A proton NMR study of the effect of a new intravasal injectable male contraceptive RISUG on seminal plasma metabolites

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Introduction

There is a need to develop a suitable male contraceptive for population control programmes. The current unavailability of an ideal male contraceptive is due to the lack of one or more essential features such as a minimally invasive drug delivery system with a one-time intervention, long-term effectiveness of the contraceptive with negligible side-effects and the option of reversal whenever desired. Rigorous research has been carried out over the past 25 years to meet these objectives, which has led to the successful development of a reversible male contraceptive that can be injected bilaterally into the lumen of the vas deferens. The contraceptive, named RISUG, consists of a specific preparation of the copolymer styrene maleic anhydride dissolved in dimethyl sulphoxide (DMSO). When spermatozoa come into contact with RISUG in the vas deferens, their membranes rupture and enzymes such as acrosin and hyaluronidase leach out (Guha, 1996). Hence, these damaged spermatozoa are rendered incapable of fertilization. The concentrations of glucose, lactate, glycerophosphorylcholine and choline were significantly lower (P < 0.01) in subjects injected with RISUG compared with controls. In addition, metabolite ratios such as choline:glucose, lactate:choline and glycerophosphorylcholine:choline were calculated. Citrate:lactate and glycerophosphorylcholine:choline ratios were significantly lower in RISUG-injected subjects than in controls (P < 0.01), thereby indicating the occurrence of partial obstructive azoospermia. The most important finding of the present study was that the intervention of RISUG in the vas deferens even for a period as long as 8 years is absolutely safe and does not lead to prostatic diseases.

Nuclear magnetic resonance (NMR) spectroscopy was used to quantify citrate, glucose, lactate, glycerophosphorylcholine and choline in seminal plasma from subjects injected with a new male contraceptive RISUG, a copolymer of styrene maleic anhydride dissolved in dimethyl sulphoxide, and in seminal plasma from normal ejaculates. No significant difference in the concentration of citrate was observed between the groups, indicating that the prostate is not affected by the contraceptive. The concentrations of glucose, lactate, glycerophosphorylcholine and choline were significantly lower (P < 0.01) in subjects injected with RISUG compared with controls. In addition, metabolite ratios such as choline:glucose, lactate:choline and glycerophosphorylcholine:choline were calculated. Citrate:lactate and glycerophosphorylcholine:choline ratios were significantly lower in RISUG-injected subjects than in controls (P < 0.01), thereby indicating the occurrence of partial obstructive azoospermia. The most important finding of the present study was that the intervention of RISUG in the vas deferens even for a period as long as 8 years is absolutely safe and does not lead to prostatic diseases.

Human seminal plasma contains a mixture of fluid from the seminiferous tubules, epididymis and accessory glands (bulbo-urethral, prostate and seminal vesicles). The composition of each secretion has characteristic biochemical features: fructose and prostaglandins are markers for seminal vesicles; prostatic fluid contains uniquely high concentrations of citrate (Costello and Franklin, 1991), zinc, polyamines and myo-inositol; and glycerophosphorylcholine, choline, neutral α-glucosidase and carnitine are markers of epididymal function (Cooper et al., 1990). High-resolution ¹H NMR spectroscopy has emerged as a powerful tool in the analysis of biofluids (Foxall et al., 1993; Spraul et al., 1994; Liu et al., 1996). Recent studies have shown that chemometric methods along with automated NMR-based analysis can be used for toxicological assessment of biofluids (Holmes et al., 2000). Lynch et al. (1994) showed that NMR could be used successfully as a diagnostic probe for altered male reproductive gland function. ¹H NMR spectroscopy has also been used to examine seminal plasma of patients in an IVF programme (Segalen et al., 1995). Recently, Hamamah et al. (1998) showed that seminal plasma metabolite ratios could be used to
differentiate between cases of spermatogenic failure and obstructive azoospermia. The main advantage that NMR spectroscopy offers over other classical biochemical analyses is that it is not biased towards a particular compound; it can simultaneously detect metabolites that are expected or unexpected or that are difficult to assay using standard biochemical methods (Patel et al., 1999). There is also no need for specific preparation such as derivatization of compounds.

The aim of the present study was to evaluate prostatic function in RISUG-injected subjects by determining the concentration of citrate in the seminal plasma ejaculates. In addition, glucose, lactate, glycerophosphorylcholine and choline metabolites were quantified. Peak area ratios of these metabolites were also estimated to assess the occurrence of obstructive azoospermia in RISUG-injected subjects.

