

Heparin enhances protamine disulfide bond reduction during *in vitro* decondensation of human spermatozoa

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BACKGROUND: Human sperm nuclear decondensation *in vivo* involves protamine disulfide bond reduction by glutathione (GSH) and protamine/histone exchange, presumably with heparan sulfate (HS) as the protamine acceptor. The aim of the present study was to test the hypothesis that these two events occur simultaneously rather than sequentially, as has been hitherto accepted, and to test for the presence of HS in the human oocyte.

METHODS: Spermatozoa and isolated sperm nuclei obtained from normal volunteers were exposed *in vitro* to heparin, the functional analogue of HS and either GSH or dithiothreitol (DTT) as the disulfide reducing agent. Decondensing reagents were added either simultaneously or sequentially. Percentage sperm nuclear decondensation was assayed by phase contrast microscopy. Thiol reduced status of isolated sperm nuclei was evaluated both indirectly [acridine orange (AO) staining of acid-denatured DNA] and directly [monobromobimane (mBBR) staining of protamine-free thiols]. The presence of HS in mature metaphase II (MII) human oocytes was analyzed by immunocytochemistry.

RESULTS: Sequential addition of reagents always resulted in significantly lower decondensation if GSH was used as the disulfide bond reducer ($P < 0.05$ for sperm and $P < 0.001$ for nuclei), but only when heparin was used first, when DTT was the disulfide reducing agent ($P < 0.05$ for sperm and $P < 0.01$ for nuclei). Both AO staining of DNA and mBBR staining of protamines revealed that the addition of heparin to GSH but not to DTT significantly increased the thiol reduced status of sperm chromatin. HS was detected in the ooplasm of zona-free MII human oocytes.

CONCLUSIONS: The results presented in this paper clearly show that heparin enhances the sperm chromatin thiol reducing activity of GSH *in vitro*, suggesting that *in vivo* thiol reduction and protamine/histone exchange could occur as simultaneous, rather than sequential, events. We also demonstrate for the first time the presence of HS in the human oocyte.

Key words: sperm nuclear decondensation / protamine disulfide reduction / glutathione / heparan sulfate / human oocyte

Introduction

During spermatogenesis, the majority of histones present in sperm chromatin are replaced by other low-molecular weight, basic proteins, namely the protamines, thereby transforming the nucleus into a highly condensed structure, which is both chemically inert and transcriptionally inactive (Pogany *et al.*, 1981). There are two types of protamine in mammalian spermatozoa, PRM1 and PRM2; both are rich in cysteine residues and thus capable of forming intra and intermolecular disulfide

bonds which contribute significantly to chromatin stability (Pongsawasdi and Svasti, 1976; Tanphaichitr *et al.*, 1978; Gusse *et al.*, 1986; Balhorn *et al.*, 1987).

Once the fertilizing spermatozoon is in the ooplasm, its nucleus undergoes a series of ultrastructural changes that eventually lead to pronuclear formation and syngamy (Tesarik and Kopečný, 1989; Lassalle and Testart, 1991). Among these, sperm nuclear decondensation occurs within the first few minutes following oocyte penetration (Berrios and Bedford, 1979; Jager, 1990) and involves the replacement

of the majority of sperm protamines by oocyte histones. This process combines two essential events: the reduction of protamine disulfide bridges (SS) to free thiols (SH) and protamine/histone exchange. It has been clearly established that protamine thiol reduction *in vivo* is carried out in a large variety of mammalian species, including human, by oocyte glutathione (GSH) (Perreault et al., 1984; Liu and Baker, 1992; Sutoovsky and Schatten, 1997) and supposedly constitutes the rate limiting step of sperm nuclear decondensation (Perreault et al., 1987). Protamine–oocyte exchange requires the participation of a high molecular weight, negatively charged molecule, functioning as protamine acceptor, which in some non-mammalian species has been identified as nucleoplamin (Ohsumi and Katagiri, 1991; Philpott et al., 1991; Rice et al., 1995). Previous work from our laboratory has led us to propose that heparan sulfate (HS), present in the mammalian (murine) oocyte, could act as protamine acceptor during human sperm decondensation *in vivo* (Romanato et al., 2008).

The molecular mechanisms of protamine disulfide bond reduction and protamine/histone exchange during mammalian nuclear decondensation have not been completely elucidated.

Traditionally, protamine disulfide bond reduction and histone-protamine exchange have been thought to occur as sequential events during nuclear decondensation (Zirkin et al., 1985). However, preliminary data from our laboratory suggested that heparin, a structural analog of HS possessing similar biological activity in all systems hitherto tested (Jackson et al., 1991; Romanato et al., 2003) and therefore extensively used as an HS substitute in *in vitro* studies, might be modulating GSH disulfide reducing activity *in vitro*. These data led us to hypothesize that both processes would be occurring simultaneously rather than sequentially *in vivo*.

The aim of the present study was to further understand the mechanism of protamine disulfide bond reduction during human sperm decondensation, particularly regarding the possible modulation of GSH disulfide reducing activity by HS, using an *in vitro* model of human sperm decondensation in the presence of GSH and heparin, which has been characterized extensively in our laboratory (Romanato et al., 2003, 2005, 2008); we also investigated the presence of HS in the human oocyte to support this contention, being aware that heparin is not present as such in the cumulus–oocyte complex.

