¹H nuclear magnetic resonance of human seminal plasma in *in vitro* fertilization attempts: use of automatic spectrum analysis

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Seminal plasma affects the quality of spermatozoa. The possible use of ¹H nuclear magnetic resonance (NMR) analysis of human seminal plasma to determine a full biochemical profile that would allow the prediction of successful *in vitro* fertilization was examined. Two seminal plasma patterns (GI and GII) were found. The main compounds in GI were glycerophosphorylcholine, citrate and lactate, and in GII, choline and amino acids. These patterns did not fit into the usual spermogram classes. However, GI exhibited the higher pregnancy rate, suggesting that the equilibrium established between the components in seminal plasma affected not only the quality of spermatozoa but also embryonic quality.

Introduction

Human seminal plasma results from combined secretions of the epididymides, seminal vesicles and the prostate and bulbourethral glands. Its function is to support and transport spermatozoa until contact is made with female cervical secretions. During this time, enzyme reactions occur in the seminal plasma that may act on the membranes of spermatozoa and decrease their quality. It is known that pregnancies obtained via *in vivo*, as well as *in vitro*, fertilization (IVF) do not strictly correlate with routine spermogram classification (Aitken *et al.*, 1984; Miesusset *et al.*, 1989), nor with all the usual biochemical markers assayed in seminal plasma (Van Uem *et al.*, 1985). It is difficult to predict the ability of a spermatozoon to fertilize an oocyte and to give a successful pregnancy.

The composition of seminal plasma is complex and, even if numerous markers are known and assayed, exact concentrations are not sufficient because of interactions, antagonisms and synergisms between compounds (Mann and Lutwak-Mann, 1981); it is possible that the relative proportions of the constituents of seminal plasma may be more important physiologically than are the exact concentrations. Thus, choosing the right markers is difficult and even then, their analysis often requires different analytical methods that are time consuming and require large volumes of ejaculate.

Nuclear magnetic resonance (NMR) was performed in ³¹P NMR on semen or on spermatozoa from individual species (boar, ram, goat and bull) (Robitaille *et al.*, 1987), on human spermatozoa (Bahl *et al.*, 1988) and on human semen (Levine *et al.*, 1987). The main interest of ³¹P NMR studies was that they could reveal the presence of phosphomonoesters, intracellular inorganic phosphate, phosphodiester, phosphocreatine and free nucleotide di- and triphosphates. By this method,

Bretan *et al.* (1989) demonstrated that the concentration of glycerophosphorylcholine was lower in seminal plasma after vasectomy and was mainly derived from the epididymis.

Bell (1992) showed, using ¹H NMR, that the spectrum of seminal plasma was rich in resonances, and assigned peaks for about 25 metabolites by one-dimensional or two-dimensional NMR. The main interest of ¹H NMR is that the profusion of hydrogen-containing compounds in biological tissues and fluids gives very complex spectra. Each component can give a spectrum consisting of a singlet or doublet according to the presence of OH, CH₂ or CH₃. As such a spectrum is obtained, peaks can be assigned and compared.

Hamamah *et al.* (1993) found significant differences in the peak intensity ratio between glyceryl phosphorylethanolamine and glycerophosphorylcholine in men with spermatogenic failure and those with obstructive failure, demonstrating the potential use of ¹H NMR in differentiating between these two types of azoospermia.

The interest of *in vitro* ¹H NMR analysis is that technically it is available as a routine analysis and is highly reproducible, fast and relatively inexpensive compared with some other biochemical methods. When used as an unrestricted method of analysis, without *a priori* knowledge, it can identify biochemical compounds that would not be detected by routine biochemical assays, and provides, in the same experiment, a full biochemical pattern for automatic pattern recognition.

The aim of the present study was to examine whether ¹H NMR analysis could identify different spectrum patterns of human seminal plasma and if these patterns could be linked to the routine spermogram classification, normozoospermic, asthenozoospermic and oligo-asthenozoospermic groups, as well as to IVF results as determined by fertilization rate and pregnancy and thus, might assist in predicting successful parameters before IVF attempts or other assisted reproduction techniques are used.

