-deficiency and to reduce the side effects of the classical supplements.

**Keywords**

anemia; mineral bioavailability; heme Fe; non-heme Fe; antioxidant enzymes; rat

Iron (Fe) deficiency is the most common nutritional disorder worldwide [1]. Many persons do not tolerate adequately the Fe therapy due to difficulties associated with the ingestion of tablets and its adverse gastrointestinal effects, present in half of the patients. Most of the oral Fe preparations contain ferrous salts, characterized by a low absorption, which is limited by the ingestion of some food and by the damage in the mucous intestine [2]. High systemic Fe burden is also associated with adverse effects arising from degradation of tissue ferritin and subsequent free radical damage of surrounding tissues [3].

Heme Fe from myoglobin and hemoglobin (Hb) intestinal absorption could occur by an endocytic process, by receptor-mediated endocytosis or by heme transporters called heme carrier protein 1 (HCP1) [4]. The uptake of non-heme Fe by enterocytes occurs through a divalent metal transmembrane receptor (DMT1) [5]. The supplementation of anemic rats and non-anemic humans with mixtures of Fe salts and bovine dehydrated Hb proved a higher mineral bioavailability than when supplemented with the Fe salt only [6, 7]. In vitro studies demonstrated that heme Fe from the native Hb forms dimers that decreased Fe intestinal absorption. However when Hb was hydrolysed, the intestinal absorption of the heme Fe was facilitated [8].

DMT1 is known to be capable of binding a variety of other divalent metals, such as Cu. However, the uptake of Cu by the enterocyte occurs predominantly via the high affinity human Cu transporter 1 (Ctr1) [9]. An ATP-dependent Cu transporter has recently been described. In addition, some Cu-dependent enzymes such as ceruloplasmin, ferrochelatase and cytochrome c play an important role in Fe metabolism. For example, ceruloplasmin is essential for the transformation of $Fe^{2+}$ to $Fe^{3+}$, an essential step for the uptake of Fe and its transfer to the circulating transferrin. Ferrochelatase and cytochrome c are

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crucial for the reduction of $Fe^{3+}$ to $Fe^{2+}$ in mitochondria, and for subsequent incorporation of Fe into protoporphyrin IX. It was reported that Cu-deficient rats presented decreased hephaestin activity, a protein that plays a role in tissue Fe efflux and which therefore regulates dietary Fe absorption. Cu and Zn are cofactors of the antioxidant enzyme superoxide dismutase (SOD). The reduction in SOD activity due to Cu or Zn deficiency could shorten the erythrocyte life span as a result of oxidative stress and anemia [10].

Fe is highly reactive, promotes the generation of oxygen radicals, which may cause oxidative damage and lead to degeneration of proteins, lipids and DNA [11]. Pathological conditions, Fe metabolism and the superoxide radical ($O_2^{-\bullet}$) are intimately linked, therefore each one can exacerbate the toxicity of the others via Fenton chemistry. Fe overload can amplify the harmful effects of the overproduction of $O_2^{-\bullet}$. In addition, the chronic oxidative stress can modulate Fe absorption and its storage, being able to lead to cytotoxic effects. The main antioxidant defence against free radicals is the enzymatic machinery that works to eliminate and neutralize the harmful reactive oxygen species (ROS), SOD, catalase (CAT) and glutathione peroxidase (GPx) [12, 13].

In spite that Fe is one of the most investigated and best known nutrients and Fe deficiency has been widely studied, the oxidative stress and the mechanisms of antioxidant defence during the recovery of the nutritional Fe deficiency anemia remain unclear. The current research was focused on the study of the nutritive utilization of Fe, Cu and Zn after the recovery of the nutritional Fe deficiency anemia with different sources and amounts of Fe from the mixture of a partially hydrolysed bovine blood, honey and propolis and $FeSO_4$. This work also investigated the main enzymatic antioxidant mechanisms (SOD, GPx and CAT) during Fe replenishing because, in situation of anemia, Fe metabolism is seriously impaired and there is a clear relationship between Fe and cell oxidative damage processes. The reason is that generation of oxidants and antioxidants is modified in this situation, so the oxidative/antioxidative balance is also impaired.

## MATERIALS AND METHODS

### Animals

The animals used for this study were female Sprague Dawley outbred albino rats ($n$ = 30), recently weaned, weighing 40–45 g (purchased from Harlan Laboratories, Correzzana, Italy). All animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada in accordance with the European Community guidelines (Declaration of Helsinki; Directive 2010/63/EU for animal experiments).