Materials and Methods

Seminal plasma preparation

Human semen samples were collected by masturbation from (i) 20 normal human volunteers (age 32–40 years; controls) who had fathered children within the previous 4 years and (ii) 17 subjects (aged 36–40 years) who had fathered children before the injection of RISUG given to them 8 years earlier. Each sample was liquefied at 37°C for 20 min to reduce viscosity. A 0.5 ml aliquot was removed to determine semen parameters using standard methods (WHO Recommended Procedure, 1999). The remaining semen sample was centrifuged at 1000 g for 15 min to remove cells and spermatozoa. The resulting supernatant was separated, diluted with D2O and NMR experiments were performed immediately, within 1 min. Even after separation from spermatozoa by centrifugation, the composition of seminal plasma undergoes progressive changes in vitro, for example, proteolysis, increase in free choline content due to dephosphorylation of phosphorylcholine, and fructolysis. Therefore, the plasma was subjected to NMR analysis immediately after separation of spermatozoa and cells. Identical conditions were maintained strictly for each sample evaluation.

NMR analysis

Seminal plasma samples were transferred into 5 mm NMR sample tubes with D2O (Aldrich Company Inc., Milwaukee, IL) for field/frequency lock. Sodium 3-(trimethylsilyl-2,2,3,3-H)-1-propionate (TSP) (E-Merck, Darmstadt) was added at a concentration of 2 mmol l–1 to (trimethylsilyl-2,2,3,3-H)-1-propionate (TSP) (E-Merck, Milwaukee, IL) for field/frequency lock. Sodium 3-derivatization of compounds.

Quantification of metabolites

Peaks or multiplets of identified metabolites, including that of internal standard TSP, were integrated to obtain signal intensity after careful baseline correction. The following formula was used to estimate the concentrations of various metabolites:

\[ [C]_X = [C]_{TSP} \frac{N_{TSP} \cdot I_X}{N_X \cdot I_{TSP}} \]

where [C]X is the concentration of biochemical X, and Ix and ITSP are the NMR signal intensities of X and TSP, respectively. NX is the number of protons per molecule giving rise to the integrated signal and N TSP = 9. Metabolite ratios were calculated from the integrated intensity of individual peaks.

Statistical analysis

Concentrations of citrate, glucose, lactate, glycerophosphorylcholine and choline are expressed as mean ± SD. Concentration and peak area ratios of specific biochemical markers from both the groups were compared using Student’s t test. The level of significance was set at P < 0.01.

Results

Various amino acids, carbohydrates and lipids present in human seminal plasma were assigned in RISUG-injected subjects using 400 MHz 1H NMR spectroscopy. Expanded regions of a typical one-dimensional proton NMR spectrum of seminal plasma of controls and of subjects injected with RISUG are shown (Fig. 1a,b). Several intense resonances were observed in the aliphatic region (0.5–4.5 p.p.m.) and aromatic region (6.5–8.5 p.p.m.). A two-dimensional double quantum filter correlation spectrum of the corresponding regions is shown (Fig. 2). Assignment of the resonances was made using the J-connectivity pattern. Resonances corresponding to CH and CH₃ protons of lactate were

\[ \text{NTSP} \cdot [C]_X = [C]_{TSP} \cdot I_X \]

for quantitative measurements. A constant receiver gain was used for all one-dimensional experiments to minimize errors in quantitative estimation. The free induction decays (FIDs) were collected with 32 K data points and line broadening of 0.3 Hz was applied before Fourier transformation.

Connectivity between protons was established with phase-sensitive double quantum-filtered correlation spectra using a standard pulse sequence. Typically, the following experimental conditions were used: spectral width 5 kHz, 2 K data points in F2 dimension with 512 t1 increments in F1 dimension. The number of scans was 32–64, the relaxation delay was 2.5 s and the receiver gain was optimized in each instance to obtain good signal:noise ratio. Data were zero filled to 1 K in F1 dimension. Squared sine bell window function was applied before Fourier transformation.
assigned at 4.11 and 1.33 p.p.m., respectively, from the cross peak observed between them in the two-dimensional double quantum filter spectrum. Similarly, the signals from citrate, glucose, glycerophosphorylcholine, myo-inositol and spermine were assigned using the connectivities. No significant difference was observed in the chemical shift positions of metabolites present in the seminal plasma samples of control and RISUG-injected subjects (Fig. 1).

