Heparan sulphate: a putative decondensing agent for human spermatozoa in vivo

Marina Romanato1, Mónica S.Cameo1, Gabriel Bertolesi3, Consuelo Baldini1, Juan Carlos Calvo2,3 and Lucrecia Calvo1,4

1Biología de la Reproducción, 2Instituto de Biología y Medicina Experimental and 3Department of Biological Chemistry, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
4To whom correspondence should be addressed. E-mail: lucrecalvo@fibertel.com.ar

BACKGROUND: Human sperm decondense in vitro upon exposure to heparin and glutathione. The aim of this study was to evaluate whether this decondensing ability of heparin in vitro is related to structural characteristics of the molecule and to test the in-vitro decondensing ability of other glycosaminoglycans. METHODS: Capacitated sperm obtained from normospermic semen samples were decondensed in the presence of heparin (or its equivalent) and glutathione. After fixation with glutaraldehyde, the percentage of decondensed sperm was determined under phase contrast. RESULTS: The decondensing ability of heparin was related to sulphation characteristics of the molecule: heparin, O-desulphated heparin and N-desulphated-N-acetylated heparin had similar decondensing abilities; N-desulphated was less active and O/N-desulphated-N-acetylated heparin was completely inactive. On the other hand, the decondensing ability of heparin was not affected by molecular weight, within the range 3000–18 000 kDa. When the decondensing ability of different glycosaminoglycans was tested, heparin and heparin sulphate were equally active, while chondroitin sulphate and hyaluronic acid were completely inactive and dermatan sulphate was slightly active. CONCLUSIONS: Our results indicate that heparin’s decondensing ability in vitro is related to sulphation characteristics of the molecule and suggest that heparan sulphate, a structural analogue of heparin, could be a sperm-decondensing agent in vivo.

Key words: fertilization/gamete biology/sperm

Introduction

Upon entering the ooplasm, the fertilizing spermatozoon must undergo a series of nuclear changes which eventually lead to the formation of the male pronucleus. Sperm nuclear decondensation, which consists of the replacement of sperm protamines by oocyte histones, is the first of these changes and is necessary to render the sperm nucleus transcriptionally active (Yanagimachi, 1994).

In mammalian species, including humans, sperm nuclear decondensation in vivo seems to involve two distinct stages: (i) reduction of disulphide bonds in protamines; and (ii) replacement of reduced protamines by histones (Zirkin et al., 1989). The role of reduced glutathione (GSH) present in the oocyte as a disulphide bond reducer has been clearly established (Perreault et al., 1988, 1994), but the nature of the protamine acceptor is still unknown. In fish, amphibians and Drosophila, nucleoplasmmin has been shown to be the protamine acceptor in vivo and thus responsible for sperm nuclear decondensation (Ohsumi and Katagiri, 1991; Philpott et al., 1991; Kawasaki et al., 1994). However, with the exception of indirect evidence of the possible involvement of nucleoplasmmin in mouse sperm decondensation (Maeda et al., 1998), these findings have not been extended to mammals.

Human sperm can be decondensed in vitro in the presence of physiological concentrations of heparin and GSH (Gaubeca-Klix et al., 1998; Reyes et al., 1989). The mechanism of action of heparin in this process is still a matter of controversy. The presence of heparin receptors on the sperm plasma membrane has been described by several groups (Delgado et al., 1982; Lasalle and Testart, 1992; Carell and Liu, 2002), and Delgado and co-workers have proposed that heparin binding to its receptors leads to the destabilization of the sperm plasma membrane, which in turn would allow the incorporation of other molecules, such as GSH, into the sperm nucleus. Alternatively, a direct effect of heparin on sperm chromatin has been suggested since heparin has a strong affinity for protamines and can combine with them to form a highly insoluble complex (Chargaff and Olson, 1938). Direct experimental evidence is lacking and why heparin is able to decondense human sperm in vitro is not clearly understood.

Heparin has also been proposed as a sperm-decondensing agent in vivo (Lalich et al., 1989; Montag et al., 1992).
However, this seems quite unlikely, since the only cell capable of synthesizing heparin in vivo is the mast cell, and no heparin has been found in the oocyte–cumulus complex. On the other hand, there is ample evidence in the literature related to the presence of other glycosaminoglycans (GAGs) in the cumulus–oocyte complex in different species, including human (Ball et al., 1982; Gebauer et al., 1978; Salustri et al., 1989). Among these, heparan sulphate (HS) seems to be the most likely candidate to be considered as a nuclear-decondensing agent in vivo, since it is a structural analogue of heparin and, in many biological systems, behaves in the same way (Delgado et al., 1982; Jackson et al., 1991).

