Original article

Identification of hyaluronic acid and chondroitin sulfates in human follicular fluid and their effects on human sperm motility and the outcome of in vitro fertilization *

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Summary — This study measured glycosaminoglycans (GAGs) in human follicular fluid (hFF) obtained from patients undergoing hormonal stimulation with combined GnRH agonist followed by gonadotropin hormone (hMG or FSH) for in vitro fertilization (IVF). The GAGs were partially characterized through the use of various mucopolysaccharidases and then their effects tested on human sperm motility. In hFF, the GAG and protein concentrations were 4.4 ± 1.3 mg/L and 32.6 ± 3.2 g/L respectively. Chondroitins (CS) and hyaluronic acid (HA) significantly stimulate sperm motion in comparison to the control. The oocytes inseminated with GAGs-pretreated spermatozoa showed a significantly higher rate of cleavage and pregnancy or delivered number. The present results suggest that hFF is a rich source of sulfated glycosaminoglycan (CS), and non-sulfated glycosaminoglycan (HA). The stimulatory effects of hFF on human sperm motility may well depend on CS and HA. GAGs pretreated sperm may enhance the fertilizing ability of spermatozoa and thus the IVF outcome.

glycosaminoglycan / human follicular fluid / spermatozoa / motility / IVF

Résumé — Identification de l'acide hyaluronique et chondroitine sulfate dans le liquide folliculaire humain et leurs effets sur la mobilité et sur le succès en fécondation in vitro. *Le rôle du liquide*

folliculaire humain (hFF) sur l'état fonctionnel de la cellule spermatique a déjà été démontré. Dans cette étude, nous avons évalué le taux des glycosaminoglycannes (GAGs) dans le hFF de patientes super-ovulées pour une fécondation in vitro. Les GAGs ont été caractérisés à l'aide des mucopoly-saccharidases. L'effet du hFF ainsi que des hFF traités par des mucopolysaccharidases des GAGs sur le pourcentage de mobilité progressive, la vitesse linéaire (VSL), l'amplitude du déplacement latéral de la tête (ALT) et le pourcentage d'hyperactivés (HPA) a été également testé. Le taux des GAGs et des protéines dans le hFF était de 4,4 ± 1,3 mg/L et 32,6 ± 3,2 g/L respectivement. Ces résultats suggèrent que le hFF est une source riche en GAGs sulfatés (chondroïtine sulfate) et des GAGs non sulfate (acide hyaluronique). Les GAGs augmentent significativement le pourcentage des spermatozoïdes mobiles par rapport au témoin. Ces résultats montrent qu'au delà du maintien et l'amélioration de la mobilité des spermatozoïdes les GAGs semblent jouer un rôle important dans l'implantation des embryons obtenus en fécondation in vitro.

glycosaminoglycannes / fluide folliculaire humain / spermatozoïde / mobilité / FIV