### Process to obtain the heme Fe source

In order to obtain the heme Fe source, a mixture of 2501 that contained 125–1301 of bovine blood, 25–281 of bee honey and 0.3751 of alcoholic extract of propolis at 15% was prepared. The remaining volume was completed by a solution of an extract of bovine pepsin obtained from the stomach of ruminants. The natural extract of the enzyme had a concentration of 1 $mg \cdot ml^{-1}$ of protein and was adjusted to pH 2.0–1.5. The preparation, with a final pH 4.5–5.0 was incubated at constant agitation at a temperature of 42–45 °C in a stainless steel reactor (Varona, Acinox, Havana, Cuba) during 5 h to partially hydrolyse proteins from bovine blood. Finally, the preparation was dehydrated by spray technology (Niro Atomizer, Soeborg, Denmark) to obtain approximately 200 $g \cdot l^{-1}$ of the dry extract as a fine, reddish-brown powder.

### Experimental design for studies of mineral bioavailability

During a pre-experimental period (PEP) of 45 days, the rats were divided into two groups: the control group ($n$ = 10) receiving the American Institute of Nutrition (AIN-93G) diet [14] with normal Fe content from $FeSO_4$, and the Fe-deficient group ($n$ = 20) receiving a low Fe diet, to induce a severe dietary Fe deficiency, according to a method developed previously [15]. After PEP, the rats entered an experimental period (EP) of 14 days. The mineral and vitamin supplements used were prepared according to the recommendations of AIN-93G, except for the fat source (corn oil instead of soybean oil, as the former is the most commonly consumed in America) and Fe source ($FeSO_4$) instead of ferric citrate, because this Fe salt is the most recommended by the World Health Organization (WHO) to be used in Fe fortification programmes [16]. Cu and Zn contents were adjusted according to the AIN recommendations in all the diets assayed. During EP, the control group received AIN-93G diet with normal Fe content (NA-$FeSO_4$), while the group that received Fe-deficient diet was sub-divided into two anemic groups ($n$ = 10 per group). One group received the same AIN-93G with normal Fe content (A-$FeSO_4$) and another group received the Fe-deficient diet AIN-93G that was supplemented with: 1 mg of Fe from the heme source and 20 mg of Fe

**Tab. 1.** Composition of experimental diets.

| Component | PEP diets | | EP diets | |
|---|---|---|---|---|
| | Normal Fe | Deficient Fe | Normal Fe | Experimental diet |
| Protein (casein) [g·kg$^{-1}$] | 200 | 200 | 200 | 200 |
| Fat (corn oil) [g·kg$^{-1}$] | 70 | 70 | 70 | 70 |
| Fibre (micronized cellulose) [g·kg$^{-1}$] | 51 | 51 | 51 | 51 |
| Mineral supplement [g·kg$^{-1}$] | 35 | 35 | 35 | 35 |
| Vitamin supplement [g·kg$^{-1}$] | 10 | 10 | 10 | 10 |
| Choline chloride [g·kg$^{-1}$] | 2 | 2 | 2 | 2 |
| Corn starch [g·kg$^{-1}$] | 530.2 | 530.2 | 530.2 | 530.2 |
| Saccharose [g·kg$^{-1}$] | 100 | 100 | 100 | 100 |
| *L*-cystine [g·kg$^{-1}$] | 1.8 | 1.8 | 1.8 | 1.8 |
| Energy [kJ·kg$^{-1}$] | 17112 | 17112 | 17112 | 17112 |
| Mineral composition | | | | |
| Fe [mg·kg$^{-1}$] | 45.7 | 6.9 | 45.7 | 27.9 |
| Cu [mg·kg$^{-1}$] | 7.1 | 7.1 | 7.1 | 7.1 |
| Zn [mg·kg$^{-1}$] | 35.0 | 35.0 | 35.0 | 35.0 |

Content of components is expressed on dry matter basis. The mineral premix was prepared according to AIN-93G for nomal Fe diet, but using $FeSO_4$ as Fe source. The mineral premix for the Fe-deficient diet was prepared by omitting Fe. The vitamin premix was prepared according to AIN-93G.
PEP – pre-experimental period; EP – experimental period.

from $FeSO_4$ per kilogram of diet (A-Heme Fe + $FeSO_4$). Tab. 1 shows the detailed composition of the experimental diets during PEP and EP.

During the study, the animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature of (22 ± 2) °C, a 12 h light-dark cycle and (55 ± 5)% humidity. Diet and mineral-free water was available ad libitum. Food intake was measured, and urine and feces were collected daily, during the second week of EP. On day 1 and 14 of EP, peripheral blood samples from the caudal vein were collected for measurement of Hb concentration and body weight was recorded.

