Human Reproduction Vol.20, No.10 pp. 2784–2789, 2005 doi:10.1093/humrep/dei124
Advance Access publication June 24, 2005.

Further evidence on the role of heparan sulfate as protamine acceptor during the decondensation of human spermatozoa

Marina Romanato[1,2], Eleonora Regueira[2], Mónica S.Cameo[1], Consuelo Baldini[1], Lucrecia Calvo[1,2] and Juan Carlos Calvo[2,3,4]

[1]Biología de la Reproducción, [2]Instituto de Biología y Medicina Experimental and [3]Department of Biological Chemistry, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[4]To whom correspondence should be addressed at: IBYME, Vuelta de Obligado 2490, (1428) Buenos Aires, Argentina. E-mail: jcalvo@dna.uba.ar

BACKGROUND: Human spermatozoa decondense *in vitro* upon exposure to heparin and glutathione. Glutathione is also the disulfide bond reducer *in vivo*, and heparan sulfate, a functional analogue of heparin, has been proposed as the protamine acceptor. The aim of this study was to evaluate the decondensing ability of chemically modified heparins and different glycosaminoglycans (GAGs) on isolated sperm nuclei *in vitro*, and to analyse the possible role of different GAGs as protamine acceptors. METHODS: Capacitated spermatozoa and isolated sperm nuclei from normospermic semen samples were decondensed in the presence of heparin (or its equivalent) and glutathione. After fixation with glutaraldehyde, the percentage of decondensed spermatozoa and nuclei was determined under phase-contrast. Proteins were extracted from sperm nuclei previously incubated in the presence of gluhathione and different GAGs by incubation with urea–β-meracptoethanol–NaCl, and analysed by acid polyacrylamide gel electrophoresis. RESULTS: The ability of desulfated heparins and other GAGs to decondense isolated nuclei mirrored exactly the decondensation of capacitated spermatozoa, the only difference being the level of maximum decondensation achieved. Heparan sulfate and heparin, but not other GAGs, were able to release protamines from sperm chromatin. CONCLUSIONS: Heparan sulfate could be functioning as protamine acceptor *in vivo* during human sperm nuclear decondensation.

Key words: heparan sulfate/protamine/sperm nuclear decondensation

Introduction

Sperm decondensation is the first visible change undergone by a spermatozoon upon entry into the ooplasm at fertilization, and is a prerequisite for male pronucleus formation and syngamy (Berrios and Bedford, 1979).

Human spermatozoa decondense *in vitro* in the presence of heparin and glutathione (GSH) (Reyes *et al*., 1989; Gaubeca-Klix *et al*., 1998). Previous results from our laboratory indicate that heparin decondensing ability *in vitro* is related to sulfation of the molecule and thus does not seem to be merely a consequence of its negative charge (Romanato *et al*., 2003). Furthermore, among a series of other glycosaminoglycans (GAGs) tested, only heparan sulfate, a functional analogue of heparin in many biological systems, is able to decondense human spermatozoa *in vitro* in the presence of GSH. These results have led us to consider heparan sulfate as a putative decondensing agent for human spermatozoa *in vivo*.

Decondensation conditions, however, are considerably different *in vivo* and *in vitro*. *In vitro*, spermatozoa are incubated in decondensing conditions simply following capacitation, while *in vivo*, after sperm egg fusion and entry into the ooplasm, the sperm nucleus is directly exposed to decondensing factors present in the oocyte (Yanagimachi, 1994). In fact, the maximum decondensation achieved with capacitated spermatozoa in our laboratory *in vitro* suggests that probably only those cells the plasma membranes of which were altered during capacitation, and are thus permeable to heparin and GSH, are able to decondense (Romanato *et al*., 2003).

It has been well established that GSH is necessary but not sufficient for decondensation to occur *in vivo* (Perreault *et al.*, 1984; Liu and Baker, 1992; Sutovsky and Schatten, 1997). Following reduction of disulfide bonds by GSH, protamines must be removed from their association to DNA and the existence of a protamine acceptor has been proposed to aid in this process. In amphibians and *Drosophila melanogaster*, nucleoplasmin has been shown to exert this role, but these findings have not as yet been extended to mammals (Ohsumi and Katagiri, 1991; Philpott *et al*., 1991; Kawasaki *et al*., 1994), although it is generally accepted that decondensation mechanisms must be quite conserved across the evolutionary scale given the ability of spermatozoa to decondense in heterologous egg extracts (Shimada *et al*., 2000; Burns *et al*., 2003).