INTRODUCTION

To fertilize the oocyte, spermatozoa must undergo a series of changes during capacitation and the acrosome reaction, which lead to the expression of their fertilizing ability. These processes, which usually take place in the female genital tract, are characterized by surface alterations in the membrane proteins, membrane component fluidity, cyclic adenosine monophosphate (AMPc) levels, and hyperactivation of spermatozoa (Yanagimachi, 1988). Some biological fluids, ie, serum (Makler et al, 1984), peritoneal fluid (Soldati et al, 1989) and follicular fluid (Tesarik, 1985; Suarez et al, 1986; Hamamah et al, 1995), have been proposed to stimulate sperm motility. Many studies have provided evidence for a positive effect by human follicular fluid (hFF), based on the changes in spermatozoa motility (Mbizvo et al, 1990; Mendoza and Tesarik, 1990; Siegel et al, 1990). These include an increase in the penetration rate in the hamster egg penetration test (Yee and Cummings, 1988) as well as in the fertilization rate of in vitro human fertilization (Ghetler et al, 1990). Hyperactivation, also observed after hFF treatment, has been described for human spermatozoa as well as other species. Such a phenomenon has been well documented, especially in hamster sperm (Suarez et al, 1991). Siiteri et al (1988) identified a 50 kDa protein in the follicular fluid which initiates the acrosome reaction. However, Osman et al (1989) and Blackmore et al (1990) suggested that progesterone and 17 α-hydroxyprogesterone from hFF are the possible stimuli for the acrosome reaction. Glycosaminoglycans (GAGs) have already been isolated from bovine follicular fluid and hFF and seem to play a role in the preservation of sperm motility as well as in the induction of the acrosome reaction of spermatozoa (Lenz et al, 1982; Eriksen et al, 1991). GAGs are composed of repeating disaccharide units, which are generally covalently attached to a protein core to form a proteoglycan. Chondroitin sulfate (CS) was identified as the predominant GAG of follicular proteoglycans. In addition, hFF appears to contain chondroitin sulfate, which improves the retention of motility of human spermatozoa (Eriksen et al, 1994). Hyaluronic acid (HA) was also identified as a component of GAGs prepared from follicular fluid. Recently, Kornovski et al (1994) found a hyaluronan receptor on the surface of human spermatozoa. The aim of this study was to evaluate the GAGs and proteins in hFF, and to elucidate the effects of hyaluronic acid and chondroitin sulfate GAGs on human sperm function and IVF outcome.

MATERIALS AND METHODS

Chemicals

Chondroitinase ABC (Proteus vulgaris), AC (Arthrobacter aurescens), leech hyaluronidase (type X), keratinase (Pseudomonas sp), heparitinase I (Flavobacterium heparium), α-L-cysteine, and BSA (type V) were obtained from Sigma. Ethyltrimethyl ammonium bromide (Prolabo), agarose LITEX HSB (Tebu), toluidine blue O, folin-ciocaleus (Merck). Chondroitin B sulfate sodium salt was obtained from Calbiochem and dimethylmethylene blue chloride from Serva.

Sperm preparation and motility analysis

Semen specimens were obtained from normozoospermic patients participating in our in vitro fertilization (IVF) program ($n = 40$). After liquefaction, 2 mL of each semen sample was layered onto a discontinuous Percoll density gradient (1 mL of 40, 55, 70, 80, 90 and 100%), and then centrifuged at 300 g for 20 min. After centrifugation, the 90 and 100% layers were washed in B2 Menezo medium (bioMerieux, Marcy-l'Étoile, France) and centrifuged at 300 g for 10 min. The spermatozoa pellet was mixed with B2 medium to obtain a final sperm concentration of 10–15 x 10^6 spermatozoa/mL.

A motility analyzer IVOS, version 7.4 G (Hamilton-Thorn Research, Inc, Denver CO, USA) was used to measure the sperm motility and movement characteristics. The measurements were made at 37 °C after 2 and 24 h of incubation on both the Percoll spermatozoa fraction incubated in B2 Menezo medium used as control (B2-Percoll sperm) and the Percoll spermatozoa fraction incubated in B2 supplemented with GAGs (GAGs-Percoll sperm). The motility parameters included motility percentage, progressive motility percentage, straight line velocity (VSL), amplitude of lateral head displacement (ALH), and hyperactivation percentage (HPA) which was characterized by a high VSL > 100 < μm/s, low linearity < 65%, and ALH > 7.5 μm. At least 100 motile spermatozoa were analyzed in three to nine different fields for each sample.

hFF preparation

hFF samples were aspirated from mature follicles obtained from patients undergoing IVF. The ovarian stimulation protocol included the combined use of GnRH agonist and gonadotropin hormone (hMG or/and FSH) (Barrière et al, 1987). The hFF obtained from several patients was centrifuged in sterile tubes (600 g, 10 min) at room temperature. The supernatant was filtered using 0.22 μm Millipore filters, and stored in aliquots at −18 °C until use.