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, 2010) semen specimens were obtained under informed consent from four normal healthy volunteers. 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 300g 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 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 23.8 mM NaHCO_3 ; 3.38 mM sodium pyruvate and 80.2 mM sodium lactate) supplemented with 0.3% bovine serum albumin (BSA). The remaining pellet was overlaid with 1 ml of 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. Subsequently, highly motile spermatozoa were incubated at a concentration of

$5-10 \times 10^6 \text{ ml}^{-1}$ in capacitating conditions for 18 h in HTF 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 de Yebra and Oliva (1993). Semen samples were washed three times by centrifugation at 1620g for 10 min in 50 mM Tris–HCl, pH 7.2 plus 0.15 M NaCl (in 10× the sample volume). Sperm pellets were resuspended in 2.6 ml of the same buffer containing 1% sodium dodecyl sulphate, incubated for 15 min at room temperature, and spermatozoa were 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 into two aliquots, each of which was placed on top of 4 ml 1.1 M sucrose in 50 mM Tris–HCl, pH 7.2 and centrifuged at 3500g for 1 h. Pellets were recovered and washed twice by centrifugation at 1620g for 10 min in 50 mM Tris–HCl, pH 7.2. Contamination of the nuclear fraction obtained by sperm tails was checked by microscopy observation. Effective removal of acrosomes was assessed by immunocytochemistry using anti-human acrosin (kind gift of Dr Mónica Vazquez Levin), as previously described (Romanato et al., 2005).

Standard sperm decondensation assay

Capacitated spermatozoa or isolated sperm nuclei were decondensed as previously described (Romanato et al., 2003, 2005) based on the original decondensation technique introduced by Reyes et al. (1989). Briefly, $3-5 \times 10^6$ spermatozoa or nuclei were incubated in HTF-26B with 46 μM heparin (Grade I-A: from porcine intestinal mucosa, Sigma Cat # H-3393) and 10 mM GSH (γ -Glu-Cys-Gly; Sigma Cat # G-4251) 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 2.5% glutaraldehyde, the percentage of decondensed spermatozoa or nuclei was determined by phase contrast in a Zeiss 47-30-11-9901 microscope at ×400 magnification, using the scoring criteria previously described (Bedford et al., 1973; Romanato et al., 2003, 2005). Spermatozoa or nuclei were classified as unchanged, moderately decondensed (M) or grossly decondensed (G), according to refringency, granular aspect and size of the nucleus. Unchanged spermatozoa or nuclei are bright and do not have an enlarged nucleus; Moderately decondensed cells or nuclei are no longer refringent but are dark and slightly enlarged; Grossly decondensed nuclei are very large, granular, gray in color and almost translucent. The percentage of decondensed spermatozoa or nuclei was calculated as the sum of the percentages of M and G. Experiments were run in duplicate and at least 200 cells or nuclei were evaluated in each sample.

Dithiothreitol versus GSH as disulfide bond reducing agent on decondensation of washed sperm and isolated nuclei

The impact of GSH on sperm nuclear decondensation was compared with that of another disulfide reducing agent, 1,4-dithiothreitol (DL-DTT, Cleland's Reagent, Sigma Cat # D-9163). The percentage of decondensation produced by different concentrations of DTT (1 and 0.1 mM) and 46 μM heparin was evaluated in whole sperm and isolated nuclei, in order to determine the optimum concentration of DTT for use in subsequent experiments. Comparison of disulfide bond reducing agents was performed on two aliquots of the same semen sample, which were incubated with 46 μM heparin plus either 10 mM GSH or the appropriate DTT concentration (1 mM for spermatozoa and 0.1 mM for nuclei) and in otherwise standard decondensation conditions. Total decondensation was determined by the usual method.

The effect of sequential use of disulfide bond reducing agent and protamine acceptor on decondensation of washed sperm and isolated sperm nuclei

Capacitated spermatozoa or isolated sperm nuclei were washed by centrifugation, and resuspended in HTF-26B medium. Aliquots of spermatozoa or nuclei were incubated with 46 μ M heparin and 10 mM GSH or DTT (1 mM for spermatozoa, 0.1 mM for nuclei) at 37°C. Decondensing agents were added either simultaneously, for 60 min, or successively, for 30 min each (heparin alone followed by GSH alone and *vice versa*). Cells or nuclei were washed by centrifugation at 2000g for 3 min with HTF-26B between incubations. Total decondensation was determined as usual.

Acridine orange staining

In order to study the effect of the disulfide bond reducer—heparin interaction on disulfide bond reduction *per se*, the thiol reduced status of human sperm chromatin was evaluated using acridine orange (AO) based on the technique introduced by Tejada *et al.* (1984). Briefly, sperm nuclei, isolated according to the protocol previously described, were centrifuged at 800g for 10 min and resuspended in HTF at a final concentration of 20–50 $\times 10^6$ nuclei/ml. Aliquots (10 μ l) were placed on slides, air-dried and fixed overnight in freshly prepared Carnoy's solution (methanol:glacial acetic acid 3:1). Slides were removed from the fixing solution and allowed to dry for a few minutes before staining. AO staining solution (final concentration 0.19 mg/ml) was prepared fresh by adding 10 ml of stock solution (1 g/l AO in distilled water) to 40 ml 0.1 M citric acid + 2.5 ml 0.3 M Na₂HPO₄ 7H₂O and adjusting pH to 2.5 (Tejada *et al.*, 1984). All solutions were kept at room temperature. Slides were covered with AO solution, kept in darkness for 5 min at room temperature and then gently rinsed in a stream of deionized water. Slides were sealed with a coverslip and analyzed by confocal fluorescence microscopy (Nikon CI) using a 490-nm excitation filter and 530-nm barrier filter. As all nuclei fluoresced both red and green, the amount of green and red present in each nucleus was quantified using ImageJ (NIH, Bethesda, MD, USA) and expressed in arbitrary units (AU). Slides were prepared in duplicate and at least 200 nuclei were evaluated in each experimental condition.

