In vivo studies regarding the anti-inflammatory activity and liver protection of bioactive complex rich in glycosaminoglycans

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NATALIA ROȘOIU1, ROXANA NITA2, MANUELA DIANA ENE2, LAURA OLARIU2, MARIANA CONSTANTINOVICI2
1“Ovidius” University Faculty of Medicine, Department of Biochemistry, Constanța, Romania; Academy of Romanian Scientists 54 Splaiul Independentei 050094, Bucharest, e-mail:natalia_rosoiu@yahoo.com
2S.C. Biotehnos S.A., Research Department, Otopeni, Romania

Abstract

In order to evaluate the anti-inflammatory activity and the liver protective effects of a bioactive complex (GAG) we performed in vivo studies on Wistar rats with carrageenan-induced paw edema and CCl4-induced liver toxicosis. The results showed that the GAG-induced anti-inflammatory activity (81.4% in 24 hours) was similar to the one of diclofenac-induced (83.7% in 24 hours). The bioactive complex induced a decrease in lipid peroxidation and a stimulation of the liver catalase activity in a dose-effect manner. Taking into account the therapeutic effects of the bioactive complex highlighted by these experiments and others described in the previously work, it may be regarded as a very good source of medicinal products with valuable therapeutic properties and minimal side effects.

Keywords: glycosaminoglycans (GAG), anti-inflammatory activity, liver protective effects, catalase, malondialdehyde (MDA)

Introduction

Sea organisms arouse a major interest for the extraction of biological active substances with multiple and valuable therapeutically applications all over the world as discussed by Serban and Rosoiu [1] and Kornprobst [2]. The aim of our researches was to investigate the therapeutic properties of biological complexes previously obtained from small sea fish in order to elaborate pharmaceuticals with high efficiency and minimum side effects.

In the previous works we route about the original complexes rich in glycosaminoglycans obtained from small sea fish [3] and certain bioactive effects of these complexes [4]. So, using an original patented technology, we have obtained a bioactive complex from small sea fish. The complex is rich in sulfated glycosaminoglycans (44-60%), essential amino acids (2-6.5%), essential fatty acids (1-2%, ω-3, ω-6) and mineral salts (Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si). It has anti-hyaluronidasic and anti-collagenasic activity, decreases the level of elastase and favors in vitro collagen fibrils formation comparable with ascorbic acid (vitamin C). Due to the high concentration of GAG, it interrupts the collagenolytic activity of the cartilage degeneration processes, recovering extracellular matrix and manifests a strong antioxidant activity [4].

Material and methods

The bioactive complexes rich in glycosaminoglycans, were obtained from small sea fish (Sprattus sprattus sprattus, Odontogadus merlangus euxinus and Engraulis encrassicolus ponticus species), according to self-developed patented technologies.
There were performed \textit{in vivo} studies regarding the anti-inflammatory activity and liver protection - denoted by: (a) lipid peroxidation, (b) catalase activity and (c) protein assay – of a bioactive complex rich in glycosaminoglycans.

\textbf{Anti-inflammatory activity}

Anti-inflammatory activity was studied by rat paw edema inhibition evaluation, induced with a solution of 2\% carrageenan \cite{5}. Wistar male rats, of 130-150 g were used for present investigation. Subsequently 30 minutes after intraperitoneal administration of GAG or diclofenac (as positive control), 0,1 ml of 2\% carragenan solution was injected subcutaneously into the planter region of right hind paw ro induce edema. Paw volumes were measured plethysmometrically before and at different times after the induction of edema. The anti-inflammatory effect was calculated using the Newbould \cite{6} formula.

\textbf{Liver protection activity}

The activity of bioactive complex of sulphated glycosaminoglycans as well as of small sea fish concentrate – raw material also for Alflutop\textsuperscript{®} injectable solution – towards lipid peroxidation and enzymatic activity was evaluated in two studies, done in normal conditions and induced oxidative stress situation obtained by carbon tetrachloride (CCl\textsubscript{4}) administration. This substance is one of the most known xenobiotics with hepatic tropism, capable to produce serious liver injuries, similarly to those appear in human hepatitis and cirrhosis \cite{7, 8, 9}. Liver induced toxicity to experimental animals through CCl\textsubscript{4} administration, determines a chemical hepatectomy, useful for studying the eventual liver regeneration under GAG action \cite{10, 11}.