Materials and Methods

Patients

Fifty-five patients (mean age: 33.8 years) were studied during IVF attempts, and classified into three groups according to semen parameters: normospermic ($n = 34$), number of spermatozoa $\geq 20 \times 10^6 \text{ ml}^{-1}$, motility $> 50\%$, normal forms $> 48\%$; asthenospermic ($n = 14$), number of spermatozoa $\geq 20 \times 10^6 \text{ ml}^{-1}$, motility $< 50\%$ or oligo-asthenoteratospermic ($n = 7$), number of spermatozoa $\leq 20 \times 10^6 \text{ ml}^{-1}$, motility $< 50\%$, normal forms $< 48\%$.

Human semen was collected by masturbation and allowed to liquefy at 37°C for 20 min. Immediately after liquefaction, the spermatozoa were homogenized and spermogram parameters were studied in an aliquot using a Makler counting chamber. An aliquot (0.3–0.5 ml) was then removed for NMR analysis. Soyabean trypsin inhibitor (1 mmol l^{-1} ; Sigma) was added to semen samples (10%, v/v) to preserve enzyme activity followed by centrifugation at 750 g for 15 min to remove spermatozoa, cells and debris. Samples were stored at -30°C , until required for analysis. Analysis was performed immediately after thawing at room temperature. An additional aliquot was prepared using the Percoll gradient method (Guerin *et al.*, 1989) for IVF. All patients had given their informed consent and ethical approval from the Scientific Committee was obtained.

NMR analysis

The ^1H NMR spectra were recorded at 24°C on a JEOL GSX 270 NMR spectrometer operating at 270 MHz for protons. The $350 \mu\text{l}$ sample of seminal plasma was supplemented with $80 \mu\text{l}$ D_2O with 0.02% trimethylsilylpropionate for chemical-shift reference at 0 p.p.m. The water peak was suppressed by a presaturation pulse sequence as previously used on other biofluids (Leray *et al.*, 1992). In each experiment, 48 free induction decay signals were accumulated using a spectral width of 3 kHz, 16 000 data points and a pulse repetition time of 3 s. A Gaussian filter of 0.5 Hz was applied to the time domain signal before the Fourier transformation. Only the aliphatic region, between 0 and 4 p.p.m. (in reference to trimethylsilylpropionate), was explored after phasing and baseline correction in a standard manner by the same operator. For relative quantification, peak height was used to avoid the problem of overlapping resonance areas (chemical noise). The measurements were performed by the same operator without knowledge of the patient's bioclinical status. Soyabean trypsin inhibitor, added during preparation of the seminal plasma samples, appeared as resonance number 17 (Fig. 1). All the resonance values (peak heights, H) were expressed as a percentage: $(Hn/(H1 + Hn + \dots H18) \times 100)$.

Resonance assignments were made by reference to the literature (Lynch *et al.*, 1994) and confirmed with two-dimensional COSY (homonuclear COrrrelation Spectroscopy) NMR experiments and standard additions of candidate compounds.

Assay of diamine oxidase activity

Diamine oxidase activity in seminal plasma was assayed according to the method of Okuyama and Kobayashi (1961),

modified by Tryding and Willert (1968), using radioactive putrescine as the substrate and by extracting the Δ^1 radioactive pyrroline formed with a toluene-based scintillator. Diamine oxidase activity is expressed as amount of Δ^1 pyrroline formed ($\text{nmol ml}^{-1} \text{ h}^{-1}$). Each measurement was performed twice using a Packard TriCarb Liquid Scintillation spectrophotometer 3310, and the result expressed as the mean value.

Statistical analysis

Given the complexity of individual ^1H NMR spectra, it is possible to simplify spectra by exploiting some of the peaks, and comparing them one by one, or by comparing their intensity ratio. Information is inevitably lost using either of these methods. Therefore, since it is possible that the spectral properties that would enable differentiation of groups of patients involve a pattern of many metabolites, analysis was performed using multiple correspondence analysis and by a hierarchical ascending classification (Software BI (c) Logiserm 1979/1987) (Jambu and Lebeaux, 1983; Howells *et al.*, 1992). With these exploratory multivariate techniques, patients could be put into groups or clusters according to their NMR pattern. Each patient is a cluster in himself. Thus, the distance between each cluster was measured and the two closest merged to form a new cluster. Merging of the two closest clusters was repeated until only one or two significant clusters were left. This method identifies the most prominent peak and quantifies the separation between groups.

