IN VITRO AND *IN VIVO* RESEARCHES OF THE IRON BIOAVAILABILITY IN FORTIFIED BAKERY PRODUCTS

Rodica STURZA, Viorel GUDUMAC, Olga DESEATNICOV and **Corina CIOBANU**

Technical University of Moldova, 168 Stefan cel Mare Bd., Chisinau; corinaciobanu@mail.ru

Received 23 June - Accepted 24 September

Abstract

During food fortification, processing and storage, numerous physical-chemical and enzymatic processes take place which may greatly influence biological value of these products. Hereby, elaboration of a technologic process of fabrication of fortified products should be based on a profound study of the evolution of these micronutrients during technological process as well as during consumption. The main object of the present study was the investigation of the influence of the bread making procedure on iron bioavailability in iron fortified bread. The enzymatic degradation of the phytates ($InsP_6$) was studied within gastro-intestinal digestion conditions in vitro. The study of iron bioavailability was performed in vivo on white laboratory rats. The study of the biochemical indices of the blood, collected from the laboratory animals, fed with fortified bread with 8 mg Fe/100 g product, made by the traditional method and by the lactic-acid fermentation method compared to the control group showed that iron intake plays conclusive role in animal nutrition. Thus, iron statute of both experimental groups, and especially the body iron reserve, was essentially improved compared to the control group.

Keywords: food fortification, iron bioavailability, bakery products, white laboratory rat, enzymatic degradation.

1. Introduction

According to WHO criteria, anaemia in the Republic of Moldova represents a major public health problem (Raport UNICEF, 1996-2000). The results of the investigations drown under UNICEF supervision over a group of 792 children under 5 years old and their mothers show anaemia presence at 47% of children between 6 and 12 months old, at 28% of children under 5 years old and at 40% of women of fertile age. According to the statistics of the Ministry of Health, anaemia rate in the case of children till one year old constitutes 20%. It is known, that one anaemia case corresponds to one case of iron nutritional deficiency, and it is possible to assume that approximately half of the children under 5 years old and 40% of fertile age women have iron deficiency. Prevalence of blood and haematopoietic system diseases increased during the last 5 years with 46.4% and for anaemia with 50.3%.

A recent study of the food intake (Motruc *et al.*, 2005) which aimed the estimation of the nutritional statute of the institutionalized children of 11-17 years old in Republic of Moldova pointed out that dialyzable iron intake is extremely low and constitutes only 0.87 mg Fe/1000 kcal (reference index is 4.67 mg Fe/1000 kcal). Average iron intake does not reach 100% level of the nutritional requirements in any population category (Motruc *et al.*, 2005).

One of the methods of micronutrient deficiency eradication the most used in developed countries is food fortification with deficient micronutrients (Bauernfeind, 1998). It is a feasible and low cost method (in the case of the mineral micronutrients) (Luten *et al.*, 1996).

However, food fortification cannot be considered as a simple mechanical administration of the additives (Turk *et al.*, 1996). In the case of vegetal origin products or combined nutrition (animal origin products consumed together with cereals) the main cause for the demineralization is considered to be phytic acid (myo-inositol hexaphosphate $InsP_6$) naturally found in wheat grain as soluble sodium and potassium salts (Hubert *et al.*, 2003). During food processing of cereal products or during digestion,

Corresponding author: corinaciobanu@mail.ru

$InsP_6$ is capable to fix metal cations and can form stable structures which are not able to diffuse through gastrointestinal wall (Haug and Lentzsch, 1983).

2. Materials and Methods

During the study, bread made of superior quality wheat flour of native production (STAS-26574-85) was used. As iron additive, iron sulfate (II) ($FeSO_4 \cdot 7H_20$) was used. This substance is accepted as food additive by "Codex Alimentarius" in the Republic of Moldova.

Bread -making procedures

Bread was made by direct (mono-phase) method, which represented the simultaneous mixing of all ingredients; indirect (bi-phase) method with the administration of additive at the kneading stage and by lactic-acid method.