\textbf{a) Lipid peroxidation}

Wistar rats, male, 200-250 g body weight, were divided in groups of six animals/lot as follows:
- Lot 1 - control, saline solution 1ml/kg b.w., i.p. (MS)
- Lot 2 - test, bioactive complex (GAG) 25 mg/kg b.w. i.p. (GAGdm)
- Lot 3 - test, GAG 50 mg/kg b.w. (GAGDM)
- Lot 4 - test, small sea fish liquid extract 0,5 ml/kg b.w. (CBPMMdm)
- Lot 5 - test, small sea fish liquid extract 1 ml/kg b.w. (CBPMMDM)
- Lot 6 - test, small sea fish liquid extract 0,5 ml/kg b.w. + GAG 25 mg/kg b.w. (CBPMMdm + GAGdm)
- Lot 7 - test, small sea fish liquid extract 0,5 ml/kg b.w. + test, small sea fish liquid extract 50 mg/kg b.w. (CBPMMdm + GAGDM)
- Lot 8 - test, Alflutop\textsuperscript{®} injectable solution 1 ml/kg b.w. (ALF)
- Lot 9 - test, Arginine \textsuperscript{®} injectable solution 50mg/ ml/kg b.w. (Hep)

Lipid peroxidation was evaluated using the method with thiobarbituric acid (TBA). The MDA-TBA adducts formed subsequent to the reaction of TBA and MDA from the biological sample was measured using a colorimetric method ($\lambda = 532$ nm).

\textbf{b) Catalase activity} was investigated according to the AEIBI protocol \cite{12}. It was monitored the decrease in absorbance (37°C, $\lambda = 240$ nm) due to the conversion of H\textsubscript{2}O\textsubscript{2} into water and oxygen \cite{12}.

\textbf{c) Protein assay} was performed according to the Bradford method \cite{13}.

\textbf{d) Statistical analysis} was done using the one-way ANOVA algorithm followed by Dunnett’s t-test.
Results and Discussion

**Anti-inflammatory activity**

Carrageenan induces an irritative edema as a result of histamine release, hyaluronidase activation, quinine formation and lock of endogenous catecholamines.

The results obtained in the experiment presented in table 1, reveals the fact that the tested extract has an antinflammatory activity (81.4 % at 24h), acting similarly to diclofenac (83.7 %). This is a nonsteroidal antiinflammatory drug having complex actions against inflammatory process: interferes with prostaglandin synthesis, has antihyaluronidase action, diminishes formation and action of some peptides (bradykinin).

**Table 1.** Anti-inflammatory activity of the GAG extract in carrageenan - induced paw edema in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-inflammatory effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>-</td>
</tr>
<tr>
<td>GAG extract</td>
<td>-</td>
</tr>
</tbody>
</table>

The observed effect is confirmed by the affirmation of other researchers who affirm that glycosaminoglycans manifest anti-inflammatory activity and are capable of reduction synovial inflammation [14, 15, 16, 17]. In clinical studies the formation of inflammatory joint effusions at patients with osteoarticular affections is significantly diminished by the perioral administration of glycosaminoglycans or chondroitin sulphate [18, 19, 20, 21]. Beren et al. [22] describe the anti-inflammatory effect of a nutritional supplement which contains glucosamine chlorhydrate, sodium chondroitin sulphate and mangan ascorbate in the case of autoimmune arthritis experimentally induced. Campo et al. [23, 24] evaluated the antioxidant activity of hyaluronic acid and chondroitin sulphate using a model of autoimmune arthritis experimentally induced and they too observed that the treatment with these substances limits the erosions of rat’s lap and paw, simultaneously with diminution of HO\(^{-}\) radical production and reduction of conjugated dienes, partially reestablishing the levels of endogenous vitamin E and catalase levels and limiting the neutrophils infiltration.

**Hepatoprotective action**

Lipid peroxidation represents a process well defined by the degradation of animal and vegetal cells [25, 26, 27, 28]. The quantification of lipid peroxidation’ final products is one of the most used methods for identification of oxidative degradation, the secondary aldehydic products of the lipid peroxidation being in general accepted markers of oxidative stress. Lipidic peroxides are unstable indicators of cell oxidative stress because they are easy decomposed, forming other compounds much more complex and reactive such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural bioproducts of lipid peroxidation.

The enzymatic activity of catalase in oxidative degradation processes is very important through its capacity of catalyzing the decomposition of other toxic compounds, such as methyl- and ethyl hydroperoxides, acting as peroxidase and contributing to detoxification of living organisms [29].
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The results of the two studies regarding the intervention of the small sea fish’ extracts at hepatic level in normal conditions and during oxidative stress, have turned out the following aspects (table 2; fig. 1 and 2).

![First Study - Liver lipid peroxidation](image1)

![Second study - Liver lipid peroxidation](image2)

**Figure 1.** Lipid peroxidation- assay of MDA from hepatic homogenate
The enzymatic activity of catalase in rat liver is significantly stimulated, in a dose-dependent manner, by the administration of marine bioactive extracts; this maximum effect obtained with the lots treated with GAG 50 mg/kg b.w. (p<0.001 vs. control) and GAG 25 mg/kg b.w., is comparable with the one induced by arginine p<0.001 vs. control), both in normal and oxidative stress conditions; there is no observable potentiation of the mentioned effect in the case of the administration of some combinations of the two types of extracts.