As previously stated, our laboratory has proposed that heparan sulfate could be a decondensing agent *in vivo*, acting

as protamine acceptor in the presence of GSH (Romanato *et al*., 2003). Because experiments leading to this conclusion were performed with intact spermatozoa, we were interested in investigating the behaviour of isolated sperm nuclei in the presence of heparin and GSH, in an attempt to better resemble *in-vivo* decondensing conditions. Therefore, the aims of this study were to: (i) compare the decondensation kinetics of capacitated spermatozoa and isolated sperm nuclei in the presence of heparin and GSH *in vitro*; (ii) analyse the effect of heparin sulfation on its decondensing ability of isolated nuclei; and (iii) test the ability of different GAGs to function as protamine acceptors *in vitro*.

Materials and methods

Semen specimens and sample processing

Normospermic (WHO, 1999) semen specimens were obtained with informed consent from normal healthy volunteers. 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, USA), unless otherwise stated.

Semen samples to be capacitated were diluted 1:5 with human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.3% bovine serum albumin (BSA), centrifuged at 300 *g* for 10 min and resuspended in 2 ml fresh HTF. This procedure was repeated twice. Afterwards, the remaining pellet was overlaid with 1 ml fresh HTF containing 2.6% BSA (HTF-26B) and sperm were allowed to swim up for 90 min at 37° C in an atmosphere of 5% CO_2 in air.

Sperm nuclei isolation

Human sperm nuclei were isolated according to the following protocol, modified from Yebra and Oliva (1993). Semen was washed three times by centrifugation at 1620 *g* for 10 min in 50 mmol/l Tris–HCl, pH 7.2, and 0.15 mol/l NaCl (10× sample volume). Sperm pellet was resuspended in 2.6 ml of the same buffer containing 1% SDS, incubated for 15 min at room temperature and spermatozoa sonicated (6× 15 s at 200 W) with a Branson sonifier cell disruptor, model W 140 (Branson Sonic Power Co., Plainview, NY, USA). Sonified cells were divided in two equal aliquots, each of which was placed on top of 4 ml 1.1 mol/l sucrose in 50 mmol/l Tris–HCl, pH 7.2, and centrifuged at 3500 *g* for 1 h. Pellets were recovered and washed twice by centrifugation at 1620 *g* for 10 min in 50 mmol/l Tris–HCl, pH 7.2. Lack of contamination of the nuclear fraction obtained with sperm tails was tested by microscopical observation. Effective removal of acrosomes was assessed by immunocytochemistry with anti-human acrosin.

Immunocytochemistry of spermatozoa and isolated sperm nuclei with anti-human acrosin

Immunocytochemistry was performed according to the protocol described by Zahn *et al*. (2002). Seminal spermatozoa and isolated sperm nuclei were washed in buffer P1 [phosphate-buffered saline (PBS), 50 mmol/l benzamidine and 2 mmol/l β-aminobenzamidine] at 400 *g* for 10 min. Pellets were fixed in 2% formaline in PBS (3× sample volume) for 10 min at room temperature. After washing twice in PBS, final concentration was adjusted to 50 000 spermatozoa or nuclei per 10 µl. Ten µl aliquots of each sample were placed on microscope slides and dried at 37° C. Slides were washed once with PBS, samples permeabilized by incubation in methanol for 10 min at 4° C, washed with PBS and dried with tissue paper. Samples were incubated for 30 min at room temperature in a blocking solution (10 µl/well) consisting of PBS + 0.02% Tween. Anti-human acrosin (C_5F_{11} Sigma; 1:500 in PBS Tween, 10 µl) was then added to each well. After incubation for 60 min at room temperature, slides were washed with PBS, dried with tissue paper and further incubated with rhodamine-labelled anti-mouse IgG (Cy3 Sigma; 1:5000 in PBS Tween, 10 µl/well) for 1 h at room temperature. Slides were washed with PBS, allowed to dry at room temperature and mounted with 0.1 mol/l *n*-propylgallate in 90% glycerol in PBS. Fluorescent labelling was assessed in an Olympus CH2 microscope with epifluorescence attachment.

Decondensation of capacitated spermatozoa and isolated sperm nuclei

Capacitated spermatozoa and isolated sperm nuclei obtained from the same semen sample were decondensed *in vitro* according to Romanato *et al*. (2003). Briefly, spermatozoa and nuclei were incubated in HTF (Irvine Scientific) with 46 µmol/l heparin (Hep) and 10 mmol/l GSH for 15, 30 and 60 min at 37° C in an atmosphere of 5% CO_2 in air. The percentage of sperm or nuclei undergoing decondensation was determined by phase-contrast in an Olympus CH2 microscope at 400× magnification.