### Chemical analysis of diets, heme source, feces and urine

The water content of the diet, in the dry extract heme Fe source and feces was determined by drying the materials at (105 ± 2) °C until the weight remained constant. These samples were mineralized by the wet method in a sand bath (J. R. Selecta, Barcelona, Spain). Fe, Cu and Zn contents in the experimental diets, feces and urine were determined by atomic absorption spectrophotometry (AAS; Analyst 1100B, Perkin Elmer, Ueberlingen, Germany) with WinLab32 for AA software (Perkin Elmer). Se levels in heme Fe source were determined by graphite furnace AAS. AAS equipped with a transversely heated graphite furnace (THGA) with longitudinal Zeeman-effect background corrector and AAS furnace autosampler (Perkin Elmer) were used. The source of radiation was a Se electrodeless discharge lamp. Samples of lyophilized bovine liver (certified reference material BCR-185; Community Bureau of Reference, Brussels, Belgium) were simultaneously used for quality control assays. The value of Se (439 ± 12) ng·g$^{-1}$ (mean ± standard deviation *SD*, $n = 5$) obtained in the analysis of this standard material agreed well with the certified content of Se (446 ± 13) ng·g$^{-1}$.

The same procedure was used for the heme source to quantify Fe, Cu, Zn, Ca and Mg contents by AAS. Analytical results were validated by comparison to standard reference materials for lyophilized bovine liver (BCR-185), which were simultaneously used to test the recovery of Fe, Cu and Zn. Mean ± *SD* of BCR-185 for Fe was (214 ± 5) $\mu$g·g$^{-1}$ versus (210 ± 3) $\mu$g·g$^{-1}$, for Cu was (189 ± 4) $\mu$g·g$^{-1}$ versus (187 ± 3) $\mu$g·g$^{-1}$ and for Zn was (143 ± 4) $\mu$g·g$^{-1}$ versus (139 ± 2) $\mu$g·g$^{-1}$.

Protein content in the heme Fe source was determined by the Kjeldahl method, and fat content was determined by extraction with petroleum ether boiling point (40–60) °C after hydrochloric hydrolysis [17]. Content of saccharides was obtained as the difference between the sum of pro-

teins and fats of the value of 100. Total ashes were determined by the method of calcination [18] and the content was assessed by the method reported by Prosky et al. [19].

### Biological indices

Percentage of bioavailability was calculated as hemoglobin regeneration efficiency (*HRE*) [6]:

$$HRE = \frac{W_{Hb} - W_{Hb0}}{W} \times 100 \quad (1)$$

where $W_{Hb}$ is final content of Fe in Hb, $W_{Hb0}$ is initial content of Fe in Hb and $W$ is consumed Fe (expressed in milligrams).

Fe content in Hb (in milligrams) was calculated by the following expression, assuming that the total blood volume was 6.7% of the rat body weight, and an average Fe content of hemoglobin was 0.335 mg.

$$W_{Hb} = \frac{BW \times Hb \times 6.7 \times 0.335}{1000} \quad (2)$$

where $BW$ is body weight (in grams), $Hb$ is expressed in grams per litre.

The nutritive utilization of Fe, Cu and Zn was measured as apparent digestibility coefficient (*ADC*) in percent [6]:

$$ADC = \frac{I - F}{I} \times 100 \quad (3)$$

where $I$ is mineral intake and $F$ is fecal excretion (in micrograms per day).

## Studies of antioxidant defence

After the studies of mineral bioavailability, EP was extended to 30 days to ensure that Fe deficiency anemia was completely recovered. On day 75 of the study, the animals were kept fasting overnight anesthetized intraperitoneally with sodium pentobarbital (Sigma-Aldrich, St. Louis, Missouri, USA), totally bled out and blood alliquots with EDTA were collected to obtain the erythrocytes cytosolic fractions. The remaining blood was centrifuged without anticoagulant to separate red blood cells from the serum, for subsequent analysis of serum Fe. The liver was removed and washed with ice-cold saline solution (9 $g \cdot l^{-1}$ NaCl). Duodenum was also removed and the samples were scraped carefully by two frozen slides to obtain the mucosa. All tissues were conserved at an ice bath.

### Serum Fe, total Fe-binding capacity (*TIBC*) and transferrin saturation

In order to calculate the rate of transferrin saturation (*TS*), serum Fe concentration ($c_{ser}$) and *TIBC* were determined (Diagnostics iron and *TIBC* reagents, Sigma). The absorbance of samples was read at 550 nm in a microplate reader (Bio-Rad Laboratories, Hercules, Canada). The percentage of *TS* was calculated from the following equation:

$$TS = \frac{c_{ser}}{TIBC} \times 100 \quad (4)$$

$c_{ser}$ and *TIBC* are expressed in micromoles per litre.