The obtained data show that there is a direct correlation between the inhibition of lipid peroxidation and the process of catalase activity stimulation, which highlights the antioxidative properties at hepatic level of bioactive compounds obtained from small sea fish [4]. The histopathological aspect (fig.3) doesn’t show irreversible structural modifications, indicating that the maximum liver protection was obtained with GAG doses of 25 mg/kg b.w. In normal conditions (before the CCl₄ administration), the marine extracts induce a lowering of lipidic peroxidation, in a dose-dependent manner, being significant in the case of 50 mg GAG/ kg b.w. (p<0.001 vs. control), comparable with that induced by perfusible arginine.

The decrease of MDA content is observed also at the CCl₄ intoxicated animals, where GAG 50 mg/kg b.w. (p<0.001 vs. control) has a superior effect compared with arginine (p<0.05 vs. control).
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<table>
<thead>
<tr>
<th>Substance</th>
<th>Type of analysis</th>
<th>Liver homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipid peroxidation</td>
<td>Catalase</td>
</tr>
<tr>
<td></td>
<td>fără CCl₄</td>
<td>cu CCl₄</td>
</tr>
<tr>
<td>Control</td>
<td>0.7825±0.047</td>
<td>1.65±0.11</td>
</tr>
<tr>
<td>Alflutop</td>
<td>0.5460±0.097</td>
<td>1.27±0.29</td>
</tr>
<tr>
<td>GAG dm</td>
<td>0.5600±0.060</td>
<td>1.03±0.18</td>
</tr>
<tr>
<td>GAG DM</td>
<td>0.2745±0.015</td>
<td>0.505±0.03</td>
</tr>
<tr>
<td>CBPMM dm</td>
<td>0.6443±0.037</td>
<td>0.98±0.04</td>
</tr>
<tr>
<td>CBPMM DM</td>
<td>0.6300±0.030</td>
<td>0.35±0.02</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.1045±0.005</td>
<td>0.91±0.02</td>
</tr>
<tr>
<td>CBPMM dm +GAG dm</td>
<td>0.4825±0.010</td>
<td>1.75±0.31</td>
</tr>
<tr>
<td>CBPMM DM +GAG DM</td>
<td>0.5470±0.0415</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1.65±0.11</td>
</tr>
<tr>
<td>MS-CBPMM DM</td>
<td>-</td>
<td>0.7000±0.05</td>
</tr>
<tr>
<td>MS-CBPMM DM +GAG DM</td>
<td>-</td>
<td>2.597±0.74</td>
</tr>
</tbody>
</table>

*** - p<0.001; ** - p<0.01; * - p<0.05;

- vs control, b – vs Alflutop, c – vs MS-CBPMM dm+GAG DM, d – GAG dm, e – GAG dm

Figure 3. Histopathological aspect of rat liver subsequent to CCl₄ intoxication and treatment with bioactive extracts

The hepatic injury induced by CCl₄ administration has as the first step its metabolization to free radical trichloromethyl in endoplasmic reticule by the oxidases system with mixed function [30, 31]. It is considered that the secondary mechanisms which produce the disturbance propagation in hepatocyte functionality imply toxic product generation.
through CCl₄ metabolization [32, 33], or through peroxidiazic degeneration of membranar lipids [34, 35, 36, 37].

For example, it was established that the oxidative degradation of lipids, which represents only 2% from crystalline mass has an important role in cataract appearance. Thus, it was demonstrated that the crystalline opacification appears in the same time with the accumulation of lipid peroxidation products, such as malondialdehyde. Thus, apart from being harmful for living organisms, the lipid peroxides provoke cell alterations through protein structure modifications as result of malondialdehyde (or other similar products) action [38]. It was established that the reaction between MDA and proteins’ primary amino groups form Schiff base compounds which lead to inter- and intramolecular cross links with the effect of enzymes’ polimerization and inactivation. The malondialdehyde’ reactivity against the amino groups also can lead to DNA and RNA synthesis inhibition [39]. It is known that chondroitin sulphate determines an increase of RNA synthesis in chondrocytes [40] which appears to be related with the proteoglicans synthesis’ increase [41, 42]. These observations confirm the hypothesis that chondroitin sulphate protects hepatic tissue through its inhibitory activity of lipid peroxidation.

The therapeutic potential of rich extracts in glycosaminoglycans (with high chondroitin sulphate content) obtained from small sea fish with inland origin is sustained by a series of clinical studies which had as objective the investigation of treatment’ effect of different origins’ chondroitin sulphate based products against degenerative osteoarticular processes [43].

