Dermatan sulfate synergizes with heparin in murine sperm chromatin decondensation.

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Short title: Dermatan sulfate and heparin in sperm decondensation

Abstract

The mammalian sperm nucleus contains an unusually condensed chromatin, due to replacement of the majority of histones by other basic proteins called protamines. However, soon after the spermatozoon penetrates the ooplasm at fertilization, decondensation of this densely packed chromatin must occur to allow formation of the male pronucleus and syngamy. Decondensation is accomplished by protamine disulfide bond reduction by oocyte glutathione and replacement of protamines by oocyte histones with the aid of an acceptor molecule. Previous results from our laboratory have demonstrated that heparan sulfate present in the ooplasm functions as protamine acceptor during human sperm decondensation *in vivo*. In the present paper, we analyze the role of heparin, structural analogue of heparan sulfate, and dermatan sulfate in murine sperm chromatin decondensation *in vitro*, including the possibility of a synergistic effect between both glycosaminoglycans. Decondensation was assessed under phase contrast microscopy following incubation of murine spermatozoa with glutathione and either heparin, dermatan sulfate or a combination of both. Ultrastructural changes taking place during decondensation were analyzed by transmission electron microscopy. Both glycosaminoglycans were able to promote the decondensation of murine spermatozoa *in vitro* but the decondensing ability of heparin was significantly higher. Use of both glycosaminoglycans together revealed the existence of a synergistic effect. Transmission electron microscopy analysis of decondensing spermatozoa supported these findings. Synergism between heparin and dermatan sulfate was observed both in capacitated and non capacitated spermatozoa but

decondensation kinetics was faster in the former. The results obtained indicate a new potential role for dermatan sulfate in murine sperm decondensation at fertilization and provide evidence of differences in the degree of chromatin condensation throughout the murine sperm nucleus.

Key words: heparin; dermatan sulfate; murine spermatozoa; chromatin decondensation; synergism

Introduction

The nucleus of the mature mammalian spermatozoa is unique, both in nucleoprotein composition and chromatin organization (McLay and Clarke 2003). During spermatogenesis, the majority of nuclear histones have been replaced by protamines (Florman and First 1988; Eddy and O'Brien 1994), relatively small and highly basic proteins, rich in arginine and cysteine, rendering a highly condensed sperm nucleus. There are two types of protamines: P1, present in all species, which possesses an arginine-rich central domain (Wouters-Tyrou, Martinage et al. 1998), and P2, present in some species, which contains less cysteine and more histidine, and may thus be expected to sustain the formation of fewer disulfide bonds (Sanchez-Vazquez, Reyes et al. 1996). There are two general models for the association of protamines with DNA, both implying that chromatin is stabilized by the formation of covalent disulfide linkages between protamines on adjacent DNA strands. In those species where P1 and P2 are present, as is the case with mouse and human, both protamines are critical for

fertility, and nuclear formation and sperm DNA stability are disrupted by a change in their ratio (Burgess and Kelly 1987; Stevens, 1993).

Upon entry into the oocyte, the sperm nucleus undergoes marked morphological changes which lead to extensive decondensation and formation of the male pronucleus, in synchrony with oocyte chromatin decondensation into the female pronucleus. This morphological remodeling involves the replacement of sperm protamines by oocyte histones which then organize into nucleosomes. Evidence in the literature indicates that protamine replacement by histones in the paternal chromatin requires the reduction of protamine disulfide bonds by reducing agents present in the egg cytoplasm, and the participation of other egg components that help protamine - histone exchange (Zirkin et al. 1989). For example, inhibition of the meiotic maturation-associated increase in oocyte glutathione (GSH) in mice (Perreault et al. 1988) prevents sperm chromatin decondensation and in amphibian, fish and Drosophila eggs, another oocyte protein, nucleoplasmin, facilitates protamine removal, chromatin decondensation, and histone replacement (Ohsumi and Katagiri 1991, Arnan et al. 2003).

