Toxicological Study of King Crab Collagenase

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A toxicological study of a preparation of king crab collagenase designed for local treatment of wounds shows that daily hypodermal administration of various concentrations of preparation (1.5 to 15 mg/ml) over 5 days does not affect erythrocyte morphology or hemoglobin content in laboratory animals (rats and rabbits). Systemic enzyme administration does not alter the histological structure of the kidneys (kidney is the target organ for enzyme) but leads to dose-dependent reversible degeneration of rat liver tissues. The milder effects of the preparation in comparison with a comparable dose of the widely used chymopsin affirm that this collagenase preparation is suitable for external use.

Key words: *collagenase; king crab; toxicology; blood components; liver; kidneys*

In previous studies we have shown that a preparation of king crab (*Paralithodes camtschatica*) collagenase expresses high necrolytic and wound-cleaning activity [1]. The most active components of this preparation are the collagenolytic proteases A and C [11], originally described by Sakharov *et al.* [7]. Crab collagenase accomplishes its cleaning activity by hydrolytic splitting of the fibrin covering the wound bottom and of the collagen strands, which hold necrotic tissue on the surface of healthy tissue [3,8]. Later, in an investigation of the pharmacokinetic properties of collagenase, it was discovered that crab proteases can penetrate into the organism through the wound surface [6]. A study of the distribution of collagenolytic proteases in organs after intravenous injection reveals isoenzyme accumulation in rat liver and kidneys, whereas the protease was not detected in other organs (heart, spleen, lungs, muscles) [6]. The data obtained form a basis for a toxicological investigation of crab collagenase.

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MATERIALS AND METHODS

The preparation known as "crab collagenase" with a specific activity of 600 units/mg protein (by Mandl [10]) was isolated from the hepatopancreas of *P. camtschatica*.

Acute collagenase toxicity (LD_{50}) was studied on male white rats weighing 180-220 g by intravenous injection of the preparation in increasing doses. Further toxicological study was carried out on male rabbits of 2.5 kg weight and male white rats of 180-220 g weight by daily hypodermal injection during 5 days of 1 ml collagenase solution (total 6 injections) in the back. The concentrations were as follows: 15 mg/ml (77 rats), 5 mg/ml (21 rats), and 1.5 mg/ml (21 rats). The control group (56 rats) was injected with 4 mg/ml chymopsin and benzoyl-arginine-p-nitroanilide (BAPNA), the activity of which was equal to the activity of collagenase in a concentration of 15 mg/ml. Seven rats received no preparation. For a comparative study of the action of the enzyme on animals of another species and weight, rabbits were injected with analogous protease doses, calculated per kg of body weight.

The erythrocyte number in the peripheral blood was estimated every day. Hemoglobin concentration was recorded using spectrophotometry

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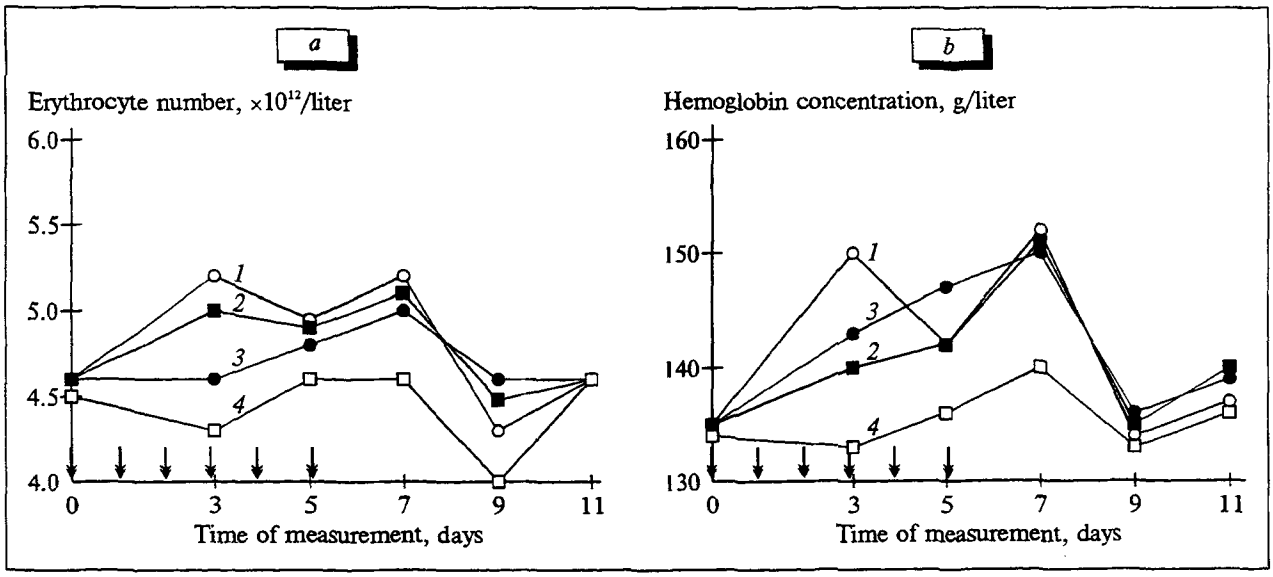

