Reprod Nutr Dev (1993) 33, 429-435

Original article

Treatment of human spermatozoa with follicular fluid can influence lipid content and motility during *in vitro* capacitation

S Hamamah [1], M Lanson [2], C Barthelemy [1], MA Garrigue [3], J Lansac [1], JP Muh [3], D Royere [1]

[1] *Unité de Biologie de la Reproduction, Département Gynécologie–Obstétrique;*
[2] *Laboratoire de Biologie des Tumeurs;*
[3] *Laboratoire de Biochimie, INSERM U316, CHU Bretonneau, F-37044 Tours, France*

(5th Franco-Czechoslovak Meeting, Jouy-en-Josas, December 1992)

Summary — In order to evaluate the role of human follicular fluid (HFF) on the fertilizing capacity of spermatozoa, we studied the effect of HFF on the lipid composition and on the movement characteristics of human spermatozoa. Spermatozoa (spz) from normospermic patients were prepared with a discontinuous Percoll gradient and incubated in Ménézo B2 medium with or without a supplement of 20% HFF (HFF-Percoll spz and B2-Percoll spz respectively) for 2 and 24 h. After 2 h HFF incubation, percentage progressive motility, straight line velocity (VSL), and amplitude of lateral head displacement (ALH) were improved in HFF-Percoll spz as compared to B2-Percoll spz ($P \leq 0.05$). After a longer incubation period (24 h), lipid changes appeared in HFF-Percoll spz with lower levels of cholesterol ($P = 0.02$) and phospholipids ($P = 0.05$). No modification of the cholesterol/phospholipid ratio after 2 and 24 h of incubation in either B2-Percoll spz or HFF-Percoll spz was observed. Such decreases in lipid content of HFF-Percoll spz may be factors which could be taken into account as constituting part of membrane modifications during the capacitation process.

human spermatozoa / lipids / motility / follicular fluid

Résumé — Effet du liquide folliculaire sur la mobilité et les lipides membranaires des spermatozoïdes humains après capacitation *in vitro*. *Pour mieux comprendre le rôle du liquide folliculaire humain (HFF) sur le pouvoir fécondant des spermatozoïdes, nous avons étudié l'effet de HFF sur la composition des lipides et la mobilité des spermatozoïdes humains. Les spermatozoïdes de sujets normospermiques ont été isolés par centrifugation sur un gradient discontinu de Percoll puis incubés dans du milieu Ménézo B2 supplémenté ou non avec 20% HFF (respectivement spz HFF-Percoll et spz B2-Percoll) pendant 2 h et 24 h. Après 2 h d'incubation avec HFF, le pourcentage de mobilité progressive, la vitesse linéaire (VSL) et l'amplitude du déplacement latéral de la tête (ALT) sont augmentés dans les spz HFF-Percoll comparés aux spz B2-Percoll ($P \leq 0,05$). Après une incubation plus longue avec HFF (24 h), des modifications de la composition en lipides apparaissent dans les spz HFF-Percoll avec une diminution du cholestérol ($P = 0,02$) et des phospholipides ($P = 0,05$). Aucune variation du rapport cholestérol/phospholipides n'est observée pour les spz HFF-*

Percoll ni pour les spz B2-Percoll après 2 h ou 24 h. Une diminution du contenu lipidique des membranes plasmiques des spermatozoïdes pourrait être un des facteurs impliqués dans les modifications membranaires de la capacitation.

spermatozoïde humain / lipides / mobilité / liquide folliculaire humain

INTRODUCTION

To fertilize the oocyte, the spermatozoa must undergo an acrosome reaction, the ultimate step for successful fertilization. Such a process needs spermatozoal modifications leading to capacitation. This process usually takes place in the female genital tract, and includes an alteration in membrane proteins, an increase in membrane fluidity, and hyperactivation movement of the spermatozoa (Yanagimachi, 1988). It has been demonstrated that treatment of human spermatozoa with human follicular fluid (HFF) can enhance their ability to penetrate zona-free hamster oocytes and the fertilization rate of human *in vitro* fertilization (Yee and Cummings, 1988; Ghetler *et al,* 1990). Similarly, this treatment can increase the percentage of capacitation, acrosome reaction and hyperactivation of human spermatozoa (Mbizvo *et al,* 1990; Mendoza and Tesařík, 1990; Siegel *et al,* 1990; Falcone *et al,* 1991). Different factors such as glycosaminoglycans from bovine follicular fluid have been considered to have effects on the function of spermatozoa (Triana *et al,* 1980). However, Osman *et al* (1989) and Blackmore *et al* (1990) proposed that HFF progesterone and 17α-hydroxyprogesterone are involved in the initiation of the acrosome reaction in human spermatozoa.

