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Short Communication

Antiproliferative heparin (glycosaminoglycans) isolated from giant clam (*Tridacna maxima*) and green mussel (*Perna viridis*)

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Heparin was isolated from two bivalve mollusks, *Tridacna maxima* (giant clam) and *Perna viridis* (green mussel). The isolated heparin was quantified in crude as well as purified samples and they were estimated as 2.72 and 2.2 g/kg (in crude) and 260 and 248 mg/g (in purified samples) in *T. maxima* and *P. viridis*, respectively. The antiproliferative activity of both the samples performed with pulmonary artery smooth muscle cells (PASMC) indicate a dose dependent manner. Among these two clams, *P. viridis* heparin showed higher antiproliferative activity than that of *T. maxima.*

Key words: heparin, antiproliferative activity, giant clam, green mussel, glycosaminoglycan.

INTRODUCTION

Glycosaminoglycans (GAGs) have been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates. Invertebrates were first shown to contain a heparin or heparan sulfate (Burson et al., 1956). An exhaustive assessment showed that the mollusks are particularly rich source of these sulfated polysaccharides (Nader and Dietrich, 1989) and it often corresponds up to 90% of the total GAG content of these organisms. Heparin and heaprin-like substances have a wide range of important biological activities including inhibition of pulmonary artery smooth muscle cell (PASMC) proliferation. In the normal physiological state, the smooth muscle cells (SMCs) are entering into quiescent growth state in pulmonary arterial walls which is regulated by a balance between inhibitory and mitogenic factors. Garg et al. (2000) have developed a method for the recovery of heparin and heparin-like substances from various marine organisms like. In the present study, the methodology of Holick et al. (1985) used for flounder, crab, mussel and clams, was followed with suitable modifications for the two bivalve mollusks, *Tridacna maxima* and *Perna viridis*, which gave good yield of heparin and heparin-like glycosaminoglycans with less impurities (Arumugam and Shanmugam, 2004).

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MATERIALS AND METHODS

Isolation

In the present study, the standard procedure of Holick et al. (1985) was followed, with suitable modification for the defating and deproteinisation, to extract heparin and heparin-like glycosaminoglycans from *T. maxima* and *P. viridis*. The purification of the crude heparin and heparin-like glycosaminoglycans was done by using the anionic resin [Amberlite IRA-900 (Cl^-)] (Nishino et al., 1989). The purified glycosaminoglycans were converted into heparin sodium salts by using cationic resin (Amberlite IR-120 Na^+) (Volpi, 1994) and the recovered precipitate was taken for further analyses.

Antiproliferative activity

The isolated bovine pulmonary artery smooth muscle cells were seeded at 1.5×10^4 cells/well into a 6-well tissue culture plate, grown for 2 days. Growth was arrested at the end of 48 h by reducing the serum concentration of the medium from 10 to 0.1%. The media was then changed to the experimental samples which contained either standard media (RPMI – 1640 with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo), growth arrest media (RPMI with 0.1% FBS) or standard media with oligomers/heparin (5 µg/ml). All media contained streptomycin (100 µg/ml), penicillin (100 U/ml) and amphotericin B (1.25 µg/ml). After 4 to 5 days of growth, the cells were lifted with trypsin/EDTA and then counted using a coulter counter. Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the STATE VIEW software package (Brainpower, In., to Calabases, CA.) for Macinotosh computers.

Table 1. The yield of crude and purified heparin complex (heparin and heparin-like glycosaminoglycans) from *T. maxima* and *P. viridis*.

