The presence of heparan sulfate in the mammalian oocyte provides a clue to human sperm nuclear decondensation *in vivo*

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BACKGROUND: Previous results from our laboratory have led us to propose heparan sulfate (HS) as a putative protamine acceptor during human sperm decondensation *in vivo*. The aim of this paper was to investigate the presence of glycosaminoglycans in the mammalian oocyte in an effort to better support this contention. METHODS: Two experimental approaches are used: oocyte labeling to identify the presence of HS and analysis of sperm decondensing ability of fresh oocytes in the presence or absence of specific glycosidases. RESULTS: Staining of mouse zona-intact oocytes with the fluorescent cationic dye, Rubipy, at pH 1.5 allowed for the detection of sulfate residues in the ooplasm by confocal microscopy. HS was detected in the ooplasm by immunocytochemistry. A sperm decondensation microassay using heparin and glutathione was successfully developed. The same level of sperm decondensation could be attained when heparin was replaced by mouse zona-free oocytes. Addition of heparinase to the oocyte/glutathione mixture significantly reduced sperm decondensation ($P = 0.0159$), while there was no effect following addition of either chondroitinase ABC or hyaluronidase. CONCLUSIONS: The results presented in this paper demonstrate for the first time that HS is present in the mammalian oocyte and show that HS is necessary for fresh oocytes to express their sperm decondensing ability *in vitro*.

Keywords: sperm decondensation; heparan sulfate; oocyte; protamine acceptor

Introduction

Once inside oocyte and in order to achieve its ultimate goal, the fertilizing spermatozoon must undergo a series of changes which eventually lead to the formation of the male pronucleus and syngamy. The first of these changes is sperm nuclear decondensation, which occurs within minutes of the spermatozoon's entry into the ooplasm (Berrios and Bedford, 1979; Jager, 1990).

Two distinct processes take place during sperm nuclear decondensation *in vivo*: protamine disulfide bond reduction and protamine replacement by oocyte histones (Perreault *et al*., 1984; Liu and Baker, 1992; Sutovsky and Schatten, 1997). Disulfide bond reduction is the rate limiting step (Perreault *et al*., 1987) of sperm nuclear decondensation and it has been clearly established in different species that reduced glutathione (GSH) present in the ooplasm is an important reducing agent in this regard (Perreault *et al*., 1984; Liu and Baker, 1992; Sutovsky and Schatten, 1997). On the other hand, the molecular basis of protamine histone exchange has not been completely elucidated. Though the participation of a negatively charged protamine acceptor molecule in the process to help remove reduced protamines from their association to DNA is generally accepted, the exact nature of this molecule is still a matter of controversy.

In amphibians, fish and *Drosophila melanogaster*, nucleoplasmin has been shown to exert the role of protamine acceptor (Ohsumi and Katagiri, 1991; Philpott *et al*., 1991; Kawasaki *et al*., 1994) but these findings have not as yet been extended to mammals. Despite the fact that a nucleoplasmin ortholog, Npm2, has been found in mouse oocytes, sperm decondense normally in oocytes obtained from Npm2 knock-out mice (Burns *et al*., 2003) following fertilization.

During the past few years, our laboratory has been studying human sperm decondensation *in vitro*, focusing on the possible role of glycosaminoglycans (GAGs) in this process. Indeed, it had previously been shown that GAGs extracted from sea urchin oocytes could decondense both sea urchin and human spermatozoa *in vitro* (Delgado *et al*., 1982). We demonstrated that heparin exhibits sperm decondensing activity and that this activity is related to its sulfation characteristics but not to molecular size (Romanato *et al*., 2003) and that both heparin and its structural analog heparan sulfate (HS) can successfully remove

protamines from the decondensing sperm nucleus (Romanato *et al.*, 2005). On the other hand, other GAGs such as chondroitin and dermatan sulfates or hyaluronic acid, showed no decondensing activity. These results, together with the fact that heparin is only synthesized by mast cells, led us to propose that HS could be acting as sperm protamine acceptor during sperm decondensation *in vivo*.

There are numerous reports on the presence of HS and other GAGs in the oocyte cumulus complex in several species, including humans (Gebauer *et al.*, 1978; Ball *et al.*, 1982; Bellin and Ax, 1984; Salustri *et al.*, 1989). However, except for hyaluronic acid which has been found in the zona pellucida and perivitelline space of the oocyte as well (Talbot and Dandekar, 2003), GAGs have usually been associated with granulosa cells and their extracellular matrix.

