Research Article

Influence of Cord Blood Fraction (below 5 kDa) on Reparative Processes during Subchronic Ulcerative Gastropathy

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The low molecular fraction (below 5 kDa) was extracted from cord blood by ultrafiltration. It has been shown that the cord blood fraction possesses antiulcer activity on the model subchronic stomach ulcer. The cord blood fraction injections caused a significant reduction in the area of ulcer lesions and promoted recovery of microcirculation, thickness, and structure of gland layer, which was accompanied by a decrease in leucocytes infiltration and an increase in glycosaminoglycans synthesis. That resulted in a faster recovery of mucus membrane of stomach as compared with Actovegin. Application of the cord blood fraction in animals with stomach ulcer normalized the alkaline phosphatase activity and thiobarbituric acid-active product content. Gel-penetrating chromatography showed that the patterns of the low molecular substances from cord blood and Actovegin differed both qualitatively and quantitatively.

1. Introduction

At present, ulcerative disease of stomach and duodenum is one of the most widespread morbi of the digestive apparatus. Mechanisms of ulcer formation are diverse. However, both ulcerogenesis and healing of ulcerative defects proceed against a background of significantly decreased cell energy levels owing to abnormalities of microcirculation, which accompany evident inflammatory changes in mucous coat of stomach [1].

Currently, the administration of medicines designated for the elimination of bioenergy disorders, reduction in inflammatory cell infiltration, and recovery of mucous coat microcirculation is among new approaches to the treatment of ulcerative disease. Actovegin belongs to such preparations. It is highly purified hemodialysate extracted from vealer blood by ultrafiltration. Molecular weights of organic substances in Actovegin do not exceed 5 kDa [2]. Actovegin only contains physiological components with high bioactivity. Its pharmacological action is based on the improvement in glucose transport and oxygen uptake in tissues. The latter results in activation of aerobic oxidation, which enhances the cell energy potential. Besides, under conditions of tissue hypoxia caused by microcirculation abnormalities Actovegin contribute to recovery of capillary network due to neogenic vessels [2].

At the same time, nowadays, extensive information on positive influence of cord (umbilical) blood on different organs, systems, cell cultures, and organism as a whole has been collected [3, 4]. Cord blood contains a variety of specific placental proteins, hormones, growth and hemopoietic factors, cytokines, interleukins, immunomodulators, opioid peptides, and enzymes [3, 4]. It was shown that due to its unique composition cord blood offered a number of benefits over donor blood [3, 5]. On the grounds of the above mentioned one can assume that cord blood and its low molecular fraction (below 5 kDa) has a higher bioactivity than Actovegin, which is obtained from vealer blood. That is why the aim of the work was to study the influence of a low molecular fraction (below 5 kDa) from cattle cord blood (CBF)
on reparative processes in mucous coat of stomach under ulcerative gastropathy in comparison with Actovegin.

2. Materials and Methods

The extraction of a fraction containing components with molecular weights below 5 kDa from the whole cattle blood was performed by the ultrafiltration method [6] using a membrane module “Sartorius” (Germany). After ultrafiltration, CBF was lyophilized in a freeze-drying chamber under the pressure of $5 \times 10^{-2}$ mmHg; the total duration of drying was 28–30 h. Lyophilized samples were stored at $-80^\circ$C. Actovegin (commercial preparation, “Nycomed”, Austria) was used as a comparator agent at the concentration of 40 mg (dry weight/mL).

Gel-penetrating chromatography was performed on a plastic column (1.6 × 40 cm) filled with polyvinyl gel Toyopeas 1HW-40 Fine (Toyo Soda, Japan). The phosphate-buffered saline (30 mM Na$_2$HPO$_4$, 200 mM NaCl, pH 7.6) was used as an eluent. Actovegin and CBF were laid onto the column in quantities 4 mg and 0.4 mg (dry weight). Relative quantitative contents of low molecular components were calculated as areas under peaks ($h \times a$, where $h$ is peak height, $a$ is width on half-height) normalized by the total area under all the peaks [7].

The content of acid mucopolysaccharides in CBF and Actovegin was determined as described in [8]. The ionic composition of the fraction and Actovegin was studied on a electrolyte analyzer AEK-01 (“Kartimed”, Russia). The levels of hormones were determined by solid-phase enzyme immunoassay using kits T3-EIA (“XEMA”, Russia) according to [9] for estradiol, [10] for testosterone, [11] for cortisol, and [12] for triiodothyronine. The total glycoproteins content was determined as described in [13]. The total peptides content was determined by the method [14]. The determination of glucose level was based on the glucose oxidase method [15].