To reproduce wheat whole bread making, reconstituted wheat flour (flour plus native large bran) was used. For preparing the leaven, 6 kg of whole wheat flour (4.65 kg of white flour plus 1.35 kg of bran) was mixed with 3.6 L of distilled water and 150 g of yeast (*Saccharomyces cerevisiae*). For lactic acid fermentation, sourdough bread was prepared by mixing 1.2 kg of whole wheat flour (0.93 kg of white flour plus 0.27 kg of bran) and 600 ml of distilled water. After one day of natural fermentation at 30°C, the sourdough was mixed with 4.8 kg of whole wheat flour and 3 L of distilled water.

For the sourdough with yeast supplement bread, the ingredients were mixed for 5 min. The resultant dough was left at 30°C to begin fermentation. It was than kneaded again to develop gluten and left to proof at 30°C (Hubert *et al.*, 2003). The dough was baked after 3 h of fermentation for yeast bread and one hour for sourdough breads. Bread was lyophilized, milled and introduced in the alimentation of rats.

In vitro analysis procedures

Iron *in vitro* digestibility, as well as investigation of enzymatic degradation process of phytates was performed in two stages: gastric stage (*p*H 2, in presence of pepsin) and intestinal stage (*p*H 8.2, in presence of trypsin) (Monsen, 1991; Miller *et al.*, 1981). A quantity of 10 g sample was maintained at 37±0.1°C for 15 min in acid environment (*p*H 2) created by addition of 1,5 N HCl solution. After pepsin administration (150 mg/100g product) the blend was incubated for two hours at 37± 0.1°C with continuous shaking. Each 30 min equal samples were taken for the analysis, centrifuged for 10 min (6000 rot/min). The intestinal stage was realized in the same conditions after the establishment of the *p*H value at 8.2, in presence of trypsin, in $NaHCO_3$ 0.08 M environment. The amount of soluble iron was determined by the spectrophotometric method (Kosse *et al.*, 2002).

Phytic acid amount ($InsP_6$) in flour and the final product was determined by spectrophotometric method (Haug and Lentzsch, 1983). The method is based on the organic phosphates capacity to interact with the ammonium molybdate and vanadate with formation of a yellowish – gold complex. The colour intensity is correlated with phosphorus amount. Phytic phosphorus amount was calculated according to:

$$Y= 0.096+0.3X \quad (1)$$

where: Y – total phosphorus X – phytic phosphorus.

Subsequently the amount of $InsP_6$ was recalculated since phytic phosphorus constitutes only 28.2% of the total $InsP_6$.

The analyses were performed on a DR-5000 spectrophotometer; all tests were made in triplicates.

In vivo analysis procedures

Animals and diets

For the researches concerning the influence of the special diets (bread prepared by different procedures with iron addition) on the main haematological parameters, an experiment on 20 white rats weighting approximately 180-210g divided in 3 groups (6-7 in each group) was designed. The animals from the first group (control sample) were fed with ordinary non fortified bread (bread prepared by traditional bi-phase method). The second group represented the rats fed with iron enriched bread prepared by traditional, bi-phase method (80 mg/kg iron addition). The III group comprised the rats fed with bread prepared by lactic-acid fermentation method enriched with iron (80 mg Fe/kg). Animals from all three groups were kept on the above mentioned diet for 21 days.

Sampling and analytical procedures

Biological material – peripheral blood samples from the laboratory animals was taken twice – at the

beginning of the experiment and at the end of the experiment by the procedure described by Ciudin *et al*. (1997).

Blood was placed in single use test tubes Ependorf type of 1.5 ml. Blood parameters have been appreciated in the heparinizated peripheral blood in the haematologic analyzer PCE-210 (ERMA, Japan).

Blood serum was obtained by centrifugation of the peripheral blood at 3000–4000 rot/min for 15 minutes. After centrifugation, blood serum was transferred in Ependorf test tubes and stored in the refrigerator at +4ºC till the end of the experiment.