Recently, Romanato et al. (2003) reported that heparan sulfate and heparin, but not other glycosaminoglycans (GAGs), used at physiological concentrations, were able to release protamines from human capacitated sperm chromatin *in vitro*, and proposed that heparan sulfate could be functioning as protamine acceptor *in vivo* during human sperm nuclear decondensation. However, the mechanism of action of heparin/heparan sulfate 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, Reyes et al. 1982), (Lassalle and Testart 1992), (Carrell and Liu 2002)), and Delgado et al. (1982) 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 (Romanato, Cameo et al. 2003). 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 *in vitro* to form a highly insoluble complex (Romanato, Cameo et al. 2003)). Direct experimental evidence is lacking, and how heparin/heparan sulfate is able to decondense human sperm *in vitro* is not clearly understood.

The first evidence on the presence of heparan sulfate (HS) in the mammalian (murine) oocyte and its requirement for human sperm *in vitro* decondensation by fresh murine oocytes was reported by our research group (Romanato et al., 2005, 2008). Recently, we have also demonstrated (Julianelli et al. 2012) that HS is present in the human oocyte as well, adding further support to the hypothesis that HS is functioning as protamine acceptor during mammalian sperm decondensation *in vivo*.

Preliminary data from our laboratory suggested that another glycosaminoglycan (GAG) present in the oocyte cumulus complex (Tirone et al., 1993), dermatan sulfate (DS), had the ability to decondense murine spermatozoa *in vitro.* This behavior, different from that which we had observed and described for human sperm decondensation, prompted us to analyze the involvement of other GAGs in murine sperm decondensation, the possibility of their differential effect on chromatin decondensation and the possible interaction between them in this process.

The aim of this study was to gain further insight on the molecular mechanisms underlying mouse sperm chromatin decondensation *in vitro*, particularly regarding the possible involvement of more than one GAG in this process. Accordingly, chromatin decondensation *in vitro* was analyzed in the presence of GSH and different GAGs, in both capacitated and non capacitated murine spermatozoa.

Results

Decondensing ability of different GAGs in capacitated spermatozoa

In the search for a putative decondensing agent *in vivo*, the ability of different GAGs usually present in the oocyte-cumulus complex to decondense capacitated murine spermatozoa, was assayed *in vitro* in the presence of GSH (Fig. 2). Both, DS and heparin readily decondensed sperm chromatin after 60 minutes of incubation (Hep: 87 ± 2 % versus DS: 67 ± 2 %, $p<0.001$, ANOVA + Tukey-Kramer Multiple Comparison Test, n = 3) while hyaluronic acid (HA) and chondroitin sulfate (CS) were completely inactive (ANOVA + Tukey-Kramer Multiple Comparison Test, not significant (NS), versus GSH or heparin alone, n = 3) in the same incubation conditions.

Heparin and dermatan sulfate dose-response curves for capacitated and non-capacitated mouse spermatozoa

In order to establish the optimum heparin and DS concentrations to be used in GAG synergism studies, mouse spermatozoa were incubated with 10 mmol/l

GSH and increasing concentrations of either heparin or DS (Fig. 3). An incubation time of 15 minutes was chosen for these experiments in order to avoid full decondensation. Fig 3A shows the heparin dose-response curves obtained with capacitated and non-capacitated spermatozoa. Sperm decondensation increased with heparin concentration until it reached a plateau (at around 5 micromol/l in non-capacitated and 15 micromol/l in capacitated spermatozoa). Percent decondensation achieved was significantly different from control (GSH alone) at all heparin concentrations tested, both for capacitated and non capacitated spermatozoa (ANOVA+ Tukey-Kramer Multiple Comparison Test, $P < 0.01$, $n = 4$). On the other hand, there was virtually no decondensation after incubating either capacitated or non-capacitated spermatozoa with GSH and DS (Fig 3B). When non-capacitated spermatozoa were used, no significant dose-response curve was obtained (data not shown) and capacitated spermatozoa showed a significant increase in % decondensation, only at the highest concentration tested (46 micromol/l, $p<0.01$ compared to GSH alone). In view of these results, 0.46 micromol/l heparin and 46 micromol/l dermatan sulfate were chosen for experiments hereafter.