Labeling of thiol groups with monobromobimane

In order to evaluate the amount of free thiols in protamines, isolated sperm nuclei were stained with monobromobimane (mBBR) (Kosower *et al.*, 1979; Kosower and Kosower, 1987; Shalgi *et al.*, 1989). Nuclei were incubated with heparin, GSH, DTT, GSH + heparin or DTT + heparin in standard decondensation conditions, centrifuged at 800g for 10 min, washed in phosphate-buffered saline (PBS) by centrifugation and resuspended in PBS at a final concentration of 4–8 $\times 10^6$ cells/ml. Aliquots (10 μ l) were placed on slides, air-dried and labeled with thiol reagent mBBR (Sigma Cat # 69898). Freshly prepared 0.5 mM mBBR in PBS [from 50 mM stock solution in acetonitrile (Shalgi *et al.*, 1989)] was added to slides and labeling was carried out in the dark for 10 min at room temperature. Labeled slides were washed twice with PBS, sealed with a coverslip and examined under the fluorescence microscope (Zeiss 426126) using a 340–380 excitation filter and an emission filter over 450 nm. Fluorescence intensity of individual nuclei was quantified in AU using the RGB function of Image J. Slides were prepared in duplicate and at least 200 nuclei were evaluated in each experimental condition.

Immunocytochemistry of human oocytes using an anti-HS antibody

To investigate the presence of HS in the human oocyte, we performed indirect immunofluorescence on metaphase II (MII) human oocytes, obtained under informed consent from patients undergoing IVF at CEGYR (Centro de Estudios en Ginecología y Reproducción, Buenos Aires, Argentina). Immunocytochemistry was performed as previously described (Romanato *et al.*, 2008). Briefly, zona-free MII human oocytes were fixed and permeabilized with PBS containing Tween 20 and formaldehyde for 60 min at room temperature. Following incubation in blocking solution for 60 min at room temperature, oocytes were immunolabeled with monoclonal anti-HS antibody (Seikagaku Corporation, Tokyo, Japan) in PBS-BSA-normal goat serum (NGS) 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 (in PBS-BSA-NGS for 60 min at 37°C). Negative controls did not contain first antibody. Oocytes were observed under confocal microscopy.

Statistical analysis

Comparison of sperm or nuclei decondensation achieved with DTT and GSH as thiol reducing agents was performed using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer's test.

Comparison of the effect of sequential use of thiol reducing agent and protamine acceptor on decondensation was performed using one-way ANOVA followed by the Student–Newman–Keuls multiple comparisons test.

The effect of heparin on thiol reducing activity of DTT and GSH, evaluated by measuring fluorescence intensity of individual nuclei following either AO or MBBR staining, was evaluated by repeated measures ANOVA followed by the Bonferroni multiple comparisons test.

All differences were considered significant when $P < 0.05$ (Instat 3.0, GraphPad Software Inc., La Jolla, CA, USA).

Results

DTT versus GSH as thiol reducing agent in decondensation of washed sperm and isolated sperm nuclei

At a concentration of 10 mM DTT, which is the GSH concentration used in our standard *in vitro* decondensation assay, the heparin + DTT mixture resulted in loss of integrity of spermatozoa but 1 mM DTT-produced decondensation levels similar to those promoted by heparin + GSH. Further dilution of DTT to 0.1 mM DTT failed to produce sperm decondensation within the time frame tested (60 min). Accordingly, 1 mM DTT was used in subsequent experiments involving whole spermatozoa. Figure 1 depicts the percentage total decondensation obtained in washed spermatozoa using DTT as the thiol reducing agent, at two different doses, in the presence of 46 μ M heparin. Results are expressed as percentage nuclear decondensation relative to decondensation achieved with 46 μ M heparin + 10 mM GSH (% RD) which are standard decondensation conditions. One millimolar DTT showed a similar decondensing activity ($93 \pm 13\%$ RD) to 10 mM GSH, while decondensation with 0.1 mM DTT was almost zero ($3 \pm 1\%$ RD, $n = 3$ ANOVA + Tukey–Kramer, $P < 0.001$). When isolated sperm nuclei were used, 0.1 mM DTT was sufficient to produce the same level of decondensation

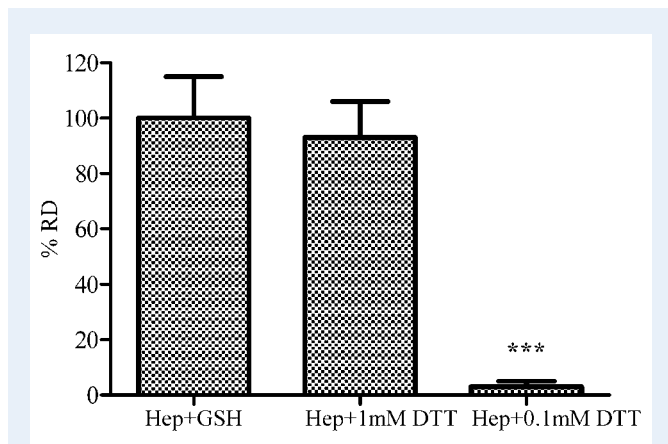


Figure 1 Dithiothreitol versus glutathione as thiol reducing agent in decondensation of washed human sperm. Washed spermatozoa were incubated in the presence of 46 μ M heparin and 10 mM GSH (standard decondensation conditions) or different concentrations of DTT and sperm decondensation evaluated by phase contrast microscopy. % RD = percentage nuclear decondensation relative to decondensation achieved with 46 μ M heparin + 10 mM GSH (maximum decondensation in standard conditions). Results are expressed as mean \pm SEM of three independent experiments. *** P < 0.001 compared to Hep + GSH

(89 \pm 6% RD, n = 3 ANOVA + Tukey–Kramer, NS) as the standard decondensation mixture while 0.25 and 1 mM DTT resulted in total loss of integrity of sperm nuclei. Thus, 0.1 mM DTT was chosen for subsequent experiments.