Such an activity may be mediated *via* lipid changes in the spermatozoa membrane. The present study was performed to analyze the lipid content and the movement characteristics of human spermatozoa when incubated in B2 medium or in B2 medium supplemented with 20% (v/v) HFF. The effect of human follicular fluid on the fertilizing ability of spermatozoa was also evaluated.

MATERIAL AND METHODS

Human follicular fluid preparation

Human follicular fluid samples were aspirated from mature follicles of patients undergoing *in vitro* fertilization. Ovulatory induction combined the GnRh agonist and gonadotropin hormone (hMG or/and FSH) in short or long protocol. The HFF were pooled and centrifuged in sterile tubes at room temperature. The supernatant was filtered and stored in aliquots at –18°C until use.

Preparation and HFF treatment of spermatozoa

Normospermic semen were collected from 19 patients participating in our *in vitro* fertilization (IVF) program for female tubal infertility. After liquefaction, the semen parameters were evaluated using the standard methods described by the World Health Organization laboratory manual (WHO, 1987): sperm count 76.5 ± 7 x 10^6/ml; motility 38 ± 3%; normal forms 24 ± 3%.

Two ml of each semen were layered onto a discontinuous Percoll gradient according to Berger *et al* (1985) in sterile polystyrene tubes and then centrifuged (300 *g;* 20 min). After centrifugation, the 80 and 90% layers were resuspended, washed in Ménézo B2 medium, and centrifuged (300 *g,* 10 min). The pellet was resuspended in B2 medium to obtain 10–15 x 10^6 spermatozoa (spz). Percoll pelleted spermatozoa were incubated in B2 medium supplement-

ed with 20% (v/v) HFF (HFF-Percoll spz) or in B2 medium (B2-Percoll spz) for 2 and 24 h.

Motility analysis

A motility Analyzer IVOS version 7.4 G (Hamilton–Thorn Research, USA) was used to measure the sperm velocity and movement characteristics of Percoll pelleted spermatozoa incubated in B2 medium or in B2 medium with HFF for 2 and 24 h. The measurements were made at 37°C. The motility parameters included motility percentage, progressive velocity percentage, straight line velocity (VSL; μm/s), amplitude of lateral head displacement (ALH; μm), and hyperactivation percentage (HA) (which was characterized by a high VCL > 100 μm/s, low linearity < 65% and ALH > 7.5 μm). At least 100 motile spermatozoa were analyzed in 3 to 9 different fields for each sample.

Extraction and quantification of lipids

Percoll pelleted spermatozoa incubated in B2 medium or in B2 medium with HFF were resuspended in 200 μl saline solution and kept frozen at –20°C. Lipids were extracted using the Bligh–Dyer method (1959) from ≈ 10 x 10^6 spermatozoa. The aqueous layer was again extracted using 2 ml chloroform according to Sugkraroek *et al* (1991). The 2 chloroform extracts were pooled and stored at –20°C. Two-thirds of the lipid extract was evaporated to dryness under a stream of nitrogen at room temperature.

Phospholipids were quantified by their phosphorus content according to the method of Bartlett (1959). The lipid residue was digested with sulfuric acid to obtain free inorganic phosphorus from phospholipids. The colorimetric phosphorus assay was then used. The final volume of the assay was reduced 4 times to increase the sensitivity of the method. The absorbance is a linear function of the phosphorus content from 5 to 40 nmol per sample. The reproducibility was 5%.