Decondensation kinetics for heparin and dermatan sulfate in capacitated and non-capacitated mouse spermatozoa.

Once the optimum GAG concentration to be used was determined, the time course of decondensation in the presence of heparin, DS or the combination of both, was analyzed in both capacitated (Fig. 4, left panel) and non-capacitated (Fig. 4, left panel) spermatozoa. Though the shape of the curve was similar for

the three experimental conditions, there were differences in $t_{1/2}$ among them, both in capacitated and non-capacitated spermatozoa. Capacitated spermatozoa (Fig. 4, left panel) decondensed somewhat faster with heparin ($t_{0.5}$ = 35.45 ± 2.17 min) than with DS ($t_{0.5}$ = 43.55 ± 2.19 min) and the combination of both molecules drastically reduced decondensation time to almost half ($t_{0.5}$ = 17.96 ± 1.20 min). However, the three curves reached the same final level of decondensation (Sigmoidal dose-response curve fit, R^2= 0.8585, preferred model different EC_{50}). The behavior of non-capacitated spermatozoa was similar (Fig. 4, right panel), although differences in $t_{0.5}$ were less marked and the final level of decondensation achieved was lower than in capacitated sperm..

Synergistic effect of heparin and dermatan sulfate on sperm decondensation

In order to analyze the possible synergism between heparin and DS on murine sperm decondensation, % decondensation was evaluated following incubation of both capacitated and non-capacitated spermatozoa with the lowest concentration of heparin (0.46 micromol/l) and the highest concentration of DS (46 micromol/l), alone or combined, for different periods of time. Results are depicted in Fig. 5. Synergism was evident following 15 min of incubation in capacitated spermatozoa (Fig. 5A, left panel) and 45 min of incubation in non-capacitated spermatozoa (Fig. 5A, right panel), but was lost after 60 min of incubation (Fig. 5B, left and right panels).

Electron microscopy of decondensed spermatozoa

The effect of GSH, heparin + GSH, DS + GSH or the combination heparin + DS + GSH on the ultrastructure of decondensing murine spermatozoa was analyzed by transmission electron microscopy (Fig. 6). Following incubation with GSH alone (control, Fig. 6A), fully condensed sperm heads were distinguishable, showing an adequate nuclear envelope conformation. Chromatin decondensation became apparent following incubation with GSH + heparin (Fig. 6B), beginning at the caudal region of the sperm head and presenting granulo-fibrillar areas. When GSH + DS were used (Fig. 6C), decondensation also began at the caudal region of the sperm head, but chromatin appeared more compact. Finally, after the addition of GSH + heparin + DS (Fig. 6D), a significantly higher degree of chromatin decondensation was observed, evidenced by the presence of “lacunar” and granulo-fibrillar areas, as well as the disarray of acrosomal and nuclear membranes. In spermatozoa where chromatin decondensation was incomplete, the cephalic region remained more compact.

Discussion

Sperm nuclear chromatin is about 6-8 times more condensed than somatic cell chromatin, as a consequence of the replacement of the majority of sperm histones by protamines during spermatogenesis. After fertilization, however, this compact chromatin must decondense in order to interact with oocyte DNA and attain syngamy.

The process of sperm decondensation in the oocyte involves the reduction of intra and intermolecular disulfide bridges in protamines by endogenous GSH, and

the removal of reduced protamines with the aid of an acceptor molecule, which our laboratory has found to be heparan sulfate, present in both murine and human ooplasm. The molecular basis of this process is not well understood in many species, including mouse and human (Romanato et al, 2008; Julianelli et al, 2012)). Previous reports from our laboratory have dealt with the role of glycosaminoglycans in human sperm decondensation; the present paper describes our first findings related to chromatin decondensation in murine spermatozoa.