The Mann–Whitney test was performed to compare resonance values; the χ^2 test was used to compare the number of pregnancies in the two groups; and Spearman's correlation was carried out between resonance values and bioclinical parameters. After the NMR-based classification, bioclinical parameters (spermogram, fertilization rate and pregnancy score in IVF, diamine oxidase concentration) were then compared.

Results

In the aliphatic part of the one-dimensional spectrum, 17 resonances were investigated between 0 and 4 p.p.m. (Fig. 1). A two-dimensional (2D COSY) spectrum was obtained to aid peak assignment (Fig. 2). Hierarchical ascending classification demonstrated two NMR patterns (GI and GII) determined by the 17 resonance values (Table 1, Fig. 3).

In group I (GI), the most prominent resonance was H15 (glycerophosphorylcholine), followed by H10, 5, 3, 13, 7. In group II (GII), the most prominent resonance was H14 (choline), followed by H6, 2, 11, 12, 9, 8, 18, 16, 4, 1. The values of 13 resonances made these two groups significantly different: H1, 4, 10, 16 ($P < 0.001$); H2, 6, 11, 15, 18 ($P < 0.01$); H5, 8, 9, 12 ($P < 0.05$). In the two groups some resonance values did not differ: H3 (valine), H14 (choline) and H7, 13 (polyamines).

For all the specimens, Spearman's correlations showed that H15 (glycerophosphorylcholine), H14 (choline) and H10 (citric acid) were independent values, not linked with any other component. Alternatively, the amino acid group was highly

Fig. 1. Water suppressed 270 Mhz ^1H NMR spectrum of the aliphatic region of human seminal plasma from 0.0 to 4.0 p.p.m. Assignments of peaks 1–18 and their chemical shifts (δ) as listed in Table 1.

linked: $\text{H2 (Leu)}\text{--}\text{H6 (Arg + Lys)}$: $r = +0.77$; $\text{H2 (Leu)}\text{--}\text{H11 (Lys)}$: $r = +0.80$; $\text{H4 (Ile)}\text{--}\text{H11 (Lys)}$: $r = +0.74$; $\text{H6 (Arg + Lys)}\text{--}\text{H11 (Lys)}$: $r = +0.72$. Some other compounds were also correlated: $\text{H7 (mainly putrescine)}$ and $\text{H13 (mainly spermine)}$: $r = +0.66$. H12 was identified as a mixture of spermidine and spermine by adding them to the semen (Lynch *et al.* (1994) had identified H12 as spermine). H12 did not correlate with H7 and H13 , but with H11 ($r = +0.61$).

The spermogram parameters for each group are given (Table 2); the only significant difference was a lower number of spermatozoa for GI ($P < 0.05$). Diamine oxidase activity was lower in GI than in GII ($P < 0.05$). In GI, all three kinds of specimens (normo-astheno- and oligo-astheno-teratospermic) were represented but in GII, oligo-astheno-teratospermic specimens were absent. A comparison is made between the IVF results of the two groups (Table 2); fertilization rate was the same for the two groups but there was a significant higher pregnancy rate in GI ($P < 0.05$).

Discussion

^1H NMR analysis is a simple reliable method that provides additional information to classical biochemical analysis by emphasizing numerous compounds at the same time in a

biofluid specimen. As peak intensity reflects active nuclei present in the solution, relative quantification is easily obtained and can give an individual seminal plasma pattern.

The study reported here demonstrated two seminal plasma patterns. In each spectral pattern, the relative proportions between components were different, undoubtedly without any abnormal decrease. In the GI pattern, the three main compounds were glycerophosphorylcholine, citric acid, lactic acid, with valine and polyamines were the next most prominent. Thus, in this group, in which most of the pregnancies occurred, markers reflecting the activity of the epididymides, prostate and indirectly, the seminal vesicles, were the most important. In the GII pattern, choline was the main marker, and all the other compounds of this pattern, except possibly H12 , were free amino acids.