Blood iron level was determined using the method with cromasurol according to the working instructions of the analyzer ("Iron Cromazurol", "Eliteh", France).

3. Results and Discussion

Technological processing of cereal products (flour moistening, fermentation and dough kneading, baking and drying) influences greatly the amount of phytates in food products. Hereby, vegetal phytase, an enzyme that degrades phytic acid and its soluble sodium and potassium salts, reduces essentially the amount of this antinutrient during technological processing of the flour especially during fermentation. This process is explained by the establishment of optimal conditions for phytase activity development during dough fermentation – at 35…40ºC, *p*H value 4–5, as well as due to the fact that bread yeast amplifies this process through its own phytase activity (Turk *et al.*, 1996).

However, recent use of fast bread making procedures, as well as the use of iron supplements at the beginning of the dough fermentation leads to rapid fixation of bivalent metal cations in chelate compounds such as $InsP_6\ xFe$, subsequently not capable to diffuse the gastro-intestinal wall and they are not subjected to enzyme hydrolysis.

3.1. *In vitro* procedures

In order to demonstrate the influence of bread making procedure (type and duration of fermentation, bread yeast quality), the results obtained during application of traditional bread making method – mono-phase and bi-phase method as well as of lactic-acid fermentation method based on the use of the wheat bran reconstituted by the addition of bran were compared (table 1).
It was established that during mono-phase method, only 15% of phytates presented in the bread were hydrolysed during 2 hours of *in vitro* gastro-intestinal digestion. Soluble iron amount (dialyzable) was, at the end of 2 hours of gastro-intestinal digestion, 0.02 mg//100 g product, which constitutes only 4.6% of the total iron amount, naturally present in the product (2.5 mg/100 g product). Phytates in the bread fabricated by bi-phase method underwent hydrolysis in 36.5%. In this case, the amount of the dialyzable iron constituted 0.2 mg/100 g product, or approximately 8% from the total iron amount.

Table 1. The influence of bread making procedure and the recipe on iron bioavailability* in bakery products**

Bread making procedure	Bakery yeast	Fe dialyzable mg %			Fe dialyz. %	Soluble phytates, mg %			Soluble phytate %
		Gastric	Intestinal	Total		Gastric	Intestinal	Total	
Tradit. (mono-phase)	100%	0.07± 0.02	0.05± 0.01	0.12± 0.02	4.6± 0.5	51.3± 1.9	33.2± 1.6	84.5± 1.9	14.6± 0.4
Tradit. (bi-phase)	100%	0.15± 0.01	0.06± 0.01	0.21± 0.01	8.1± 0.4	125.2± 1.7	89.5± 1.3	214.7± 1.7	36.5± 0.3
Lactic-acid	-	0.29± 0.02	0.25± 0.02	0.54± 0.02	21.6± 0.8	217.5± 2.2	116.8± 1.9	334.3± 2.2	56.8± 0.4
	25%	0.32± 0.03	0.24± 0.02	0.56± 0.03	22.4± 1.2	212.6± 2.7	87.5± 1.6	300.1± 2.7	51.0± 0.5
	50%	0.26± 0.02	0.08± 0.01	0.34± 0.02	13.6± 0.8	197.9± 2.5	99.7± 2.2	297.6± 2.5	50.6± 0.4
	75%	0.22± 0.01	0.24± 0.02	0.46± 0.02	18.4± 0.8	195.5± 2.9	131.3± 3.1	326.8± 3.1	55.6± 0.5

*III - Fe total – 2,5 ± 0,2 mg %

** phytates –total phytates amount in the product 578,0 ± 5,0 mg phytic acid / 100 g product

Obviously, this difference was due to bread making procedure, because the ingredient composition is the same in all the cases. In the first case, fermentation procedure went rapidly while during bi-phase procedure in the starter an important phytase activity will develop due to the phytase presence in flour as well as to the enzymes present in the bread yeast. The addition of flour at the dough formation stage was followed by a considerable phytate enzymatic hydrolysis, which led to a more intense release of the iron from the insoluble phytate complexes.