The remaining lipid extract was evaporated to dryness and used for cholesterol assay. The residue was dissolved with isopropyl alcohol and the assay was carried out following the technical instructions for the A-Gent cholesterol test (Abbott, USA). Cholesterol esters were hydrolyzed to free cholesterol by cholesterol ester hydrolase, and the free cholesterol content was measured using a colorimetric assay method with 4 amino antipyrine and phenol in the presence of peroxidase (Allain *et al,* 1974). Cholesterol and phospholipids were expressed as nmol/10 x 10^6 spermatozoa. The values were expressed as mean ± standard error of the mean (SEM). The statistical significance of differences was assessed using a nonparametric test (Mann–Whitney *U* test).

RESULTS

Sperm motility parameters

The motility parameters of B2-Percoll spz and HFF-Percoll spz after 2 and 24 h incubation are presented in table I. Motility percentage, and ALH values of the HFF-Percoll spz were not significantly different from those of the B2-Percoll spz. However, after 2 h incubation with HFF progressive motility percentage, VSL, and HA percentage were significantly higher in the HFF-Percoll spz than in the B2-Percoll spz ($P \leq 0.05$). No significant differences were observed after 24 h incubation.

Level of lipids in human pelleted Percoll spermatozoa with or without HFF

In B2-Percoll spz, no difference was noted in the cholesterol and phospholipid contents between 2 and 24 h (fig 1). After 2 h incubation with HFF, similar concentrations of cholesterol and phospholipids were observed in the HFF-Percoll spz and in the B2-Percoll spz. However, after 24 h HFF incubation, there were lower levels of cholesterol ($P = 0.02$) and phospholipids ($P = 0.05$) in the HFF-Percoll spz compared with B2-Percoll spz. Therefore, the treatment of Percoll pelleted spermatozoa with HFF induced a time-dependent decrease

Table I. Motility parameters of pelleted Percoll spermatozoa with and without human follicular fluid.

	Spermatozoal motility (%)	*Progressive motility (%)*	*VSL (μm/s)*	*ALH (μm)*	*HA (%)*
B2-Percoll spz (2 h)	60.0 ± 4.1	28.0 ± 4.0[a]	45.3 ± 4.6[b]	3.9 ± 0.2	2.6 ± 0.2[c]
HFF-Percoll spz (2 h)	70.0 ± 5.1	44.0 ± 8.2[a]	60.2 ± 9.2[b]	4.2 ± 0.4	4.2 ± 1.0[c]
B2-Percoll spz (24 h)	49.0 ± 5.3	25.0 ± 5.9	42.0 ± 4.9	3.8 ± 0.2	1.7 ± 0.7
HFF-Percoll spz (24 h)	59.0 ± 7.0	31.0 ± 9.3	43.3 ± 6.5	4.0 ± 0.2	3.0 ± 1.3

Values are means ± SEM (n = 10); values with the same superscripts are significantly different : $P < 0.05$.

in cholesterol as well as in phospholipids. The cholesterol/phospholipid ratio was similar for the B2-Percoll spz and for the HFF-Percoll spz both after 2 and 24 h.

When the spermatozoa were divided into groups on the basis of fertilization rates (fertilization percentage < 50 or ≥ 50 established after *in vitro* fertilization), differences in spermatozoa phospholipid contents ($P = 0.04$) between the 2 groups were observed (table II). There was lower level of phospholipids, particularly after 24 h incubation, in spermatozoa incubated with HFF in the ≥ 50 percentage fertilization group as compared to those < 50 (12.1 nmol/10^7 spz *vs* 21.3 nmol/10^7 spz, respectively). No significant differences were observed in the sperm cholesterol levels between the 2 groups. Therefore, the cholesterol/phospholipids ratio was increased in the ≥ 50 percentage fertilization group.

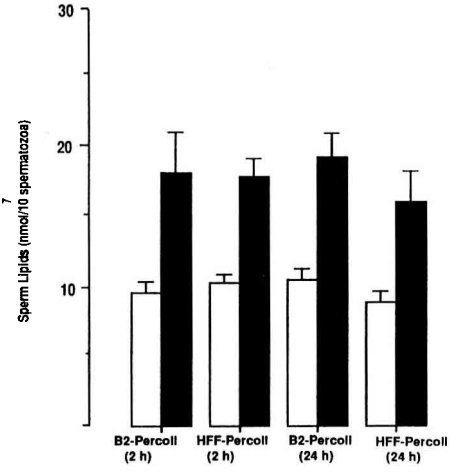

Fig 1. Cholesterol (□) and phospholipid (■) levels of Percoll pelleted spermatozoa with or without human follicular fluid.