Extraction of nuclear proteins

Isolated sperm nuclei were decondensed for 15 and 30 min as described previously, but without BSA in the culture medium (modified from Romanato *et al*., 2003). Incubated nuclei were washed for 8 min at 8000 *g* and nuclear proteins were extracted according to the methodology described by Montag *et al*. (1992). Washed pellets were resuspended in 1.1 mol/l NaCl, 6 mol/l urea and 0.1 micromol/l β-mercaptoethanol and incubated for 2 h in a water bath at 37° C. Following addition of an equal volume of 0.32 mol/l HCl, samples were further incubated for 30 min in ice and centrifuged at 13 000 *g* for 15 min at 4° C. TCA was added to the supernatants in order to attain a 20% final concentration and incubated for 48 h at 4° C. Samples were washed twice by centrifugation (13 000 *g* for 15 min at 4° C) with ice-cold 90% acetone and evaporated to dryness. Evaporated samples were resuspended in polyacrylamide gel electrophoresis (PAGE) sample buffer.

PAGE of extracted nuclear proteins

Acid-urea PAGE was performed on a Protean III vertical electrophoresis unit (Bio-Rad, Hercules, CA USA) according to a modification of the technique proposed by Panyim and Chalkley (1969). Fifteen percent acrylamide gels containing 43.2% v/v glacial acetic acid and 10 mol/l urea were prerun with sample buffer (0.9 N acetic acid, 1 mol/l urea, 0.1 mol/l β-mercaptoetanol, 15% sucrose and Pyronin Y) at 200 V at 4° C until dye disappeared from gel, with 0.9 N acetic acid as running buffer. Samples were applied so that the amount of protein per well corresponded to the extraction of 10^6 sperm or nuclei and run at 100 V. Gels were stained with Coomassie Brilliant Blue and destained with acetic acid and methanol using standard methodology.

Sulfation characteristics of heparin and decondensing ability

To evaluate the effect of sulfation characteristics of heparin on its nuclear decondensing ability, isolated nuclei from the same semen sample were decondensed in the presence of 10 µmol/l GSH and 46 micromol/l heparin, or each of the following chemically modified structures (Syntex S.A., Buenos Aires, Argentina): partially *N*-desulfated (N-des), partially *O*-desulfated (O-des), partially *N*-desulfated-*N*-acetylated (N-des-N-Ac) and partially *O*/*N*-desulfated-*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 (Romanato *et al.*, 2003). An aliquot of capacitated spermatozoa from the same semen specimen was used as internal control for the decondensation assay.

Decondensing ability of different GAGs

Isolated sperm nuclei 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: heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid. Total decondensation in each sample was determined as usual, following 15, 30 and 60 min of incubation in decondensing conditions (Romanato *et al.*, 2003). Capacitated spermatozoa, incubated with heparin and GSH, were used as an internal control for the decondensation assay.

Statistical analysis

Statistical analysis was performed using Instat Mathpad.

Comparison of decondensation kinetics of capacitated spermatozoa and isolated nuclei was performed by Student's *t*-test for paired samples.

The effect of heparin sulfation on nuclei decondensation and decondensing ability of different GAGs were evaluated by repeated measures ANOVA followed by Tukey–Kramer's multiple comparisons test.

Differences were considered statistically significant when $P < 0.05$.

Results

Nuclei isolation

The fraction of isolated sperm nuclei obtained according to the protocol described in Materials and methods consisted solely of sperm heads and showed no contamination with sperm tails (Figure 1). Fluorescent labelling of isolated nuclei with anti-human acrosin demonstrated that acrosomes too had been efficiently removed by the procedure, since no fluorescence could be detected on sperm nuclei preparations (Figure 2).

Comparison of decondensation kinetics of intact sperm and isolated sperm nuclei

Upon observation under phase-contrast, the same stages of decondensation described for intact spermatozoa (Romanato *et al.*, 2003) could be identified in isolated sperm nuclei: unchanged (U), moderately decondensed (M) and grossly decondensed (G) (Figure 3).

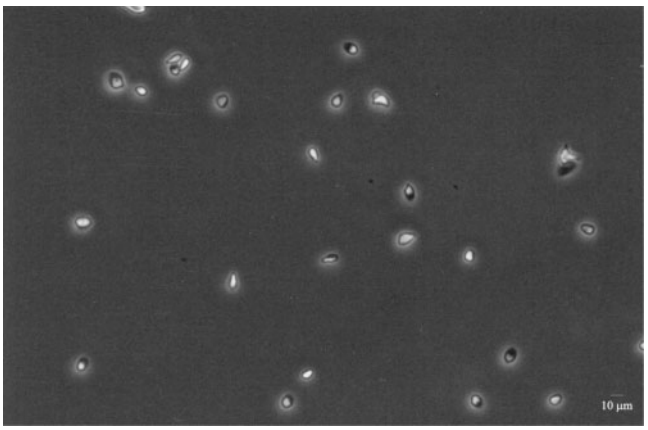

Figure 1. Isolated sperm nuclei obtained according to the procedure described in Materials and methods. The fraction of isolated sperm nuclei consists solely of sperm heads and shows no contamination with sperm tails. Original magnification 400×.