A number of previous studies showed that lactic-acid fermentation of the dough, obtained using bran from the reconstituted wheat flour, contributes greatly to an essential decrease of the phytate amount (Hubert *et.al*, 2003). Lactic-acid microflora, isolated from the dough obtained by natural fermentation is capable to degrade phytates. Together, phytic degradation and formation of lactic acid could contribute to a more intense release of the minerals in the products.

Hereby, during this study the influence of lactic-acid fermentation, established on the basis of bran from the reconstituted wheat flour, was investigated. In order to show the important role of yeast in this process, bakery products were fabricated. After lactic-acid fermentation development (with the duration of fermentation of 24 hours), in the traditional recipe for the bi-phase method 25, 50 and 75% of yeast was added.

According to experimental results, in the case of the sourdough fermentation application, 50-57% of the present phytates were hydrolyzed after 2 hours of *in vitro* gastro-intestinal digestion. The addition of the bread yeast did not seem to have an important effect (table 1). This fact indicated that even though phytate decrease during traditional bread making procedure was identified, it cannot be referred to the phytase activity of the bread yeast but it was due to endogen flour phytase activity.

Dialyzable iron amount in this case was higher (0.34 – 0.56 mg/100 g product), and represented 13-22% from the total iron amount. It was obviously due to an important degradation of phytates, which made this mineral insoluble. Apparently, the naturally present iron intake from wheat flour was insufficient for diets, which included few products containing heme iron.

The study of iron bioavailability in fortified bread products, prepared by the bi-phase and lactic-acid method showed that in the case of bi-phase method only 8-12% of the iron was presented in soluble form after 2 hours of gastro-intestinal digestion (table 1). In the case of bread prepared by lactic-acid method, the rate of the dialyzable iron constituted 20-22%.

Evidently *in vitro* researches do not reflect the multitude of the factors that may influence the mineral availability in the gastro-intestinal tract. First, there are subjective factors that reflect body reserve of iron. According to the literature data, *in vitro* bioavailability is correlated to *in vivo* digestion in a proportion of 60-76% (Monsen, 1991**].**

3.2. *In vivo* procedures

The analysis of the peripheral blood parameters collected from the animals

The leukocyte number in 10^9/L (WBC) increases in case of animals from control group (group I) and those fed with bread fortified with iron prepared by bi-phase method (group II). In case of group fed with sourdough bread (group III), leukocyte number slightly varies (Figure 1).

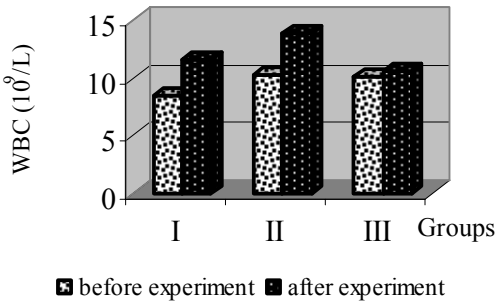

Figure 1. Evolution of the leukocyte parameter during the experiment

Erythrocyte number (RBC) in 10^{12}/L increases for the control group (I) as well as for the groups fed with fortified bread (II and III) but this variability is more essential for the animals fed with fortified bread prepared by lactic-acid method (figure 2).

Haemoglobin (Hb) did not differ essentially in the examined three groups of rats (figure 3). However, for all three groups a tendency for HB increase was noticed, which is more significant for the rat groups fed with fortified products (a increase of 2.2 and 2.9 g/L respectively for the groups fed with fortified bread prepared by classic method and lactic-acid fermentation method).