Analysis of the effect of different GAGs on *in vitro* murine sperm decondensation resulted in an interesting finding: contrary to observations on human sperm decondensation (Romanato et al, 2003), DS was shown to be almost as active as heparin in decondensing mouse sperm chromatin, in the conditions normally used: 46 micromol/l of the GAGs and a 60 min incubation period (Figure 2). One cannot help but wonder whether this differential response in murine and human spermatozoa might be related to the percentage of histones and/or the relative amount of P1 and P2 present in mature spermatozoa in both species. Murine spermatozoa organize about 1-2% of their genome in nucleosomes, whereas up to 15 % of human sperm DNA is packaged in this manner (Johnson, Lalancette et al. 2011).

Alternatively, this differential decondensing ability of DS and heparin on human and mouse sperm nuclei could be explained in terms of the molecular characteristics of protamines P1 and P2 and the P1/P2 ratio in both species. Mouse and human protamines have similar physicochemical characteristics, such as percentage of basic aminoacids (namely lysine and arginine), isoelectric

point, number of total residues (conserved between human and mouse, although differing in sequence), with P2 including histidine in the primary structure. However, a major difference between species is the 1:1 ratio of P1/P2 found in the human sperm nucleus in comparison to a 1:2 ratio in mouse nuclei. We have previously mentioned that P2 has a lower content of cysteine residues than P1, allowing for a diminished possibility of disulfide bond formation. Additionally, P2 contains a larger amount of the acidic aminoacid, glutamate, which incorporates a negative charge into its structure and could therefore lessen its interaction with the negatively charged DNA molecule. Considering all these characteristics, it could be expected that DS, a less sulfated glycosaminoglycan compared to heparin, would show a stronger decondensing activity in mouse than in human spermatozoa and that a synergistic effect could occur between both molecules, with DS acting where P2 is more abundant and heparin in those regions of the chromatin where the more tightly bound P1 is present.

The results presented in this paper suggest that decondensation does not take place in the same way when heparin/HS or DS are used; not only was the system more sensitive to heparin than to DS (Figure 3), the maximum level of decondensation achieved was higher with heparin than with DS (Figures 2 and 4), and also the time course of the process indicated a faster response to heparin (Figure 4). Doubtlessly, these differences could well be related to the physicochemical characteristics of the molecules. Heparin and DS not only possess a different distribution of charged groups throughout the molecule, but also DS is less sulfated than heparin, and therefore has a smaller net negative charge. Consequently, a differential ability to diffuse through the sperm

membranes and to interact with chromatin could be expected for both GAGs; this paper, however, does not provide any direct evidence to support this contention.

Although murine sperm decondensation occurred faster in the presence of heparin than in the presence of DS alone, it was almost twice as fast when both GAGs were used together, an observation that prompted us to propose a possible synergism between both molecules (Figure 5). Such a synergistic behavior suggests that both GAGs would be acting together and interacting with each other in neighboring regions of the sperm chromatin rather than independently at distant places. If the latter were true, a simple additive effect on decondensation would be expected when both molecules were used simultaneously.

Although the concentrations of heparin and DS used in this experiment differ considerably, the rationale for choosing these experimental conditions was to force the system to reveal a possible synergistic behavior which could not be observed otherwise. Both glycosaminoglycans (DS and HS, the molecular equivalent of heparin and naturally present in both human and mouse oocytes) appear to be synthesized by granulose cells in response to FSH (Ax and Bellin, 1988; Tirone et al, 1993) but, to our knowledge, there is no data available on the amount present inside the oocyte. Future experiments in our laboratory will focus on the quantitation of both molecules in human and murine oocytes and, until then, the results presented herein represent the first indication of a possible interaction between these glycosaminoglycans and sperm chromatin decondensation.