DISCUSSION

It has been demonstrated that the preovulatory follicular fluid creates a microenvironment which is of importance for normal sperm function. The composition of this microenvironment includes the presence of steroids, pituitary hormones, proteins, and glycosaminoglycans. Despite the limited data concerning the physicochemical properties of this fluid, the use of HFF for *in vitro* studies can improve the motility and initiate the acrosome reaction of capacitated human sperm (Tesařík, 1985; Suarez *et al*, 1986). The present results demonstrate that the Percoll-pelleted spermatozoa incubated with HFF over a

Table II. Cholesterol and phospholipid contents of original semen and pelleted Percoll spermatozoa with and without human follicular fluid (HFF) according to fertilizaton percentage.

	Cholesterol (nmol/10^7 spermatozoa)		*Phospholipids (nmol/10^7 spermatozoa)*		*Cholesterol/ phospholipid ratio*	
	Fertil % < 50	*Fertil % ≥ 50*	*Fertil % < 50*	*Fertil % ≥ 50*	*Fertil % < 50*	*Fertil % ≥ 50*
B2-Percoll spz (2 h)	9.3 ± 0.8	9.5 ± 1.1	18.0 ± 1.8	17.8 ± 4.5	0.54 ± 0.06	0.66 ± 0.1
HFF-Percoll spz (2 h)	10.0 ± 1.1	10.2 ± 0.8	19.9 ± 0.9	16.2 ± 1.9	0.50 ± 0.05	0.65 ± 0.08
B2-Percoll spz (24 h)	10.0 ± 1.0	10.4 ± 1.0	21.3 ± 3.0	17.6 ± 2.1	0.51 ± 0.07	0.57 ± 0.05
HFF-Percoll spz (24 h)	8.3 ± 0.3	9.0 ± 1.2	21.1 ± 4.5[a]	12.9 ± 2.1[a]	0.45 ± 0.05[b]	0.74 ± 0.14[b]

Values are means ± SEM; Fertil: fertilization % < 50, $n = 6$; fertilization % ≥ 50, $n = 11$; values with the same superscripts are significantly different: [a] $P = 0.04$; [b] $P = 0.02$.

short incubation period show a specific motility pattern characterized by higher progressive motility percentage, VSL and HA percentage.

The lipid concentrations in the pelleted spermatozoa obtained in the present study agree with the results of other researchers (Hoshi *et al,* 1990; Sugkraroek *et al,* 1991). However, it is important to observe that the concentration of cholesterol and phospholipids varied considerably between samples. Such high inter-individual variations in semen from fertile subjects are often observed during the capacitation process in human spermatozoa.

Although we did not observe any significant effect on the cholesterol and phospholipid contents after a short incubation in percoll-HFF spz as compared to B2-Percoll spz, a longer incubation period (24 h) induced a significant decrease in the levels of both cholesterol and phospholipids.

The relative stability of the cholesterol/ phospholipid ratio found in our study can probably be attributed to the concomitant decrease in cholesterol and phospholipids. Changes in cholesterol and phospholipid contents in HFF-treated spermatozoa could alter their membrane physical properties. Since spermatozoal membranes are directly involved in the capacitation and acrosome reaction processes (Langlais and Roberts, 1985), lipid depletion could be necessary to enhance these processes. Furthermore, Tesařík and Fléchon (1986) have described a decrease in the cholesterol/anionic lipid ratio, topographically restricted to the fusogenic anterior part of the acrosome region in human spermatozoa undergoing *in vitro* capacitation.

The comparison of lipid levels according to fertilization percentage (< 50 or ≥ 50) shows that the HFF induced a decrease in phospholipid concentrations after 24 h incubation when the fertilization percentage was ≥ 50, but no effect was observed in the < 50% group. The beneficial effect of HFF after a long incubation period was observed to induce the acrosome reaction of abnormal sperm when used for subzonal insemination (Palermo *et al,* 1992). Such results might suggest that there is a relationship between the fertilizing capacity and the influence of HFF on modifications of spermatozoal membranes. However,

this finding has to be confirmed in a larger series. The simultaneity of the increase in motile spermatozoa and the decrease in cholesterol and phospholipid concentrations after incubation with HFF remains a task for further research. The results obtained in this and previous studies seem to show that treatment with HFF may maintain and improve motility, velocity and the fertilization rates in IVF of human sperm.