The aim of this study was to investigate the presence of HS in the mammalian oocyte and to assess the sperm decondensing ability of fresh oocytes in the presence of different glycosidases, including heparinase.

Materials and Methods

All chemicals and reagents used were obtained from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated.

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.

Samples were washed twice by centrifugation at $300 \times g$ for 10 min in human tubal fluid (HTF: 4.6 mM KCl; 0.37 mM KH_2PO_4; 90.7 mM NaCl; 1.3 mM $MgSO_4$; 2.78 mM glucose; 1.6 mM $CaCl_2$ $2H_2O$; 23.8 mM $NaHCO_3$; 3.38 mM sodium pyruvate and 80.2 mM sodium lactate) supplemented with 0.3% bovine serum albumin (BSA) and resuspended in 2 ml fresh HTF. 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. Highly motile spermatozoa were incubated in capacitating conditions for 18 h in HTF-26B at $37°C$ in an atmosphere of 5% CO_2 in air at a concentration of $5–10 \times 10^6 ml^{-1}$.

Mice superovulation and oocyte collection

Animals were fed *ad libitum* and kept in air-conditioned rooms at $20 \pm 2°C$ with a 12 h light–dark period. Animal care and manipulation was in agreement with institutional guidelines and the guiding principles in the care and use of animals (DHEW Publication, NIH 80–23).

Eight-week-old CF1 female mice were stimulated with 5 IU pregnant mare's serum gonadotrophin (Novormon, Syntex, Buenos Aires, Argentina) followed by 5 IU hCG 48 h later. Animals were sacrificed 12–14 h post hCG by cervical dislocation. Oocytes were recovered from the oviduct according to the following procedure: each oviduct was placed in a 60 µl drop of phosphate-buffered saline (PBS) and cumuli released into the medium by puncturing the ampulla. Oviducts were discarded and cumuli pooled into a 300 µl drop of PBS. Cumulus cells were removed by incubation for 5–10 min in 0.1% mg/ml hyaluronidase (Type IV). Denuded oocytes were washed three times by transfer into PBS to remove

hyaluronidase. When zona-free oocytes were required, oocytes were treated with acid Tyrode's, pH 2.5 until the zona disappeared and then washed three times (10 min each time) in PBS.

Staining of oocytes with rubipy

To evaluate the presence of sulfated GAGs, denuded oocytes were stained with the fluorescent cationic dye Rubipy (Tris (2,2'-bipyridine) Ruthenium (II) Chloride). Zona-intact oocytes were fixed in methanol for 2 min and incubated with Rubipy (1000 µg/ml in distilled water, pH 1.5) for 5 min. Under these pH conditions, Rubipy binds to sulfate groups but not to carboxylic or phosphate residues (Rozenberg *et al.*, 2001), and can therefore be used as an indicator of the presence of sulfate containing macromolecules, such as HS and other sulfated GAGs. In particular, Rubipy has been used for the cytochemical identification of heparin containing mast cells under these experimental conditions (Bertolesi *et al.*, 1995). After staining, oocytes were washed three times with PBS (10 min/ wash), dried at room temperature, mounted with glycerol 50% in PBS and observed under confocal microscopy (Nikon C1 equipped with a 570 LP emission filter). Oocytes incubated under the same conditions in the absence of the dye were used as negative control. Fluorescence intensity was quantified in control and labeled oocytes using the Image J software (NIH; Bethesda, MD, USA).

Immunocytochemistry of mouse oocytes using an anti-HS antibody

Zona-free mouse oocytes were fixed and permeabilized with 0.5% Tween 20, 2% formaldehyde in PBS for 60 min at room temperature. After incubation in blocking solution (PBS containing 0.5% BSA, 0.1% Tween, 5% Normal Goat Serum) for 60 min at room temperature, oocytes were immunolabeled with monoclonal anti-HS (Seikagaku Corporation, Tokyo, Japan) 1:20 in PBS −0.5% BSA, 5% normal goat serum for 60 min at $37°C$. A fluorescein isothiocyanate—conjugated goat polyclonal anti-mouse immunoglobulin M (Chemicon International Inc., Temecula, CA, USA) was used as second antibody (1:100 in PBS −0.5% BSA, 5% NGS for 60 min at $37°C$). Negative controls did not contain first antibody. Oocytes were observed under confocal microscopy. Fluorescence intensity was quantified in control and immunolabeled oocytes using the Image J software (NIH; Bethesda, MD, USA).