Results presented here comparing chromatin decondensation in capacitated and non-capacitated spermatozoa are interesting and probably a consequence of changes in membrane composition and protein distribution that are associated with sperm capacitation. Capacitated spermatozoa attained higher levels of decondensation and also showed faster decondensation kinetics than non-capacitated spermatozoa, probably as a reflection of the role of the sperm membrane in regulating the entry of GAGs and/or GSH into the sperm cell (Romanato et al, 2005).

Another possible explanation for these differences between nuclear decondensation of capacitated and non- capacitated spermatozoa, not to be ruled out at this point, involves the perinuclear theca, a cytoskeletal structure intercalated between the inner acrosomal membrane and the nuclear envelope of the mammalian sperm head (Sutovsky et al, 1997; Oko and Sutovsky, 2009). This structure is rich in disulfide bonds and appears to be involved in chromatin stabilization; its disappearance when the sperm head is incorporated into the oocyte would allow for chromatin decondensation and, in this way, the perinuclear theca might play a role in the regulation of this process. Though the perinuclear theca seems to remain unaltered until the spermatozoon enters the oocyte (Ramalho-Santos et al, 2000), subtle modifications in its structure could be occurring during sperm capacitation and these might, in turn, alter the spermatozoon's sensitivity to decondensing reagents. This is an interesting hypothesis which will be further analyzed in future experiments.

Electron microscopy of decondensing spermatozoa enabled us to gain insight on the ultrastructural changes that take place in the sperm head during nuclear

306 decondensation. The results obtained revealed that the decondensation process
307 begins at the caudal region of the sperm head, where the tail is inserted, and
308 continues all the way up until full chromatin decondensation is attained. In
309 agreement with decondensation kinetics observed for both GAGs, DS + GSH
310 promoted a lower degree of decondensation than heparin + GSH and the
311 addition of both GAGs + GSH resulted in a fully decondensed chromatin. In
312 somatic cells undergoing cell division, it has been well established that different
313 degrees of condensation along the chromatin are responsible for uneven timing
314 when chromosomes duplicate; our findings could reflect a similar situation for
315 sperm chromatin, although at present this remains pure speculation.

316 Electron microscopy also revealed that as decondensation progressed,
317 membranes were disorganized and eventually disappeared, leaving bulks of
318 condensed chromatin on the periphery of the cell. This observation is in
319 agreement with the findings by Sanchez-Vazquez et al. (1996) who showed
320 similar results with heparin and GSH on mouse spermatozoa.

321 The fact that the decondensation process starts at the caudal region of the sperm
322 head probably reflects the lack of perinuclear theca overlying this region of the
323 nuclear membrane and is in agreement with the findings of Ramalho-Santos et al
324 (2000) who showed, using the rhesus monkey model, that persistence of the
325 perinuclear theca after ICSI prevents sperm chromatin decondensation. Once
326 again, these results could be indicative of the involvement of the perinuclear
327 theca in the regulation of the chromatin decondensation process.

328 Taken together, our findings support the idea of differences in chromatin
329 condensation throughout the murine sperm nucleus, also recognized in other

species such as the human, and probably due to the fact that not only protamines but also a certain amount of histones (variable according to the species) remain attached to DNA. This undoubtedly confers very particular and specific characteristics to sperm chromatin, with probably important effects on epigenetics and early embryo development (Pittoggi et al. 1999, Arpanahi et al. 2009, Miller et al. 2010).

In conclusion, this paper presents, for the first time, evidence that two molecules that share certain physicochemical characteristics and that are normally present in follicular fluid and the cumulus – oocyte complex, heparan sulfate and dermatan sulfate, act synergistically on murine sperm decondensation *in vitro*. This cooperative behavior in such a crucial event in mammalian reproduction - sperm decondensation is a prerequisite for male pronucleus formation - is possibly reflecting differential degrees of condensation at different regions of the sperm chromatin. This, in turn, might be of physiological relevance to the developing embryo. Studies are currently under way in our laboratory to determine whether dermatan sulfate is present in the murine ooplasm, as has already been demonstrated for heparan sulfate. As we do not know the existence or concentration of dermatan sulfate inside the oocyte, we cannot assess the relative importance for this synergism in an *in vivo* situation, but it could certainly prove useful for *in vitro* studies of sperm chromatin decondensation as a tool to discriminate between patients that show a non decondensed sperm chromatin after ICSI procedure.