The effect of sequential use of thiol reducing agent and protamine acceptor on decondensation of washed sperm and isolated sperm nuclei

The effect of sequential versus simultaneous exposure to decondensing agents was evaluated in both spermatozoa and isolated sperm nuclei.

The sequential use of heparin and GSH significantly decreased the percentage of total decondensation achieved, regardless of the order in which they were used, both in intact spermatozoa (Fig. 2A) and isolated sperm nuclei (Fig. 2B). Exposure of washed sperm to both reagents simultaneously resulted in 46 \pm 8% decondensation, while exposure first to heparin and then to GSH, reduced decondensation to 27 \pm 6% and exposure to reagents in reverse order resulted in 19 \pm 7% decondensation (n = 4, ANOVA + Student–Newman–Keuls multiple comparisons test, P < 0.05) (Fig. 2A). Exposure to GSH (control) alone produced 7 \pm 4% decondensation. In isolated nuclei, 47 \pm 5% decondensation was achieved when both reagents were used simultaneously compared with only 10 \pm 3% when heparin was used first and 17 \pm 4% when GSH was used first (n = 11, ANOVA + Student–Newman–Keuls multiple comparisons test, P < 0.001) (Fig. 2B). Exposure to GSH (control) alone produced 6 \pm 4% decondensation.

In contrast, when DTT was used as a thiol reducing agent, a significant decrease in percentage decondensation following sequential versus simultaneous use of reagents was only observed when

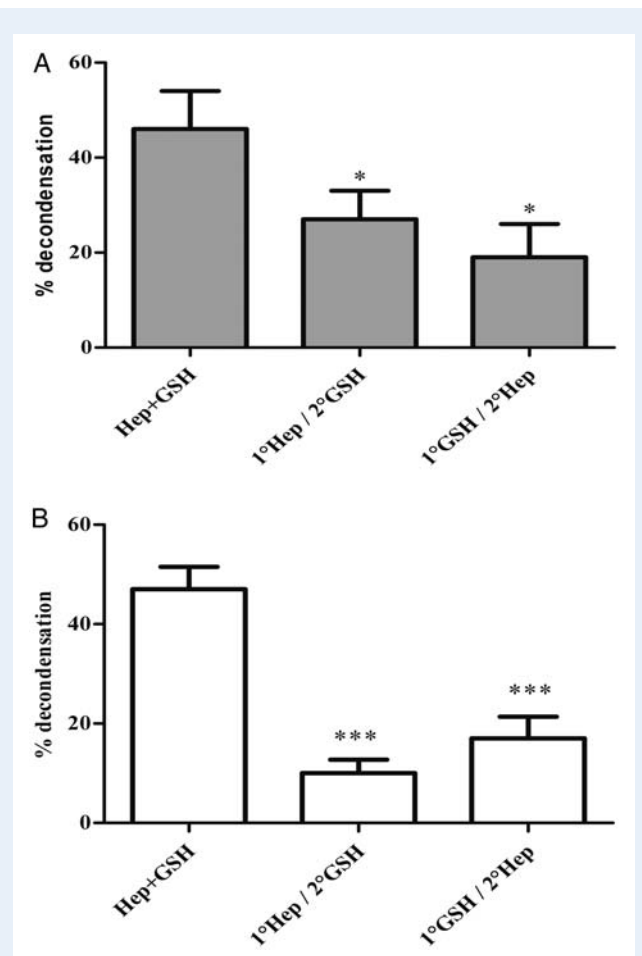
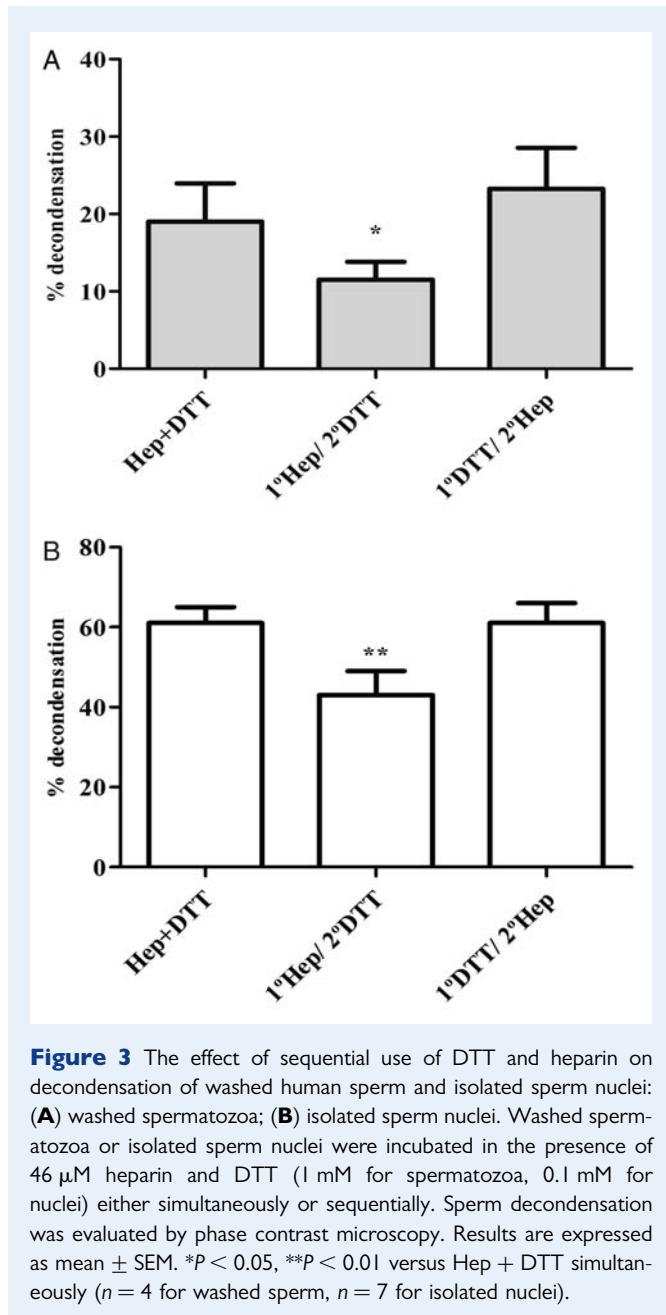