Standard sperm decondensation assay

Capacitated spermatozoa were decondensed as previously described (Romanato *et al.*, 2003) based on the original decondensation technique introduced by Reyes *et al.* (1989). Briefly, $3–5 \times 10^6$ spermatozoa were incubated in HTF with 46 µM heparin and 10 mM GSH, in a 0.3 ml final volume, for 1 h at $37°C$ in an atmosphere of 5% CO_2 in air. After fixation in 2% glutaraldehyde, the percentage of decondensed spermatozoa was determined by phase contrast in a Zeiss microscope at $400 \times$ magnification, using the scoring criteria previously described (Bedford *et al.*, 1973; Romanato *et al.*, 2003). Briefly, spermatozoa are classified as unchanged, moderately decondensed or grossly decondensed, according to the refringency, granular aspect and size of the nucleus. Unchanged spermatozoa are bright and do not have an enlarged nucleus; moderately decondensed cells are no longer refringent, but dark and slightly enlarged; grossly decondensed cells have a very large, granular nucleus, gray in color and almost translucent. The percentage of decondensed spermatozoa is the sum of both moderately and grossly decondensed cells. Experiments were run in duplicates and at least 200 cells were evaluated in each sample.

Sperm decondensation assay: micromethod

A micromethod to assay sperm decondensation was developed based on the standard decondensation assay described above and on a micromethod for mouse sperm chromatin decondensation in cell-free extracts described by Maleszewski (1990). One microlitre of heparin (46 μM final concentration), 1 μl GSH (10 mM final concentration) and 1 μl HTF containing 20 000 capacitated spermatozoa were mixed and incubated between slide and cover slip for 3.5 h at 37°C in a humidified chamber, as a safeguard to prevent evaporation. Slides were sealed in order to avoid drying during incubation. Controls consisted of parallel incubations with heparin or GSH alone. The percentage of decondensed spermatozoa was evaluated as usual by phase contrast microscopy. Comparison of this new micromethod and the standard decondensation protocol was performed using aliquots of the same sperm sample. Nine independent experiments were performed and within each experiment assays were run in duplicate.

Sperm decondensation in the presence of fresh oocytes

The decondensation microassay described above was used to evaluate the decondensing ability of fresh mouse oocytes. One microliter HTF containing 20 000 capacitated spermatozoa and 1 μl HTF containing 30 zona-free oocytes were placed on microscope slides. One microliter GSH was added and a cover slip placed on top and gently pressed to crush the oocytes. Incubation was carried out for 3.5 h at 37°C in a humidified chamber as described above. Controls consisted of parallel incubations containing spermatozoa plus 46 μM heparin, 10 mM GSH or both. Each of five independent experiments was performed using aliquots of the same sperm sample and within each experiment assays were run in duplicate.

Effect of different glycosidases on oocyte-induced sperm decondensation

To evaluate the possibility that the sperm decondensing activity of fresh oocytes might be due to the presence of HS, capacitated spermatozoa were decondensed with fresh mouse oocytes using the microassay described above, with or without the addition of different glycosidases. The enzymes used were heparinase III (E.C.: 4.2.2.8; 10 mIU/ml), chondroitinase ABC (E.C.: 4.2.2.4; 100 mIU/ml; Seikagaku Corporation, East Falmouth, MA, USA) and hyaluronidase (E.C.: 3.2.1.35; 2 IU/ml; Calbiochem, Darmstadt, Germany). Enzymes were added to incubation mixture in 1 μl HTF to achieve the final concentrations mentioned in the 3 μl incubation volume. Five independent experiments were performed and assays were run in duplicate.

Statistical analysis

Comparison of standard and microdecondensation assays and comparison of decondensing ability of fresh oocytes versus the standard heparin/GSH mixture were performed using Student's Paired t-test (two-tail) because each individual experiment used spermatozoa obtained from one semen sample.

Effect of glycosidases on oocyte-induced decondensation was evaluated by One Way Analysis of Variance (ANOVA) followed by Dunnett's test to compare each enzyme treatment against control.

Differences were considered significant when $P < 0.05$.

