Relationship between heparin binding characteristics and ability of human spermatozoa to penetrate hamster ova*

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Summary. Heparin binding site affinity and density on human spermatozoa were compared between fertile and infertile men with normal or abnormal results in the zona-free hamster ova–sperm penetration assay (SPA). A portion of fresh semen from fertile donors and potentially infertile men was processed through the SPA while the remainder of the ejaculate was used to quantitate heparin binding on spermatozoa. Saturation binding assays with [3H]heparin (15–375 nM) were analysed for 3 groups of men: (1) infertile patients with abnormal SPA results, (2) infertile patients with normal SPA results and (3) fertile donors. The heparin binding site density was significantly higher in men who possessed normal SPA results (infertile men and fertile donors) than in infertile men with abnormal scores in the SPA. There was no difference in heparin binding affinity between the three groups. These findings suggest that the heparin binding-site density may be related to the ability of human spermatozoa to undergo successfully the acrosome reaction.

Keywords: glycosaminoglycans; fertility; sperm penetration assay; man

Introduction

It has been proposed that glycosaminoglycans (GAGs) may be important molecules in the regulation of reproductive function (Delgado et al., 1980, 1984, 1988; Parrish et al., 1980; Lenz et al., 1983; Reyes et al., 1984; Meizel & Turner, 1986). GAGs are ubiquitous components of human tissues with detectable levels found in the genital tracts of men (Sampaio et al., 1985) and women (Lee & Ax, 1984) and in follicular fluid (Yanagishita et al., 1979; Grimek et al., 1984). These molecules are negatively charged polysaccharides composed of repeating disaccharide units with or without sulphate groups attached to the carbohydrate backbone. In tissues, GAGs usually exist in the proteoglycan form. Hyaluronate, chondroitin sulphate, dermatan sulphate, heparin sulphate, keratin sulphate and heparin are all examples of GAG molecules.

In connection with spermatozoa, GAGs have been associated with the following effects: increased capacitation and induction of the acrosome reaction in spermatozoa from bulls, boars, hamsters and humans (Lenz et al., 1983; Reyes et al., 1984; Meizel & Turner, 1986; Delgado et al., 1988); conversion of boar proacrosin into acrosin (Parrish et al., 1980); and sperm-chromatin decondensation and subsequent release of DNA-template restrictions in the human spermatozoa (Delgado et al., 1980, 1984). Most importantly, specific, saturable binding sites for GAGs have been demonstrated...
on spermatozoa from many species, including the human (Delgado et al., 1982). While these sites are best quantitated by their in-vitro ability to associate tightly with [3H]heparin, they have the capacity to bind tightly to several types of GAGs.

Recently, the quality of semen was compared to heparin binding-site density and affinity in human and bovine spermatozoa. In the dairy bull, heparin binding-site affinity but not density was positively correlated with the fertility status (non-return rates) of these animals (Marks & Ax, 1985). In the human, binding affinity and density were separately correlated with specific parameters of the semen analysis (Miller et al., 1988). Adding heparin to human in-vitro fertilization culture medium, however, did not improve fertilization or pregnancy rates (Boyers et al., 1987). An explanation for this may be that the physiologically active GAGs are those which are hydrolysed in the follicular fluid before ovulation (Delgado et al., 1987).

The zona-free hamster oocyte–human sperm penetration assay (SPA) was introduced in 1979 as an adjunct to semen analysis for the diagnosis of male factor infertility (Rogers et al., 1979). While the SPA does not directly measure the ability of spermatozoa to fertilize a human ovum, it will, at a minimum, determine whether spermatozoa can capacitate, acrosome react and fuse with the vitelline membrane. As such, results of this bioassay can be predictive of the in-vivo fertility potential of humans (Rogers, 1985).

The objective of the present investigation was to determine whether sperm heparin binding-site characteristics could be related to the fertility status in the human. Due to the fact that male factor infertility can be the result of multiple and perhaps unrelated causes, and since convincing evidence has associated GAG binding with the acrosome reaction, we chose to correlate heparin binding activity specifically with the results of the SPA.

**Materials and Methods**

*Specimens.* Semen was obtained by masturbation from potentially infertile males visiting our Fertility Unit. These men were partners in a barren marriage (> 1 year unprotected intercourse) in which a female factor was not apparent and a possible male factor was under investigation. Fertile donors from the donor insemination programme served as controls. After liquefaction, a portion of each semen sample was processed for the SPA while the remaining spermatozoa were frozen in TEST-CI buffer as previously described (Weidel & Prins, 1987) until assayed for heparin binding sites.

