Original Article

Milk fortification with a complex of iron with ascorbic acid for control of iron deficiency anemia

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ABSTRACT

The aim of this work was the fortification of milk with a bioavailable complex of iron with ascorbic acid and in vivo evaluation of its effect on the hematological parameters of laboratory rats. To do this, we produced sterilized milk with iron and ascorbic acid supplementations. Fortification of milk increased the iron content to 1.0 mg/100 mL and ascorbic acid to 3.0 mg/100 mL, which meets the requirements for iron and ascorbic acid levels in children's nutrition. *An in vivo* experiment was performed on 42 male rats of the Wistar line with an initial body weight of 120-240 g. As a result of the study, it was found that administration of milk fortified with iron and ascorbic acid to rats with severe anemia against the background of an iron-deficient diet at a dose of 1.7 mL per 100 g of body weight for 20 days helps to prevent the development of the condition of IDA in animals. The results of the study show the great prospects of using milk fortified with iron and ascorbic acid for the prevention of iron deficiency anemia.

Keywords: Fe, Vitamin C, Fortification, Hematological parameters, Biochemical parameters

Introduction

Iron deficiency anemia (IDA) is one of the most common hematological diseases in the world, that covers up to 80% of all types of anemia [1, 2]. According to WHO data, the prevalence of IDA varies from country to country and largely depends on environmental, industrial, climatic, and geographical living conditions, as well as on gender and age [3, 4]. Young children, as well as pregnant and lactating women, suffer most often from this pathology [5-7]. Notably, the incidence of IDA has increased several times over the past 10 years, and, unfortunately, it is most typical for children [8].

The high prevalence of IDA necessitates the development of methods for its treatment and prevention. In this regard, the following main directions can be distinguished: medicinal and non-medicinal (dietary) [9, 10]. Even though currently there are a significant number of preparations containing various iron compounds, the number of people suffering from IDA is constantly increasing [11-13]. Among the various causes of IDA in children, alimentary disorders occupy an important place [14, 15]. Insufficient iron in the diet of a pregnant woman is one of the main causes of IDA in breastfed and artificially-fed children [16]. Another reason is the untimely and irrational introduction of complementary foods. Despite the high digestibility of iron from breast milk, a breastfed child, due to the iron that comes

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from the mother's milk, needs additional iron intake from other sources [17, 18]. Therefore, it is important to widely use in the diet both traditional complementary foods and specialized products fortified with bioavailable iron. In this case, one of the most suitable food matrices for iron fortification is milk [19].

Thus, the aim of this work was the fortification of milk with a bioavailable complex of iron with ascorbic acid and in vivo evaluation of its effect on the hematological parameters of laboratory rats. To do this, we produced sterilized milk with iron and ascorbic acid supplementations. According to the scientific principles of micronutrient fortification of food products, their effectiveness must be proven by experimental studies confirming the claimed profile of the developed product [20, 21], which was the main focus of this study.

Materials and Methods

For the experiment, we used 2.5% fat-sterilized milk produced by the Nalchik Dairy Plant (Nalchik, Russia). Fortification of milk increased the iron content to 1.0 mg/100 mL and ascorbic acid to 3.0 mg/100 mL, which meets the requirements for iron and ascorbic acid levels in children's nutrition [22].

An in vivo experiment was performed on 42 male rats of the Wistar line with an initial body weight of 120-240 g. From the total batch of selected animals, equivalent groups were formed, which were placed in separate cages. On the eve of the experiment, the animals were adapted to laboratory conditions with standard climatic parameters and unlimited access to food and water [23].

The experiment consisted of two stages. In the first stage, we conducted the itemization of animals using a special diet with a minimum iron content [24]. In the second stage, we performed the direct oral administration of two samples to animals with feed. Sample 1 was sterilized milk fortified with iron and ascorbic acid, while sample 2 was sterilized milk fortified only with ascorbic acid. To create an alimentary system, the animals were randomized into 2 groups. Group A (n = 12) were intact animals on a standard vivarium diet [25]. Group B (n = 30) consisted of animals fed with a minimum iron content. The duration of the experiment was 7 weeks, which is consistent with previous similar works [26-28].