Previously, none of the individual components of seminal plasma studied was found to determine *in vitro* fertilization success with certainty; it is only when they are considered in combination, as here, that it is possible to link their NMR pattern with the occurrence of pregnancy.

In GI, the main component, glycerophosphorylcholine, is a relatively stable marker in seminal plasma (Mann and Lutwak-Mann, 1981); in man, the head of the epididymis is its major source and the seminal vesicles its minor one (Dadoue, 1987). An abnormal decrease in concentrations of

Fig. 2. Resonance identification by 270 Mhz two-dimensional homonuclear correlation spectroscopy ^1H NMR spectrum of human seminal plasma. The x and y axes are the chemical shift expressed in p.p.m. The diagonal represents the one-dimensional proton spectrum in contour lines. Cross-peaks correspond to the coupling between the different resonances. TSP: trimethylsilylpropionate; Leu: leucine; Val: valine; Glu: glutamate; Gln: glutamine; Spe: spermine; Lac: lactate; Cit: citrate; Cho: choline; Ala: alanine.

glycerophosphorylcholine reflects epididymal dysfunction, regardless of the cause (Arrata *et al.*, 1978). Jeyendran *et al.* (1989) suggested that glycerophosphorylcholine influences the fertilizing ability of spermatozoa, as it was found at a higher concentration in the fertile group in an IVF programme. The next two most prominent markers were citric acid, a prostatic marker and the main anion of seminal plasma (Tobelem, 1989), and lactic acid which results from glycolysis of the epididymal tissue (Dadoune, 1987).

The assignment of the polyamines (H7, H13) were resolved by adding each polyamine to seminal plasma samples. H12 apparently contains only polyamines as demonstrated by Spearman's correlations. Polyamines are assumed to come mostly from the prostate (Mann and Lutwak-Mann,

1981) and might be important for fertility: spermine incubated with human sperm cells increases adenylylase activity (Shah *et al.*, 1975) and has a beneficial effect on fertilization of zona-free mouse ova *in vitro* (Quinn *et al.*, 1982). In previous studies, we demonstrated a significant negative correlation between number of spermatozoa and the two polyamines: concentrations of spermidine, and particularly spermine, were significantly higher in the group with the higher fertilization rate (Le Calvé *et al.*, 1990). A significant positive correlation between number of spermatozoa and individual diamine oxidase activity in seminal plasma was noted by Le Calvé *et al.* (1991). In the present study, diamine oxidase activity and number of spermatozoa were lower in GI than in GII. It is known that the possible

Table 1. Assignments and intensity values of resonances (H1 to H18) detected in proton NMR spectra of human seminal plasma

Peak number	Compound	Chemical shift (δ)	Intensity values (AU)	
			GI	GII
H1	Isoleucine	0.88	3.79	2.79***
H2	Leucine	0.94	5.39	4.42**
H3	Valine	1.06	1.29	1.42
H4	Isoleucine	1.21	1.95	1.45***
H5	Lactate	1.35	2.63	3.45*
H6	Arginine + lysine	1.70	2.64	2.07**
H7	Putrescine + spermine	1.75	1.58	1.61
H8	Glutamine	2.06	4.48	3.95*
H9	Glutamate	2.35	2.77	2.47*
H10	Citrate	2.66	5.43	8.49***
H11	Lysine	3.00	3.09	2.43**
H12	Poly +	3.08	3.49	2.90*
H13	Spermine	3.12	2.20	2.65
H14	Choline	3.20	43.18	42.36
H15	Glycerophosphorylcholine	3.23	7.46	10.79**
H16	Glycine	3.50	4.66	3.63***
H18	α -Glucose + methionine	3.82	3.97	3.13**

The chemical shift (δ) expressed in p.p.m. and the intensity values are in arbitrary units (AU).

GII value significantly different from GI value: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

H17: Soyabean trypsin inhibitor.

