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In vitro and *in vivo* anti-coagulant activity and toxicological studies of marine sulfated glycosaminoglycans

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ABSTRACT

The present study aimed to characterize and evaluate the *in vitro* and *in vivo* anticoagulant activity of sulfated glycosaminoglycans from the skins of smooth hound (SHSG) and grey triggerfish (GTSG). The analysis of SHSG and GTSG with acetate cellulose electrophoresis in Zn-acetate revealed the presence of hyaluronic acid (HA), chondroitin sulfate (CS) and dermatan sulfate (DS). Both glycosaminoglycans were evaluated for their *in vitro* anticoagulant activities using activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombine time (PT) tests. SHSG and GTSG and calciparin were tested as *in vivo* anticoagulants by subcutaneous (s.c) injection to adult female Wistar rats in a concentration of 75 mg/kg of body weight. The administration of SHSG, GTSG and calciparin to rats induced a significant decrease of platelet rates compared to the control. The aPTT assay of SHSG and GTSG was prolonged 1.3 and 1.23-fold respectively compared with the control. Toxicity studies were performed to investigate whether or not SHSG and GTSG can cause pathological changes in the liver, proteins and DNA. The concentration and catalytic activity of liver oxidative stress markers and enzymes, respectively, as well as the observed hepatic morphological changes indicated that calciparin induced hepatic toxicity and oxidative damage in the liver. The higher activity and lower toxicity of SHSG and GTSG recommended these compounds as a better drug candidate than calciparin.

1. Introduction

The physiological process involving blood coagulation and its interruption is the second steps of haemostasis. The imbalance in this process is the origin of many ischemic and hemorrhagic diseases.

Heparin is far used as an anticoagulant and antithrombotic drug, mainly by binding to antithrombin (AT) and heparin cofactor II (HCII), accelerating the rate at which these protease inhibitors form complexes with enzymes implied in blood clotting, particularly – factor Xa and thrombin, inactivate them (Casu, 1985; Bourin et al., 1993; Mourão and Pereira, 1999). Recently, the low molecular weight heparin became an alternative for prophylaxis of venous thromboembolism because of its longer half-life in blood circulation system, higher bioavailability and lower variability in coagulation response. The big advantage is the possibility of administration this drug subcutaneously at fixed doses without need for diagnostic laboratory monitoring (Pawlaczyk et al., 2011). Although, the clinical use of heparin has limitations since its anticoagulant effect is unpredictable, resulting in risk of bleeding (Warkentin, 2006). Heparin suffers other disadvantages including the problem of heparin-induced thrombocytopenia, poor bioavailability, and risk of animal diseases which can possibly affect human health such as Mad Cow disease.

So there is still a clinical need for new parental anticoagulants that are effective and safe when used in conjunction with either fibrinolytic therapy or antiplatelet agents in patients with cardiovascular diseases (Hirsh, 2001). Therefore, the replacement of heparin by natural or chemical sulfated polysaccharides may have benefits due to health (Mourão and Pereira, 1999; Maas et al.,

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2012; Araújo et al., 2013). Both anticoagulant and antithrombotic effects are related to the presence of sulfate groups, and their distribution along the polysaccharide chain.

Glycosaminoglycans (GAGs) are highly sulfated, complex, linear and negatively charged polysaccharides, which present in all animal tissues (Heinegard and Sommarin, 1987; Tovar et al., 2005). Their structure consists of a repeated disaccharide unit containing hexosamine residues and uronic acid. GAGs can be sulfated (chondroitin-sulfate, dermatan-sulfate, heparin/heparan-sulfate, keratan-sulfate) or not (hyaluronic acid).

Recently, many studies revealed the presence of polysaccharides with novel structures and anticoagulant activities. It is well known that sulfated GAGs exhibit anticoagulant effect through interactions with the serine proteinases of the coagulation system and their physiological inhibitors antithrombin (AT) and heparin cofactor II (HCII) (Barbbucci et al., 1996; Athukorala et al., 2007). In this study we evaluated the *in vitro* and *in vivo* anticoagulant activity of sulfated glycosaminoglycans obtained from grey triggerfish (GTSG) and smooth hound (SHSG) skins.

2. Materials and methods

2.1. Reagents

The chemicals and solvents used in the present study were purchased at the analytical grade or highest level of purity available. Alcalase® 2.4 l serine-protease from *Bacillus licheniformis* was obtained from Novozymes® (Bagsvaerd, Denmark). Calciparin (12500 UI/0.5 ml) was purchased from sanofi-aventis, France. Chondroitin sulfate from bovine trachea and hyaluronic acid were from Fluka, Germany. Chondroitin sulfate B sodium salt (dermatan sulfate) was purchased from Sigma, USA.