The development and severity of anemia were monitored by hematological and biochemical parameters: hemoglobin level, the average hemoglobin content in the erythrocyte, the average concentration of hemoglobin in the erythrocyte, the number of red blood cells, mean cell volume, the width of the distribution of red blood cells by volume, hematocrit, the concentration of iron in the blood serum, the total iron binding capacity of the serum (TIBCS), the level of ferritin.

Blood for hematological analyses was taken from the lateral caudal vein [29]. An automatic hematology analyzer Humacaunt (Germany) was used to determine the morphological parameters of blood. For biochemical analysis of rat blood serum, peripheral blood was taken from decapitated animals [30]. Diagnostic kits P.Z.Cormay (Poland) were used to determine biochemical parameters [31]. The study was carried out using a Hitachi 902 biochemical analyzer (Germany).

After a hematological and biochemical diagnosis of anemic condition, 3 groups were formed from anemized animals of the second group to assess the effect of fortified milk on blood parameters. The first group of experimental animals (n = 10), while continuing to be kept on an iron-deficient diet, received daily sample 1. The second group of experimental animals (n = 10) consisted of anemized animals, which additionally intragastrically received sample 2. The amount of administrated milk was calculated according to the formula of intraspecific dose recalculation generally accepted in experimental pharmacology, based on the average amount of milk recommended for daily intake to a person, taken equal to about 200 mL [32]. The received dose for rats was 1.7 mL/100 g of body weight. The administration of milk was continued for 20 days of the experiment, while the animals continued to receive feed with a minimum iron content. The third group (n = 10) was the control consisted of anemized animals that continued to be kept on an iron-deficient diet but did not receive prepared samples. The fourth group (n = 12) was formed additionally and consisted of intact animals on a stationary vivarium diet. To determine the dynamics of body weight during the experiment and the associated correction of the amount of administrated milk, the animals were regularly weighed.

Results and Discussion

The results of the experiment to recreate the experimental alimentary IDA showed that animals of group B (n = 30) fed with a minimum iron content had a decrease in body weight by 27% compared with the initial value. In anemized animals, lethargy, a change in the color of the coat, and a weight lag of more than 50% from healthy animals were noted. At the same time, the body weight of intact rats receiving the usual vivarium diet increased by 34% **(Table 1)**.

Table 1. Dynamics of body weight in rats of control and experimental groups

Group	Body weight, g			
	Initial value		After 7 weeks	
Group A	212.08 ± 8.06	100 %	285.00 ± 8.02 $^{\#}$	134.4 %
Group B	188.88 ± 5.03	100 %	138.54 ± 4.76 $^{*\#}$	73.3 %

Note: * – The differences are statistically significant compared to group A at $P < 0.001$,

$^{\#}$ – The differences are statistically significant compared to the initial value at $P < 0.001$.

The development of IDA in animals was judged primarily by a decrease in blood hemoglobin levels. The initial hemoglobin level in rats before the start of the experiment ranged from 116 to 145 g/L. In group A, the hemoglobin level increased by an average of 6%, whereas in group B rats, the hemoglobin level significantly decreased by 12% compared to the initial value. Notably, compared with group A, the hemoglobin index in Group B decreased by 17% **(Table 2)**.

Table 2. The effect of an iron-deficient diet on the level of hemoglobin in the blood of rats

Group	Hemoglobin, g/L			
	Initial value		Initial value	
Group A	143.33 ± 1.15	100%	1 51.71 ± 1.74 *	105.8%
Group B	120.20 ± 2.07	100%	106.46 ± 3.23*#	88.6%

Note: # – The differences are statistically significant compared to group A at $P < 0.001$,

* – The differences are statistically significant compared to the initial value at $P < 0.001$.

The initial number of red blood cells in the blood of animals of the two experimental groups at the beginning of the studies fell within the range of the norm of this indicator for this type of animal [33]. The transfer of group B animals to an iron-deficient diet caused a decrease in the number of red blood cells and hematocrit levels by 29% compared with group A, which confirms the development of an iron deficiency condition in rats on an iron-deficient diet **(Table 3)**.

