¹H nuclear magnetic resonance studies of seminal plasma from fertile and infertile men

S. Hamamah¹, F. Seguin², C. Barthelemy¹, S. Akoka², A. Le Pape²,
J. Lansac¹ and D. Royere¹

¹Unité de Biologie de la Reproduction, Département de Gynécologie-Obstétrique, Faculté de Médecine, CHU Bretonneau, F-37044, Tours cedex, France; and ²Laboratoire de Biophysique Cellulaire et RMN-INSERM U316, Faculté de Médecine, F-37032, Tours cedex, France

Glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), citrate, and lactate content of human seminal plasma was analysed by measuring the peak area of ¹H nuclear magnetic resonance (NMR) spectra in samples from four groups of patients: 21 spermatogenic failure subjects; 14 obstructive azoospermic subjects (vasectomized); seven patients presenting very severe oligoasthenozoospermia (OAT) and 18 normozoospermic subjects (control). The peak areas for GPC, citrate and lactate in seminal plasma were significantly smaller for patients with azoospermia than for the control group: 16.79, 8.18 and 2.28 versus 23.38, 10.58 and 4.30, respectively ($P < 0.01$). The peak area ratios for citrate:lactate and GPC:lactate were significantly different ($P < 0.01$) between the control group and the spermatogenic failure or obstructive azoospermia groups. A significant difference was also found in GPE:GPC peak intensity ratio between spermatogenic failure and obstructive azoospermia subjects ($P < 0.001$). These results provide some quantitative markers which may be used for examining infertility by using ¹H NMR of seminal plasma samples.

Introduction

Human seminal plasma contains a mixture of secretions from the seminiferous tubules, the epididymis and the accessory glands (bulbourethral, prostate, seminal vesicles). Information about some biochemical markers of the seminal plasma such as fructose for seminal vesicle secretion, zinc or acid phosphatase for prostate gland secretion and L-carnitine or α -1,4-glucosidase to determine epididymal function was obtained by classical biochemical analysis with differences in the sensitivity and intra-assay coefficients of variation (Soufir *et al.*, 1981; Soufir, 1985; Guérin *et al.*, 1986; Yeung *et al.*, 1990); but such analyses provide limited information.

The use of nuclear magnetic resonance (NMR) in biochemical exploration of seminal plasma in infertile subjects provides additional information and is technically simple as well as a highly reproducible and sensitive procedure.

The purpose of this study was to investigate whether the measurement by ¹H NMR of some biochemical markers (glycerylphosphorylcholine, glycerylphosphorylethanolamine, citrate and lactate) in human seminal plasma could provide more information and allow the differentiation of the various types of azoospermia.

Materials and Methods

Preparation of seminal plasma

Human seminal plasma samples were obtained from 21 men with spermatogenic failure, 14 had obstructive azoospermia after vasectomy and seven with severe oligoasthenozoospermia (OAT) were presenting for infertility evaluation, and 18 normozoospermic controls. Samples were liquefied at room temperature and semen parameters were determined by standardized methods (WHO recommended procedure, 1987). The semen was then centrifuged at 750 g for 15 min to remove cells and spermatozoa. Azoospermia was confirmed by the absence of a sperm sediment. Seminal plasma was stored at -20°C and analysed immediately after thawing.

Identification of spermatogenic failure

Human follicle-stimulating hormone (FSH) was systematically assayed in sera of patients; a radioimmunoassay kit (IRE FSH-RIA, Fleurus, Belgium) was used with the international MRC standard 69/104 and was complemented by a testicular biopsy if the concentration was in normal ranges ($\leq 8.8 \text{ mIU ml}^{-1}$).

NMR analysis

All proton NMR spectra were obtained without knowledge of the specimen group. For analysis, a BRUKER AM 200 WB spectrometer (4.7 T) was used, operating at 200.13 MHz and equipped with a 5 mm $^1\text{H}/^{13}\text{C}$ probe thermostated at 25°C with the sample spinning.