Incubation test of spermatozoa in hFF or enzyme-treated hFF

Sperm was adjusted to 15–20 x 10^6 mL after Percoll preparation and divided into three aliquots: one aliquot was diluted in B2 medium and used as a control; the second was incubated with hFF 20% (final concentration); and the third was incubated with 20% pretreated various mucopolysaccharidases hFF (treated hFF). All the tests were performed for 2 and 24 h at 37 °C.

Preparation of hFF after enzyme hydrolysis

Individual hFF ($n = 8$) was diluted 1:10 in 1 M sodium acetate buffer containing 1.5 M NaCl (pH 7.3), and divided into four aliquots. One aliquot was used as a control and the others were enriched with hyaluronidase (HYase), chondroitinase ABC (ABCase), and chondroitinase AC (ACase), at the rate of 50, 0.5, and 0.5 mU, respectively, in 800 μL of hFF. The samples were incubated for 16 h at 37 °C. Inactivation of the enzyme was undertaken using a 100 °C water bath for 1 min. The samples, including the control, were frozen-thawed. After thawing the samples were incubated with 1 mg of pronase for 800 μL hFF under similar conditions and the enzyme activity was stopped as above. All samples were centrifuged at 4 000 rpm for 5 min, and the supernatants were frozen until use.

Quantitative determination of GAGs

GAGs were estimated according to Taylor and Jeffree (1969) adapted on a centrifugal analyzer (COBAS FARA II F Hoffmann-La-Roche and Akjtiengesselschaft CH-Basel) by De Jong et al (1989), and stained by 1,9-dimethyl blue chloride. The standardizing scale (GAGs) was carried out with successive dilution's of chondroitin B sulfate at the rate of 2.5, 5, 10 and 20 mg/L.

Electrophoresis

Sample preparation

The hFF samples ($n = 8$) were, before electrophoretic migration, treated by reduction with α-L-cysteine (1 mL hFF with 10 mg cystein at 37 °C for 4 h) and stored at 4 °C; 500 µL of hFF was treated with hyaluronidase (HYase) diluted at 10 mU for 50 µg GAGS in 100 µL of enzyme solutions, incubated overnight at 37 °C, and stored at 4 °C. The Hyase solution was prepared in 1 M sodium acetate buffer containing 1.5 M NaCl (pH 5.5).

Electrophoresis

Samples were identified by agarose gel 0.9% in 0.1 M veronal buffer (pH 8.2) on glass plates 10 x 10 cm at 35 V for 30 min. The plates were fixed 1 h with 0.1% Cetavlon, dried overnight at 37 °C, stained with toluidine blue O (4 g/L) of distilled water/acetone (1 vol/4 vol) and finally pure water.

Protein estimation in hFF

The quantification of proteins was carried out using a spectrophotometric method (UVICON 930 Kontron Instruments, Saint-Quentin, France) according to Lowry et al (1951).

IVF procedure

Abnormal semen from patients participating in the IVF program was prepared via a discontinuous Percoll gradient, divided into two aliquots. One aliquot was preincubated with B2 medium supplemented with 20% commercialized GAGs and the second was preincubated in B2 medium (bioMerieux, France). Oocyte-corona-cumulus (OCC) retrieval was carried out by vaginal ultrasound-guided puncture 36 h after hCG. The OCC were transferred directly into a Falcon tube with B2 medium and were incubated at 37 °C in an humidified atmosphere of 5% CO_2. The aspirated OCC samples from each patient were randomly divided into two groups with respect to morphological signs of maturation. One group of OCC was inseminated with spermatozoa preincubated with B2 medium supplemented with GAGs (SPZ-GAGs). The second group of OCC was inseminated with spermatozoa preincubated in B2 medium and used as (controls). The IVF outcome was analyzed in terms of fertilization percentage, cleavage percentage and pregnancy (ongoing) or delivered.

Statistical analysis

The values were expressed as mean ± standard error of the mean (SEM). Data were analyzed by the non-parametric Wilcoxon signed-rank test. Comparison between groups was carried out using the Mann-Whitney U test.