Three groups of semen samples were assayed for heparin binding sites: (1) patient samples with abnormal SPA results (N = 13), (2) patient samples with normal SPA results (N = 13) and with semen characteristics (sperm count, motility, morphology) that matched within 10% of the abnormal SPA samples, and (3) samples from fertile controls (N = 10).

*Sperm penetration assay.* The SPA technique of Rogers et al. (1979) was used with minor alterations as recommended by WHO. This involved a 'swim-up' separation of spermatozoa, an 18-h sperm pre-incubation period in BWW medium containing 0.3% human serum albumin at 37°C in air, and incubation of at least 30 zona-free hamster oocytes with spermatozoa adjusted to 2.5 × 10⁶ motile cells/ml. At least one fertile donor was used as a positive control in each assay. The intra-assay coefficient of variance was 3.2% and the inter-assay coefficient of variance was 14.6%. Results are reported both as a percentage penetration (no. of eggs penetrated/total no. of eggs) and as a penetration index (no. of penetrating spermatozoa/total no. of eggs). Abnormal (subfertile) SPAs were considered to be those with <10% penetration and a penetration index of <0.10. Patients who failed or scored poorly on the SPA had a repeat assay and those with normal second SPA results were excluded from this study.

*Heparin-binding assay.* A saturation binding assay (described below) was used to quantitate heparin binding sites and binding affinity. In 8 preliminary assays, spermatozoa were incubated with increasing amounts (15-1500 nM) of [3H]heparin (sp. act. 0.43 mCi/mg: New England Nuclear, Boston, MA, USA) in the presence and absence of 100-fold molar excess radioinert heparin to determine specific binding. Saturation analysis suggested the presence of two types of specific binding sites, one which was saturable and the other which was non-saturable at 1500 nM-[3H]heparin (Fig. 1a). Scatchard analysis of these data produced a curvilinear plot (Fig. 1b) which could be vectorially resolved into 2 distinct binding components; a high affinity component (Kd, 6.0 × 10⁻⁸ M) corresponding to the saturable sites and a lower affinity component (Kd, 1.3 × 10⁻⁶ M). To quantitate the high-affinity saturable sites only, further assays used a saturation range between 15 and 375 nM-[3H]heparin. A Hill plot revealed no co-operativity between the high affinity receptors.

The heparin-binding microassay of Miller & Ax (1988) for bovine spermatozoa was modified for use with human spermatozoa in the present study. Briefly, frozen semen was thawed at 21°C, washed twice (300 g; 10 min) with room
temperature Tris buffer (40 mM-Tris(hydroxymethyl)aminomethane, 2 mM-CaCl, 0.01% NaN₃, pH 7.4) and resuspended to a concentration of 1 x 10⁶ spermatozoa/ml using a Makler Counting Chamber. Microtitre 96-well filtration plates (Millititer-GV, Millipore, Bedford, MA, USA) were washed by vacuum filtration, first with assay buffer and then by 4% BSA in buffer to reduce subsequent non-specific binding. Spermatozoa (50 000/well) were incubated with increasing concentrations (15-375 nM) of [³H]heparin in the presence or absence of 50 µM radioinert heparin for 2 h at 37°C. All assay points were run in duplicate. The assays were terminated by vacuum filtration and the wells were washed twice with ice-cold Tris buffer to remove unbound [³H]heparin. The filters were punched from the microtitre plates and the cell-bound radioactivity on each filter was counted by liquid scintillation spectrometry (Beckman LS 7000) in mini-vials with 5 ml scintillation cocktail (3a70B: RPI, Mt Prospect, IL, USA). Specific binding was defined as total binding minus binding not displaced by radioinert heparin. Total specific binding sites and binding affinity were calculated by the method of Scatchard (1949). The intra-assay coefficient of variance ranged between 3.9 and 8.7% while the inter-assay coefficient of variance was 15.4%.

To validate the use of frozen spermatozoa for heparin binding assays, a preliminary study was performed comparing the binding-site quantity and affinity in fresh and frozen sperm samples. Semen from 4 donors was obtained and separated into 2 equal portions. One portion was processed immediately for heparin binding quantitation while the other portion was extended in TEST-CI buffer (Weidel & Prins, 1987), frozen, thawed 2–3 weeks later and subsequently processed through the assay. Results were analysed by a paired Student's t-test.

Statistics. Analysis of variance was used to determine statistically significant differences in semen parameters, SPA results and heparin-binding characteristics between the three groups of men in this study. After analysis of variance indicated overall differences, comparisons between specific parameters in different groups were evaluated by partial F-tests.