Fig. 1. Erythrocyte number (*a*) and hemoglobin concentration (*b*) in the blood for daily hypodermal injections of crab collagenase at 1.5 mg/ml (*1*) and 15 mg/ml (*2*), chymopsin at 4 mg/ml (*3*) for rats, and crab collagenase at 15 mg/ml (*4*) for rabbits. Here and in Fig. 2: arrows indicate days of injection of the preparation.

[4]. Cytological investigation of rat and rabbit blood and histological study of rat spleen and liver were carried out using the light microscopy method. Every day blood smears were prepared and stained with azure-eosin [4]. Serial hystological 7-μ sections of internal organs, prepared after 6-fold parenteral protease injections in concentrations of 15, 5 and 1.5 mg/ml (collagenase) and 4 mg/ml (chymopsin), were stained with hematoxylin-eosin [5]. Histidase activity was estimated on a spectrophotometer, as described elsewhere [12].

RESULTS

Earlier we demonstrated a high necrolytic activity of the new enzyme preparation "crab collagenase"

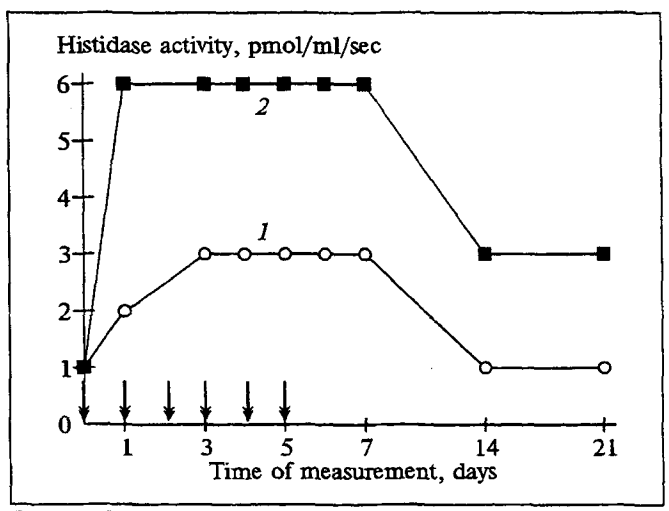

Fig. 2. Histidase content in rat blood for daily hypodermal administration of crab collagenase at 15 mg/ml (*1*) and chymopsin at 4 mg/ml (*2*).

[1,11]. The discovery of such an obvious therapeutic effect forced us to assess the possible side effects of the preparation. Acute toxicity for intravenous injection in rats amounted to 216.7 mg/kg body weight, which exceeds the optimal concentrations for wound necrolysis many times [11].

Investigation of crab collagenase pharmacokinetics using the application method showed that collagenolytic proteases can penetrate through granulation tissue into the blood stream and be inactivated by blood protein inhibitors [6]. In view of this it was of great importance to estimate the possible effect of the preparation on blood components. We selected the hypodermal method of administration, since in this case the entire dose has a systemic effect, in contrast to the case with application to the wound.