Fig. 3. Dendrogram showing cluster analysis of ^1H NMR spectra of human seminal plasma. H1–H18 as listed in Table 1.

degradation of spermine by diamine oxidase *in vivo* may give rise to cytotoxic aldehyde formation which interferes with motility (Hölttä *et al.*, 1975).

In the group II pattern, choline was the main marker and all the other compounds of this pattern, except possibly H12, were free amino acids. Choline arises from phosphorylcholine,

Table 2. Bioclinical data and NMR classification (GI and GII) of human seminal plasma

Parameter	GI	GII
Number of patients	33	22
Sperm classification		
Normospermic	18	16
Asthenospermic	8	6
Oligo-astheno-teratospermic	7	0
Spermogram values		
Count (10^6 ml^{-1})	56.06 $\pm$ 45.64*	76.14 $\pm$ 28.03*
Motility (%)	47.27 $\pm$ 16.35	51.14 $\pm$ 13.09
Normal forms (%)	52.00 $\pm$ 11.73	52.00 $\pm$ 14.73
Diamine oxidase ($\text{nmol } \Delta^1 \text{ pyrroline ml}^{-1} \text{ h}^{-1}$)	189 $\pm$ 220*	309 $\pm$ 171*
<i>In vitro</i> fertilization results		
Fertilization rate (%)	50.1 $\pm$ 31.1	49.8 $\pm$ 27.5
Number of pregnancies	11**	2**
Pregnancy rate (%)	33	9

* $P < 0.05$, ** $\chi^2 P < 0.05$.Values are expressed as means $\pm$ SEM.

the conversion carried out by prostatic acid phosphatase early after ejaculation (Mann and Lutwak-Mann, 1981). The other markers in GII, amino acids, serve mostly as oxidizable substrates for spermatozoa and act as buffers with a protective influence on the plasma membrane (Mann and Lutwak-Mann, 1981).

Analysis of biochemical markers is usually carried out in azoospermia or oligo-astheno-teratospermic cases to localize the precise origin of obstruction or dysfunction of the epididymis or accessory glands (Levine *et al.*, 1987; Bretan *et al.*, 1989; Hamamah *et al.*, 1993). Lynch *et al.* (1994) showed considerable differences between the NMR pattern of seminal plasma from a patient with non-obstructive azoospermia and a patient with obstructive azoospermia and claimed that these studies provided the basis for the use of NMR as a diagnostic probe of altered male reproductive gland function.

In the present study, we wanted to determine whether, in patients with normal or sperm counts sufficient to allow IVF to be attempted, it was possible to demonstrate different seminal patterns. These patterns, reflecting various equilibria between secretions that might interfere with sperm maturation, could then be used in the evaluation of sperm fertility.

The two patterns observed in the work reported here do not correspond with the usual spermogram classes; in GI, all three classes were present, and when spermogram parameters were examined, the only significant difference between the two groups was the lower number of spermatozoa in GI, most probably due to the presence of the several oligo-astheno-teratospermic cases in this group. These results were not surprising as it is known that the sperm characteristics of fertile and infertile men show a wide overlap (Feneux *et al.*, 1986) and that there is no exact point at which fertilization fails (Edwards *et al.*, 1984).

The GI NMR pattern correlated with a higher pregnancy rate, suggesting that the equilibrium established between

the components of seminal fluid in this group affects, not the fertilizing ability of spermatozoa (no differences occurred in fertilization rate between the two groups), but the quality of the resulting embryos, as has been demonstrated in animals under various conditions (Orgebin-Crist and Jahad, 1977; Martin-Deleon and Boice, 1985).

In conclusion, this is the first time that ^1H NMR has been used to examine the seminal plasma of patients in an IVF programme. This analysis method, associated with the exploratory multivariate technique, demonstrated two NMR patterns. The examination of bioclinical data of the two groups of patients showed that one pattern was linked with ongoing pregnancies. Thus, despite the development and success of micromanipulation of gametes for severe male infertility, the implications for male fertility of changes in the equilibrium of seminal plasma are such that a greater understanding and consideration of these factors is warranted for future progress in this field.

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