The antiulcer activity of the fraction was studied on the model stomach aspirin-induced ulcer in rats, which clinically corresponds to chronic ulcer in people taking aspirin [16]. The experiments were carried out according to the actual EU Directive (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes) [17]. The experiments were carried out in outbred rat males weighing 180–220 g. The animals had preliminary been deprived of food for 24 hours with free access to water. Acetylsalicylic acid solution (10 mg/mL) was given per os by gavage at the dose of 150 mg/kg 5 times within 3 days.

The animals were divided into 4 groups. The number of animals in each group was 6. Group 1 comprised intact rats. The other animals after formation of the model stomach ulcer were intramuscularly injected daily 12 days running: group 2 (control)—with physiological saline, group 3—with CBF, and group 4—with Actovegin (as a comparator agent). The dose of the fraction below 5 kDa was calculated according to the curative dose of Actovegin, which was 1.2 mg of dry weight of the fraction per 100 g of animal weight. The antiulcer activity of the low molecular fraction was assessed on the 3rd, 7th, and 12th days.

Mucous coat of stomach was macroscopically assayed on the 12th day after the formation of ulcer defect. Animals were decapitated under the diethyl ether narcosis. The indices of severity of ulcer lesion were the average area of defects presented as points and the percentage of rats with ulcer in a group, which allowed calculating an integral parameter of antiulcer activity—the ulcer index (UI), and, on its basis the antiulcer activity per se (AUA, %). The average area of lesions was estimated by the following scoring system [16], which allows qualitative and quantitative considering of all the types of destruction in mucous coat of stomach. According to this system, 0 points meant no ulcer lesions; edema, hemorrhages, and 1–3 small ulcers were scored as 1 point; more than three small ulcers or a big one as 2 points; ulcer of a significant size (its diameter can amount to 4 mm) as 3 points. The ulcer index and antiulcer activity were calculated by the following formulas:

\[
UI = \frac{\text{average area of ulcer lesions} \times \% \text{of animals with ulcer in a group}}{100},
\]

\[
\text{AUA, } \% = 100\% - \frac{\text{UI of animals injected with the preparation} \times 100\%}{\text{UI of the control group}}.
\]

Histological specimens for morphological assay of the tissue from an ulcer region were prepared by the established procedure [18, 19]: tissue excised from an ulcer region was fixed in 10% formalin. After fixation, specimens were washed off, dehydrated in increasing concentrations of ethanol, decolored in xylene, and embedded in paraffin. Histologic sections of stomach tissue at the thickness of 4-5 µm after

Ehrlich’s hematoxylin and eosin stain were analyzed on a light microscope.

Functional activity of regenerating tissues was estimated by a number of histochemical reactions. The reaction with N,N’-diethyl pseudoisocyanine by Modis’s method [20, 21] was used for the polarization optical study of total glycosaminoglycans (GAGs) in affected mucous coat of stomach.
Sulfated GAGs (chondroitin sulfate) were determined with toluidine blue as described in [20–22]. GAGs total content and sulfated GAGs content were determined by a semiquantitative method according to the 4-point scale. Specimens were studied and photographed on a confocal laser scanning microscope LSM 510 META (“Carl Zeiss”, Germany).

The development of inflammatory and regenerative processes was judged by alkaline phosphatase (AP) (EC 3.1.3.1) activity determined by hydrolysis of phenylphosphate in neutrophilic granulocytes of rats’ peripheral blood [23]. The content of thiobarbituric acid-reactive substances (TBARS) in rats’ sera was determined by Mihara’s method [24].

The experimental data were statistically processed with the program package “Statgraphic plus for Windows”, version 2.1. The results are presented as mean ± standard error. Mann-Whitney nonparametric test was used for comparison of groups. The confidence level is 0.05.

### 3. Results

A comparative analysis of chromatographic profiles of CBF and Actovegin allowed to detect a significant difference in localization of the peaks observed and in quantitative contents of substances corresponding to these peaks. Figure 1 and Table 1 show that CBF and Actovegin have only four peaks in common from fourteen. In particular, these are peaks 2, 4, 6, and 7. Nevertheless, substance contents of these peaks for CBF and Actovegin differ considerably (Table 1). The data obtained indicate that the low molecular fraction and Actovegin differ qualitatively; however, the differences discovered do not exclude the fact that they can share the same active component.