Figure 2 The effect of sequential use of GSH and heparin on decondensation of washed human sperm and isolated sperm nuclei: (A) washed spermatozoa; (B) isolated sperm nuclei. Washed spermatozoa or isolated sperm nuclei were incubated in the presence of 46 μ M heparin (Hep) and 10 mM GSH either simultaneously or sequentially. Sperm decondensation was evaluated by phase contrast microscopy. Results are expressed as mean \pm SEM. * P < 0.05, *** P < 0.001 versus Hep + GSH simultaneously (n = 4 for washed sperm, n = 11 for isolated nuclei).

heparin was used first, both in intact spermatozoa (Fig. 3A) and isolated sperm nuclei (Fig. 3B). Intact spermatozoa exposed to heparin and DTT simultaneously attained 19 \pm 5% decondensation versus 12 \pm 2% for exposure to heparin first (n = 4, ANOVA + Student–Newman–Keuls multiple comparisons test, P < 0.05) and 23 \pm 5% for DTT first (n = 4, ANOVA + Student–Newman–Keuls multiple comparisons test, NS) (Fig. 3A). Isolated sperm nuclei exposed to heparin and DTT simultaneously achieved 61 \pm 4% decondensation versus 43 \pm 6% upon exposure to heparin first (n = 11, ANOVA + Student–Newman–Keuls multiple comparisons test, P < 0.01) and 61 \pm 5% upon exposure to DTT first (n = 11, ANOVA + Student–Newman–Keuls multiple comparisons test, NS) (Fig. 3B).

AO labeling of isolated sperm nuclei

Isolated sperm nuclei were labeled with AO after exposure to thiol reducing agents GSH and DTT, with or without the addition of



heparin, following acid denaturation of DNA. AO binds to DNA and fluoresces green or red with double (non-denatured) or single-stranded (denatured) DNA, respectively. Highly reduced chromatin is more susceptible to denaturation (fluorescing red or yellow) than poorly reduced chromatin (fluorescing green) because of weak chromatin packaging. Thus, AO staining of spermatozoa following acid denaturation has been used as an indirect measure of thiol reduced status of sperm chromatin (Kosower *et al.*, 1992).

Results of AO labeling of human sperm nuclei are depicted in Fig. 4A. Untreated nuclei (control) and nuclei exposed to GSH fluoresced mostly green, those exposed to heparin alone fluoresced yellow, and those exposed to both agents simultaneously were mostly orange or red. On the other hand, in the presence of DTT, nuclei fluoresced mostly yellow and when exposed to DTT and

heparin simultaneously they fluoresced mostly red (Fig. 4A). Because the color of each cell is actually a mixture of different amounts of red and green, individual cells were characterized by quantifying the intensity (AU) of red and green fluorescence separately, and the results plotted as green versus red intensity for at least 200 cells in each experimental condition tested (Fig. 4B). The scattergrams clearly show that following exposure to GSH or medium (control), nuclei fluoresced predominantly green in color while after exposure to GSH and heparin simultaneously there was a definite shift toward red fluorescence, indicating a higher level of chromatin SS reduction. A similar shift was observed after exposure to DTT and heparin simultaneously. Interestingly, exposure to DTT alone or heparin alone also produced a shift toward red fluorescence, though not as pronounced as when both reagents were added simultaneously.

Labeling of thiol groups with mBBr

Results obtained after labeling isolated sperm nuclei with the thiol reagent mBBr are depicted in Fig. 5A. Nuclei exposed to heparin alone or buffer (not shown) appeared small and slightly fluorescent. Exposure to either GSH or DTT alone apparently increased overall fluorescence intensity due to SS reduction, but there was no change in nuclear size.

Simultaneous incubation with heparin and thiol reducing agent for 30 min. (standard decondensing conditions) promoted nuclear decondensation, revealed by the increase in nuclear size. After exposure to GSH and heparin for 30 min, 76% nuclei were large in size and after exposure to DTT and heparin, 66% nuclei increased in size; these percentages coincided with percentage decondensation evaluated on a separate unstained aliquot of each sample (results not shown). Concomitant with nuclear decondensation, there was a corresponding decrease in overall mBBr fluorescence owing to the release of protamines from DNA. Therefore, to analyze whether heparin affected SS reducing activity of GSH and DTT, nuclei were exposed to each thiol reducing agent for 30 min with the addition of heparin for the last 5 min, in order to avoid nuclear decondensation. Fluorescence intensity of each condensed nucleus (S stage, small) was measured and average fluorescence intensity calculated for each experimental condition (Fig. 5B). Fluorescence intensity after exposure to thiol reducing agent alone was similar for GSH and DTT (101 ± 14 versus 90 ± 20 AU, repeated measures ANOVA + Bonferroni Multiple Comparisons Test, NS, $n = 8$), implying that, at the concentrations used, GSH and DTT are equipotent as protamine SS reducing agents. Fluorescence intensity after exposure to heparin alone was significantly lower: 47 ± 8 AU (repeated measures ANOVA + Bonferroni Multiple Comparisons Test, $P < 0.005$, $n = 8$). Addition of heparin to GSH for 5 min significantly increased the fluorescence intensity of small nuclei compared with GSH alone (132 ± 12 versus 101 ± 14 AU, repeated measures ANOVA + Bonferroni Multiple Comparisons Test, $P < 0.05$, $n = 8$), while addition of heparin to DTT had no such effect (82 ± 22 versus 90 ± 20 AU, repeated measures ANOVA + Tukey, NS, $n = 8$), suggesting that heparin favors SS reduction by GSH but not by DTT.