### Liver, erythrocyte and duodenal mucosa cytosolic preparations

The liver, erythrocyte and duodenal mucosa samples were thawed and homogenates were freshly prepared on the same day in a Potter homogenizer after the addition of 1.0 ml of phosphate buffer per 0.1 g of tissue. Successive differential centrifugations were performed to separate cytosolic fractions, according to a procedure reported by DeSandro et al. [20]. The erythrocyte cytosolic fractions were also freshly prepared on the same day by successive differential centrifugations with hypotonic hemolysis according to the method of Hanahan and Ekholm [21]. The final fractions were alliquoted, snap-frozen in liquid nitrogen, and stored at –80 °C until analysis. Cytosolic protein contents were measured as described by Lowry et al. [22].

### Measurement of the activity of the antioxidant enzymes

CAT activity was determined by the Aebi's method [23], by monitoring at 240 nm the $H_2O_2$ decomposition, as a consequence of the catalytic activity of CAT. The activity was calculated from the first-order rate constant $K$ (expressed as reciprocal seconds).

SOD activity was determined according to the method of Crapo et al. [24]. It was based on the inhibition by SOD of the reduction of cytochrome *c*, measured by spectrophotometry at 550 nm. One unit of the SOD activity is defined as the amount of enzyme required to produce 50% inhibition of the rate of reduction of cytochrome *c*.

GPx activity was measured by the technique of Flohé and Gunzler [25]. That method is based on the instantaneous formation of oxidized glutathione during the reaction catalysed by GPx. The oxidized glutathione is continually reduced by an excess of glutathione reductase and NADPH present in the cuvette. The subsequent oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm. Cumene hydroperoxide was used as reaction substrate.

**Statistical analysis**

Statistical analyses were performed using the SPSS computer programme (SPSS, version 20.0, SPSS, Chicago, Illinois, USA). Differences between groups (control versus each of the two anemic groups or between the two anemic groups) were tested for statistical significance by Student's *t*-test. A level of $p < 0.05$ was considered to indicate statistical significance. All the data are reported as mean values with their standard deviations (*SD*).

## RESULTS AND DISCUSSION

The constant increase of Fe-deficiency and anemia prevalence worldwide [1, 16] highlights the importance to continue searching for new strategies to palliate and prevent this common nutritional deficiency. Bovine blood is one of the subproducts of the food industry with a major nutritional value, in particular in the treatment of the Fe-deficiency anemia [26] due to the high bioavailability of heme Fe [27]. This natural product is used for different purposes, among which the development of nutritional supplements [28] can be mentioned, as well as the preparation of fortified foods for the prevention of Fe-deficiency anemia [27, 29, 30].

The approximate chemical analysis of the dry extract of the heme Fe source is a basis to explain its effects on the anemia recovery and its antioxidant potential. The proteins and the saccharides are the main components as the fundamental constituents of the preparation are bovine blood, extract of pepsin and bee honey. Other minor components are fats, ashes and fibre (Tab. 2).

In order to interpret the composition of minerals in the heme Fe source, we compared our results with those reported by Duarte et al. [26]. In that study, the mineral composition of different fractions of bovine blood, in particular the cellular fraction, was obtained from centrifugation (5 000 ×*g*, 10 °C, 15 min) and the precipitate was dissolved in distilled water and subsequently dehydrated by spray-dryer. The authors reported for this fraction a higher content (Fe 2.53 $g \cdot kg^{-1}$ and Zn 0.038 $g \cdot kg^{-1}$) than in the heme Fe source assessed in the current study. This result was in agreement with the content of proteins in the cellular fraction of bovine blood of 78.2% compared with the heme Fe source used in the current study. In the cellular fraction of bovine blood, the contents of Cu (0.001 $g \cdot kg^{-1}$), Ca (0.082 $g \cdot kg^{-1}$) and Mg (0.042 $g \cdot kg^{-1}$) were lower, however, the mineral content in the heme Fe source preparation was higher (Tab. 2). With regard to Se content, though we do not have reference to the values reported for to this mineral in blood, we consider its presence really noteworthy, since it could have an antioxidant effect due to its presence as a cofactor in the antioxidant enzymes, such as GPx [3, 12]. The variable composition of the heme Fe source, prepared from several raw materials of natural origin, make difficult any comparison with other formulations prepared from bovine blood [6, 26–28, 31–32].

**Tab. 2.** Approximate chemical analysis and mineral content in heme Fe source.

| Component [$g \cdot kg^{-1}$] | | Minerals content [$g \cdot kg^{-1}$] | |
|---|---|---|---|
| Water | 26.0 | Fe | 1.089 |
| Protein | 493.0 | Cu | 0.002 |
| Fat | 32.4 | Zn | 0.010 |
| Ash | 34.1 | Ca | 0.228 |
| Carbohydrates | 410.6 | Mg | 0.065 |
| Dietary fibre | 4.0 | Se | 0.0002 |

Mineral content was determined by atomic absorption spectrophotometry, except for Se, which was measured by graphite furnace atomic absorption spectrometry.