Results

Staining of oocytes with rubipy

To evaluate the presence of sulfated GAGs, zona-intact oocytes were stained with the cationic dye Rubipy at pH 1.5. Analysis by confocal microscopy showed a cytoplasmic red fluorescence
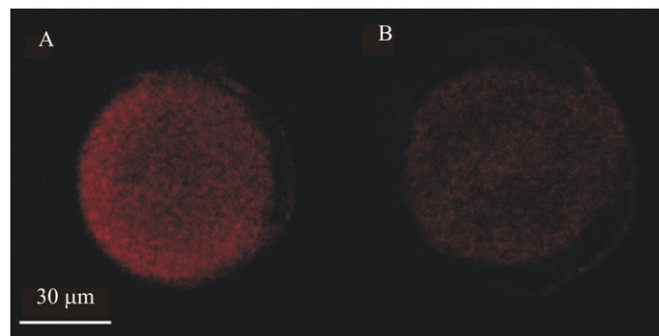
Figure 1: Confocal microscopy of mouse oocytes stained with Rubipy.
(**A**) Oocyte incubated with Rubipy 1 mg/ml in distilled water at pH 1.5. (**B**) Oocyte incubated in distilled water at pH 1.5 (negative control). Images shown are representative of 100 oocytes observed in four different experiments

in all stained oocytes (Fig. 1A) which was markedly stronger than the slight autofluorescence observed in oocytes which had not been incubated with the dye (Fig. 1B). Overall, 60 stained oocytes and 40 control oocytes were analysed in four different experiments. Quantification of fluorescence revealed that label intensity was significantly higher in Rubipy incubated oocytes (25 433 ± 1885 arbitrary units) than in control oocytes (13 913 ± 1449 arbitrary units; Unpaired Student, $P = 0.0167$).

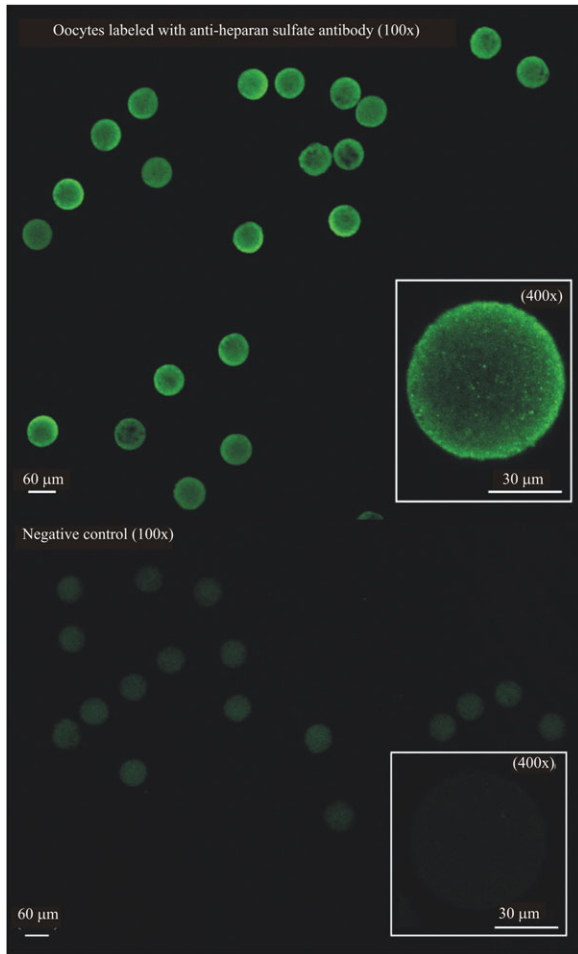### Immunocytochemistry of mouse oocytes using an anti-HS antibody

To investigate directly the presence of HS in the oocyte, we performed indirect immunofluorescence of mouse oocytes using a specific anti-HS monoclonal antibody. Analysis by confocal microscopy clearly revealed a strong fluorescent label in all oocytes incubated with the monoclonal which could not be seen when the first antibody was omitted (Fig. 2). Overall, 150 immunolabeled oocytes and 120 control oocytes were analysed in four different experiments. Quantification of fluorescence revealed that label intensity increased almost 3-fold in immunolabeled oocytes (17 667 ± 883 arbitrary units) compared to negative controls (6668 ± 378 arbitrary units; Unpaired Student, $P < 0.0001$).