Decondensation kinetics of capacitated spermatozoa and isolated sperm nuclei are depicted in Figure 4. Total decondensation of isolated nuclei was significantly higher than decondensation of capacitated spermatozoa at each time point studied ($n = 8$; Student's *t*-test for paired samples, $P < 0.05$). In contrast to capacitated spermatozoa, for which maximum decondensation after 60 min of incubation was 10 ±1% ($n = 8$), 92 ±4% ($n = 8$) sperm nuclei were already decondensed after 30 min. Surprisingly, isolated nuclei were decondensed by heparin in the absence of GSH. However, the maximum decondensation attained in this condition (50 ± 3%; $n = 8$) was significantly lower than the corresponding value following incubation in heparin and GSH (100 ±0.5%; $n = 8$; Student's *t*-test for paired samples, $P < 0.05$). Incubation with GSH but no heparin did not produce decondensation of either capacitated spermatozoa or isolated nuclei.

Sulfation characteristics of heparin and decondensing ability

To analyse the relationship between structural characteristics of heparin and decondensing ability of isolated sperm nuclei *in vitro*, four chemically modified heparins were tested as decondensing agents in the presence of GSH. As previously stated (Romanato *et al.*, 2003), *O*- or *N*-desulfation and *N*-acetylation alter both the net charge of the disaccharide and the localization of positively and negatively charged groups. Figure 5 depicts isolated sperm nuclear decondensation kinetics for heparin and its four analogues, and shows clearly that heparin's decondensing ability was strongly affected by sulfation characteristics of the molecule. Heparin, O-des and N-des-N-Ac had similar decondensing abilities at each time point studied (repeated mesures ANOVA, $P > 0.05$; $n = 7$). N-des, although less active after 30 min of incubation (repeated measures ANOVA + Tukey–Kramer, $P < 0.05$; $n = 7$), induced a similar level of nuclear decondensation after 60 min. ON-des-N-Ac was inactive at all times.

Decondensing ability of different GAGs

In search of a putative decondensing agent *in vivo*, the decondensing ability of different GAGs that can be found in the oocyte–cumulus complex was tested on isolated sperm nuclei *in vitro* in the presence of GSH (Figure 6). At each time point studied, decondensing abilities of heparin and heparan sulfate were similar (ANOVA + Tukey–Kramer, $P > 0.05$; $n = 7$). Hyaluronic acid and chondroitin sulfate were completely inactive (ANOVA + Tukey–Kramer, $P < 0.01$; $n = 7$) throughout the incubation. Although dermatan sulfate appeared to be slightly active after 60 min of incubation, its decondensing ability was not significantly different from that of chondroitin sulfate and hyaluronic acid ($P > 0.05$).

Protamine acceptor ability of different GAGs

In order to confirm that heparin/heparan sulfate are indeed capable of removing protamines from sperm chromatin, basic proteins were extracted from isolated sperm nuclei previously

Figure 2. Immunocytochemistry of (**A**) spermatozoa and (**B**) isolated sperm nuclei using anti-human acrosin and rhodamine-labelled IgG as secondary antibody. Acrosomes were efficiently removed by the procedure, since no fluorescence can be detected on sperm nuclei preparations. (**C**) Phase-contrast image of (B). Original magnification c1000×.