The mixture of both chemical forms of Fe in the animal group supplemented with the heme Fe source and $FeSO_4$ can be compared with studies by Campos et al. and by Lisbona et al. [6, 32], in which the diet supplied to the anemic rats was supplemented to reach Fe requirements (35 $mg \cdot kg^{-1}$ of diet) [14]. The Fe ratio in the diets (non-heme Fe/heme Fe) was 50%/50% and 80%/20%, respectively. These authors reported that *HRE* in the anemic rats was higher than when the proportion of Fe was 80%/20% [6, 32]. Heme Fe bioavailability is known to be 20–35%, whereas that of the non-heme Fe is 1–15% [27]. We considered that heme Fe source contained a high amount of Fe in a highly bioavailable form (Tab. 2). The analysis of all these facts led us to evaluate the heme Fe source and $FeSO_4$ with an amount of elemental Fe which was 61% of the nutritional requirements for this species [14] and a proportion of non-heme/heme Fe, which was approximately 96%/4%, with the objective to obtain a similar hematological response during anemia recovery. Therefore we achieved a high Fe bioavailability and mineral nutritive utilization, with lower oxidative damage, compared with the conventional Fe source from ionic Fe.

Body weight, Hb concentration, Fe content in Hb, serum Fe, *TIBC* and percentage of transferrin saturation were different for the anemic groups:

**Tab. 3.** Body weights, hemoglobin and hemoglobin Fe contents in control and anemic rats fed with different diets.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| Body weight [g] | day 46 (Initial) | 208.44 ± 8.31 | 190.10 ± 14.06*[a] | 190.50 ± 10.02*[a] |
| | day 60 (Final) | 223.56 ± 7.24 | 207.80 ± 10.52*[a] | 212.30 ± 10.43*[a] |
| Hb [$g \cdot l^{-1}$] | day 46 (Initial) | 148.89 ± 6.19 | 69.90 ± 5.72*[a] | 69.52 ± 5.53*[a] |
| | day 60 (Final) | 166.67 ± 7.16 | 130.50 ± 4.39*[a] | 126.30 ± 5.21*[a] |
| Fe in Hb [mg] | day 46 (Initial) | 6.97 ± 0.28 | 2.98 ± 0.30*[a] | 2.97 ± 0.29*[a] |
| | day 60 (Final) | 8.37 ± 0.41 | 6.09 ± 0.46*[a] | 6.02 ± 0.45*[a] |

Values are mean ± *SD* for group, $n = 10$. Fe in Hb was calculated using Eq. 2.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t* test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t*-test).
Hb – hemoglobin.

**Tab. 4.** Serum Fe, total Fe binding capacity, transferrin saturation and Fe content in liver in control and anemic rats fed with different diets (day 75).

| Group | Control | Anemic | |
|---|---|---|---|
| | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| $c_{ser}$ [$\mu mol \cdot l^{-1}$] | 25.15 ± 1.02 | 35.17 ± 0.86*[a] | 35.73 ± 0.55*[a] |
| *TIBC* [$\mu mol \cdot l^{-1}$] | 84.27 ± 3.65 | 110.75 ± 3.35*[a] | 114.18 ± 3.62*[a] |
| *TS* [%] | 30.4 ± 0.8 | 31.9 ± 0.6[a] | 31.2 ± 0.8[a] |
| Liver Fe [$\mu g \cdot g^{-1}$] | 805.21 ± 22.61 | 554.34 ± 21.08*[a] | 312.18 ± 24.33*[b] |

Values are mean ± *SD* for group, $n = 10$. Content of Fe in liver is expressed on dry matter basis.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t* test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t*-test).
*TIBC* – total iron binding capacity, $c_{ser}$ – serum Fe concentration, *TS* – transferrin saturation, calculated using Eq. 4.

(A-$FeSO_4$) and (A-Heme Fe + $FeSO_4$) in relation with the control (non-anemic) rats (NA-$FeSO_4$; Tab. 3 and Tab. 4; $p < 0.001$).

We analysed the relation of body weight, Hb content and Fe intake to evaluate Fe bioavailability using the *HRE* index. Fe bioavailability was higher in the anemic groups with respect to the control group and if both anemic groups were compared, *HRE* was higher in the (A-Heme Fe + $FeSO_4$) group than in the (A-$FeSO_4$) group ($p < 0.001$; Tab. 5). The differences observed between the anemic groups and the control group were the consequence of the severe Fe deficiency induced during PPE [33, 34].