Results

Preliminary experiments comparing heparin-binding characteristics between fresh and frozen sperm samples revealed no difference in number or affinity of heparin-binding sites between the
two groups. Fresh spermatozoa contained $234 \pm 40 \times 10^5$ binding sites/cell with a dissociation constant of $6.41 \times 10^{-8} \text{M}$. After freezing–thawing, those same specimens contained $228 \pm 41 \times 10^5$ heparin-binding sites/cell with a dissociation constant of $7.75 \times 10^{-8} \text{M}$. It was therefore concluded that the process of freezing–thawing does not markedly affect the heparin-binding capacity of human spermatozoa. Similar findings have been reported for repeated freeze–thawing of bovine spermatozoa if cells are extended in egg yolk–citrate buffer (Miller & Ax, 1988).

The characteristics of the sperm samples from the 3 groups of men used in this study are shown in Table 1. While the semen parameters did not differ between the normal and abnormal SPA patient samples, the fertile donors showed a significantly greater mean sperm motility and post swim-up density than did those of the other two groups.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of sperm samples in potentially infertile men with normal and abnormal SPAs and in fertile donors</th>
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<tr>
<td><strong>Infertile men</strong></td>
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<tr>
<td>Abnormal SPA (N = 13)</td>
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<tr>
<td>Ejaculate volume (ml)</td>
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<tr>
<td>Sperm concentration (×10^9/ml)</td>
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<tr>
<td>Motility (%)</td>
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<td>Morphology (% normal)</td>
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<td>Post swim-up sperm density (×10^9/ml)</td>
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<td>Post swim-up sperm motility (%)</td>
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<td>Post thaw motility (%)</td>
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<td>Ova penetration (%)</td>
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<td>Penetration index</td>
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Values are mean ± s.e.m.

* $P < 0.001$, donor compared with abnormal SPA and normal SPA values.
* $b P < 0.05$, donor compared with abnormal SPA and normal SPA values.
* $c P < 0.01$, donor compared with normal SPA value.
* $d P < 0.05$, donor compared with normal SPA value.
* $e P < 0.001$, abnormal SPA compared with normal SPA and donor values.

The results of the heparin binding assays are shown in Table 2. The dissociation constant ($K_D$) for specific heparin binding was not statistically different for the 3 groups. However, the heparin binding-site density per spermatozoon was significantly higher in the normal SPA patients ($P < 0.05$) and fertile donors ($P < 0.01$) when compared to the abnormal SPA patients.

**Discussion**

Although in-vivo association of GAGs in the female reproductive tract with heparin-binding sites on spermatozoa is speculative, this putative interaction may play an important role in the process of fertilization. The formation of GAG–heparin binding site complexes could modify the acrosome of capacitated spermatozoa in a manner which drives the acrosome reaction, thereby permitting sperm–oocyte fusion. The results presented herein provide biological support for this interaction in the human by demonstrating a decreased heparin binding-site density on spermatozoa from infertile men who specifically show evidence of capacitation/acrosome reaction dysfunction. Male
partners in a barren marriage who have normal SPA results possessed heparin binding site levels similar to those of the fertile donor control group, further suggesting that the heparin binding-site density may be related to the ability of spermatozoa to capacitate and undergo the acrosome reaction. Factors other than an inadequate acrosome reaction are the likely cause of infertility in these men (Rogers, 1985).

Previous work has shown that spermatozoa from bulls with high fecundity possessed heparin-binding sites with higher affinity for $[^3]$Hheparin than did spermatozoa from bulls with low non-return rates (Marks & Ax, 1985). However, these results cannot be compared to those in the present study since Marks & Ax (1985) evaluated high- and low-affinity binding sites on a single Scatchard plot whereas only high-affinity sites were evaluated in the present study. We found no variation in heparin-binding affinity for receptor sites in spermatozoa from humans with normal or abnormal SPA results. Alternatively, species variation may account for these observed differences.

Curvilinear Scatchard plots for heparin binding have been previously presented which suggest two types of binding sites in bull, monkey and rabbit spermatozoa (Handrow et al., 1984). An additional report provides further chromatographic evidence for multiple heparin-binding domains on monkey and bull spermatozoa (Lavin et al., 1986). We have obtained similar results with human spermatozoa which show distinct high-affinity and lower affinity binding components. The significance or interdependence of these two types of heparin-binding sites is not known. While the location of heparin-binding sites on human spermatozoa has not been reported, studies with monkey and bull spermatozoa (Handrow et al., 1984) showed that $[^3]$Hheparin was associated with the sperm head whereas fluorescent heparin was associated primarily on the tail and midpiece. Perhaps this is related to the multiple types of heparin-binding sites found on spermatozoa.

In conclusion, heparin binding-site density has been positively correlated with the capacity of human spermatozoa to fuse with zona-free hamster oocytes. This further supports the hypothesis that GAGs play a role in the capacitation/acrosome reaction process.

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References


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