The aim of this study was to evaluate whether the human sperm-decondensing ability of heparin in vitro is related to structural characteristics of the molecule and to test the hypothesis that HS could be a human sperm-decondensing agent in vivo.

Materials and methods

Semen specimens and sample processing
Normospermic (World Health Organization, 1999) semen specimens were obtained under informed consent from normal healthy volunteers. Specimens were used solely for research and not for clinical purposes. Donor data were kept confidential. Samples were collected by masturbation after 36–48 h of abstinence, allowed to liquefy and processed within 1 h of collection.

All chemicals and reagents used were obtained from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. Samples were washed twice by centrifugation at 300 g for 10 min in human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA) supplemented with 0.3% bovine serum albumin (BSA). Washed sperm were swam-up in HTF containing 2.6% BSA (HTF–26B) for 90 min at 37°C in an atmosphere of 5% CO2 in air. Specimen concentration after swim-up was adjusted to 3–5 × 10^6/ml and sperm were incubated in capacitating conditions in HTF–26B for 18 h at 37°C in 5% CO2/95% air.

Sperm nuclear decondensation assay
Capacitated sperm were decondensed in the presence of 10 mmol/l GSH and 46 μmol/l heparin (mol. wt 13 500 Da, 170 IU/mg) in HTF–26B at 37°C for 15, 30 and 60 min (Romanato et al., 2001). Controls consisted of parallel incubations with heparin or GSH alone. After each time period, a 20 μl aliquot was removed and fixed with an equal volume of 2.5% glutaraldehyde in phosphate-buffered saline (PBS).

Two 5 μl aliquots were transferred onto a microscope slide, a coverslip was placed on top and nuclear status was assessed under phase contrast in an Olympus CH2 microscope at 400×. Sperm were classified (Bedford et al., 1973) as unchanged (U), moderately decondensed (M) or grossly decondensed (G) (Figure 1). At least 200 sperm were evaluated in each aliquot. Total decondensation achieved, % (M + G), was determined as the sum of %M and %G.

Heparin dose–response curve
The optimum concentration of heparin was determined by incubating sperm in 10 mmol/l GSH and increasing concentrations of heparin: 0.46, 4.6, 9.2, 23, 46, 230 and 460 μmol/l. Total decondensation was determined as previously described after 15, 30 and 60 min of incubation.

Sulphation characteristics of heparin and decondensing ability
To evaluate the effect of sulphation characteristics of heparin on its nuclear-decondensing ability, capacitated sperm from the same semen sample were decondensed in the presence of 10 mmol/l GSH and 46 μmol/l heparin or each of the following chemically modified structures (Syntex S.A., Buenos Aires, Argentina): partially N-desulphated (N-des), partially O-desulphated (O-des), partially N-desulphated-N-acetylated (N-des-N-Ac) and partially O/N-desulphated-N-acetylated (ON-des-N-Ac).

Total decondensation in each sample was determined as usual, following 15, 30 and 60 min of incubation in decondensing conditions.

Heparin molecular weight and decondensing ability
To determine the effect of molecular size on heparin nuclear-decondensing ability, capacitated sperm from the same semen specimen were decondensed in the presence of 10 mmol/l GSH and 46 μmol/l of one of three different molecular weight heparins: 13.5, 18.0 and 3.0 kDa (Syntex S.A., Buenos Aires, Argentina). Total decondensation in each sample was determined as usual, following 15, 30 and 60 min of incubation in decondensing conditions.

Decondensing ability of different GAGs
Capacitated sperm from the same semen specimen were decondensed in the presence of 10 mmol/l GSH and 46 μmol/l heparin or each of the following GAGs: HS, chondroitin sulphate (CS), dermatan sulphate (DS) and hyaluronic acid (HA). Total decondensation in each sample was determined as usual, following 15, 30 and 60 min of incubation in decondensing conditions.

Statistical analysis
Statistical analysis was performed using Instat Mathpad.