RESULTS

Chemical and biochemical characterization of GAGs

The quantitative determination of GAGs and proteins concentrations for hFF were 4.4 ± 1.3 mg/L and 32.6 ± 3.2 g/L, respectively.

Using agarose gel 0.9%, the electrophoretic data of hFF before, used as control (No 1 to 8), and after HYase treatment (No 1' to 8') of each hFF sample are shown in figure 1. After this treatment, a faster band appeared, which corresponded to hydrolysis of HA.
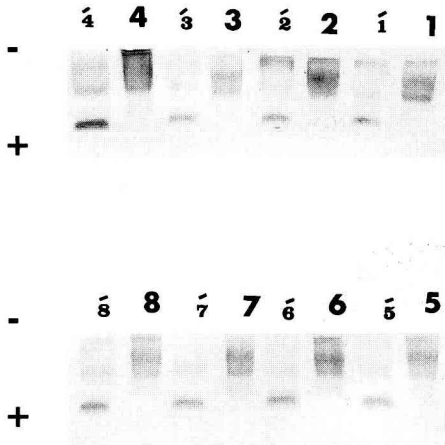
Fig 1. Electrophoretic data of human follicular fluid before (No 1 to 8), and after hyaluronidase treatment (No 1' to 8'). After this treatment, a faster band appeared which corresponds to the hydrolysis of HA. GAGs were stained with toluidine blue O.

The major components of the GAGs were identified as HA and chondroitin sulfates A and C, based on the finding that the HYase and ABCase or chondroitinase AC (ACase) have hydrolyzed the GAGs present in hFF. However, the keratanase (KSase) and heparan sulfatase (HSase) did not hydrolyze any constituents of hFF in these samples. These observations were supported by studying the GAGs which remained undigested during the KSase and HSase action (table I). Chondroitin sulfates AC, and HA were digested by ABCase, ACase and HYase respectively and represent the major constituents of hFF GAGs.

hFF, CS and HA effects on spermatozoa motility

After sperm preparation by Percoll gradient, the spermatozoa incubated in B2 medium supplemented with untreated hFF were more motile than raw semen ($p < 0.05$). When the spermatozoa were incubated in B2 medium supplemented with 20% (final concentration) treated hFF (hFF was treated before use with chondroitinase or HYase), we observed a significant decrease in the percentage of motile spermatozoa in comparison with the sperm incubated in B2 medium (fig 2). In order to pro-

Table I. Characterization of GAGs in hFF from patients undergoing ovarian stimulation for in vitro fertilization.

hFF sample	GAGs characterized				
	HA	CSAC	CSB	KS	HS
6	+	+	–	–	–
1	+	+	–	–	–
2	+	+	+	–	–
3	+	+	+	–	–
7	+	+	+	–	–
4	+	+	+	–	–
5	+	+	–	–	–
8	+	+	–	–	–