### Table 1: Relative component contents in Actovegin and low molecular (below 5 kDa) CBF determined as areas under peaks.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Actovegin Content in the preparation, %</th>
<th>CBF Content in the preparation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.86</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>15.71</td>
<td>12.97</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>12.94</td>
</tr>
<tr>
<td>4</td>
<td>34.92</td>
<td>5.16</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>8.98</td>
</tr>
<tr>
<td>6</td>
<td>9.75</td>
<td>4.65</td>
</tr>
<tr>
<td>7</td>
<td>4.38</td>
<td>39.56</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>1.59</td>
</tr>
<tr>
<td>9</td>
<td>24.67</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>13.81</td>
</tr>
<tr>
<td>11</td>
<td>0.56</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>0.34</td>
</tr>
<tr>
<td>13</td>
<td>0.49</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>0.67</td>
<td>—</td>
</tr>
</tbody>
</table>

As one can see from Table 2, CBF comprises a number of components which were also found in Actovegin. However, there are some quantitative differences in their contents, which is quite understandable taking into account the fact that the sources of CBF and Actovegin differ ontogenically. Much higher levels of estradiol, cortisol, testosterone, calcium and sialic acids in CBF as compared to Actovegin are noticeable.

The macroscopic assay of rats’ stomachs after formation of aspirin-induced ulcer showed that the administration of aspirin caused a great number of small and large hemorrhagi-
Ulcers

Figure 2: Stomach mucous coat of rats on the 12th day of the experiment: (a) normal structure; (b) control group (physiological solution injections); (c) group 3 (CBF injections); (d) group 4 (Actovegin injections). Hematoxylin-eosin stain. Magnification: 200x.

ges, edema, hyperemia, ulcer lesions of different size and depth and abnormal stomach rugosity. Ulcer lesions were found in 100% of animals. The average area of lesions was $2.50 \pm 0.20$ points; UI was 2.5 (Table 3).

On the 12th day after injections of physiological solution in the control group 2, we observed hemorrhages, edema, moderate hyperemia, and abnormal stomach rugosity in the lesser curvature of stomach. Ulcer lesions were found in 100% of the control animals. The average area of lesions was $1.8 \pm 0.15$ points; UI was 1.8 (Table 3).

The histological study of the control rats’ group 2 stomachs on the 12th day revealed that all the animals had numerous erosions, the depth of which ran up to $1/3$ of the thickness of mucous coat of stomach and in some cases up to muscle endplate (Figure 2(b)). The floor of erosions was a layer of fibroid necrosis. The glands were shortened in comparison with the norm (Figure 2(a)), and their structure was damaged to the extent of atrophy. There were clusters of desquamated epithelium on the gland surface. Lymphocytoplasmonic infiltration was seen in the mucous coat plate. There were a lot of dilated vessels filled with aggregated erythrocytes above the muscle endplate. The glands lost specialized cells for a large space.

GAGs play an important role in the reparative function of connective tissue [25]. Excessive proteolysis activation under pathologies sets conditions for distortions of mucus glycoproteins and proteoglycans structure and for a rise in their degradation. That leads to a reduction in the resistance of mucous coat of stomach and is a metabolic precondition for the development of ulcer lesions [1]. The total GAGs test
in histologic sections of the control group 2 was slightly positive (+) (Figures 3(b) and 4(b)). Hyaluronic acid was only located on the epithelium surface (Figure 3(b)). A significant decrease in chondroitin sulfate content was noticed in the lower part of the glands (Figure 4(b)). Thus, ulcerogenesis leads to an intensified degradation of GAGs. Consequently, aspirin affection of stomach in rats morphologically corresponds chronic erosive atrophic gastritis.

On the 12th day after injections of CBF in group 3, the macroscopic assay revealed no ulcer lesions (UI = 0.1), only minor hyperemia and few hemorrhages. The colour and stomach rugosity approximated to those of intact animals. The average area of ulcer lesions was 0.30 ± 0.19 points, which was significantly lower than the value of the control group (Table 3); AUA was 92.86%.