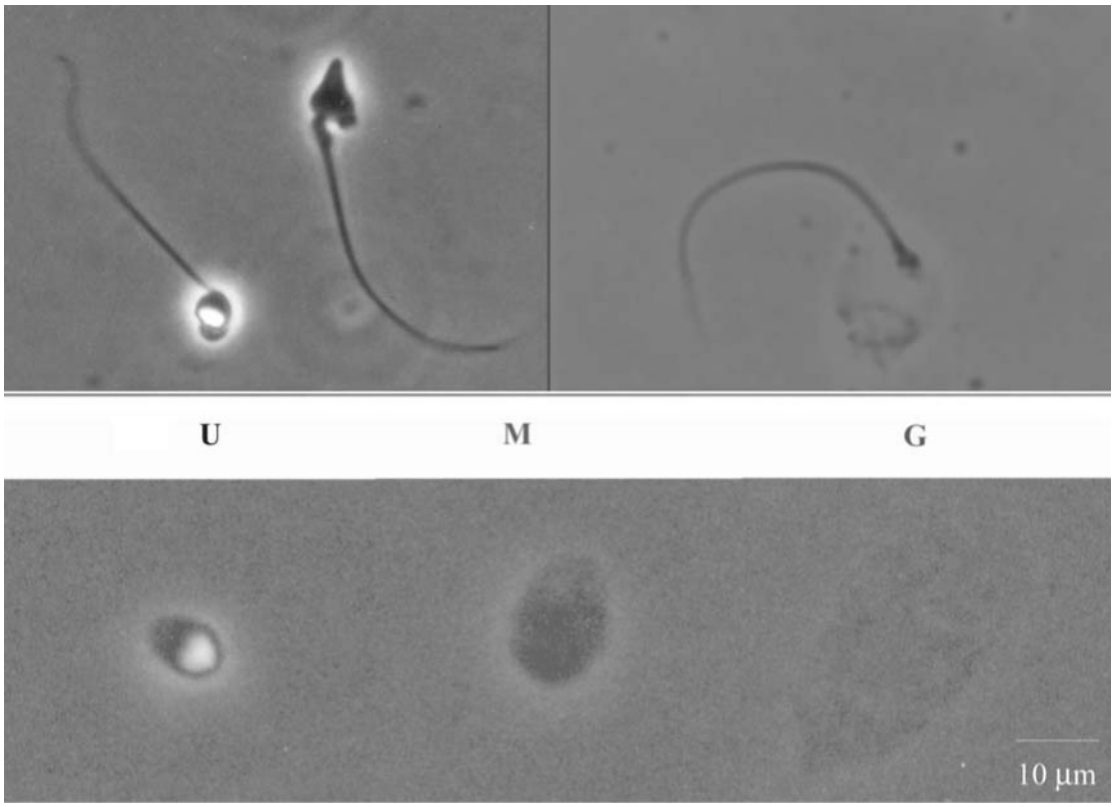

Figure 3. Nuclear decondensation status of human spermatozoa and isolated sperm nuclei visualized under phase contrast. U = unchanged; M = moderately decondensed; G = grossly decondensed. Original magnification 400×.

incubated in the presence of GSH plus different GAGs, and electrophoresed as described in Materials and methods. Results are depicted in Figure 7. Bands corresponding to protamines 1 and 2 (Montag *et al*., 1992) can be seen clearly in lanes 3–6, which contain proteins extracted from nuclei previously incubated with GSH alone (control), GSH + chondroitin sulfate, GSH + dermatan sulfate or GSH + hyaluronic acid; however, these are absent from lanes 1 and 2, which contain proteins extracted from nuclei previously incubated with heparin + GSH or heparan sulfate + GSH, respectively. Experiments were repeated five times using different semen samples, with identical results.

Discussion

It is well established that protamine disulfide bond reduction by GSH is necessary but not sufficient for mammalian sperm decondensation to occur *in vivo* (Perreault *et al*., 1984; Maeda *et al*., 1998). An additional molecule seems to be required to remove protamines from DNA and allow their replacement by oocyte histones. Although it has been demonstrated that nucleoplasmin plays this role in amphibians (Philpott *et al*., 1991) and that p22 protein does so in *D. melanogaster* (Kawasaki *et al*., 1994), the protamine acceptor in mammals has not yet been identified. Members of the nucleoplasmin family have been indeed identified in different mammalian species, including man (Shackleford *et al*., 2001), but sperm decondensation seems to occur normally in nucleoplasmin-depleted oocytes (Burns *et al*., 2003).

Previous results from our laboratory involving decondensing human spermatozoa *in vitro* in the presence of heparin and GSH (Romanato *et al*., 2003) led us to propose heparan sulfate as a putative protamine acceptor *in vivo*.

In the present paper we have reinforced this contention by studying the ability of heparin and other GAGs, including heparan sulfate, to decondense isolated sperm nuclei *in vitro*.