HA: hyaluronic acid; CSAC: chondroitin sulfate A and C; CSB: dermatan sulfate; HS: heparan sulfate; KS: keratan sulfate. (+) Presence, (–) absence.
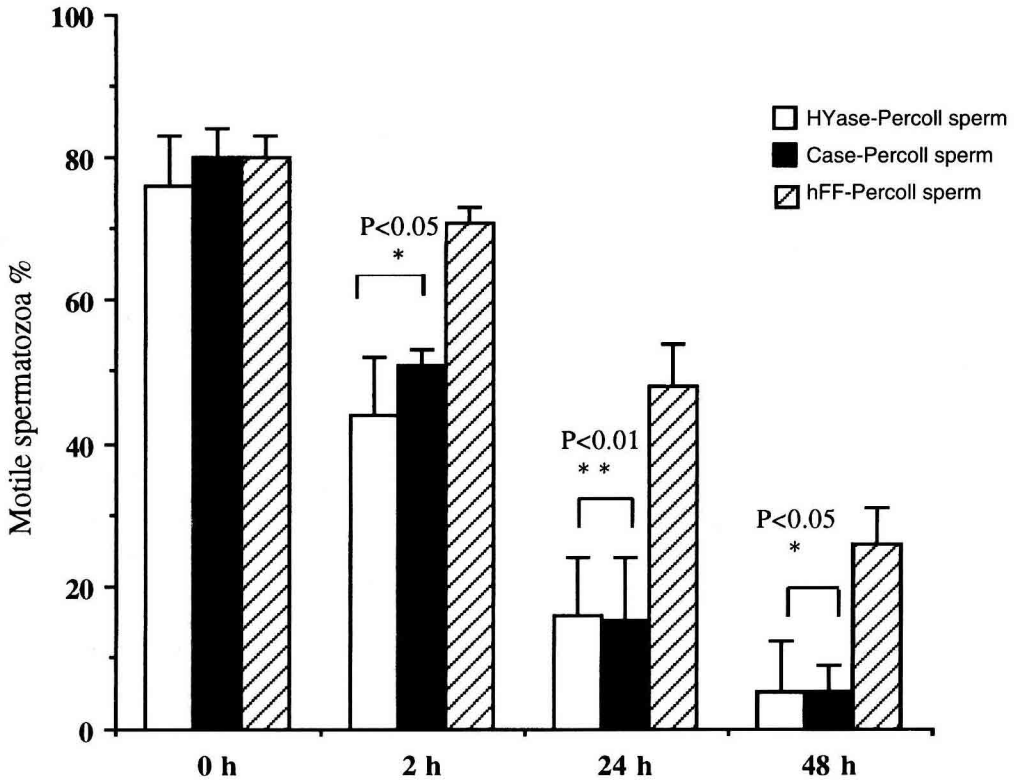
Fig 2. Percentage of motile spermatozoa after 2, 24 and 4 h of incubation in B2 medium supplemented with hFF or hyaluronidase and chondroitinase. The data represent the mean ± SEM. * $P < 0.05$, ** $P < 0.01$ compared to hFF-Percoll sperm.

Table II. Motility parameters of spermatozoa incubated either in B2 medium (control) or B2 medium supplemented with GAGs (chondrointin sulfate and HA)*.

Treatment	Spermatozoa motility (%)	Progressive motility (%)	VSL (µm/s)	ALH (µm)	HPA (%)
B2 medium (2 h)	59 ± 2	28 ± 4	54 ± 3	4.0 ± 0.3	3 ± 1
B2-GAGs (2 h)	69 ± 3[a]	33 ± 2	59 ± 2[a]	4.8 ± 0.4[a]	5 ± 1[a]
B2 medium (24 h)	48 ± 4	23 ± 3	48 ± 5	3 ± 0.2	1 ± 1
B2-GAGs (24 h)	54 ± 7	29 ± 6[a]	53 ± 3[a]	4 ± 0.3	3 ± 1[a]

* Values are means ± SEM (n = 12). [a] $P < 0.05$ compared with control.

vide evidence for CS and HA effects on sperm motility, we compare, in table II, the motility parameters of spermatozoa incubated either in B2 medium (control) or B2 medium supplemented with GAGs (CA, HA). After a short incubation period (2 h), the motility percentage VSL, ALH and HPA were significantly increased after incubation in the treated group in comparison with the control. After a long incubation period (24 h), there were no significant differences between the two groups in terms of percentage motility and ALH values. However, the percentage of progressive motility, VSL, and HPA were significantly higher in the treated group than in the control one ($P <$ 0.05).

GAGs effect on IVF outcome

The IVF outcomes are summarized in table III. Transfer was possible in 19 versus 5 of 31 cycles with the GAG-pretreated spermatozoa and control groups, respectively. The proportion of transferable cleaved embryos was slightly higher in the treated group in comparison to the control. There was no difference in fertilization rate betwen the treated and control groups. However, the cleavage rate as well as the pregnancy (ongoing) or delivered were significantly different for the treated group as compared with the controls.