Physiological modifications taking place during the instauration of anemia feature stimulation of the intestinal Fe absorption mechanism, which leads to an increase in the bioavailability of the dietary Fe and increases Fe utilization for the Hb synthesis [3]. If we take into account that one milligram of the heme Fe source was included in about one gram of the hydrolysed heme Fe preparation that contained about 49 mg of protein (Tab. 2), we suppose that this source of proteins was mainly supplied by the peptides of Hb derived from the partial hydrolysis with pepsin. These protein fractions would enhance the absorption of the heme and non-heme Fe present in the diet of the animal group supplemented with the mixture of heme/non-heme Fe to obtain the higher *HRE*, in spite of the fact that this group received a lower Fe amount (Tab. 1 and Tab. 6). This result could be also influenced by honey and propolis, which were previously reported to increase Fe bioavailability [35].

*HRE* of the anemic group fed with the mixture of heme Fe/non-heme Fe was lower compared with the results obtained by Campos et al. (*HRE* = 66.8%) and by Lisbona et al. (*HRE* = 77.5%) [6, 32]. We can explain these results by the fact that, in those studies, male Wistar rats were used, unlike female Sprague Dawley rats that were used in the current study. The initial body weight of the animals was higher (265 ± 5.3) g

**Tab. 5.** Hemoglobin regeneration efficiency in control and anemic rats fed with different diets.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| Food intake [g·d$^{-1}$] | | 12.90 ± 0.96 | 12.52 ± 0.46$^{a}$ | 12.67 ± 0.88$^{a}$ |
| Gain (day 60) | Body-weight [g] | 15.00 ± 6.14 | 19.88 ± 7.72$^{a}$ | 22.56 ± 7.18*$^{a}$ |
| | Hb [g·l$^{-1}$] | 15.22 ± 6.26 | 61.22 ± 7.74*$^{a}$ | 56.42 ± 7.54*$^{a}$ |
| | Fe in Hb [mg] | 1.40 ± 0.71 | 3.17 ± 0.56*$^{a}$ | 3.03 ± 0.50*$^{a}$ |
| *HRE* [%] | | 14.96 ± 6.30 | 41.24 ± 5.99*$^{a}$ | 61.20 ± 6.56*$^{b}$ |

Values are mean ± *SD* for group, $n = 10$.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t*-test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t* test).
Hb – Hemoglobin, *HRE* – hemoglobin regeneration efficiency, calculated using Eq. 1.

**Tab. 6.** Digestive utilization of Fe, Cu and Zn in control and anemic rats fed with different diets.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| Fe (day 60) | Intake [$\mu$g·d$^{-1}$] | 582.46 ± 19.72 | 572.92 ± 20.86$^{a}$ | 356.93 ± 21.42*$^{b}$ |
| | Fecal [$\mu$g·d$^{-1}$] | 284.44 ± 8.72 | 127.80 ± 6.73*$^{a}$ | 84.04 ± 5.97*$^{b}$ |
| | ADC [%] | 51.17 ± 2.33 | 77.69 ± 2.78*$^{a}$ | 76.45 ± 3.13*$^{a}$ |
| Cu (day 60) | Intake [$\mu$g·d$^{-1}$] | 90.91 ± 6.86 | 89.80 ± 4.14$^{a}$ | 89.70 ± 6.35$^{a}$ |
| | Fecal [$\mu$g·d$^{-1}$] | 76.01 ± 4.30 | 60.20 ± 4.87*$^{a}$ | 60.05 ± 5.28*$^{a}$ |
| | ADC [%] | 16.38 ± 1.74 | 33.04 ± 3.19*$^{a}$ | 33.11 ± 3.67*$^{a}$ |
| Zn (day 60) | Intake [$\mu$g·d$^{-1}$] | 457.80 ± 23.51 | 447.10 ± 24.55$^{a}$ | 447.70 ± 26.54$^{a}$ |
| | Fecal [$\mu$g·d$^{-1}$] | 344.20 ± 28.57 | 367.50 ± 26.26$^{a}$ | 368.05 ± 28.56$^{a}$ |
| | ADC [%] | 24.81 ± 2.69 | 17.78 ± 1.71*$^{a}$ | 17.79 ± 1.96*$^{a}$ |

Values are mean ± *SD* for group, $n = 10$.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t*-test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t*-test).
ADC – apparent digestibility coefficient, calculated using Eq. 2.

and (267 ± 7) g, respectively, the amount of daily food intake was also higher and, therefore, the increase in the body weight was higher (2.9 g·d$^{-1}$ and 3.8 g·d$^{-1}$, respectively). Taking into account that body weight increase is directly proportional to *HRE*, this relationship explains the differences obtained in the current study. Nevertheless, when we compared the results obtained with those reported by HERNÁNDEZ et al. in groups of Sprague Dawley rats of mixed genders (male and female), *HRE* for the anemic group fed with $FeSO_4$ was 28.8% [36]. Therefore, *HRE* results are in agreement with those obtained by other authors using experimental designs similar to that of the current study.