The double-blind clinical studies’ results, done on 300 pacients with knee’ osteoarthritis treated with 800 mg/ day doses of chondroitin sulphate, during two years , suggest that such a treatment can stop the structural evolution typical for this illness. Even if the precise mechanism of this substance’ action is not fully elucidated, the long term effects are attributed to chondroitin sulphate’s intervention on cartilage metabolism. These effects in part can be the results of competitive inhibition done by degradative enzymes such as hyaluronidase as well as of leukocyte elastase inhibition [40].

There is a series of data which shows that drugs with slow symptomatic action represent valuable therapeutical instruments for osteoarthritis. Studies made by Palmieri et al. (1990) and Conte et al. (1995), demonstrate that chondroitin sulphate is absorbed after oral administration and induce positive modifications of clinical aspect specific to osteoarthritis, acting as a drug with slow symptomatic action [44]. The pharmacological properties and the importance of chondroitin sulphate based products are confirmed also by the results obtained by the Morreale et al. (1996). They show that diclofenac, a well known nonsteroidal antiinflamatory drug used in the joint pathology, presented a quick analgesic/ antiinflamator response during treatment period; however, after treatment interruption the clinical aspect presented a regressive progression towards initial status, sustaining the fact that nonsteroidal antiinflamatory drugs are not capable to modify the natural course of this illness. On the other hand, the chondroitin sulphate administration was associated with a relatively reduced variation of symptoms (evident modifications where observed after the 30th day of treatment), presenting in time a global efficiency comparable with diclofenac; more of that, the therapeutical effects are long lasting, even after treatment interruption. The illness’ symptoms tend to reappear only at the end of the 6th month of observation.

There are several hypotheses for explai ning the glycosaminoglycans’antioxidative mechanisms. For example, Karlsson et al. (1988) suggested that the complex formed by glycosaminoglycans with extracellular superoxide-dismutase could protect mammalian cells against free radicals. However the most plausible explanation appears to be the one of Dundstone in 1960 namely that, because of carboxyl groups presented in glucuronic acid and
being always in the same positions, the chondroitin sulphate is capable to chelate metallic ions such as Cu$^{2+}$ and Fe$^{2+}$ which are responsible for the initiation of Fenton and Haber-Weiss reactions [45]. The ability of these polysaccharides to chelate different transitional metallic ions was reported by other authors too [46] and seems to be related by the antioxidative effect because the caption of these metallic ions will certainly reduce their disponibility for oxidative processes.

The molecular characteristics of chondroitin sulphate and its regulatory functions at cell metabolism level through growth factors, hormones and other extracellular matrix molecules’ binding suggests that the antioxidative mechanism of action could be expressed through in principal by the chelating activity and also against antioxidants biosynthesis [24].

The clinical studies done for the purpose of proving the therapeutic potential of glycosaminoglycans demonstrated the total lack of secondary effects or of the overdoses risk, sustaining the long term administration security of these products. The Committee of European League against Rheumatism (EULAR) granted to chondroitin sulphate a level of toxicity of 6 on a scale from 0 to 100, confirming that is one of the safest drugs for osteoarthritis [47]. More of that, the security of its administration is sustained by the absence of interactions with other drugs and by the lack of other sure alternatives for the patients with multiple treatment for osteoarthritis, diabetes, hypertension, dislipidemy, etc. [48].

Conclusions

• The results show that the GAG-induced anti-inflammatory activity (81.4% in 24 hours) is similar to the diclofenac-induced one (83.7% in 24 hours).

• The bioactive complex rich in glycosaminoglycans induces a decrease in lipid peroxidation and a stimulation of the liver catalase activity in a dose-effect manner. This data demonstrates that there is a direct correlation between inhibition of lipid peroxidation processes and stimulation of the catalase activity and proves the hepatic antioxidant properties of the bioactive extract.

• Through in vivo studies on Wistar rats with carrageenan-induced paw edema and CCl$_4$-induced liver toxicosis no irreversible histopathological alterations have been revealed, the results obtained demonstrating that the maximum protection was due to a 25 mg/kg b.w. GAG dose.

• Taking into account the presented results, the chemical composition (glycosaminoglycans, essential amino acids, essential fatty acids and micro-elements: Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si) of this bioactive complex and its therapeutic effects highlighted by these experiments and others described in previous works (a significant inhibition action of hyaluronidase, collagenase and elastase, the enzymes implicated in the pathology of conjunctive, cartilaginous and bone tissue, an increasing activity of the in vitro formation of collagen fibrils and a strong antioxidative activity), it may be regarded as a very good source of medicinal products with valuable therapeutic properties and minimal side effects. Thus, the bioactive extract rich in glycosaminoglycans obtained from small sea fish is very useful to prevent the disturbances of the macromolecular structure and to keep the functionality of the extracellular matrix from conjunctive, cartilaginous and bone tissue with simultaneously antioxidative and liver protection activity.

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