DISCUSSION

During the sperm transport in the female genital tract, spermatozoa are exposed to various fluids such as peritoneal fluid and follicular fluid. It has become evident that the hFF creates a micro-environment which is important to the normal sperm function of fertilizing the oocyte. This micro-environment includes the presence of steroids, pituitary hormones, proteins, and GAGs. The GAGs from hFF have been shown in vitro to stimulate numerous sperm functions that are involved in the fertilization process and enhance the influx Ca^{2+} during the acrosome reaction (Triana et al, 1980). However, Osman et al (1989) proposed that hFF progesterone and 17α-hydroxyprogesterone induce the acrosome reaction. Furthermore, Blackmore et al (1990) and Tesarik et al (1992) have reported plasma membrane binding sites for progesterone on human sperm. Recently, Zamir et al (1993) and Anderson et al (1994) have characterized an atrial natriuretic peptide in hFF as the stimulus of the human acrosome reaction.

In this report, the presence of chondroitin sulfates and HA in untreated hFF were observed by electrophoretic analysis. The treatment of hFF with chondroitinase, and HYase resulted in a significant decrease of the percentage of motile spermatozoa after 24 and 48 h incubation in comparison to the control. These results indicate that the stim-

Table III. Glycosaminoglycan supplementation effects on IVF outcome [a].

Treatment	No of oocytes	Fertilization (%)	Cleavage (%)	No of transfered embryos	No of pregnancy (ongoing) or delivered
SPZ-GAGs	127	58 ± 6	58 ± 7b	19	10[b]
Controls	132	49 ± 6	46 ± 6	5	1

Values are mean ± SEM. [a] IVF attempts n = 31. [b] $P \leq 0.05$ compared with control.

ulatory effects of hFF may depend on chondroitin sulfates and HA. Such results are in agreement with other studies on bovine follicular fluid or hFF that demonstrate that the chondroitin sulfates proteoglycan and HA influence sperm capacitation as well as acrosome reaction (Grimek and Ax, 1982; Lenz et al, 1983; Bushmeyer et al, 1985; Eriksen et al, 1994). On the other hand, in human sperm, HA has been shown to induce capacitation (Karlstrom et al, 1991) and to maintain the sperm motility (Huazar et al, 1990). More recently, Kornovski et al (1994) and Ranganathan et al (1994) have described the hyaluronan receptor on the surface of human, rat and bull sperm. However, we observed considerable inter-hFF sample variation in terms of chondroitin sulfate concentrations, particularly when the hFF was obtained after ovarian stimulation with or without GnRH agonist and gonadotropin hormone (unpublished data).

Chondroitin sulfates as well as HA have already been found in cumulus cells surrounding the human and bovine oocyte (Drahorad et al, 1991; Ball et al, 1982, respectively). Although Hurst et al (1989) demonstrated that the bovine sperm surface contains receptors that are specific for glycosaminoglycans, the origin of GAGs in hFF remains as yet unknown. It is possible that the GAGs from hFF may be transported from the cumulus-oophorus oocytes cells to the follicular fluid via the granulosa cells.

The percentage of cycles with embryo replacement was high in the treated group in comparison with controls. The implantation rate was high for the embryos of the treated group in spite of the fact that the mean number of embryos transferred was similar in the two groups. The improved cleavage rate and particularly the ongoing pregnancy rate or delivered number was high in the treated group in comparison with controls. While it is difficult to propose a valid explanation for these results, this study suggested that the pretreatment of spermatozoa with GAG may produce better embryos to be transferred or involved in the implantation process. However, Hook et al (1984) reported that GAGs may exert their influence on cell behavior via an effect on cell-to-cell adhesion. Recently, Hemmings et al (1994) reported that the hFF supplementation under IVF conditions has been associated with a significantly higher proportion of pre-embryos reaching the morula and blastocyste stage. As follicular fluid contains many of the growth factors that have been involved in early mammalian embryo development the currently accepted idea is that growth factors bind or interact with GAGs to exert a cell proliferation effect (Sato et al, 1991). However, the molecular basis for the action by which GAGs promote embryonic development remains to be determined.

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