ACKNOWLEDGMENTS

The authors wish to thank MH Saussereau, N Bignon, J Poindron for their technical assistance and the CECOS/Tours for their support.

REFERENCES

Allain CC, Poon L, Chan SG, Richmond W, Fu P (1974) Enzymatic determination of total cholesterol. *Clin Chem* 20, 470-475

Bartlett GR (1959) Phosphorus assay in column chromatography. *J Biol Chem* 234, 466-468

Berger T, Marrs RP, Moyer DL (1985) Comparison of techniques for selection of motile spermatozoa. *Fertil Steril* 43, 268-273

Blackmore PF, Beebe SJ, Danforth DR, Alexander N (1990) Progesterone and 17α-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem* 25, 1376-1380

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37, 911-917

Falcone L, Gianni S, Piffaretti-Yanez A, Marchini M, Eppenberger U, Balerna M (1991) Follicular fluid enhances sperm motility and velocity *in vitro*. *Fertil Steril* 55, 619-626

Ghetler Y, Ben-Nun I, Kaneti H, Jaffe R, Gruber A, Fejgin M (1990) Effect of preincubation with follicular fluid on the fertilization rate in human *in vitro* fertilization. *Fertil Steril* 54, 944-946

Hoshi K, Aita T, Yanagika K, Yoshimatsu N, Sato A (1990) Variation in the cholesterol/phospholipid ratio in human spermatozoa and its relationship with capacitation. *Hum Reprod* 5, 71-74

Langlais J, Roberts KD (1985) A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res* 12, 183-224

Mbizvo M, Burkman LJ, Alexender NJ (1990) Human follicular fluid stimulates hyperactivated motility in human sperm. *Fertil Steril* 54, 708-712

Mendoza C, Tesařík J (1990) Effect of follicular fluid on sperm movement characteristics. *Fertil Steril* 54, 1134-1139

Osman RA, Andria ML, Jones DA, Meizel S (1989) Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun* 160, 828-833

Palermo G, Joris H, Devroey P, Van Steirteghem AC (1992) Induction of acrosome reaction in human spermatozoa used for subzonal insemination. *Human Reprod* 7, 248-254

Siegel MS, Paulson RJ, Graczykowski JW (1990) The influence of human follicular fluid on the acrosome reaction, fertilizing capacity and proteinase activity of human spermatozoa. *Hum Reprod* 5, 975-980

Suarez SS, Wolf DP, Meizel S (1986) Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res* 14, 107-121

Sugkraroek P, Kates M, Leader A, Tanphaichitr N (1991) Levels of cholesterol and phospholipids in freshly ejaculated sperm and Percoll-gradient-pelleted sperm from fertile and unexplained infertile men. *Fertil Steril* 55, 820-827

Tesařík J (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophor A23187 in human sperm population of proven fertilizing ability *in vitro*. *J Reprod Fertil* 74, 383-388

Tesařík J, Fléchon JE (1986) Distribution of sterols and anionic lipids in human sperm membrane: effects of *in vitro* capacitation. *J Ultrastruc Res* 97, 227-237

Triana LR, Babcock DF, Lorton SP, First NL, Lardy HA (1980) Release of acrosomal hyaluronidase follows increased membrane permeability to calcium in the presumptive capacitation sequence for spermatozoa of the bovine and other mammalian species. *Biol Reprod* 23, 47-59

World Health Organization (1987) *Laboratory Manual for the Examination of Human Semen and Semen – Cervical Mucus Interaction.* Cambridge Univ Press, Cambridge, MA

Yanagimachi R (1988) Mammalian fertilization. *In: The Physiology of Reproduction* (Knobil E, Neill JD, Ewing L, Market CL, Greenwald GS, Pfaff DW, eds). Raven Press, NY, 135 p

Yee B, Cummings LM (1988) Modification of the sperm penetration assay using human follicular fluid to minimize false negative results. *Fertil Steril* 50, 123-128