Materials and methods

354 Sperm collection and capacitation

355 Animal care and manipulation was in agreement with institutional guidelines and
356 the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH
357 80–23). Animals (3 to 4 eight week old CF-1 male mice per experiment) were fed
358 *ad libitum* and kept in air-conditioned rooms at 20-28°C with a 12 h light–dark
359 period.
360 Epididymis from each mouse were removed and transferred to a dish containing
361 300 microliters of In Vitro Fertilization Medium (IVFM, 99.3 mmol/l NaCl, 2.70
362 mmol/l KCl, 0.50 mmol/l $MgSO_4$·6H2O, 1 mg/ml glucose, 0.31 mmol/l
363 Na_2HPO_4·$2H_2O$, 1.80 mmol/l $CaCl_2$·H_2O). Medium pH was adjusted to 7.3 with
364 25.07 mmol/l NaOH, and 0.0055 mg sodium pyruvate and 0.35 ml L-Na-lactate
365 (60% syrup) were added to a final volume of 100 ml. Spermatozoa were
366 recovered by cutting the isolated caudae into fragments and allowing mature
367 sperm to “swim out” into IVFM at 37ºC during 10 minutes. Tissue fragments were
368 removed and the remaining sperm suspension incubated in capacitating
369 conditions for 1 h at a concentration of 30-50 x 10^6 spermatozoa/ml. Sperm
370 motility and viability were checked in each experiment on a 10 microliter aliquot
371 of the sperm suspension, under phase contrast, in an Olympus CH2 microscope
372 at 400x magnification.
373

374 Evaluation of sperm capacitation

375 Capacitation status of spermatozoa was assessed by CTC assay and by tyrosine
376 phosphorylation (data not shown) (Barbonetti et al. 2010).

A 100 microliter aliquot of sperm suspension, containing $10x10^6$ spermatozoa/ml, was mixed rapidly with CTC stock solution and 30 s later fixed by addition of 1.6 microliters of 12.5% glutaraldehyde in 1 M Tris buffer (pH 7.8). The mixture was centrifuged for 30 s at 9500 xg and the resulting pellet washed by centrifugation with 400 microliters distilled water (three times). A 50 microliter aliquot was placed on a prewarmed slide (37°C) and left to dry overnight in the dark. One drop of DABCO mounting medium was carefully added and a coverslip placed on top. Cells were observed under the fluorescence microscope. Three main patterns of CTC fluorescence could be identified: F, with uniform fluorescence over the entire head, characteristic of non-capacitated, acrosome-intact cells; B, with a fluorescence-free band on the postacrosomal region, characteristic of capacitated, acrosome-intact cells; and AR, with dull or no fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. A bright fluorescence over the midpiece of spermatozoa could be seen at every stage. (Kong et al. 2009).

Decondensing ability of different GAGs.

Capacitated and non capacitated sperm were decondensed in the presence of 10 mmol/l GSH and 46 micromol/l heparin (13500 Da, 170 IU/mg) or each of the following GAGs: HS, CS, DS and HA in IVFM at 37°C for 15, 30, 45 and 60 min (Romanato et al. 2003). Controls consisted of parallel incubations with GAGs or GSH alone. After each time period, a 20 microliter aliquot was removed and fixed with an equal volume of 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Two 5 microliter aliquots were transferred onto a microscope slide, a coverslip

placed on top and nuclear status assessed under phase contrast in an Olympus CH2 microscope at 400x. Spermatozoa were classified (Bedford et al. 1973) as unchanged (U), moderately decondensed (M) or grossly decondensed (G) (Fig. 1). At least 200 cells were evaluated in each aliquot. Total decondensation achieved (%M+G) was determined as the sum of %M and %G. Note: heparin, structural analogue of HS, has been demonstrated to possess the same biological activity in the *in vitro* decondensation of sperm chromatin (Romanato et al, 2003) and, due to its accessibility, has been used as a substitute for heparan sulfate , in the present experiments.