The effect of the mixture of heme/non-heme Fe during anemic recovery on the nutritive utilization of Fe, Cu and Zn was also investigated. Fe intakes in (NA-$FeSO_4$) and (A-$FeSO_4$) groups were higher, when compared with the (A-Heme Fe + $FeSO_4$) group ($p < 0.001$). The anemic effect was demonstrated by the differences in fecal Fe and Cu excretion that were lower for the (A-$FeSO_4$) and (A-Heme Fe + $FeSO_4$) groups in comparison with (NA-$FeSO_4$) group ($p < 0.001$; Tab. 6).

In addition, the effect of anemia was demonstrated because values of Fe and Cu *ADC* were higher in the anemic groups than in the control group. However, Zn *ADC* was lower in both anemic groups ($p < 0.001$; Tab. 6). The decrease of Fe fecal excretion in the group fed with the mixture of heme/non-heme Fe indicates that when Fe intake diminished, the organism was adapted, diminishing also the Fe digestive utilization. This response allowed dietary Fe to be used mainly in Hb synthesis and, in this way, diminished the undesirable effects produced by the severe Fe deficiency during the depletion period. Fe nutritive utilization in the group fed with the mixture of heme/non-heme

Fe was higher than that reported by CAMPOS et al. and LISBONA et al. (*ADC* = 21.6% and 30%, respectively) [6, 32]. Fe ADC obtained in the current study was similar to that reported by PALLARÉS et al., in which Fe *ADC* for $FeSO_4$ was 69.7% [15]. Therefore, the results obtained indicate that Fe utilization in the anemic groups is in agreement with those reported by other authors using $FeSO_4$ as Fe source.

*ADC* for Cu obtained in this study was in agreement with the results reported elsewhere [37]. The results obtained in the current study, with no differences between the two anemic groups in *ADC*, corroborated the hypothesis that the nutritive utilization of different Fe sources with Fe supplied below dietary requirements for the rat [14] for the (A-Heme Fe + $FeSO_4$) group did not affect the nutritive utilization of Cu. The higher digestive and metabolic utilization of Cu observed in the anemic rats compared with the controls (Tab. 6) could be caused by the fact that deficiency of a divalent cation, such as Fe, in the intestine may increase the absorption of other divalent cations, such as Cu [38].

**Tab. 7**. Superoxide dismutase activity in the cytosolic fractions of tissues in control and anemic rats fed with different diets.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| SOD [U·mg$^{-1}$] (day 75) | Liver | 17.539 ± 3.007 | 16.944 ± 3.754$^{a}$ | 15.995 ± 3.269$^{a}$ |
| | Erythrocytes | 14.817 ± 1.973 | 16.739 ± 1.783*$^{a}$ | 16.333 ± 2.178$^{a}$ |
| | Plasma | 5.353 ± 1.252 | 6.015 ± 1.451$^{a}$ | 6.140 ± 1.092$^{a}$ |
| | Duodenal mucosa | 74.601 ± 6.368 | 97.858 ± 6.410*$^{a}$ | 69.093 ± 8.198$^{b}$ |

Values are mean ± *SD* for group, $n = 10$. SOD activity is expressed per milligram of protein.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t*-test). Within a column, means followed by the same letter are not significantly different ($p < 0.05$, Student's *t*-test).

**Tab. 8**. Glutathione peroxidase activity in the cytosolic fractions of tissues in control and anemic rats fed with different diets.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| GPx [mmol·mg$^{-1}$ml$^{-1}$] (day 75) | Liver | 0.372 ± 0.126 | 0.696 ± 0.125*a | 0.773 ± 0.130*a |
| | Erythrocytes | 0.891 ± 0.142 | 1.244 ± 0.136*a | 1.070 ± 0.138*b |
| | Plasma | 0.029 ± 0.005 | 0.046 ± 0.005*a | 0.022 ± 0.004*b |
| | Duodenal mucosa | 1.411 ± 0.154 | 1.823 ± 0.147*a | 1.019 ± 0.122*b |

Values are mean ± *SD* for group, $n = 10$. GPx activity is expressed per milligram of protein and millilitre.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t*-test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t*-test).t

**Tab. 9.** Catalase activity in the cytosolic fractions of tissues in control and anemic rats fed with diets containing different sources of Fe for 30 days.