Investigation of rat blood showed an increase of the erythrocyte count (from 4.6±0.3 to 5.5±0.4×10¹² cells/liter) and a rise in the hemoglobin concentration (from 134±0.2 to 163±0.8 g/liter) on the 6th day after the first collagenase and chymopsin administration, regardless of protease concentration and specificity. On days 8-9, i.e., 2-3 days after withdrawal of the preparations, the parameters under study returned to the initial level (Fig. 1). No reliable changes of erythrocyte and hemoglobin concentrations were observed after administration of collagenase solution to rabbits. Dynamic cytological investigation of blood cell morphology (the presence of immature forms, anisopoikilocytosis, polymorphism, hypochromia, etc.) revealed no morphological alterations in either rats or rabbits.

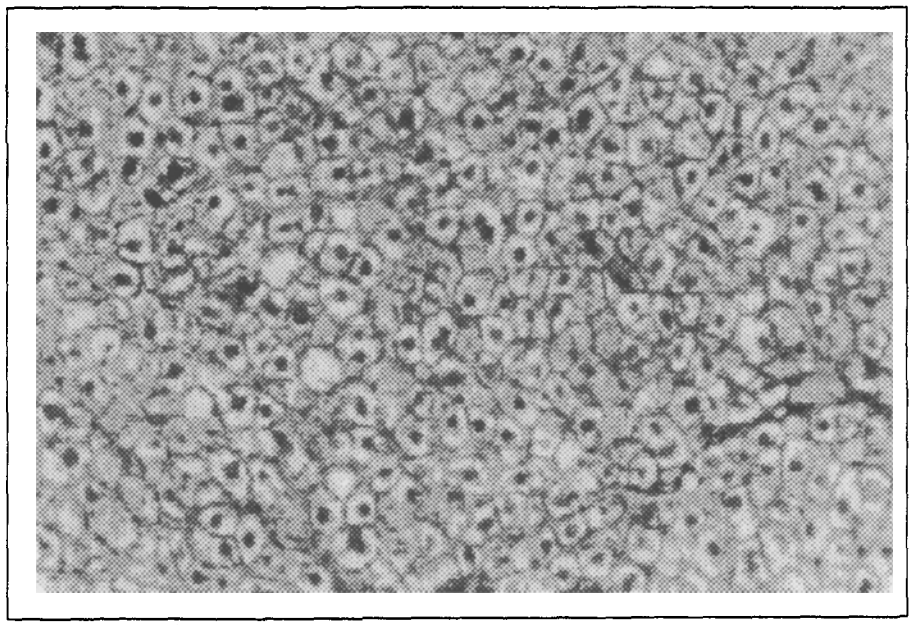

Fig. 3. Histophotogram of rat liver on the 6th day after 6 daily hypodermal crab collagenase injections at 15 mg/ml. Note marked hepatocyte vacuolization and the presence of many cells with "depleted" cytoplasm. Cell boundaries distinct. Necrotic alterations absent. Here and in Fig. 4: staining with hematoxylin–eosin; ×160.

However, histidase determination in rat blood showed an increase of its activity against the background of crab collagenase injection (Fig. 2). The histidase content began to decrease immediately after the preparation was discontinued and within a week returned to the baseline level in intact animals. The same effect was observed when the well-known proteolytic enzyme chymopsin was injected into rats. This enzyme, like collagenase, manifests trypsin and chymotrypsin activity. Nonetheless, the histidasemia response to chymopsin was almost twice as high as that to collagenase injection, and the initial histidase level was restored only 2 weeks after the abolition of chymopsin administration (Fig. 2). Since it is known that histidase is an intracellular enzyme of mammalian hepatocytes, histidase detection in the blood may reflect the influence of parenterally administered compounds on liver cell status [2]. Thus, the detection of a reliably high specific hepatocyte marker concentration in the blood as a result of systemic collagenase and chymopsin application suggests a hepatotoxic action of intravenous enzyme injections.

In order to confirm or refute the above hypothesis, we performed a histological analysis of rat liver after parenteral collagenase administration. Thus, after 6 daily enzyme administrations in a dose of 1.5 mg/ml we observed clearing and vacuolization of the hepatocyte cytoplasm. Raising the collagenase concentration to 5 mg/ml caused more