Immunocytochemistry of human oocytes using an anti-HS antibody

Indirect immunofluorescence of MII human oocytes using a specific anti-HS monoclonal antibody clearly revealed a strong cytoplasmic

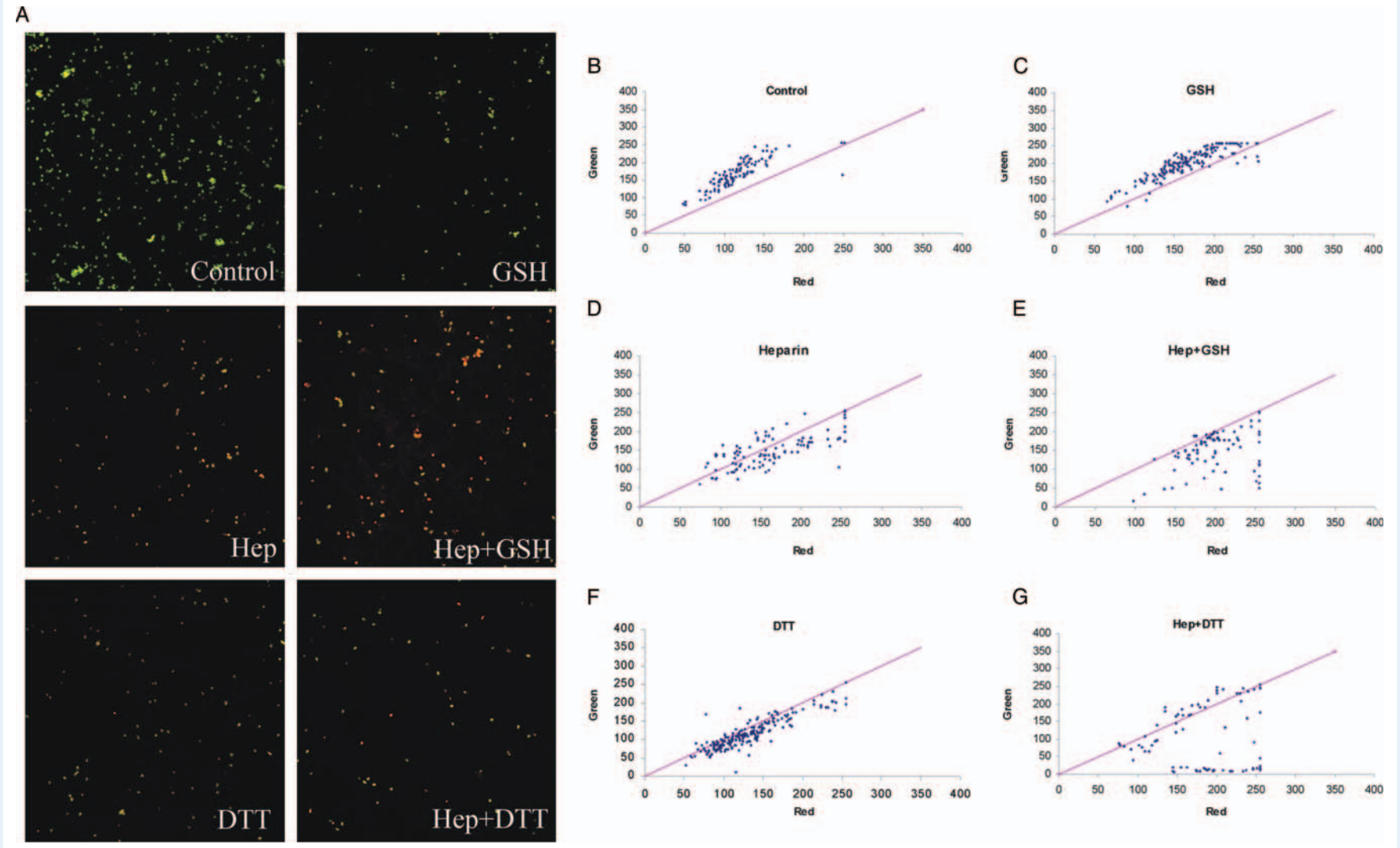


Figure 4 Acridine Orange staining of isolated human sperm nuclei. **(A)** Fluorescence microscopy of acridine orange (AO) labeled isolated sperm nuclei previously incubated in the presence of Hep, GSH, DTT or a combination of thiol reducing agent and Hep. **(B–G)** Scattergrams of AO-stained nuclei expressing green versus red fluorescence intensity (AU) for each nucleus. At least 200 nuclei were analyzed in each experimental condition. (B) control, (C) Hep, (D) GSH, (E) Hep + GSH, (F) DTT, (G) Hep + DTT.

