Effect of Lactoferrin on Consequences of Acute Experimental Hemorrhagic Anemia in Rats

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The effect of human lactoferrin on the arrest of experimental hemorrhagic anemia consequences was studied in rats. After six blood losses (days 1-4 and 7-8 of the experiment), the rats developed acute anemia: hemoglobin concentration decreased to 59% of the initial level, serum iron level decreased 3-fold. Intraperitoneal injections of lactoferrin (10 mg/day) for 4 days starting from day 7 led to an increase in hemoglobin level to 109% and of serum iron to 125% on day 14. In controls, hemoglobin level on day 14 was 70% and iron content 49% of the initial level. Ferroxidase activity of ceruloplasmin in blood serum decreased after 5 blood losses returned to normal only in rats receiving lactoferrin. The results indicate that lactoferrin modified ceruloplasmin activity *in vivo*, promoting normalization of iron metabolism

Key Words: *hemorrhagic anemia; lactoferrin; ceruloplasmin*

Iron is one of the most important trace elements essential for oxygen transfer and deposition and hence, for all metabolic reactions in vertebrates. Iron deficiency and its extreme degree (iron deficiency anemia) are highly prevalent; about 12% population suffer from these conditions. The incidence of iron deficiency in the high risk groups (pregnant and nursing women, newborns, vegetarians, patients suffering from hemorrhages) reaches 50%. Correction of diets and prescription of iron preparations do not always lead to improvement of the status, because metabolic disorders can be due to iron absorption or deposition.

A close relationship between iron and copper metabolism is known. Correction of experimental anemia with copper salts was studied in rats and mice fed whole milk diet [4]. Ceruloplasmin (CP), a plasma and nervous tissue copper-containing protein in mammals, the main serum ferroxidase, is a common component in the metabolism of these trace elements [4,9]. Physiological role of CP-catalyzed oxidation of Fe(II) to Fe(III) for iron incorporation into transferrin and further transport in the body was shown.

The CP gene defect leads to the development of aceruloplasminemia, a degenerative disease associated with iron deposition in the nervous and other tissues [9]. CP gene knock-out mice serve as a model of this disease. It was shown using this model that CP is essential for discontinuation of anemic status caused by acute blood loss [2]. Despite the presence of iron in the diets of animals with experimental CP gene knock-out, normal hemoglobin level in them is attained only after CP injections. The same study showed that acute blood loss in the controls did not modify CP expression: mRNA content in the liver and concentration serum protein did not change during the experiment. The authors proposed a scheme according to which CP can stimulate iron absorption by oxidizing iron ions in the vicinity of entherocyte basal membrane. In addition, it is known that CP promotes iron release from hepatocytes, reticuloendothelial cells, astrocytes, and macrophages [3,5,6].

CP is now used as a drug for correction of some pathologies including anemia [4,9]. Ceruloplasmin for this purpose is isolated from donor material. This prac-

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tice is fraught with some drawbacks, because donors often develop anemia in response to regular donation of blood. Modulation of CP ferroxidase activity can become a principally new approach to anemia correction. We showed *in vitro* that CP ferroxidase activity is stimulated by lactoferrin (LF; a milk transferrin) in both the apo-form and iron-saturated form [1]. Normally, LF and CP form a stable complex; other proteins do not prevent the complex formation [8,10]. These data suggested that LF can modify CP ferroxidase activity *in vivo,* thus preventing iron deficiency.

We previously showed the possibility of the formation of heterogeneous complex consisting of rat CP and human LF [10]. Thirty minutes after injection of LF (10 mg) to rats, the entire CP was in the complex with human LF. Preliminary *in vitro* experiments showed that human LF stimulated rat CP ferroxidase activity.

We studied the effect of apo-LF on the arrest the experimental acute hemorrhagic anemia consequences in rats. In addition to iron metabolism parameters, CP concentration and ferroxidase activity were measured.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (210±15 g). The animals were divided into 2 groups, 6 rats in each. The blood (3 ml) was collected under ether narcosis in rats of both groups over 4 days and on days 7 and 8 of the experiment. Hemoglobin concentration, serum iron level, CP concentration, and CP ferroxidase activity were measured daily. Starting from day 7, the animals of the experimental group were intraperitoneally injected with 10 ml human apo-LF in 1 ml saline during 4 days. Controls were injected with 1 ml saline. The blood (0.2 ml) measuring of hemoglobin level, CP concentration, and CP ferroxidase activity was collected on days 9-13 of the experiment. On day 14, 2 ml blood was collected for the analysis of the above parameters and serum iron content.

Analytical reagents from Sigma, Serva, and Pharmacia were used in the study. Spectrophotometry was carried out on an SF-2000-02 device (OKB Spectrum).

Human LF was isolated from breast milk by ion exchange chromatography on carboxymethyl sephadex and by gel filtration on sephadex G-100 [10].