Table 3. The effect of an iron-deficient diet on hematological parameters in rats

Group	Number of red blood cells, 10^{12}/L		Hematocrit, %	
Group A	9.94 ± 0.37	100 %	51.71 ± 1.51	100 %
Group B	7.02 ± 0.35 *	70.6 %	36.89 ± 1.82 *	71.3 %

Note: * – The differences are statistically significant compared to group A at $P < 0.001$

The main biochemical markers characterizing the presence of IDA are a decrease in the concentration of iron in the blood serum, an increase in the total iron-binding capacity of the blood serum, and a decrease in the level of ferritin [34, 35]. The combination of these indicators most fully reflects the presence, absence, and nature of IDA. Data presented in **Table 4** confirmed the development of IDA in rats **(Table 4)**. Thus, in the group of animals on an iron-deficient diet, the concentration of iron decreased by 38%, and the level of ferritin decreased by 27% compared to animals of group A. Accordingly, an increase in the indicator characterizing the ability of blood serum to bind iron was found by 21%.

Table 4. The effect of an iron-deficient diet on biochemical parameters in blood serum

Group	Iron content, µmol/L		The total iron binding capacity of serum, µmol/L		Ferritin content, ng/mL	
Group A	11.35 ± 1.17	100 %	8.51 ± 0.31	100 %	246.32 ± 8.74	100 %
Group B	7.02 ± 0.19 *	61.9 %	10.32 ± 0.26 *	121.3 %	179.10 ± 11.08 *	72.7 %

Note: * – The differences are statistically significant compared to group A at $P < 0.001$

Thus, the obtained value of the hemoglobin level, a decrease in the number of red blood cells and the value of hematocrit, a decrease in the concentration of iron and ferritin, as well as an increase in the total iron-binding capacity of blood serum allowed us to state the development of IDA in rats 7 weeks after staying on an iron-deficient diet [36, 37].

The results of the second stage of the experiment showed a change in animal body weight, hemoglobin levels, and biochemical parameters of animal blood compared with the initial level and these indicators in the control group. The results obtained are in line with data reported by other researchers [38, 39]. Thus, administration of samples 1 and 2 to animals for 20 days (Group 1 and Group 2, respectively) against the background of an iron-deficient diet led to a significant increase in body weight compared with control (Group 3). Thus, in Group 1, body weight gain was 36% compared to Group 3. In group 2, body weight gain increased by 28% **(Table 5)**.

Table 5. The effect of fortified milk on body weight and hemoglobin levels in rats with IDA

Group	Body weight, g	Hemoglobin, g/L
Group 1	163.78 ± 1.05 *#	133.33 ± 4.92 *#
Group 2	154.00 ± 3.93* #	118.60 ± 3.82 *

Group 3	120.63 ± 2.58 *#	106.00 ± 9.19 *
Group 4	281.67 ± 8.23	170.83 ± 2.48

Note: * – The differences are statistically significant compared to group 4 at P < 0.001,
– The differences are statistically significant compared to group 3 at P < 0.001.

During the experiment, significantly high hemoglobin levels were established in rats receiving samples 1 and 2, compared with this indicator in the group of control animals. The highest level of hemoglobin was recorded in animals of group 1 receiving sample 1. In this group, this indicator increased by 25% compared with the control group. In group 2, which received sample 2, this indicator increased by 11%. In group 3 of anemized animals, the hemoglobin level did not differ from the initial value (106.00 ± 9.19 g/L versus 106.46 ± 3.23 g/L). Simultaneously with the diet, the administration of samples 1 and 2 to the animals for 20 days had a noticeable effect on the number of red blood cells and the level of hematocrit **(Table 6)**.