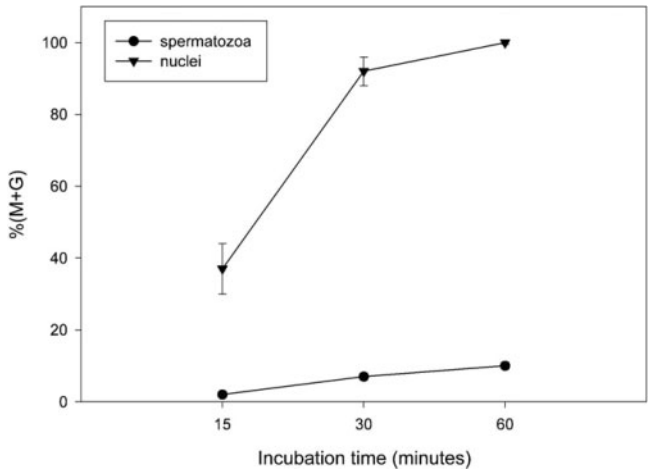

Figure 4. Decondensation kinetics of capacitated spermatozoa and isolated sperm nuclei in the presence of 46 μmol/l heparin and 10 mmol/l GSH. % M+G = total decondensation. Results are expressed as mean ± SEM (n = 8).

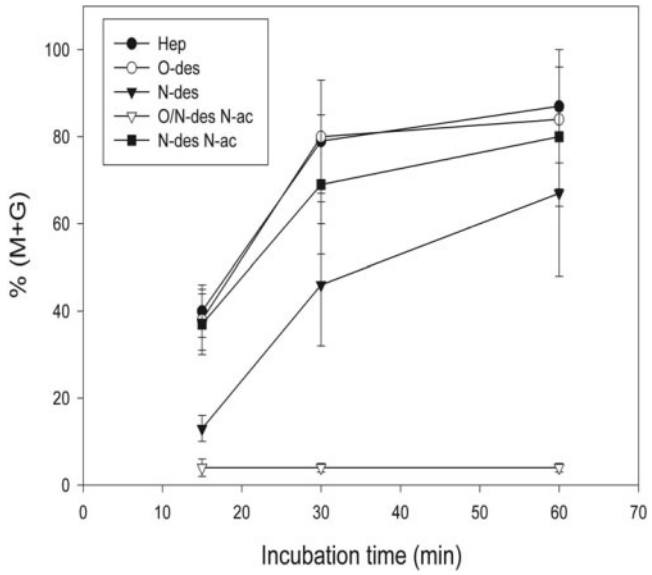

Figure 5. Decondensation kinetics of isolated sperm nuclei in the presence of 46 μmol/l heparin or its chemically modified analogues and 10 mmol/l GSH. N-des = partially *N*-desulfated; O-des = partially *O*-desulfated; N-des-N-Ac = partially *N*-desulfated-*N*-acetylated; ON-des-N-Ac = partially *O*/*N*-desulfated-*N*-acetylated. % M+G = total decondensation. Results are expressed as mean ± SEM (n = 7).

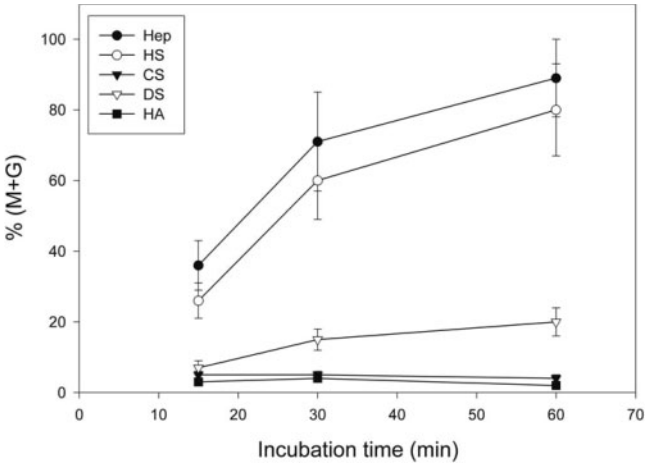

Figure 6. Decondensation kinetics of isolated sperm nuclei incubated with different GAGs (46 μmol/l) in the presence of 10 mmol/l GSH. HS = heparan sulfate; CS = chondroitin sulfate; DS = dermatan sulfate; HA = hyaluronic acid. % M+G = total decondensation. Results are expressed as mean ± SEM (n = 7).