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**Figure 1.** Nuclear decondensation status of human spermatozoa, as visualized under phase contrast. U = unchanged, M = moderately decondensed, G = grossly decondensed. Original magnification 400× (bar = 10 μm).
The effect of heparin concentration, heparin sulphation and heparin molecular weight on total decondensation was determined by repeated-measures ANOVA followed by Tukey–Kramer’s multiple comparisons test. The decondensing ability of different GAGs was evaluated by one-way ANOVA followed by Tukey–Kramer’s multiple comparisons test. Differences were considered statistically significant when \( P < 0.05 \).

Results

**Heparin dose–response curve**

In order to establish the optimum heparin concentration for human sperm decondensation in our experimental conditions, sperm were incubated with 10 mmol/l GSH and increasing concentrations of heparin. Figure 2 shows the corresponding dose–response curves after 15, 30 and 60 min of incubation. There was virtually no decondensation after 15 min of incubation at any of the doses tested. After 30 and 60 min, there was a dose–response effect (ANOVA, \( P < 0.05 \), \( n = 4 \)). Maximum decondensing ability was achieved at ~20 \( \mu \)mol/l heparin, and no significant differences were observed upon further increasing the heparin concentration (Tukey, NS). To ensure optimum decondensing conditions, 46 \( \mu \)mol/l heparin was used in all experiments hereafter.

**Sulphation characteristics of heparin and decondensing ability**

To analyse the relationship between structural characteristics of heparin and decondensing ability in vitro, four chemically modified heparins were tested as decondensing agents in the presence of GSH. The structure and net negative charge of the disaccharidic units of the five molecules tested is shown in Figure 3, where it can be clearly seen that \( O \)- or \( N \)- desulphation and \( N \)-acetylation alter both the net charge of the disaccharide and the localization of positively and negatively charged groups. Figure 4 depicts nuclear decondensation kinetics for heparin and its four analogues and clearly shows that heparin’s decondensing ability was strongly affected by the sulfation characteristics of the molecule. Heparin, O-des and N-des-N-Ac had similar decondensing abilities at each time point studied (ANOVA, NS, \( n = 6 \)). N-des, though less active after 30 min of incubation (ANOVA + Tukey, \( P < 0.05 \), \( n = 6 \)), induced a similar level of nuclear decondensation after 60 min. ON-des-N-Ac was inactive at all times.

**Heparin molecular weight and decondensing ability**

To address the question of whether the molecular weight of heparin might have an effect on its decondensing ability in vitro, sperm were incubated with control heparin (mol. wt 13.5 kDa) and two additional heparins of 3.0 and 18.0 kDa. Nuclear decondensation kinetics were not affected by heparin molecular size within the tested range (Figure 5). Total decondensation was similar for the three heparins tested at 15, 30 and 60 min of incubation (ANOVA, NS, \( n = 5 \)).

**Decondensing ability of different GAGs**

In search of a putative decondensing agent in vivo, the decondensing ability of different GAGs which can be found in the oocyte–cumulus complex was tested in vitro in the presence of GSH. Nuclear decondensation kinetics in the presence of heparin, HS, DS, CS and HA can be seen in Figure 6. At each
time point studied, the decondensing abilities of heparin and HS were similar (ANOVA + Tukey, NS, \( n = 6 \)). HA and CS were completely inactive (ANOVA + Tukey, \( P < 0.01 \) versus heparin, \( n = 6 \)) throughout the incubation. Though DS appeared to be slightly active after 60 min of incubation, its decondensing ability was not significantly different from that of CS and HA.

Discussion

Human sperm nuclear decondensation, which is already evident as early as 60 min after insemination, is necessary for the development of the male pronucleus (Tesarik and Kopecky, 1989).

This study presents evidence that the human sperm-decondensing ability of heparin in vitro is related to structural characteristics of the molecule rather than simply due to the fact that this molecule is a polyanion that can compete efficiently with DNA for positively charged protamines. Moreover, the results presented in this study suggest that HS, a structural analogue of heparin which can be found in the oocyte–cumulus complex (Bellin et al., 1986; Jackson et al., 1991; Eriksen et al., 1997), could be involved in in-vivo human sperm decondensation.