Table 6. The effect of fortified milk on hematological parameters in rats with IDA

Group 1	Group 2	Group 3	Group 4
Number of red blood cells, 10^{12}/L			
8.10 ± 0.29 *#	7.04 ± 0.24 *#	6.46 ± 0.44 *#	10.49 ± 0.17
Mean cell volume, fL			
52,02 ± 0,32	52,76 ± 0,42	51,74 ± 0,81	52,83 ± 0,10
Hematocrit, %			
42,16 ± 1,629 *#	37,13 ± 1,29 *	33,55 ± 2,46 *	55,17 ± 0,82

Note: * – The differences are statistically significant compared to group 4 at P < 0.001,
– The differences are statistically significant compared to group 3 at P < 0.001

In Group 1, along with the restoration of hemoglobin levels, normalization of the number of red blood cells was noted. This indicator significantly increased by 33% in Group 1, whereas by 16% in Group 2 compared with Group 3. However, it should be noted that the number of red blood cells in all three groups of itemized animals remained at a low level compared with animals of Group 4. This fact is consistent with results reported in previous studies [40, 41]. Analysis of hematocrit level revealed its significant dynamics during 20 days of the experiment in Group 1 and Group 2. This change was most clearly observed in the group receiving sample 1 (an increase of 24%) compared with the itemized animals of Group 3. It is worth noting that after administration of samples 1 and 2, there was a clear upward trend in mean cell volume and its approximation to that value in the intact animals of Group 4 **(Table 6)**. It is worth noting that the results obtained are in the line with results of recent studies [42, 43]. Interestingly, at the same time, administration of samples 1 and 2 for 20 days did not lead to a sharp improvement in the biochemical parameters of blood serum **(Table 7)**.

Table 7. The effect of fortified milk on biochemical parameters in blood serum

Group	Biochemical parameters		
	iron content, μmol/L	the total iron binding capacity of serum, μmol/L	Ferritin content, ng/mL
Group 1	7.93 ± 0.26 *	8.78 ± 0.13 #	158.85 ± 8.21 *
Group 2	6.97 ± 0.25 *	10.19 ± 0.22 #	138.36 ± 5.74 *
Group 3	6.96 ± 0.58 *	10.920 ± 0.17 *	139.68 ± 6.76 *
Group 4	10.26 ± 0.24	9.15 ± 0.29	250.66 ± 9.75

Note: * – The differences are statistically significant compared to group 4 at P < 0.001,
– The differences are statistically significant compared to group 3 at P < 0.001

According to **Table 7**, the concentration of serum iron in Group 2 and Group 3 remained almost at the same level. At the same time, serum iron content in animals of Group 1 increased slightly compared to the control (Group 3). However, these differences were unreliable (P > 0.05), which contradicts with results of recent similar works [44, 45].

It was found that administration of milk fortified with iron and ascorbic acid to experimental animals (Group 1), led to the prevention of further decrease in serum ferritin levels. Thus, the ferritin content in Group 1 remained quite low compared to the initial level (158.85 ± 8.21 ng/mL versus 179.10 ± 11.08 ng/mL). However, compared with that value in the control (Group 3), this indicator significantly increased by 14%. In this group of animals, due to the constant intake of iron from milk, the total iron binding capacity of serum significantly decreased in comparison with this indicator in control (Group 3). Notably, the results obtained are confirmed by the results of other authors [46-48]. In Group 2, there were no changes in the studied biochemical parameters of blood

serum, the latter remained at the level of indicators of Group 3, which is consistent with similar studies [49, 50].

Conclusion

As a result of the study, it was found that administration of milk fortified with iron and ascorbic acid to rats with severe anemia against the background of an iron-deficient diet at a dose of 1.7 mL per 100 g of body weight for 20 days helps to prevent the development of the condition of IDA in animals. In particular, the obtained value of the hemoglobin level, a decrease in the number of red blood cells and the value of hematocrit, a decrease in the concentration of iron and ferritin, as well as an increase in the total iron-binding capacity of blood serum allowed us to state the development of IDA in rats 7 weeks after staying on an iron-deficient diet. It was found that administration of milk fortified with iron and ascorbic acid to experimental animals (Group 1), led to the prevention of further decrease in serum ferritin levels. Thus, the ferritin content in Group 1 remained quite low compared to the initial level (158.85 ± 8.21 ng/mL versus 179.10 ± 11.08 ng/mL). However, compared with that value in the control (Group 3), this indicator significantly increased by 14%. In this group of animals, due to the constant intake of iron from milk, total iron binding capacity of serum significantly decreased in comparison with this indicator in control (Group 3).

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Conflict of interest: None

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Ethics statement: The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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