Sperm decondensation assay: micromethod

A micromethod to evaluate sperm decondensation in the presence of heparin and GSH on a microscope slide as described in Materials and Methods was successfully developed. Using this methodology, the percentage of decondensed spermatozoa was significantly higher (49 ± 5%) than the one observed with the standard assay (24 ± 6%, $n = 9$, Paired Student, $P = 0.007$) (Fig. 3). Spermatozoa incubated in either heparin or GSH alone showed no signs of decondensation by either method (not shown).

Sperm decondensation in the presence of fresh oocytes

The decondensing ability of fresh oocytes was assessed using the microdecondensation assay described above (Fig. 4). Decondensation was almost null when spermatozoa were

Figure 2: Immunocytochemistry of mouse oocytes using an anti-HS monoclonal antibody.
Denuded and permeabilized oocytes were incubated with (top panel) or without (negative control, bottom panel) anti-HS antibody followed by fluorescein isothiocyanate-labeled anti-mouse immunoglobulin M. Images shown are representative of 270 oocytes observed in four different experiments

incubated with crushed oocytes alone $(4 \pm 1\%)$, but the addition of 10 mM GSH allowed a level of decondensation $(42 \pm 7\%)$ which was similar ($n = 9$, Paired Student, $P = 0.558$) to the one achieved with the classical decondensation mixture containing heparin and GSH $(47 \pm 4\%)$.

Effect of different glycosidases on oocyte-induced sperm decondensation

To evaluate the possibility that the sperm decondensing activity of fresh oocytes might be due to HS present in the ooplasm, spermatozoa were decondensed in the presence of oocytes, with or without the addition of different glycosidases (Fig. 5A). The percentage of decondensed spermatozoa decreased significantly ($n = 4$, One Way ANOVA+Dunett's Test, $P = 0.0159$) with the addition of heparinase: 3 ± 1 versus $42 \pm 7\%$ control while addition of either chondroitinase ABC or hyaluronidase had no effect on the level of sperm decondensation achieved: $48 \pm 11\%$ for chondroitinase and $34 \pm 7\%$ for hyaluronidase (Fig. 4A). Specificity of the enzymes was checked by decondensing spermatozoa with the
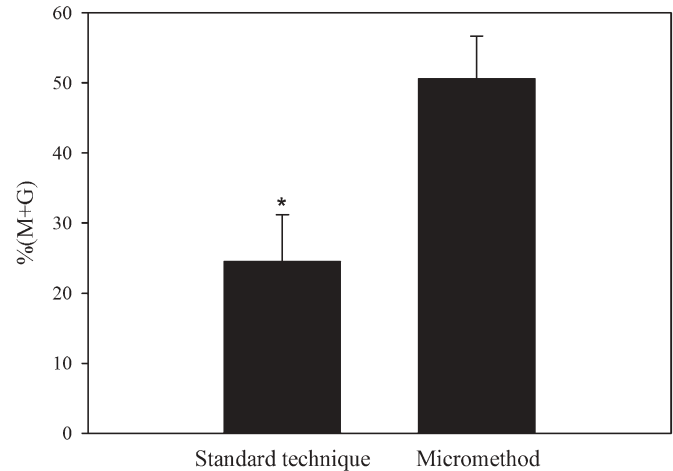

Figure 3: Comparison of standard and micro sperm nuclear decondensation assays.
Capacitated spermatozoa were decondensed in the presence of 46 μM heparin and 10 mM reduced glutathione (GSH) using both the standard decondensation assay and the newly developed micromethod. % M+G=total sperm decondensation (sum of moderately and grossly decondensed cells). Results are expressed as mean $\pm$ SEM; $n = 9$. *$P < 0.05$ versus standard assay

standard heparin/GSH mixture with and without the addition of each enzyme (Fig. 5A). Once again, addition of heparinase significantly decreased heparin/GSH-induced decondensation (19 ± 5 versus $47 \pm 4\%$; $n = 4$, One Way ANOVA+Dunett's Test; $P = 0.0092$) while chondroitinase ABC and hyaluronidase had no effect: 39 ± 7 and $33 \pm 7\%$, respectively.