Cooperative effect of heparin and dermatan sulfate on nuclear sperm decondensation.

The optimum concentrations of heparin and DS were determined by incubating spermatozoa in 10 mmol/l GSH and increasing concentrations of heparin or dermatan sulfate (0.46, 1.2, 2.3, 4.6, 9.2, 46 micromol/l). Total decondensation was determined as previously described after 15, 30, 45 and 60 min of incubation.

To evaluate the possible cooperative effect between both GAGs, chromatin decondensation kinetics in capacitated and non capacitated spermatozoa was determined in the presence of 10 mmol/l GSH and 0.46 micromol/l heparin, 46 micromol/l dermatan sulfate or the combination of both GAGs. Aliquots were drawn after 15, 30, 45 and 60 min of incubation and sperm decondensation determined as previously described.

Electron microscopy

Spermatozoa were decondensed in the presence of heparin and GSH for 60 min at 37°C and prepared for electron microscopy. Samples (heparin, GSH and heparin + GSH) were diluted 1:4 in 0.1 mol/l PBS (pH 7.4) at room temperature, thoroughly mixed, transferred to conical tubes and centrifuged at 380 xg for 10 min. Pellets (5 x 10^6 spermatozoa) were carefully fixed using 3% glutaraldehyde in PBS, at 4°C. After 18 h, fixed samples were treated with osmium tetroxide (1.3%), dehydrated with increasing concentrations of ice-cold ethanol and washed with propylene oxide at room temperature. Pellets were embedded in Eponate 12 - Araldite (Pelco, Redding, CA, USA) and sliced in an ultramicrotome with a diamond blade. Slices were analyzed with a Zeiss EM 109T electron microscope (Laboratorio Nacional de Investigación y Servicios de Microscopía Electrónica, LANAIS-MIE, Buenos Aires, Argentina) after double staining with uranyl acetate and lead citrate.

Statistical analysis

Data were analyzed using one-way analysis of variance and the corresponding post-test as indicated in each case, using the Instat 3.0 software program or GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA, USA). A P value < 0.05 was considered significant.

Declaration of interest.

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported in the present manuscript.

Author's Contributions.

Sanchez M performed the research, Alvarez Sedó C performed the electron microscopy, Julianelli V and Romanato M coached Sanchez M with the experiments, Calvo L helped with the data analysis and thoroughly revised the manuscript, Fontana V and Calvo JC had equal responsibilities in designing the experiments and wrote the paper.

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Legends to Figures

Figure 1: Morphology of decondensed murine spermatozoa. Decondensation of murine sperm nucleus as visualized with Hoechst stain (A, D, G), under phase contrast (B, E, H) and merged image (C, F, I). Panels A, B and C= unchanged, panels D, E and F= moderately decondensed, panels G, H and I= grossly decondensed. Original magnification: 400x. Scale bar: 10 micrometers.

Figure 2: Effect of GAGs on sperm decondensation. Capacitated murine spermatozoa were decondensed in the presence of 10 mmol/l GSH and each of the following GAGs (46 micromol/l each): heparin (Hep), chondroitin sulfate (CS), dermatan sulfate (DS) or hyaluronic acid (HA). Decondensation is expressed as %(M+G) and results correspond to mean ± SEM of 3 independent experiments. Decondensation achieved with Hep + GSH was significantly higher than with DS + GSH, and so was decondensation with each pair compared to GSH alone ($p<0.001$, ANOVA + Tukey-Kramer Multiple Comparison Test, n = 3). HA + GSH and CS + GSH were completely inactive (ANOVA + Tukey-Kramer Multiple Comparison Test, NS, versus GSH or heparin alone, n = 3).