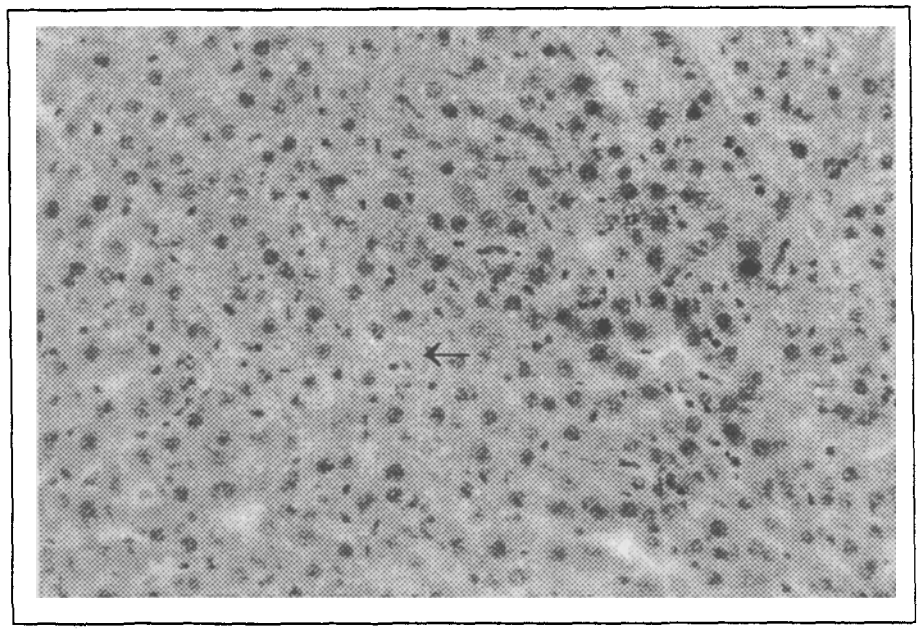

Fig. 4. Histophotogram of rat liver on the 6th day after 6 daily hypodermal chymopsin injections at 4 mg/ml. Note local discomplexation of liver trabeculae and marked cytoplasm vacuolization. Cell boundaries indistinct. Monocellular necrosis in parenchyma visible (arrow).

marked hydropic degenerative phenomena in liver samples. There were cells with diminished nuclei and strongly eosinophilic cytoplasm. Further augmentation of the enzyme concentration to 15 mg/ml led to the appearance of large number of dystrophic cells with "depleted" cytoplasm. At the same time, initial phenomena of focal discomplexation of liver trabeculae and hepatocyte cytoplasm vacuolization were detected on the 3rd day after the first injection. Cell boundaries were clearly defined and necrotic changes were not observed (Fig. 3). On the 8th-11th days of observation (3-5 days after collagenase abolition) the degenerative processes in the liver began to diminish, and the cytoplasm became more basophilic and contained only a few small vacuoles. On the 14th day of observation the liver histostructure was much the same as that in intact animals.

On the 6th day after daily chymopsin injection in a dose of 4 mg/ml a marked hydropic dystrophy and hepatocyte clearing and vacuolization in rat liver were also observed. Moreover, monocellular necrosis developed in the liver parenchyma. Cell boundaries were indistinct (Fig. 4). The 8th-14th days saw hydropic degeneration of various degrees, monocellular necrosis, and cell discomplexation phenomena, along with alterations of hepatocyte contacts. The hepatocyte cytoplasm was of irregular density. The phenomena described disappeared in this group of rats only toward the 21st day of observation.

Since liver hydropic degeneration reflects the proteolysis level in hepatocytes [9], we can explain the discovered effect either by activation of hepatocellular enzyme systems by blood-circulating enzyme-inhibitor complexes, or by reactivation of inactive proteases in hepatocytes. The more marked degeneration and presence of necrotic lesions in rats against the background of chymopsin administration suggest that the sensitivity threshold of rat liver to chymopsin is lower than that to collagenase administration.

Morphological investigation of rat kidneys was carried out in order to assess the effect of intravenous injection of proteases on other target organs. In this case even the use of maximal collagenase doses (15 mg/ml) induced no significant histostructural changes in liver tissues either before or on the 6th, 8th, and 14th days after the beginning of the experiment.

Thus, the toxicological investigations of king crab collagenase showed that the preparation has no influence on blood cell morphology or on the hemoglobin and erythrocyte concentration in experimental animals. However, the enzyme provokes reversible degenerative alterations in rat liver. Collagenase administration causes less marked alterations in comparison to the widely used chymopsin, allowing to us conclude that the new enzyme preparation is suitable for external wound treatment.

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