Discussion

It has been 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.*,
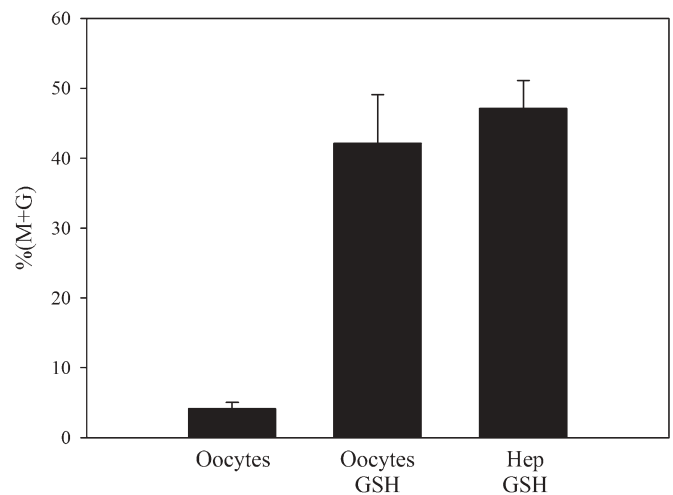
Figure 4: Oocyte sperm decondensing ability *in vitro*.
Capacitated human spermatozoa were decondensed in the presence of fresh mouse oocytes+10 mM GSH or 46 μM heparin (Hep)+10 mM GSH. %M+G=total sperm decondensation. Results are expressed as mean $\pm$ SEM; $n = 9$
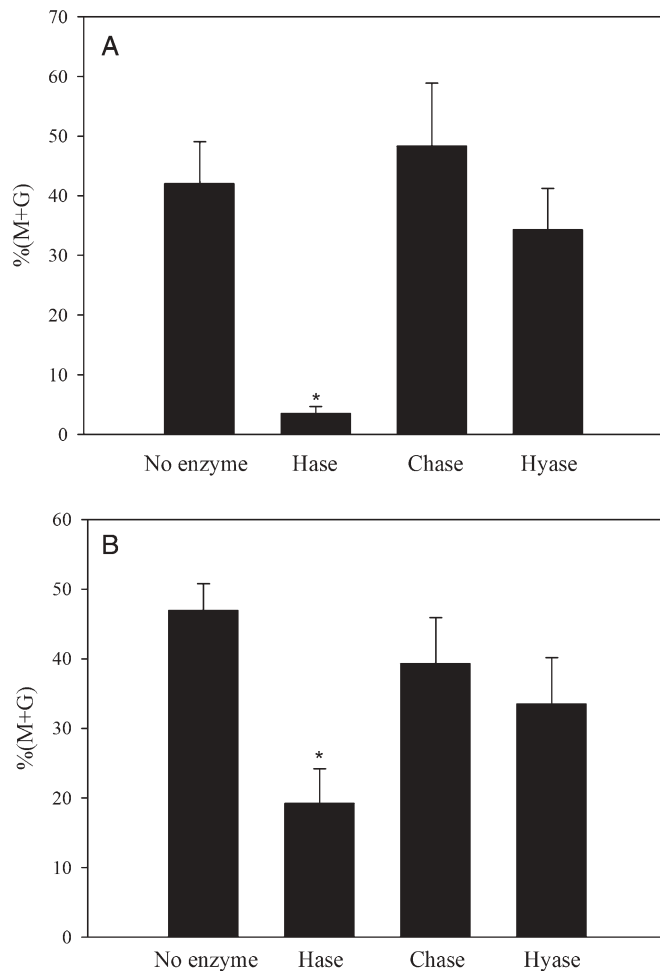
Figure 5: Effect of different glycosidases on oocyte-induced sperm decondensation.
(**A**). Capacitated human spermatozoa were decondensed in the presence of fresh mouse oocytes with or without the addition of specific glycosidases: heparinase III (Hase), chondroitinase ABC (Chase) and hyaluronidase (Hyase). % M+G: total sperm decondensation. Results are expressed as mean ± SEM ($n = 4$). *$P < 0.05$ versus control (No enzyme). Enzyme specificity was confirmed by decondensing human spermatozoa with heparin+GSH in the presence of each glycosidase (**B**)

1984; Liu and Baker, 1992; Sutovsky and Schatten, 1997; Maeda *et al*., 1998). An additional molecule seems to be required to act as protamine acceptor, facilitating removal of protamines from DNA and thus enabling their replacement by oocyte histones. The nature of this protamine acceptor in mammals is still unknown, but previous results from our laboratory have demonstrated that human spermatozoa can decondense *in vitro* in the presence of HS and GSH (Romanato *et al*., 2003) and that heparin and HS, but not other GAGs, can remove protamines from decondensing human spermatozoa *in vitro* (Romanato *et al*., 2005). These findings have led us to propose HS as a putative protamine acceptor during sperm decondensation *in vivo*. In the present paper, we have reinforced this contention by demonstrating that HS is indeed present in the mammalian oocyte and providing evidence which strongly suggests that HS is necessary for fresh oocytes to express sperm decondensing ability *in vitro*.