In the histological sections of mucous coat of stomach from group 3 (rats injected with CBF) on the 12th day, we could observe restoration of mucous coat thickness (Figure 2(b)) and the stomach glands were complete and narrow. The vessels above muscle endplate contained few erythrocytes. The number of lymphocytes in mucous coat of stomach was considerably lower than in the control (Figure 2(b)). Stomach epithelium was high, cylindrical (Figure 2), and had minor injuries. All the types of specialized cells were present in the main glands. However, as compared to the norm, a certain predominance of accessory

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**Figure 3:** GAGs in stomach mucous coat of rats on the 12th day of the experiment: (a) in the norm (++++); (b) control group (physiological solution injections) (+); (c) group 3 (CBF injections) (++++); (d) group 4 (Actovegin injections) (++). N,N’-diethyl pseudoisocyanine stain. Laser scanning microscopy. Excitation wave 470 nm. Magnification: 200x.
cells with enhanced mucus production over main cells was observed. Judging by a higher intensity of the stain, the administration of CBF stimulated GAGs biosynthesis in cells of stomach mucous coat in rats with ulcer (Figures 3(b) and 4(b)). Presumably, CBF considerably accelerated reparative processes by stimulating GAGs and their derivatives (including glucosamine) biosynthesis, since glucosamine as a building block of fibronectin positively affects reparation of ulcer [25]. On the grounds of the above stated, one can conclude that rats which were injected with CBF only had catarrhal gastritis with a slight reduction in secretory function of glands and simultaneously demonstrated activation of regenerative processes. This attests to suppressed development of aspirin-induced ulcer in rats under the influence of CBF.

On the 12th day after injections of Actovegin in group 4 the macroscopic assay revealed a lesser intensity of the ulcerous process: hemorrhages were few in number; they were small; a minor hyperemia was visualized; color and stomach rugosity were similar to those of intact animals (UI = 0.3). The average area of ulcer lesions was 0.50 ± 0.20 points, which was significantly lower as compared to the control group and significantly higher than this index in rats after CBF injections (group 3). AUA in group 4 was 78.57%.

In the histological sections of mucous coat of stomach of rats injected with Actovegin on the 12th day, we observed...
Ulcers

![Graph](image1.png)

**Figure 5**: Dynamics of the AP activity in rats’ peripheral blood after the low molecular fraction injections. *Significant difference (P < 0.05) in comparison with the control.

![Graph](image2.png)

**Figure 6**: Dynamics of TBARS content in rats’ peripheral blood after the low molecular fraction injections. *Significant difference (P < 0.05) in comparison with the control; **significant difference (P < 0.05) in comparison with Actovegin.

A pattern similar to that in group 3 after CBF injections. Nevertheless, unlike the results obtained after CBF injections, in group 4 in most cases, we noticed an obliterated inflammatory infiltrate in surface parts of stomach mucous coat (Figure 2(a)). The total GAGs test was moderately (+++) positive after the administration of Actovegin (Figures 3(d) and 4(d)). Thus, in the group of rats which were injected with Actovegin in stomach mucous coat there were symptoms of surface chronic gastritis with simultaneous activation of regenerative processes.

Changes of AP activity in neutrophilic granulocytes of peripheral blood are known to be an informative index of development of inflammatory processes in the organism [26]. An enhanced AP activity under ulcerative gastropathy reflects the prevalence of inflammatory processes, and the dynamics of the enzyme activity gives the disease development estimate [26].

The AP activity was increased in rats’ blood on the 3rd day after ulcer formation in comparison with the normal value (Figure 5). The normalization of this index was noticed both after Actovegin and after CBF injections (Figure 5). Hence Actovegin and CBF injections promote a decline in the inflammatory response in stomach mucous coat.

At present it has been established the excessive activation of lipid peroxidation (LPO) processes is an important factor in the pathogenesis of ulcerative gastropathy [1]. LPO products are able to inhibit proliferation processes, and this property is one of the causes of a fall in the reparative capacity of stomach mucous coat. Augmentation of LPO in stomach mucous coat leads to a variety of macroscopic and histological changes such as vessel damage, hemorrhages, stasis in arterioles and venules, epithelium desquamation, which in its turn leads to necrosis and ulcer formation. For objective appraisal of severity and functional magnitude of ulcer lesions TBARS content in animals’ sera was determined.

After formation of ulcer TBARS content was significantly increased in peripheral blood of group 2 (control) in comparison with the norm over the term of the experiment (Figure 6).