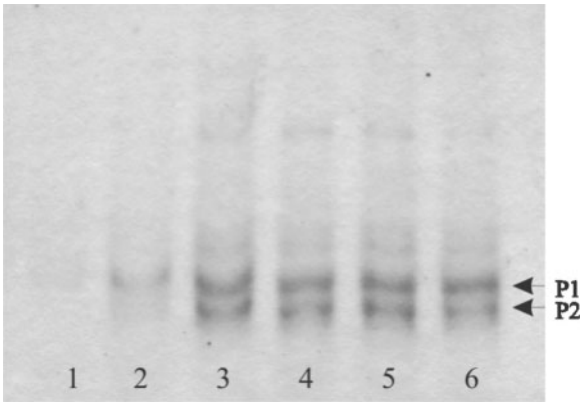

Figure 7. PAGE of sperm nuclear proteins extracted from isolated sperm nuclei previously incubated in the presence of GSH and different GAGS. Lane 1 = spermatozoa incubated with heparin; lane 2 = spermatozoa incubated with heparan sulfate; lane 3 = spermatozoa incubated with GSH alone (negative control); lane 4 = spermatozoa incubated with chondroitin sulfate; lane 5 = spermatozoa incubated with dermatan sulfate; lane 6 = spermatozoa incubated with hyaluronic acid. Arrows indicate electrophoretic bands for protamines P1 and P2. Shown is one experiment representative of five different experiments performed with different semen samples.

Comparison of decondensation kinetics of isolated sperm nuclei and capacitated spermatozoa revealed that the sperm plasma membrane is a powerful barrier against decondensation by heparin and GSH. While the maximum sperm decondensation attained by capacitated spermatozoa rarely exceeded 20%, isolated sperm nuclei consistently achieved 100% decondensation. These results are in agreement with previous data from our laboratory (Romanato *et al*., 2003), which suggested that only spermatozoa the plasma membrane of which had been altered during capacitation were able to decondense in the presence of heparin and GSH.

Interestingly, in the present study, isolated sperm nuclei were able to decondense with heparin alone, although not to such an extent as they did in the presence of GSH. This phenomenon is not observed in intact spermatozoa, where heparin on its own is unable to promote sperm decondensation. Although oocyte GSH is acknowledged as being necessary for sperm decondensation, it has been suggested that the human spermatozoon possesses an intrinsic thiol-reducing mechanism for nuclear sperm decondensation (Kvist, 1982). Such a mechanism would be dependent on the presence of free thiols in protamines, which would be able to change -S-S- bonds from inter to intrachromosomal fibers and thus favour decondensation. Free thiols would be normally stabilized by reversibly bound zinc ions, but following cellular fractionation and in the absence of zinc-containing fluids from either male or female

genital tracts (Kvist, 1980), nuclei could be depleted of zinc. Thus, nuclear decondensation could take place to a certain extent in the presence of heparin, without the addition of GSH.

Previous results from our laboratory showed that the sulfation characteristics of heparin are important for its ability to decondense capacitated human spermatozoa *in vitro* (Romanato *et al*., 2003). The results obtained in this paper show that the ability of partially desulfated heparins to decondense isolated sperm nuclei mirrors exactly their ability to decondense capacitated spermatozoa. Thus, decondensing ability of isolated nuclei is also dependent on quantity and localization of negative charges in the heparin molecule.

Isolated nuclei react to each chemically modified heparin in the same way as capacitated spermatozoa, except for the level of maximum decondensation achieved. We have already discussed that maximum decondensation achieved by capacitated spermatozoa is low because the plasma membrane acts as a barrier impeding access of decondensing agents to the nucleus. Once the barrier is removed, decondensation takes place readily and 100% decondensation is attained. Taken together, these results support the idea that decondensation of capacitated spermatozoa merely reflects interaction of decondensing agents directly with the nucleus in those cells in which the plasma membrane has been damaged. In this way, decondensation of capacitated spermatozoa *in vitro* would be a good model to study sperm nucleus decondensation *in vivo*. This, in turn, could have important implications as a diagnostic tool in the evaluation of male infertility.

In a similar fashion to partially desulfated heparins, the effect of different GAGs on isolated nuclei decondensation mirrored decondensation of capacitated spermatozoa. Only heparan sulfate was able to decondense isolated nuclei *in vitro*; the remaining GAGs were inactive. These results reinforced our hypothesis that heparan sulfate, in the presence of GSH, could be the decondensing agent of human spermatozoa *in vivo*.

If heparan sulfate were indeed a decondensing agent of human spermatozoa *in vivo*, its role in this process would be as protamine acceptor to enable histone protamine exchange. The results obtained in the present paper, analysing using PAGE proteins extracted from sperm nuclei previously incubated in the presence of GSH and different GAGs, clearly support this contention. Protamines P1 and P2 were absent in extracts obtained from nuclei previously incubated with heparin or heparan sulfate, indicating that they had been removed from sperm chromatin during incubation. On the other hand, both protein bands could be clearly seen in extracts from nuclei incubated either with GSH alone or with GAGs that had previously proved inactive as decondensing agents, confirming that P1 and P2 were still associated with sperm chromatin following incubation.