In order to measure hemoglobin concentration, 10 μl whole blood (no clotting allowed) was added to 1 ml 0.04% aqueous solution of ammonium and D_{575} was measured (ammonium solution served as the reference sample). Hemoglobin level was expressed in arbitrary units, the initial value was taken as 100%.

Serum iron was measured in the supernatant after precipitation of serum proteins. Trichloroacetic acid (30%; 0.25 ml) was added to 0.5 ml serum and the mixture was centrifuged at 13,000*g* for 10 min. Then 0.5 ml 10% $NH_2OH{\times}HCl$ and 0.5 ml 5 mM ferrozine (forming a complex with Fe^{2+} recorded at D_{564}) were added to 0.5 ml supernatant; the sample with distilled water instead of the serum served as the control. Iron concentration was determined by the calibration curve plotted using ferric ammonium sulfate solution with Fe^{3+} concentrations of 10 to 100 μM.

Serum CP concentration was measured by *p*-phenylene diamine oxidation. The reaction mixture contained 10 μl serum and 1.79 ml 0.05% *p*-phenylene diamine dihydrochloride in 0.4 M sodium acetate buffer (pH 5.5). After 1-h incubation at 37°C the reaction was stopped by adding 0.2 ml 0.5% sodium azide, and D_{530} was measured (to control sample sodium azide was added before incubation). Ceruloplasmin concentration was estimated in mg/100 ml by multiplying D_{530} by 64. The coefficient was evaluated by measuring oxidase activity of purified rat CP towards *p*-phenylene diamine. In preliminary experiments we confirmed that LF in the studied concentrations did not modify CP activity towards *p*-phenylene diamine.

CP ferroxidase activity was evaluated by Fe^{2+} decrease. The serum (10 μl) was added to 0.9 ml 0.25 mM $Fe(NH_4)_2(SO_4)_2{\times}6H_2O$ solution in 0.45 M sodium acetate buffer (pH 5.8) and incubated during 8 min at 37°C. Ferrozine (50 μl 18 mM solution) and sodium azide (40 μl 0.5% solution) were then added into the sample and D_{564} was measured (sodium azide was added to the control sample before incubation). Activity was expressed in mM iron oxidized over 1 h.

The results were statistically processed using Student's *t* test ($p<0.05$).

RESULTS

The decrease in hemoglobin level during the experiment served as the criterion of anemia development (Fig. 1, *a*). In order to confirm that hemoglobin deficit was caused by iron deficiency, we evaluated serum level of this trace element (Fig. 1, *b*). On day 4 of the experiment, hemoglobin level dropped to 59% of the basal level. After the next blood loss (day 7) hemoglobin level virtually did not change and was 58%. By this time, the serum iron level decreased to 20 μM (3-fold in comparison with the basal level). Starting from this time the experimental rats were injected with human LF. One day after the first injection, hemoglobin level in the experimental group increased to 72% *vs.* 61% in the control group. On day 14, hemoglobin level in animals treated with LF increased to 109% *vs.* 70% in control animals. Iron level was not measured on days 9-13 of the experiment, because this test requires at least 2 ml blood. By day 14, this parameter