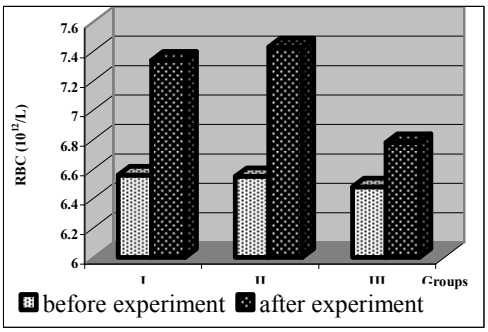

Figure 2. Evolution of the erythrocyte parameter during the experiment

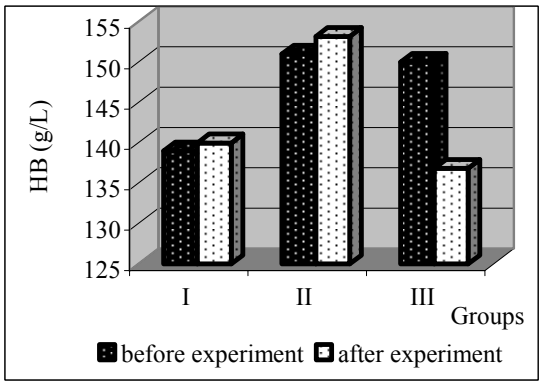

Figure 3. Evolution of the haemoglobin parameter during the experiment

Hematocrit (Ht) corresponded to the volume occupied by the red globules reported to the total blood volume (L/L), providing, thus, information about the circulating haemoglobin concentration. For control group as well as for the groups fed with iron fortified bread, Ht increased insignificantly and reached values of 0.44–0.48, which corresponded to the established standard values (figure 4).

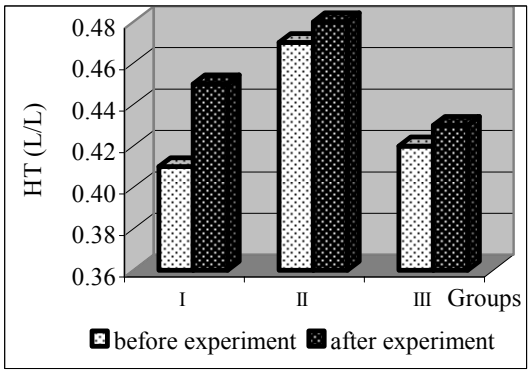

Figure 4. Evolution of the hematocrit parameter during the experiment

Analysis of blood iron transport indicators

Serum iron concentration was an indicator of the amount of iron in plasma, fixed to a specific protein – transferin. The majority of iron which is part of plasma derived through catabolism of reticulo-endothelial system, and iron that leaves plasma derived from bone marrow. Regardless the fact that plasmatic iron reserve was not significant (approximately 3 mg), it was very active because it transported about 30 mg of iron daily.

Generally, transport parameters were not modified till body reserve of iron was not completely depleted. In the convalescence period, after a severe anaemia, namely serum iron is the recovery degree indicator of the body iron statute.

In the experimental groups, it was established that after the administration of the special diet, the level of serum iron was of 36.48±13.8 µmol/L for the control group (I), of 41.55±19.58 µmol/L (II group) and of 40.14±18.78 µmol/L for group III (figure 5).

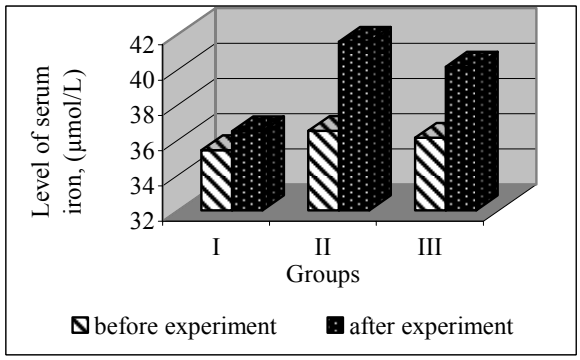

Figure 5. Serum iron evolution during the experiment

During *in vivo* investigations, it was established that for all animals the parameters of peripheral blood after 21 days of special diet administration were within established limits.