2.2. Enzymatic extraction of sulfated glycosaminoglycans

Glycosaminoglycans were extracted according to a slightly modified version of the method described by Ben Mansour et al. (2009). In brief, the fish skins were cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). An amount of 5 g of sample was dissolved in 250 ml sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH6. Alcalase® was added, and the mixture was kept for 24 h at 50 °C. The mixture was then left to cool down at room temperature and then filtered. The residue was washed with distilled water and filtered again. The filtrates were mixed, and polysaccharides were precipitated with cetylpyridinium chloride 10% (w/v). The mixture was kept for 24 h at room temperature and centrifuged for 30 min at 5869 g and 4 °C. The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and then dissolved in 200 ml NaCl solution (2 M) in ethanol (100:15, v/v). An amount of 700 ml ethanol was added. The polysaccharide containing solution was left for 24 h at 4 °C and then centrifuged for 30 min at 5869g and 4 °C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. After that, the pellet was redissolved in desionised water and lyophilized in a freeze dryer (CHRIST, ALPHA 1–2 LD plus, Germany).

2.3. Cellulose acetate electrophoresis

The cellulose acetate electrophoresis was performed as following. 2 μl of the GAG solution standard containing Dermatan Sulfate (DS), Chondroitin Sulfate (CS) and Hyaluronic Acid (HA) and 6 μl of the sulfated glycosaminoglycans from fish skins were placed at the origin (10 mm from the cathode side) of a cellulose acetate strips (Sartorius). Electrophoresis was carried out in Zn-acetate 0.1 M pH 6 buffer and run at 200 V at room temperature, for 1 h. After electrophoresis, the cellulose acetate strip was stained by alcian blue (Wegrowski and Maquart, 2001).

2.4. In vitro *anticoagulant activity of sulfated glycosaminoglycans*

The activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombine time (PT) assays were performed using a semi-automatic line STA (Diagnostica Stago). The sample was dissolved in physiological serum. All analyses were performed in triplicate and mean values were taken.

2.4.1. APTT assay

For the aPTT assay, 45 μl of normal citrated platelet poor plasma (PPP) were mixed with 5 μl of glycosaminoglycans at various concentrations and incubated for 3 min at 37 °C. Then, 50 μl of aPTT reagent (CK-PREST) were added and the mixture was incubated for 3 min at 37 °C. The clotting time was immediately saved after the addition of 100 μl of 25 mM $CaCl_2$. The clotting time is expressed in seconds and as a ratio, with the average value of a normal subject less than 1.2. The enzyme activity control (C) is measured by replacing the 5 μl of sulfated polysaccharides with physiological serum.

2.4.2. PT assay

For the prothrombin time assay, 5 μl of glycosaminoglycans at various concentrations were incubated with 45 μl of PPP for 3 min at 37 °C. The clotting time was determined after the addition of 100 μl of Neoplastine® CI (DIAGNOSTICA-STAGO). The prothrombin time value is expressed in seconds.

2.4.3. TT assay

For the TT assay, volumes of 10 μl of glycosaminoglycans from fish skins at different concentrations were incubated with 90 μl of PPP for 3 min at 37 °C. The clotting time was recorded after the addition of 100 μl of thrombin (80 NIH). The Thrombin time value is expressed in seconds.

2.5. In vivo *anticoagulant activity of sulfated glycosaminoglycans*

2.5.1. Animals

The investigation was conducted in accordance with the International Principles for Laboratory Animal Use and Care as found in the guidelines (Council of European Communities, 1986), and in accordance with the guidelines of the Animal Care Committee at the Faculty of Medecine, Sfax University (Tunisia). Experiments were performed on 3 month-old adult female Wistar rats, weighing approximately 180 g. Twenty-four female adult Wistar rats were purchased from the Central Pharmacy (Tunisia) and were maintained under conditions of controlled temperature (22 °C ±1), relative humidity of 40% and a daily 12 h photoperiod.

2.5.2. Experimental procedure

Glycosaminoglycans were dissolved in physiological serum and injected subcutaneous (s.c) using an administration volume of 1 ml/kg. The animals were allocated randomly to four groups of six animals each.

- [Control group], group A: rats received a subcutaneous dose of physiological serum and served as negative controls.
- [Calciparin treated group], group B: rats received a subcutaneous dose of calciparin (0.9 mg/kg of body weight) and served as positive controls.
- [GTSG treated group], group C: rats received a subcutaneous dose of GTSG (75 mg/kg of body weight).
- [SHSG treated group], group D: rats received a subcutaneous dose of SHSG (75 mg/ kg of body weight).