Source	Net yield	
	Crude (g/kg)	Purified (mg/g)
Tridacna maxima	2.72	260
Perna viridis	2.2	248

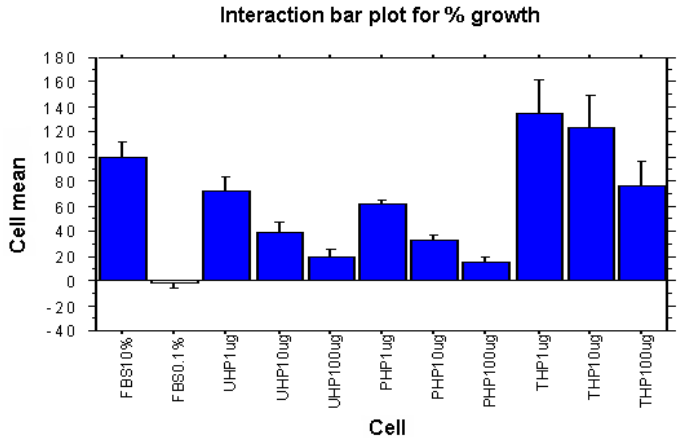

Figure 1. Showing the antiproliferative effect of heparin and heparin-like glycosaminoglycans from *P. viridis*, *T. maxima* and Upjohn heparins. FBS = 10% fetal bovine serum, UHP = Upjohn heparin, PHB = *P. Viridis* heparin, and THP = *T. maxima* heparin.

RESULTS

Isolation

The amount of heparin and heparin-like glycosaminoglycans (heparin complex) (crude) was estimated as 2.72 g/kg of dry tissue in *T. maxima* and 2.2 g/kg in *P. viridis*. After purification by using the amberlite anion exchange resin, the yield was found as 260 and 248 mg/g in *T. maxima* and *P. Viridis*, respectively (Table 1).

Antiproliferative activity

The effect of heparin and heparin-like glycosaminoglycans (GAGs) isolated from *P. Viridis*, *T. maxima* and Upjohn heparin grown in RPMI medium containing 0.1 and 10% FBS is depicted in Figure 1. The heparin and heparin-like GAGs isolated from *P. viridis* recorded increasing inhibition over the growing cells when the concentration increased from 1.0 to 100 µg. The percentage cell mean in the case of 1.0 µg was found as 61.764 ± 3.660, in 10 µg it was 33.064 ± 3.507 and 100 µg concentration it was 15.071 ± 4.609 showing an increasing influence over the cell growth. The inhibition of *P. viridis* heparin is comparatively more even than that of Upjohn heparin, the standard used in the present study, which showed the percentage mean cell growth as follows. On the contrary, the heparin and heparin-like glycosaminoglycans extracted from *T. maxima* was found to promote cell growth in lower concentrations of 1 µg (134.939 ± 26.468%) and 10 µg (123.934 ± 25.325%). But at higher concentrations, it was also found to reduce the growth (at 100 µg concentration the mean cell growth was only 77.429 ± 18.923%).

DISCUSSION

Heparin is a large linear molecule with a molecular weight of approximately 6,000 – 20,000 Daltons and is highly negatively charged due to many carboxylate and sulfate groups (Barlow, 1964; Jaques, 1967; Ehrlich and Stivala, 1973; Lasker, 1977; Wesler and Gatel, 1979). Different methodologies are available for the isolation of heparin / glycosaminoglycans/sulphated mucopolysaccharides. But in most of the methodologies the isolation involves proteolytic enzymes (Kim et al., 1996; Hovingh and Linker, 1998). But, the methodology described by (Holick et al., 1985) is relatively faster, simple and also does not require the expense of large quantities of proteolytic enzymes.

In the present investigation, the yield of crude heparin complex (heparin and heparin-like glycosaminoglycans) was 2.72 and 2.2 g/kg in *T. maxima* and *P. Viridis*, respectively. Whereas Dietrich et al. (1989) quantified the heparin yield as 2.2 to 2.8 g/kg, 1.8 to 2.5 g/kg and 2.7 to 3.8 g/kg in *Aplysia brasiliana*, *Dermatobranchus striatus* and *Tivela mactroides,* respectively. But Straus et al. (1981) recorded the yield of heparin and other sulphated mucopolysaccharides from thymus as 274 µg/kg. Likewise Cassaro and Dietrich (1977) isolated the sulfated mucopolysaccharides by using quaternary ammonium salts and reported the yield as 170.0, 174.0, 843.0, 307.0 and 1,090.0 µg/kg dry tissue in different molluscan species of *Aulocombia ater*, *Perna perna*, *Mesodesma donacium*, *Loligo brasiliense* and *Octopus* sp., respectively.