| Group | | Control | Anemic | |
|---|---|---|---|---|
| | | (NA-$FeSO_4$) | (A-$FeSO_4$) | (A-Heme Fe + $FeSO_4$) |
| CAT [s$^{-1}$mg$^{-1}$] (day 75) | Liver | 0.289 ± 0.020 | 0.297 ± 0.017$^{a}$ | 0.290 ± 0.025$^{a}$ |
| | Erythrocytes | 0.398 ± 0.033 | 0.346 ± 0.035*$^{a}$ | 0.298 ± 0.032*$^{b}$ |
| | Duodenal mucosa | 0.128 ± 0.014 | 0.105 ± 0.015*$^{a}$ | 0.107 ± 0.011*$^{a}$ |

Values are mean ± *SD* for group, $n = 10$. CAT activity is expressed per milligram of protein.
* – Mean values were significantly different from the group (NA-$FeSO_4$) ($p < 0.05$, Student's *t*-test). Within a column, means followed by the same letter are not significantly different ($p > 0.05$, Student's *t*-test).

In a situation of Fe deficiency, a greater absorption of Cu occurs, while that of Zn remains unchanged [39]. *ADC* for Zn in the anemic groups was lower than that reported by Díaz-Castro et al. [33]. Metabolic studies and supplementation trials suggest an antagonistic relationship between Fe and Zn, in which Zn reduces the positive effect of Fe supplementation and vice versa [40]. We hypothesize that the higher utilization of Fe in this study (Tab. 6) could contribute to the inhibition of Zn absorption.

We analysed the activities of the antioxidant enzymes SOD, GPx and CAT in different tissues after 30 days of Fe supplementation.

The SOD activity in the anemic group supplemented with $FeSO_4$ was altered in the erythrocytes ($p < 0.01$) and in duodenal mucosa ($p < 0.001$) in relation to the control group. A difference in the SOD activity between both anemic groups was recorded in duodenal mucosa, being higher in the group supplemented with $FeSO_4$ ($p < 0.001$; Tab. 7).

The highest activity in GPx was observed in erythrocytes, plasma and duodenal mucosa of anemic rats supplemented with $FeSO_4$ ($p < 0.001$), and in liver and erythrocyte of rats supplemented with the mixture heme/non heme Fe ($p < 0.001$). In the liver, GPx activity in both anemic groups was higher compared with the control group ($p < 0.001$; Tab. 8).

CAT activity was lower in erythrocytes and duodenal mucosa in the anemic groups ($p < 0.05$) and was not affected in liver neither by the anemia nor the type of Fe supplementation (Tab. 9).

SOD and GPx activities found in the group supplemented with the mixture of heme/non-heme Fe can be explained partly by the lower amount of Fe in this diet compared with the anemic group supplemented with $FeSO_4$ only. The feedback mechanisms that control Fe absorption and trafficking include several Fe regulatory proteins, such as DMT1, transferrin and ferritin, between many others. This regulatory function is mediated by Fe responsive elements [41], which would be better recovered with the supply of heme Fe. The reason is that heme internalization involves either direct transport of heme or receptor-mediated endocytosis [4] in a very efficient process, limiting the Fe available to catalyse the generation of free radicals. Nevertheless, non-heme Fe provided by the $FeSO_4$ diet would result in an excessive Fe trafficking in the intestinal lumen, which is reflected by an increased antioxidant defence by SOD and GPx [42].

CAT is a ferric heme-containing enzyme and, therefore, Fe deficiency could reduce the activity of Fe-dependent enzymes. However, under our experimental conditions, CAT activity in liver of rats with Fe deficiency anemia and after Fe replenishment was similar to control rats. These results are in agreement with our previously reported ones [34], however, CAT activity was lower in erythrocyte and duodenal mucosa, indicating a protective effect of Fe deficiency on the oxidative stress. The positive antioxidant balance in all the tissues of animals fed the diet containing the mixture of heme/non-heme Fe indicates an adequate nutritive utilization of Zn and Cu, which are minerals that act as cofactors in SOD [10].

Normally a balance between free radical production and antioxidant defence system exists in the organism. Studies are on par with the fact that antioxidant status is lowered in Fe deficiency [43–45]. The results for the antioxidant enzymes in the group supplemented with the mixture of heme Fe/non-heme Fe indicate that the anemic animals have recovered.

## CONCLUSIONS

The recovery of the hematological parameters in the group supplemented with the mixture of heme Fe/non-heme Fe showed a high Fe bioavailability and an adequate nutritive utilization of Fe, Cu and Zn. These results suggest that the amount of Fe consumed was sufficient to cover the physiological needs of the mineral, although the rats received just approximately 61% of the recommended amount of Fe for the species. The recovery of the anemia was demonstrated too by the recuperation in the antioxidant enzymatic defence in the group that received the mixture of heme/non-heme Fe source. The supplementation with the mixture of heme/non-heme Fe could be considered an alternative therapy to palliate the Fe deficiency and to reduce the side effects of the classical supplements based on non-heme Fe alone.

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