Figure 3: Heparin and DS dose-response curves for sperm decondensation of capacitated and non-capacitated murine spermatozoa. Dose-response curves were obtained following 15 min incubation of murine spermatozoa in the presence of different concentrations of heparin or DS and 10 mmol/l GSH. Decondensation is expressed as %(M+G) and results correspond to mean ± SEM of 4 independent experiments. Panel A: heparin dose response curve in capacitated (•) and non-capacitated (o) spermatozoa. Decondensation was significantly higher for all heparin concentrations tested when compared to GSH alone (ANOVA+ Tukey-Kramer Multiple Comparison Test, $P < 0.01$, n = 4). Panel B: DS dose response curve in capacitated spermatozoa. Only the highest DS concentration tested resulted in a significant increase in decondensation

compared to GSH alone (ANOVA+ Tukey-Kramer Multiple Comparison Test, $P < 0.01$, $n = 4$).

Figure 4: Sperm decondensation kinetics in the presence of heparin and DS. Time course of decondensation in capacitated (left panel) and non-capacitated (right panel) murine spermatozoa, was analyzed following incubation with heparin, DS or a combination of both, in the presence of 10 mmol/l GSH. Decondensation is expressed as %(M+G) and results correspond to mean ± SEM of 8 independent experiments. Both capacitated spermatozoa and non capacitated spermatozoa showed a significant decrease in $t_{0.5}$ when 0.46 micromol/l heparin and 46 micromol/l dermatan sulfate were used together. Differences were analyzed by Sigmoidal dose-response curve fit, $R^2= 0.8585$, with preferred model being different EC_{50}.

Figure 5: Synergistic effect of heparin and DS on murine sperm decondensation. Spermatozoa were incubated with either heparin (0.46 micromol/l), DS (46 micromol/l) or a combination of both, in the presence of 10 mmol/l GSH, for 15, 45 or 60 min. Decondensation was assessed as %(M+G). Figure 5A shows the decondensation achieved by capacitated (15 min incubation, left panel, n= 5) and non-capacitated (45 min incubation, right panel, n= 4) spermatozoa. The last bar in each panel indicates the sum of the corresponding values for heparin and DS alone. In both types of spermatozoa, incubation with Hep+DS resulted in a significant increase in decondensation compared to incubation with each GAG

separately ($p < 0.01$) or to the sum of %(M+G) achieved separately (last bar in each panel, $p < 0.05$). Figure 5B shows that the synergistic effect was lost after 60 min of incubation, both in capacitated (left panel, n= 5) and non-capacitated (right panel, n= 4) spermatozoa. (ANOVA+ Tukey-Kramer Multiple Comparison Test).

Figure 6: Transmission electron microscopy of decondensing murine spermatozoa. The effect of 10 mmol/l GSH (A), 10 mmol/l GSH + 0.46 micromol/l Heparin (B), 10 mmol/l GSH + 46 micromol/l DS (C) or both GAGs + 10 mmol/l GSH (D) was examined by electron microscopy. Micrographs shown are representative of 200 spermatozoa analyzed for each experimental condition. Sperm nuclei treated with GSH (A) were uniformly electron-dense and fully condensed, with intact nuclear envelope and outer membranes. Following incubation with heparin + GSH (B) decondensation could be observed, starting at the caudal region of the sperm head (arrow); membrane disarray was evident. Incubation with DS + GSH (C) also produced decondensation starting at the caudal region (arrow), but chromatin appeared more condensed than with heparin. When both GAGs were used together (D) a higher degree of decondensation was observed (presence of “lacunar” and granulo-fibrillar areas, thin arrow), with totally disorganized membranes, including acrosomal membranes (short arrow). On the cephalic region, residues of packed chromatin still remain. Scale bars: A, 0.2 micrometers; B-D, 0.5 micrometers.

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