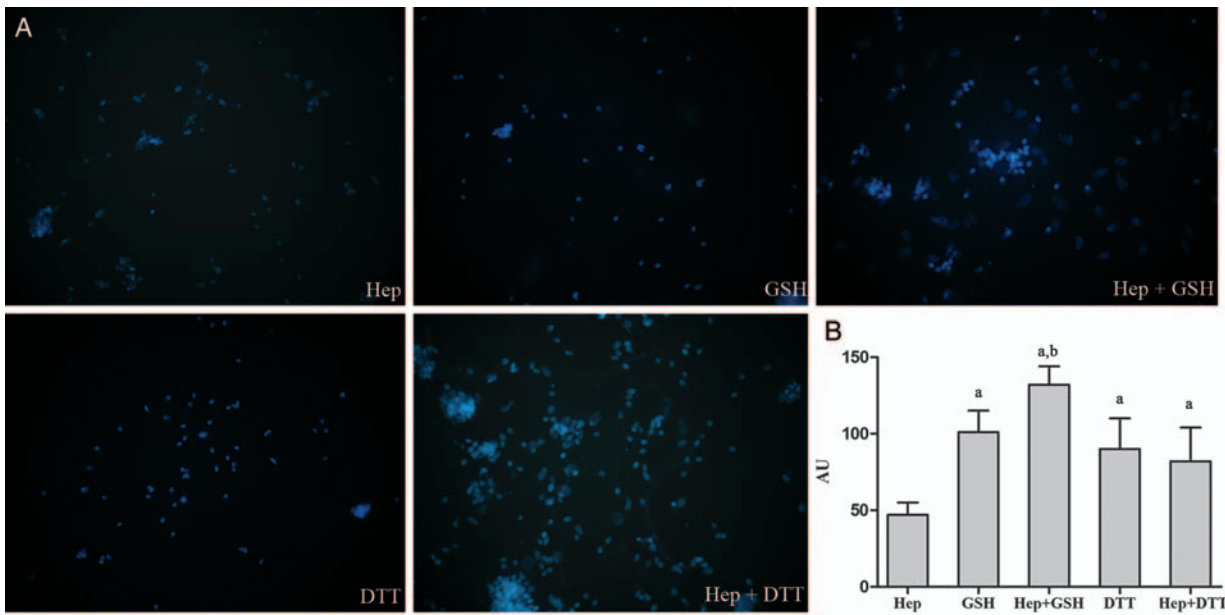


Figure 5 Monobromobimane staining of isolated human sperm nuclei. **(A)** Fluorescence microscopy of mBBR labeled isolated sperm nuclei previously incubated in the presence of Hep, GSH, DTT or a combination of thiol reducing agent and Hep. **(B)** Mean fluorescence intensity (AU) of condensed nuclei following treatment with GSH or DTT with or without the addition of Hep for the last 5 min. Results are expressed as mean \pm SEM of eight independent experiments. At least 200 nuclei were analyzed in each experimental condition. ^a $P < 0.05$ compared with Hep; ^b $P < 0.05$ compared with GSH.

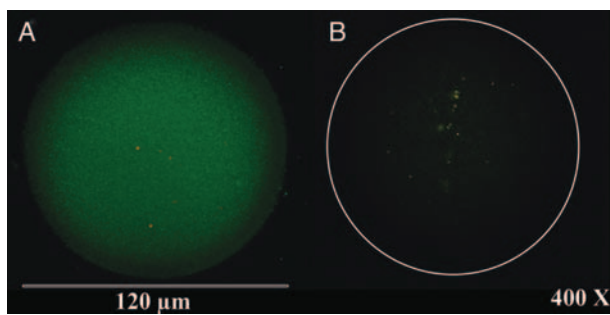


Figure 6 Immunocytochemistry of human MII oocytes using an anti-heparin sulfate monoclonal antibody. Denuded and permeabilized oocytes were incubated with **(A)** or without **(B, negative control)** anti-heparin sulfate antibody followed by fluorescein isothiocyanate labeled anti-mouse immunoglobulin M. Image shown is representative of results from 31 human MII oocytes observed in three different experiments.

fluorescent label (Fig. 6 in all oocytes incubated with the anti-HS antibody (a total of 33 oocytes evaluated in three different experiments), which could not be seen when this antibody was omitted (a total of nine oocytes evaluated = negative control).

Discussion

The present paper constitutes an attempt at elucidating the molecular mechanisms involved in human sperm decondensation, particularly

regarding protamine disulfide bond reduction by GSH and its possible modulation by heparin, and demonstrates for the first time that HS is present in the cytoplasm of human oocytes.

Initially, we compared the *in vitro* decondensing ability of GSH to that of DTT, another thiol reducing agent with a lower standard reduction potential and a completely different molecular structure: GSH is a tripeptide (γ -Glu-Cys-Gly) and DTT a thiolic derivative of the C₄ monosaccharide, threitol. The results obtained suggest that DTT is a much stronger reducing agent than GSH, particularly in isolated sperm nuclei. This behavior cannot be solely explained by the fact that DTT has a lower standard reduction potential than GSH at pH 7: -0.33 V for DTT (Cleland, 1964) compared with -0.24 V for GSH (Aslund *et al.*, 1997). Accessibility of oxidized protamines to thiol reagent must undoubtedly affect its SS reducing ability too, and this accessibility would in turn be determined by certain characteristics of the reagent itself, such as structure, size and membrane permeability, and by the nature of the protamine source. Our results show that isolated sperm nuclei, which lack the protection of extranuclear structures such as the acrosome and sperm outer membranes, required the use of significantly lower concentrations of DTT in order to achieve chromatin decondensation without affecting sperm nuclear integrity. This effect, however, was not observed when GSH was used as SS reducing agent, whose molecular structure and thus membrane permeability doubtlessly differ from those of DTT. Accessibility of heparin and GSH to sperm chromatin was already discussed in a previous report from our laboratory (Romanato *et al.*, 2005), where comparison of decondensation kinetics of isolated sperm nuclei and capacitated spermatozoa led us to conclude that the sperm plasma membrane is a powerful barrier against decondensation by these agents. Moreover, additional data (Romanato *et al.*, 2003) suggested

that only those spermatozoa whose plasma membrane had been altered during capacitation were able to decondense in the presence of heparin and GSH.