Although the decondensation of human spermatozoa is the focus of our study, experiments presented in this paper have been performed using mouse rather than human oocytes for ethical considerations. Ample evidence suggests that the protamine acceptor in the oocyte is not species-specific because spermatozoa readily decondense *in vitro* and *in vivo* using oocyte extracts from different species (Maleszewski, 1990; Shimada *et al*., 2000; Burns *et al*., 2003).

Our first approach toward demonstrating the presence of HS in the oocyte, used a simple histochemical technique which allows for the identification of sulfate residues (Rozenberg *et al*., 2001). Sulfate residues in the cell are mainly found in GAGs, and therefore the positive stain observed in zona-intact oocytes was taken as indicative of the possible presence of HS in the ooplasm. Subsequently, immunocytochemistry of zona-free oocytes using a specific anti-HS antibody unequivocally confirmed this result. Undoubtedly, these results do not rule out the possibility of the presence of other sulfated GAGs in the ooplasm as well, such as chondroitin and dermatan sulfates. However, this would be of no consequence whatsoever as far as our working hypothesis is concerned, since previous results from our laboratory have demonstrated that these GAGs are unable to decondense human spermatozoa *in vitro* (Romanato *et al*., 2003, 2005).

Having demonstrated that HS was indeed present in the ooplasm, we were interested in evaluating its involvement in sperm nuclear decondensation by studying the effect of different glycosidases on the decondensing ability of fresh oocytes *in vitro*.

For this purpose, it was necessary to develop a micromethod to assess sperm decondensation and this was successfully achieved using the standard heparin/GSH decondensation mixture routinely used in our laboratory (Romanato *et al*., 2003). In the final experimental conditions, the percentage of decondensed spermatozoa was significantly higher with the microtechnique than with the standard assay, probably due to the presence of a larger number of dead spermatozoa at the end of the incubation period. Indeed, previous results from our laboratory showed that the percentage decondensation observed in capacitated human spermatozoa correlates highly with the percentage of Eosin Y positive cells, implying that sperm membrane needs to be damaged for decondensation to occur (Romanato *et al*., 2003). However, this discrepancy in maximum decondensation achieved between standard and microassays did not defeat our purpose of obtaining a reliable system which would allow the evaluation of sperm decondensation at a smaller scale, using very small volumes.

With this micromethodology, capacitated spermatozoa were then successfully decondensed in the presence of fresh oocytes and GSH, attaining a level of decondensation similar to the one achieved with heparin and GSH. The fact that incubation with oocytes in the absence of GSH did not allow decondensation to occur, though surprising at first, was interpreted as a consequence of both the rapid oxidation of existing GSH and a lack of a functional GSH regeneration system upon crushing of the oocytes for the experiment. It is noteworthy that the maximum decondensation achieved with fresh oocytes was around 50%. There is ample evidence in the literature regarding

the limited capacity of the ooplasm to allow normal pronuclear formation and chromosome remodeling following fertilization (Witkowska, 1981; Clarke and Masui, 1987; Wakayama and Yanagimachi, 1998). Under our experimental conditions, considering that 30 oocytes were incubated with 20 000 spermatozoa, a 50% decondensation implies that about 30 spermatozoa were decondensed per oocyte. Interestingly, a previous report by Hirao and Yanagimachi (1979) showed that one zona-free hamster oocyte could decondense as many as 50 sperm nuclei following fertilization *in vitro*.

The ability of mouse oocytes to decondense human spermatozoa *in vitro* was significantly reduced by the addition of heparinase III and not by other glycosidases, indicating that the observed oocyte-induced sperm decondensation could be attributed to the presence of HS.

In summary, the results presented in this paper constitute the first report on the presence of HS in the mammalian oocyte and provide evidence that this GAG is necessary for oocytes to successfully decondense spermatozoa *in vitro*, supporting our hypothesis that HS is functioning as protamine acceptor during sperm decondensation *in vivo*.

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