Antiproliferative activity of isolated heparin from *P. viridis* could also be compared with that of Dahlberg et al. (1996) in which they reported such inhibition over the cell growth in Elkins-Sinn heparin on PASM cells. But at the same time, they also observed a stimulatory effect on PASM cell growth (1.4% increase) at 1.0 µg/ml concentration of Choay heparin which also has less antiproliferative activity of 29 ± 5% at 10 µg/ml concentration like the heparin and heparin-like GASs extracted from *T. maxima*. Furthermore, it is interesting to note that the same commercial standard Upjohn heparin showed varying antiproliferative activity on the PASM cells in a medium containing 0.1% FBS. In the present study, it produced 27.36 and 61.19% of inhibition on the PASM cells grown in RPMI medium containing 0.1 and 10% FBS; whereas it showed an antiproliferative activity of 48 ± 5 and 80 ± 4 at the concentration of 1 or 10 µg/ml (Dahlberg et al., 1996).

Thus in the present study, the results reveal that there is a dose dependant decrease in the percentage of viable

cells when treated with the heparin and heparin-like GAGs isolated from *T. Maxima*, *P. viridis* and standard heparin. There was a significant reduction in the number of live cells in the case of all the concentrations of heaprin and heparin-like GAGs from *P. viridis* in an ascending order with increasing concentration. But, at the same time, the isolated compound from *T. maxima* did not show any inhibitory effect in 1 and 10 µg concentration, but it showed some inhibitory effect in 100 µg concentration. From the above results, it is quite evident that the heparin and heparin-like GAGs isolated from *P. viridis* has more antiproliferative effect than even the commercial standard Upjohn heparin and that of *T. maxima*.

Apart from the above, the previous investigations have shown variable effects of heparin on cell growth. Guyton et al. (1980) and Zaragosa et al. (1990) demonstrated the inhibition over the aortic smooth muscle cell growth. Castellot et al. (1986), using rat aortic smooth muscle cells, reported varying antiproliferative activity of 10 different commercial heparins. Among them, two of the heparins stimulated the cell growth at low concentration as the heparin and heparin-like GAGs isolated from *T. maxima*. Therefore, both the cell type to be studied and the source of heparin are important in determining the antiproliferative activity of heparins. Furthermore, for as yet uncertain chemical reasons, not all heparins have the antiproliferative activity to the same degree (Dahlberg et al., 1996). Therefore the foregoing account may shed light on the previous paradoxes in which different commercial heparin and heparin-like GAGs isolated from different sources have had variable antiproliferative effects even on the same cell types.

Further, the inhibiting effect of heparin on smooth muscle cell growth *in vitro* has been shown to be independent of its anticoagulant activity (Castellot et al., 1987; Greyfin et al., 1995; Tiozzo et al., 1993). This concept is very well supported by the results of the present study also. Though the purified heparin and heparin-like GAGs extracted from *T. maxima* reported more anticoagulant activity (30,212I U/kg) than that of *P. viridis* (16,144I U/kg), the heparin and heparin-like GAGs from *P. viridis* showed more antiproliferative activity than that of *T. maxima* which showed stimulatory effect in lower concentrations (1 and 10 µg). This suggests that the anticoagulant activity does not correlate with the ability of a given heparin and heparin-like GAGs to inhibit cell growth. It can be concluded that the heparin and heparin-like GAGs isolated from *P. viridis* and *T. maxima* possess an anti-cell proliferative component which needs to be further studied.

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