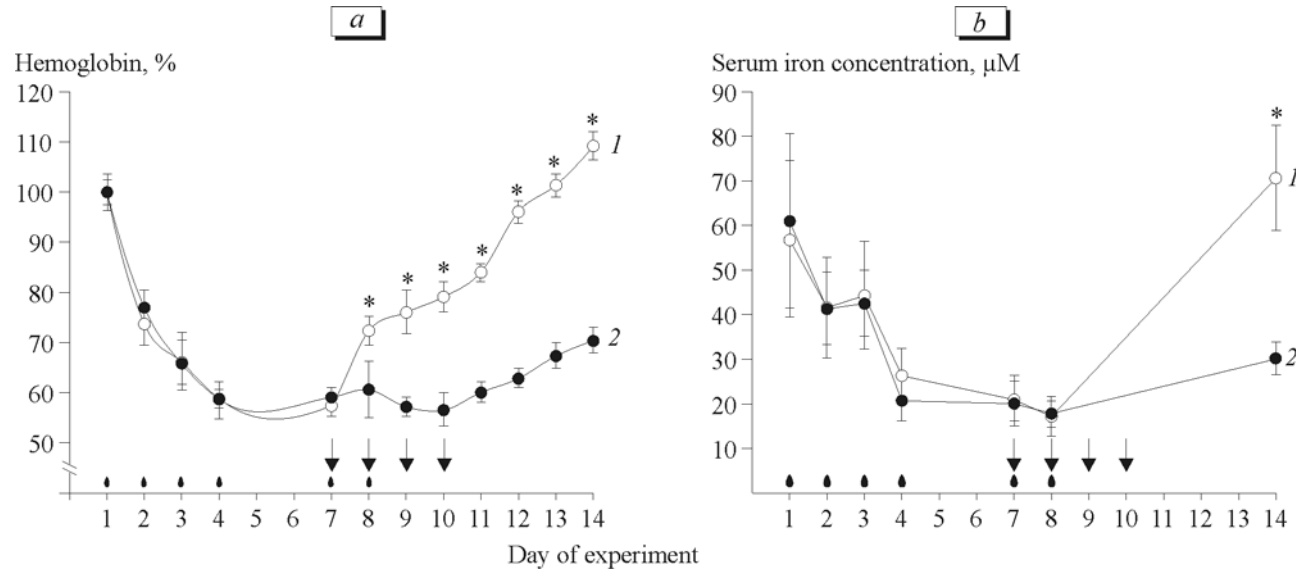

Fig. 1. Effect of LF on hemoglobin concentration (*a*) and serum iron level (*b*) in rats after blood loss. Here and in Fig. 2: *1*) rats treated by LF; *2*) rats injected with saline. Droplets show the periods of blood collection. Arrows show injections of LF or saline. **p*<0.05 compared to the control.

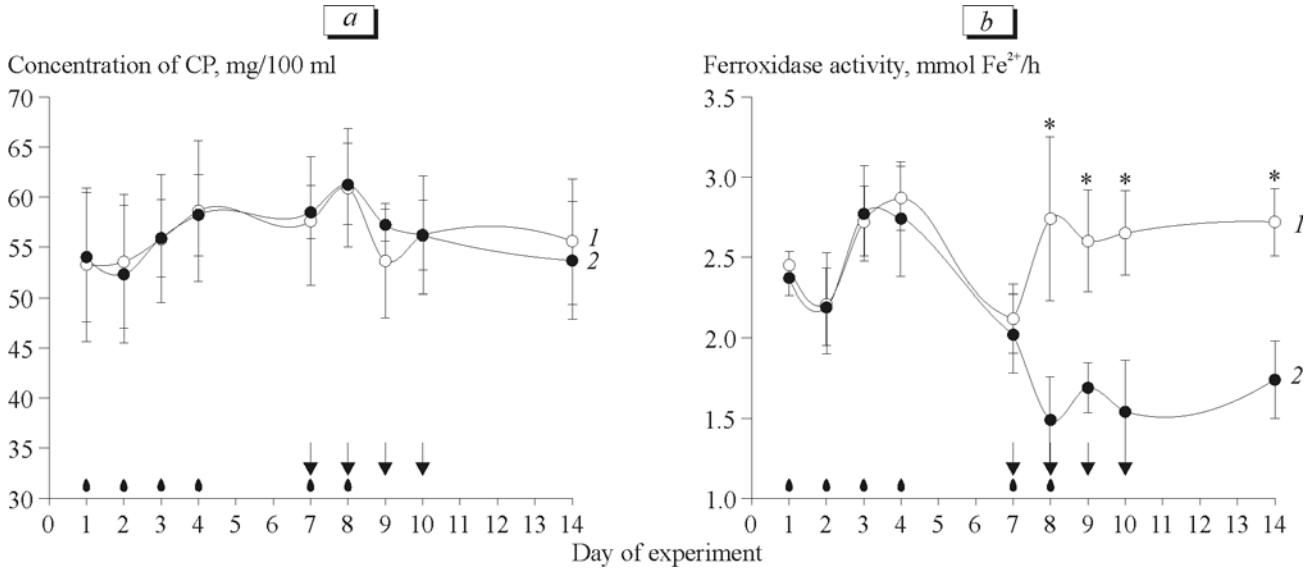

Fig. 2. Effect of LF on CP activity (*a*) and ferroxidase activity (*b*) in rats after blood loss.

differed significantly in the two groups: serum iron in LF-treated animals increased to 125% *vs.* just 49% of the initial level in controls.

Ceruloplasmin ferroxidase activity virtually did not change before LF injections. However, we can speak about a trend to a reduction of this activity after the first collection of blood and about a compensatory increase of this activity on days 3-4 of the experiment. By day 7, the mean ferroxidase activity decreased by 14%. After the start of LF treatment, ferroxidase activity remained low in the control group and returned to normal in experimental rats. Statistically significant differences persisted until day 14 of the experiment.

Serum CP concentration measured by its activity towards *p*-phenylene diamine did not change throughout the experiment (accuracy of measurements to an error of measurements).

Our data indicate that injection of human LF to rats with iron deficiency anemia resulting from regular blood losses induced normalization of the parameters of iron metabolism. This was probably caused by stimulation of CP ferroxidase activity under the effect of LF.

Presumably, a similar mechanism was realized by using cow LF for the treatment of anemia in pregnant women. Oral therapy with this drug was significantly more effective than iron sulfate therapy [7].

Hence, approaches to therapy of anemia by modulation of CP ferroxidase activity during CP reactions with other proteins or polypeptides seem to be effective. This suggests the development of recommendations/approaches to the therapy of anemia in patients.

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