In order to test the hypothesis of a cooperative effect between heparin and GSH during sperm decondensation, we evaluated the effect of simultaneous versus sequential use of the thiol reducing agent and heparin on decondensation of washed spermatozoa and isolated sperm nuclei, using both DTT and GSH as thiol reducing agents.

Results obtained were similar for whole spermatozoa and isolated sperm nuclei, indicating, as we have previously suggested (Romanato et al., 2005), that interaction of decondensing agents with whole spermatozoa reflects their direct interaction with the nucleus. Thus, the decondensation of whole spermatozoa *in vitro* would be a good model to study sperm nuclear decondensation *in vivo* and could eventually be useful for monitoring nuclear sperm decondensation in a clinical setting.

The effect of simultaneous versus sequential incubation with thiol reducing agent and heparin differed according to which thiol reducing agent was used and according to which reagent was added first. Addition of heparin first resulted in a significant reduction of nuclear decondensation regardless of which thiol reducing agent was added second, suggesting that a compact-oxidized chromatin would not allow for maximum protamine removal by heparin. Addition of GSH first also significantly reduced nuclear decondensation while addition of DTT did not. It could be speculated that a small molecule like DTT would be able to reach protamine molecules deeply embedded in chromatin structure, while the tripeptide GSH would require the partial removal of protamines by heparin in order to do so, speaking in favor of a cooperative effect between heparin and GSH.

In an attempt to evaluate whether this cooperative effect was a consequence of the modulation of protamine SS reduction by GSH, we evaluated the thiol reduced state of sperm chromatin following the use of SS reducing agent alone or SS reducing agent + heparin. To this purpose, two different fluorescent probes were used: AO, which binds to DNA and constitutes an indirect measure of sperm chromatin thiol reduced status, and mBBr, which binds directly to free thiols in sperm protamines. The results obtained in both cases revealed that heparin enhances the ability of GSH to reduce protamine SS bridges but does not affect SS reduction by DTT, further supporting our hypothesis of a cooperative effect between GSH and heparin during sperm decondensation *in vitro*. This effect appears to be related to the chemical nature of GSH because it was not observed when GSH was replaced by DTT.

It is noteworthy that condensed nuclei that were not exposed to a thiol reducer showed different degrees of fluorescence upon mBBr staining, indicating that a certain amount of protamine thiol reduction had taken place spontaneously, without the need of a thiol reducing agent. These results are in agreement with the hypothesis that the human spermatozoon possesses an intrinsic thiol-reducing mechanism (Kvist, 1982) dependent on the presence of free thiols in protamines. Free thiols would normally be 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 and Eliasson, 1980), nuclei would be depleted of zinc and thus free thiols exposed and become susceptible to labeling by mBBr. Also, mBBr fluorescence of condensed nuclei increased significantly following exposure to either DTT or GSH alone, indicating that disulfide

reduction can indeed take place in the absence of heparin, regardless of the nature of the SS reducing agent.

Interestingly, the association between GSH and HS has also been observed in another process involving the spermatozoon during its journey toward the oocyte within the female genital tract: the interaction with oviductal cells. Such an interaction has been demonstrated in several species, including human (Baillie et al., 1997), and is fundamental for the formation of a functional sperm reservoir in the oviduct to ensure that a suitable number of viable, potentially fertile spermatozoa are available for fertilization (Talevi and Gualtieri, 2010). Direct evidence of the ability of molecules present in the oviductal fluid to release spermatozoa bound to the oviductal epithelium has been provided in cattle, where it has been postulated that heparin, like glycosaminoglycans and GSH, both of which increase in concentration at estrus (Lapointe and Bilodeau, 2003; Bergqvist and Rodriguez-Martinez, 2006), may directly modulate the affinity of sperm surface proteins for the oviductal epithelium (Talevi and Gualtieri, 2010).

Immunocytochemistry of MII human oocytes revealed that HS is present in the ooplasm with a similar distribution pattern (i.e. cytoplasmic) to that previously observed in murine oocytes (Romanato et al., 2008), emphasizing its possible involvement in human sperm decondensation *in vivo*, as has been hypothesized by our laboratory (Romanato et al., 2003, 2005, 2008).

In summary, the results presented in this paper favor the contention that during human sperm decondensation *in vivo* HS, whose presence is here documented for the first time in human mature oocytes, not only acts as protamine acceptor but also co-operates with GSH in protamine SS reduction. Accordingly, protamine SS reduction and protamine exchange with oocyte histones could be considered as simultaneous, rather than sequential, events as hitherto suggested.

Authors' roles

V.J. designed and performed the experiments, which are included in her PhD thesis, and participated in result discussion and manuscript preparation. B.F. is a graduate student and helped with the experimental work. C.A.S. provided MII zona free and permeabilized human oocytes for immunocytochemistry. M.R. performed immunocytochemistry of MII human oocytes. L.C., M.R. and J.C.C. had equal participation in experimental design, result discussion and manuscript preparation.

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Conflict of interest

The authors have no potential conflicts of interest in the present work.

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