Seminal plasma (500 μl) was introduced into 5 mm (o.d.) NMR sample tubes. The signal from water was used to optimize the homogeneity of the magnetic field before the data acquisition. Data were obtained without a field lock, using a water suppression by a 4 s selective decoupler pulse applied on the H_2O signal, before a 7 μs nonselective excitation pulse (90°). The selective pulse of 4 s allowed the complete relaxation of protons of the metabolites as a relaxation delay (longitudinal relaxation time T_1 of these metabolites varied between 400 and 800 μs in these conditions). The acquisition parameters were: sweep width of 5000 Hz, 16 K data points and 64 accumulations. Fourier transformation was performed after a 0.3 Hz exponential line broadening. Proton chemical shifts were determined with respect to the external NMR standard sodium 3-trimethylsilylpropionate. Two-dimensional NMR experiments were performed using the homonuclear shift-correlated (COSY) technique (Bax, 1984). The peak areas were determined using the integration software of the spectrometer (DISNMR, BRUKER). Despite the precautions taken to obtain quantitative measures of area peaks, the use of a selective pulse for water suppression could disturb these analyses. To prevent this problem, we therefore used the ratio of peak areas to compare results from different seminal plasma samples.

Transferrin assay

Transferrin was measured by the technique of Khalfoun *et al.* (1986), modified as followed: the radioimmunoassay used antibody-coated polystyrene tubes (Nunc, Inter Med, Denmark) as the solid phase; after incubation (4°C, 48 h) in 0.05 mol carbonate-bicarbonate buffer l^{-1} (pH 9.6), tubes were kept at 4°C in the same buffer containing 0.1% bovine serum albumin (BSA, Sigma, France). Nitroloacetic acid trisodium salt solution was used for iron saturation of transferrin with FeCl_3 (Merck, France). The iodination of transferrin (5 μg per 20 μl) was performed with 4.44 MBq (120 μCi) of Na^{125}I (Amersham, UK) in the presence of chloramine T (10 μg per 10 μl) and the reaction was stopped by the addition of sodium metabisulfite (10 μg per 10 μl). For the radioimmunoassay, tubes were dried overnight before measuring in a gamma counter. The assay sensitivity ranged from 3.1 to 800 ng ml^{-1} and the logit-log plot was linear with a regression coefficient of $r=0.99$. Results were expressed as μg transferrin per ml of seminal plasma.

Statistical analysis

Concentrations of glycerylphosphorylcholine (GPC), citrate, lactate and transferrin and the peak ratios of GPC:citrate, citrate:lactate; GPC:lactate, glycerylphosphorylethanolamine (GPE)/GPC were expressed as means $\pm$ SEM. Comparison between groups was carried out using the Mann-Whitney U test.

Fig. 1. ^1H : ^1H correlation NMR spectra of human seminal plasma at 200 MHz. GPE: glycerylphosphorylethanolamine; GPC: glycerylphosphorylcholine; CIT: citrate; LAC: lactate.

Results

^1H -NMR spectra of the seminal plasma of normozoospermic men and men with spermatogenic failure or obstructive azoospermia (post-vasectomy)

GPC, GPE, citrate and lactate were identified in a typical ^1H -NMR spectrum of seminal plasma from normozoospermic men and the spectrum compared with that of seminal plasma from men with obstructive azoospermia. There was a lower signal of lactate in seminal plasma from men with obstructive azoospermia, spermatogenic failure or OAT than in normozoospermia. The resonance assignments were made using the analysis of the chemical shifts and coupling and using the COSY map (Fig. 1). In this way, the methyl derivatives of lactate (1.28 and 1.34 ppm), the methylene derivatives of citrate (2.42, 2.50, 2.59 and 2.67 ppm), the methyl derivatives of glycerylphosphorylcholine (3.13 ppm) and the methylene derivatives of glycerylphosphorylethanolamine (3.16 ppm) were identified. The ratios of the species were calculated with the peak area in arbitrary units (AU), except for the GPE:GPC ratio for which, because of the proximity of the GPE and GPC peaks, we used the peak intensity.

To evaluate the freezing-thawing effect on metabolic activity of seminal plasma, we assayed the same sample before and after repeated freezing-thawing cycles. No change was observed in the individual values or in the ratios even after many freezing-thawing cycles.

Table 1. Mean values of glycerylphosphorylcholine (GPC), citrate and lactate from NMR analysis and transferrin from radio-immunoassay analysis in seminal plasma from men with spermatogenic failure, obstructive azoospermia or normozoospermia

Patient definition	Sperm concentration (10^6 ml^{-1})	Volume of ejaculate (ml)	Peak area from NMR spectra (AU)			Transferrin ($\mu\text{g ml}^{-1}$)
			GPC	citrate	lactate	
Spermatogenic failure ($n = 21$)	0	2.15 ± 0.3	16.79 ± 1.2^a	8.18 ± 0.8^b	2.28 ± 0.1^a	25.29 ± 4.7
Oligoasthenoazoospermia ($n = 7$)	0.32 ± 0.2	5.22 ± 0.4	18.18 ± 1.4^a	10.2 ± 1.2	2.33 ± 0.3	25.60 ± 6.5
Obstructive azoospermia ($n = 14$)	0	4.46 ± 0.4	19.72 ± 1.5^a	9.58 ± 1.3	2.43 ± 0.2	24.50 ± 5.4
Normozoospermia ($n = 18$)	74 ± 13	3.87 ± 0.6	23.38 ± 1.4^a	10.58 ± 0.8	4.30 ± 0.3	30.21 ± 8.1

Values are expressed as means $\pm$ SEM.