In summary, the results presented in this paper strongly reinforce the proposal that heparan sulfate could be acting as protamine acceptor during human sperm decondensation *in vivo*. Identification of heparan sulfate in the oocyte would definitely confirm this hypothesis and is currently under way in our laboratory.

Acknowledgements

The authors would like to thank Dr Monica Vazquez-Levin for the anti-acrosin antibody and the rhodamine-labelled anti-mouse IgG. M.R. is a CONICET fellow. This work was supported by BID 1201-OC/AR PICT 98 No. 05-03511 from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina.

REFERENCES

Berrios M, Bedford JM (1979) Oocyte maturation: aberrant post-fusion responses of the rabbit primary oocyte to penetiating spermatoza. J Cell Sci 39: 1–12.

Burns K, Viveiros M, Ren Y, Wang P, DeMayo F, Frail D, Eppig J and Matzuk M (2003) Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. Science 300,633–636.

Filicori M and Flamingni C (eds) Communications Media for Education, Inc., New Jersey, USA. p. 70.

Gaubeca-Klix E, Marin-Briggiler C, Cameo M and Calvo L (1998) In vitro sperm nuclear decondensation in the presence of heparin/glutathione distinguishes two subgroups of infertile patients not identified by conventional semen analysis. In Treatment of Infertility: The New Frontiers (Abstract book). Abstract PO-51. Boca Raton, FL, USA.

Kawasaki K, Philpott A, Avilion AA, Berrios M and Fisher PA (1994) Chromatin decondensation in Drosophila embryo extracts. J Biol Chem 269,10169–10176.

Kvist U (1980) Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. Acta Physiol Scand 109,79–84.

Kvist U (1982) Spermatozoal thiol–disulphide interaction: a possible event underlying physiological sperm nuclear chromatin decondensation. Acta Physiol Scand 115,503–505.

Liu DY and Baker MD (1992) Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. Fertil Steril 58,1178–1184.

Maeda Y, Yanagimachi H, Tateno H, Usui N and Yanagimachi R (1998) Decondensation of the mouse sperm nucleus within the interphase nucleus. Zygote 6,39–45.

Montag M, Tok V, Liow S, Bongson A and Nicolle JC (1992) In vitro decondensation of mammalian sperm and subsequent formation of pronuclei-like structures for micromanipulation. Mol Reprod Dev 33,338–346.

Ohsumi K and Katagiri C (1991) Characterization of the ooplasmic factor inducing decondensation of and protamine removal from toad sperm nuclei: involvement of nucleoplasmin. Dev Biol 148,295–305.

Panyim S and Chalkley R (1969) High resolution acrylamide gel electrophoresis of histones. Arch Biochem Biophys 130,337–346.

Perreault SD, Wolff RA and Zirkin BR (1984) The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vitro. Dev Biol 101,160–167.

Philpott A, Leno GH and Laskey RA (1991) Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin. Cell 65,569–578.

Reyes R, Rosado A, Hernández O and Delgado NM (1989) Heparin and glutathione: physiological decondensing agents of human sperm nuclei. Gamete Res 23,39–47.

Romanato M, Cameo M, Bertolesi G, Baldini C, Calvo JC and Calvo L (2003) Heparan sulphate: a putative decondensing agent for human spermatozoa in vivo. Hum Reprod 18,1868–1873.

Shackleford G, Ganguly A and MacArthur C (2001) Cloning, expression and nuclear localization of human NPM3, a member of the nucleophosmin/nucleoplasmin family of nuclear chaperones. BMC Genomics 2,8.

Shimada A, Kikuchi K, Noguchi J, Nakano M and Kaneko H (2000) Protamine dissociation before decondensation of sperm nuclei during in vitro fertilization of pig oocytes. J Reprod Fertil 120,247–256.

Sutovsky P and Schatten G (1997) Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronucler apposition during fertilization. Biol Reprod 56,1503–1512.

WHO (1999) WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge, UK.

Yanagimachi R (1994) Mammalian fertilization. In Knobil E and Neill JD (eds) The Physiology of Reproduction. 2nd edn, Raven Press, New York, NY, USA, pp. 189–317.

Yebra L and Oliva R (1993) Rapid analysis of mammalian sperm nuclear proteins. Anal Biochem 209,201–203.

Zahn A, Furlong LI, Biancotti JC, Ghiringhelli PD, Marín-Briggiler CI and Vazquez-Levin MH (2002) Evaluation of the proacrosin/acrosin system and its mechanism of activation in human sperm extracts. J Reprod Immunol 54,43–63.

Submitted on March 21, 2005; Resubmitted on May 6, 2005; Accepted on May 10, 2005