For the II and III group, where the special diet was used (iron fortified bread prepared by traditional and sourdough procedures) an essential increase of hemoglobin, erythrocyte number was observed compared to the control group. That was obviously due to an increased iron intake from the administered special diet. But the most significant influence was noticed in the case of the supplement administration on the serum iron level, which reflected body iron reserve. For those 2 experimental groups, fed with fortified bread, body

iron reserve was considerably higher compared to the control group.

The comparison of biochemical parameters of the blood for the two groups fed with fortified bread did not allow revealing the influence of bread making method. Hereby, although *in vitro* investigations showed that iron dialysability from the sourdough bread is considerably higher (≈20%) compared to the iron dialysability from traditionally prepared bread (≈8%), *in vivo* investigations did not testify any significant difference for the biochemical indices for those 2 experimental groups.

The reason might probably be due to the considerable iron intake, which allowed to satisfy the requirements and to complete the body iron reserve.
An essential role played by the initial iron statute of those 3 rat groups that was within the normal limits at the beginning of the experiment.

4. Conclusions

The investigations established that the procedure applied for fortified bread production had a key influence on iron bioavailability degree in "*in vitro"* gastro-intestinal conditions.
This fact was directly related with a high level of phytate degradation from the bread, due to applied bread making procedure and also due to a longer duration.

The study of biochemical blood parameters, in samples collected from the laboratory animals fed with fortified bread (8 mg/100 g product), that was prepared by traditional and by lactic-acid methods and compared to the control group, showed that iron intake plays a key role in animal nutrition. Hereby, iron status in both experimental groups especially body iron reserve was significantly improved compared to the control group.

In case of sufficient iron intake, the bread making procedure did not have any influence on iron status in examined groups. In order to show the influence of bread-making procedure on iron status, it is necessary to investigate the influence of the special diet on the laboratory animals with induced anaemia.

References

Bauernfeind, J.C. 1988. Nutrification of food. In *Modern nutrition in Health & Disease*, 7th Ed.M.E.Shils and Y.R.Young (eds), Lea & Febiger, Philadelphia.

Ciudin, E. and Marinescu, D. 1997. *Patologia animalelor de laborator şi tehnica experimentală*. Editura Moldogrup, Iaşi, 203-205.

Haug, W. and Lentzsch, H.G. 1983. Sensitive method for the rapid determination of phytate in cereals and cereal products. *J.Sci. Food Agric*., 34, 1423-1426.

Hubert, W., Lopez, V. and Duclos Coudray, C. 2003. Making bread with sourdough improves mineral biovailability from reconstituted whole wheat flour in rats, *Nutrition*, 19, 524-530.

Kosse, S., Young, A.C., Gil, A.I. and Miller, D.D. 1996. A rapid method for iron determination in fortified foods, *Food Chemistry*, 75, 371-376.

Luten, J., Crews, H. and Flynn, A. 1996. Interlaboratory trail on the determination of the *in vitro* iron dialysability from food, *J. Sci Food Agric*, 72, 415-424.

Miller D.D., Schricker B.R. and Rasmussen, R.R. 1981. An *in vitro* estimation of iron availability from meals. *Am. J. Clin. Nutr*., 34, 2248-2256.

Monsen, S. 1991 A model for feed evaluation based on *in vitro* digestible dry matter and protein. *CAB International*, Vollingford, UK, 1991, 135-145.

Motruc, N., Ciobanu, C., Deseatnicov, O. and Sturza, R. 2005. Aportul nutriţional în micronutrimenţi în alimentaţia copiilor instituţionalizaţi şi posibilităţi de optimizare. *Proceedings of the 30th Annual Congress of the American Romanian Academy of Arts and Sciences* (ARA), July 5-10.

Turk, M., Carlsson, N.G and Sandberg, A.S. 1996. Reduction in the levels of phytate during wholemeal bread making: effect of yeast and wheat phytases, *J. Cereal Sci*., 23, 257.

* * * 2000. Starea de nutriţie în Republica Moldova, Raport UNICEF 1996- 2000, 38.