^a $P < 0.01$, ^b $P < 0.03$ compared with normozoospermia.

AU: arbitrary units.

Fig. 2. ^1H -NMR peak ratios for seminal plasma from men with spermatogenic failure ($n = 21$), obstructive azoospermia ($n = 14$) and normozoospermia ($n = 18$). Values are means $\pm$ SEM. (a) ■ Glycerylphosphorylcholine: citrate, □ citrate: lactate and ▨ glycerylphosphorylcholine: lactate. (b) GPE: GPC peak intensity ratio. GPE: glycerylphosphorylethanolamine; GPC: glycerylphosphorylcholine. **Fig. 3.** ^1H -NMR peak intensity ratio (GPE:GPC) from men with spermatogenic failure (■), obstructive azoospermia (▲) or normozoospermia (○). GPE: glycerylphosphorylethanolamine; GPC: glycerylphosphorylcholine. Results are individual values.

^1H -NMR intensity of human seminal plasma

No significant difference was observed between samples from men with obstructive azoospermia, spermatogenic failure or OAT for GPC, citrate, lactate and transferrin (Table 1). When we compared these three groups with samples from normozoospermic men, GPC, lactate and transferrin concentrations were significantly higher ($P < 0.01$), whereas citrate concentrations were significantly higher in normozoospermia than in obstructive azoospermia ($P < 0.03$).

The mean peak area ratios of GPC: citrate, citrate: lactate and GPC: lactate show significant differences ($P < 0.01$, Fig. 2a) between men with obstructive azoospermia or spermatogenic failure and men with normozoospermia.

A significant difference in the mean GPE: GPC peak intensity ratio was also found between men with spermatogenic failure and men with obstructive azoospermia ($P < 0.001$, Fig. 2b) and also for individual values (Fig. 3). However, no significant difference was observed between these two groups of patients in terms of peak area ratios for GPC: citrate, citrate: lactate and GPC: lactate.

Table 2. Determination of sensitivity and specificity for different GPE:GPC ratio thresholds in seminal plasma to differentiate between men with spermatogenic failure ($n = 17$) and obstructive azoospermia ($n = 14$)

Threshold of GPE:GPC ratio	Percentage sensitivity	Percentage specificity
< 0.04	43	100
< 0.08	64	82
< 0.12	86	71
< 0.16	86	41
< 0.20	100	24

GPE: glycerylphosphorylethanolamine; GPC: glycerylphosphorylcholine.

The ability of the GPE:GPC peak intensity ratio to differentiate spermatogenic failure from obstructive azoospermia was established by calculating the percentage sensitivity and percentage specificity of the discrimination using several cut-off points (Table 2). Subsequently, the cut-off point chosen was the GPE:GPC ratio of < 0.12, allowing a sensitivity of 86% and a specificity of 71%, with a relative risk: 4.9 (95% confidence interval: 1.3–18.5).

A significant difference ($P < 0.01$) was also found in citrate:lactate and GPC:lactate peak area ratios between men with obstructive azoospermia, spermatogenic failure or OAT and normozoospermia. There were no significant differences for GPC:citrate peak area ratio between these groups.

Discussion

The use of NMR in the biochemical exploration of seminal plasma of infertile subjects provides additional information, is economical and technically simple, and gives reproducible results for the detection of some compounds present in seminal plasma.

During the last few years, several *in vivo* studies showed the potential for the use of NMR in the study of human testicular function and male fertility (Chew and Hricak, 1989; Grond *et al.*, 1991). A few reports have been published on the use of ^{31}P -NMR on human or mammalian spermatozoa *in vitro* (Robitaille *et al.*, 1987; Bahl *et al.*, 1988; Bretan *et al.*, 1989).

In this study, ^1H -NMR analysis shows that the peak area of GPC, citrate and lactate in seminal plasma from normozoospermic men is greater than in seminal plasma from azoospermic or OAT subjects. In all groups, we observed considerable inter-individual variation in terms of GPC and citrate, but not of lactate. In addition, we observed that seminal plasma from vasectomized men, men with spermatogenic failure or OAT contains less lactate than that of normozoospermic men, since the amount of lactate is directly related to the sperm concentration.