TBARS content significantly (P < 0.05) decreased related to the control on the 12th day after CBF or Actovegin injections (Figure 6), but normalization of this index only occurred after CBF injections. This can be attributed to the fact that cord blood comprises higher contents of substances, participating in the antioxidant protection system [3, 27, 28].

### Table 2: Compositions of the cord blood low molecular fraction (below 5 kDa) and Actovegin.

<table>
<thead>
<tr>
<th>Components</th>
<th>CBF</th>
<th>Actovegin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total peptides</td>
<td>1.0 ± 0.01 mg/mL</td>
<td>0.5 ± 0.03 mg/mL</td>
</tr>
<tr>
<td>Estradiol</td>
<td>54.0 ± 3.0 nmol/L</td>
<td>21.2 ± 2.0 nmol/L</td>
</tr>
<tr>
<td>Testosterone</td>
<td>15.0 ± 1.0 nmol/L</td>
<td>0.7 ± 0.6 nmol/L</td>
</tr>
<tr>
<td>Cortisol</td>
<td>133.6 ± 8.0 nmol/L</td>
<td>98.4 ± 6.0 nmol/L</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>3.3 ± 0.1 nmol/L</td>
<td>N/A</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.2 ± 0.2 nmol/L</td>
<td>67.8 ± 3.0 nmol/L</td>
</tr>
<tr>
<td>Sodium</td>
<td>206.0 ± 14.0 mmol/L</td>
<td>298.0 ± 10.0 mmol/L</td>
</tr>
<tr>
<td>Calcium</td>
<td>3.2 ± 0.1 nmol/L</td>
<td>0.7 ± 0.2 nmol/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>9.2 ± 0.2 mmol/L</td>
<td>7.1 ± 0.3 mmol/L</td>
</tr>
<tr>
<td>Total glycoproteins</td>
<td>5.8 ± 0.6 mmol/L</td>
<td>4.9 ± 0.3 mmol/L</td>
</tr>
<tr>
<td>Hexuronic acids</td>
<td>0.02 ± 0.09 mg/mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Free hexuronic acids</td>
<td>0.03 ± 0.11 mg/mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>0.6 ± 0.1 µg/mL</td>
<td>0.4 ± 0.2 µg/mL</td>
</tr>
<tr>
<td>Free hyaluronic acid</td>
<td>0.1 ± 0.1 mg/mL</td>
<td>N/A</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>5.1 ± 0.2 mmol/L</td>
<td>2.9 ± 0.1 mmol/L</td>
</tr>
</tbody>
</table>

4. Conclusions

The fraction below 5 kDa from cord blood was shown to have the antituulcer activity against the model stomach subchronic ulcer. CBF contributed to the significant reduction in the area of ulcer lesions, which correlated with the recovery of micro-circulation, thickness and structure of the gland layer accompanied by the decline in leukocyte infiltration of affected...
tissues, stimulation of GAGs synthesis, which resulted in a more efficient shortening the recovery time of stomach mucous coat as compared to Actovegin. The composition of CBF was discovered to differ qualitatively and quantitatively from Actovegin components. It was revealed that the administration of CBF in animals with stomach ulcer promoted the normalization of TBARS content and AP activity in peripheral blood, which attested to the antiinflammatory activity of CBF.

Conflict of Interests

The authors have no financial interests in the substances used in the study.

References

[22] D. S. Sarkisov and Y. L. Persova, Microscopic Equipment, Meditsina, Moscow, Russia, 1996.

Table 3: Antiulcer activity of the cord blood fraction (below 5 kDa) and Actovegin.

<table>
<thead>
<tr>
<th>Experimental conditions (n = 6)</th>
<th>Percentage of animals with ulcer lesions in the group</th>
<th>The average area of ulcer lesions, points</th>
<th>Ulcer index</th>
<th>Antiulcer activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day after the formation of ulcer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>100</td>
<td>2.5 ± 0.20</td>
<td>2.5</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>1.8 ± 0.15</td>
<td>1.8</td>
<td>—</td>
</tr>
<tr>
<td>Actovegin</td>
<td>50</td>
<td>0.5 ± 0.2*</td>
<td>0.3</td>
<td>78.57</td>
</tr>
<tr>
<td>Cord blood fraction</td>
<td>33</td>
<td>0.3 ± 0.19**</td>
<td>0.1</td>
<td>92.86</td>
</tr>
</tbody>
</table>

*Significant difference (P < 0.05) in comparison with the control; **significant difference (P < 0.05) in comparison with Actovegin.


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