Upon optimizing assay conditions, it was surprising to see that maximum sperm decondensation achieved was ~25% despite the fact that most sperm readily decondense following ICSI. This fraction of decondensing sperm may well correspond to those cells whose plasma membrane suffered alterations during overnight capacitation. There was a significant correlation (Spearman, \( P = 0.005, n = 14 \)) between the percentage decondensed and the percentage of eosin Y-stained cells after overnight capacitation (data not shown). However, this does not affect the validity of the results presented herein since the aim of the present study was to understand the mechanism whereby heparin functions as a sperm-decondensing agent in vitro, with no implications on the use of the assay as a diagnostic tool.

There is ample evidence in the literature regarding the effect of structural modifications of the heparin molecule on its biological activity (Jackson et al., 1991; Bertolesi, 1998). As previously stated in the Results, heparin desulphation affects both the net charge of the molecule’s disaccharidic unit and the localization of positively and negatively charged groups. Consequently, electrostatic interactions between charged groups in the molecule will be affected. This, in turn, will induce conformational changes in the molecule which may or may not be reflected on its biological activity. In our study, ON-des-N-Ac, which possesses the lowest negative charge, showed no decondensing ability, and might lead us to believe that decondensing ability is simply related to net negative charge. However, heparin, N-des-N-Ac and O-des behaved similarly despite the fact that their net negative charges are different. On the other hand, O-des and N-des have the same
net charge but did not show the same decondensing ability. Thus, the position of negatively charged groups on the disaccharidic structure appears to be more important than net negative charge per se.

Interestingly, N-des was less active than O-des, in agreement with the general idea that N-sulphations are important for the biological activity of heparin (Lindahl and Kjellén, 1991; Bertolesi, 1998). Furthermore, Bertolesi (1998), studying a series of biological activities of the same chemically modified heparins, also found that ON-des-N-Ac was completely inactive and that the activity of N-des-N-ac was similar to that of heparin.

The biological activity of GAGs has been shown to be influenced by molecular size. Fedarko and Conrad (1986) have proposed that this might be due to the fact that certain specific fragments of the molecule could be incorporated into the cell and eventually reach the nucleus. In this study, we tested the decondensing ability of two additional heparins, with lower and higher molecular weight than control heparin, and observed no significant differences between them, suggesting that there is no effect of molecular size (within the range 3000–18 000 Da) on this particular biological activity. These results are in agreement with those of Delgado et al. (1988) who tested heparin fragments obtained by treatment of heparin with heparanases and found that heparin decasaccharides (mol. wt ~3000 Da) were as active as heparin in inducing nuclear swelling. However, smaller molecular weight fragments were progressively less active, with tetrasaccharides being the smallest fragments showing any activity.

Having concluded that the decondensing ability of heparin in vitro is related to structural characteristics of the molecule, we decided to test the decondensing ability of other GAGs known to be present in the cumulus–oocyte complex in an attempt to find heparin’s equivalent in vivo. It is important to keep in mind that heparin is not present in the cumulus–oocyte complex and is, thus, not likely to play this role as previously suggested. On the other hand, HA, CS, HS, DS and keratan sulphate have been found in the female genital tract, particularly in follicular fluid, of various species (Yanagishita et al., 1979; Grimel et al., 1988) including human (Bellin et al., 1986).

Among the different GAGs tested in this study, only HS, a structural analogue of heparin, possessed sperm nuclear-decondensing ability in vitro, while HA, CS and DS were inactive. Moreover, the decondensing abilities of HS and heparin were similar, despite the fact that the former is significantly less sulphated than the latter, and thus possesses a smaller net negative charge. These results once again support the contention that heparin’s decondensing ability in vitro is related to structural characteristics of the molecule, rather than merely a consequence of its elevated negative charge.

The similarity between the biological activities of heparin and HS in vitro has already been observed in a variety of biological systems, where it has been demonstrated that HS is the active agent in vitro (Yanagishita and Hascall, 1992). Therefore, it is tempting to speculate that HS could be responsible, together with GSH, for human sperm nuclear decondensation in vivo. There is no evidence as yet indicating that GAGs are present inside the oocyte, an issue which the authors plan to address in the near future. In the meantime, the involvement of HS in sperm decondensation in vivo remains a plausible hypothesis.

Preliminary data from our laboratory show that some human follicular fluids obtained from women undergoing IVF possess sperm nuclear-decondensing ability in vitro when GSH is added to the incubation medium. Whether this activity is due to the presence of HS in these fluids remains yet to be proven and is the subject of current research in our laboratory.

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