No GPC would be expected in the seminal plasma of vasectomized patients, since GPC is synthesized by the epididymis. However, some vasectomized patients had concentrations of GPC not significantly different from those of men with

spermatogenic failure but significantly different from normozoospermic men used as control. Significant differences between men with spermatogenic failure, obstructive azoospermia or OAT and normozoospermia are found for citrate:lactate and GPC:lactate peak area ratios, whereas no significant differences were found in GPC:citrate peak area ratio.

We found significant differences in GPE:GPC peak intensity ratio between men with spermatogenic failure and obstructive azoospermia patients. This ratio appears to be an important parameter in differentiating between spermatogenic failure and obstructive azoospermia. The sensitivity and specificity of such a parameter varies with the cut-off value. However, when the cut off is set at < 0.12, this parameter shows a high sensitivity (86%) with no important loss of specificity (71%).

It was difficult to correlate the values for GPE, GPC, citrate and lactate obtained in this study with those obtained for transferrin. Some previous studies that attempted to differentiate between obstructive azoospermic and spermatogenic failure were conducted solely on the basis of transferrin concentrations (Barthélémy *et al.*, 1988). This parameter of Sertoli cell function cannot be used to differentiate between obstructive and non-obstructive azoospermia.

In conclusion, this preliminary work opens a promising field of investigation and demonstrates the potential use of ^1H -NMR in differentiating between obstructive and non-obstructive azoospermia in patients presenting for male infertility evaluation. The combination of biochemical and NMR methods could determine the molecular basis of pathologic disturbances in human seminal plasma.

We are indebted to J. Poindron, R. Bourreau and J. M. Guillaumin for their assistance in this study and B. Hubert for his help in the statistical analysis.

References

- Bahl S, Bawa SR, Srivastava S, Phadke RS and Govil G (1988) Magnetic resonance studies of intact human spermatozoa *Physiological Chemistry and Physics and Medical NMR* **20** 183–188
- Barthélémy C, Khalfoun B, Guillaumin JM, Lecomte P and Bardos P (1988) Seminal fluid transferrin as an index of gonadal function in men *Journal of Reproduction and Fertility* **82** 113–118
- Bax A (1984) *Two-dimensional Nuclear Magnetic Resonance in Liquids* pp 50–97 Delft University Press, Holland
- Bretan PN, Vigneron DB, Hricak H, Tom RA, Moseley M, Tanagho EA and James TL (1989) Assessment of male infertility: correlation between results of semen analysis and phosphorus-31 magnetic resonance spectroscopy *Urology* **33** 116–119
- Chew WM and Hricak H (1989) Phosphorus-31 MRS of human testicular function and viability *Investigative Radiology* **24** 997–1000
- Grond J, Laven JSE, Lock MTWT, de Velde ER and Mali WPTH (1991) ^{31}P magnetic resonance spectroscopy for diagnosing abnormal testicular function *Fertility and Sterility* **56** 1136–1142
- Guérin JF, Benal H, Rollet J, Soucher C and Czyba JC (1986) Alpha-glucosidase as a specific epididymal enzyme marker – its validity for etiologic diagnosis of azoospermia *Journal of Andrology* **7** 156–162
- Khalfoun B, Barthélémy C, Crouzat-Reynès G and Bardos P (1986) A simple and sensitive solid-phase radioimmunoassay for measuring the transferrin content of human biological fluid: its application to seminal plasma *International Journal of Biochemistry* **18** 1135–1139
- Robitaille PM, Robitaille PA, Martin PA and Brown G (1987) Phosphorus-31 nuclear magnetic resonance studies of spermatozoa from the boar, ram, goat and bull *Comparative Biochemistry and Physiology B* **87** 285–296

- Soufir JC** (1985) Azoospermie, asthénozoospermie et biochimie séminale *Annales de Biologie Clinique* **43** 67–70
- Soufir JC, Marson J and Jouannet P** (1981) Free L-carnitine in human seminal plasma *International Journal of Andrology* **4** 388–390
- World Health Organization** (1987) *Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction* pp 3–15 Ed. WHO. Cambridge University Press, Cambridge
- Yeung CH, Cooper TG and Senge T** (1990) Histochemical localization and quantification of α -glucosidase in the epididymis of men and laboratory animals *Biology of Reproduction* **42** 669–676