The calculated concentrations of specific biochemical markers of prostate, seminal vesicles and epididymis for control and RISUG-injected subjects are shown (Table 1). Most of these metabolites had reasonably distinct resonance peaks in the one-dimensional spectrum, thereby facilitating reliable estimation of their concentration. The concentration of citrate was estimated from its two doublets at 2.55 and 2.67 p.p.m. No significant difference in the concentration of citrate in the seminal plasma ejaculates was observed between control and RISUG-injected subjects.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** The expanded regions of one pulse 400 MHz \(^1\)H nuclear magnetic resonance spectra of human seminal plasma diluted in D\(_2\)O of (a) subjects injected with the contraceptive RISUG into the lumen of the vas deferens and (b) normal controls. Expanded regions are shown: 0.6–2.9 p.p.m. showing lactate and citrate resonances, scale of signal intensity is magnified by four times; the region 3.10–3.30 p.p.m. shows glycerophosphorylcholine (GPC) and choline resonances, scale is unchanged; and the region 5.0–6.1 p.p.m. shows the resonance of \(\alpha\)-glucose resonance with scale magnified by eight times.

**Table 1.** Concentrations of metabolites from the seminal plasma of normal controls and men treated with the intravasal injectable contraceptive RISUG

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sperm concentration ((\times 10^{-6}) ml(^{-1}))</th>
<th>Volume of ejaculate</th>
<th>Concentration of metabolites (mmol l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Citrate</td>
</tr>
<tr>
<td>Normal controls ((n = 20))</td>
<td>75 ± 10</td>
<td>4.0 ± 0.6</td>
<td>55.8 ± 11.0</td>
</tr>
<tr>
<td>RISUG-injected ((n = 17))</td>
<td>&lt; 1 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>54.4 ± 9.0</td>
</tr>
</tbody>
</table>

GPC: glycerophosphorylcholine.
Values are mean ± sd.
\(^*\)Value is significantly different from corresponding control value (\(P < 0.01\)).
Citrate:lactate and glycerophosphorylcholine:choline ratios were also lower in RISUG-injected subjects ($P < 0.01$) compared with controls (Table 2).

Discussion

The concentrations of prostatic markers vary in different prostatic diseases. Citrate concentrations are reported to be lowered significantly in prostatic carcinoma (Cornel et al., 1993). Vasectomy, the most commonly used method of male contraception, is suspected to be linked with subsequent prostate cancer (Comhaire, 1994). Therefore, it was considered essential to evaluate the prostatic function in RISUG-injected subjects. In the present study, the prostate biochemical marker citrate, which can be assigned specifically from NMR spectra, was quantified. No
significant difference was observed in the concentration of citrate in control and RISUG-injected men. This finding is of great importance as it implies that intervention of RISUG in the vas deferens, even for a period as long as 8 years, is absolutely safe and does not lead to prostatic diseases. The glucose content determined in normal ejaculates in the present study was in agreement with the biochemical assay results reported by Diamandis et al. (1999). A significant decrease in the glucose content in seminal plasma of RISUG-injected men was observed: the feedback mechanism for decreased synthesis of glucose in seminal vesicles of RISUG-injected subjects cannot be ruled out.

The estimated concentration of lactate in normal ejaculates in the present study was slightly higher than that reported after biochemical analysis by Mann and Mann (1981); this finding may be the result of the overlapping of the methyl resonance peak of threonine with the methyl protons of lactate. The decrease in the amount of lactate in the seminal plasma of RISUG-injected men may be attributed to insufficient secretion of the enzyme lactate dehydrogenase (S. K. Guha, unpublished) in the seminal plasma of RISUG-injected subjects.

It is well established that glycerophosphorylcholine originates mainly in the epididymis (Brown-Woodman et al., 1980). The physiological significance of glycerophosphorylcholine in epididymal secretion is not yet clear. Hartree and Mann (1960) suggested that glycerophosphorylcholine and phospholipids significantly affect respiration and motility of spermatozoa. There are controversial reports regarding the relationship between sperm motility and total seminal amounts of glycerophosphorylcholine (Arrata et al., 1978; Mieusset et al., 1988). The concentration of glycerophosphorylcholine and choline estimated in the present study was slightly higher than that reported after biochemical analysis by Mann and Mann (1981). The increased content of glycerophosphorylcholine may be attributed to the indistinguishibility of methyl resonances of glycerophosphorylcholine and phosphorylcholine. Even at a high field of 600 MHz, the glycerophosphorylcholine signal is nearly coincident with that of phosphorylcholine at 3.23 p.p.m. (Tomlins et al., 1998). Tomlins et al. (1998) have also shown that unlike glycerophosphorylcholine, which is stable for several hours in human seminal fluid, phosphorylcholine is converted to choline with a half-life ($t_{1/2}$) of 9 min. There is also a partial overlapping of the NCH$_2$ signal of spermine with the choline signal. These two factors are probably responsible for the higher concentration of choline recorded in the present study compared with standard biochemical data. The concentration of glycerophosphorylcholine was significantly lower in the seminal plasma of RISUG-injected men compared with control men. In RISUG-injected men, the partial obstruction of the vas deferens may slow down the flow of spermatozoa through the epididymis. The significant decrease in the sperm count of RISUG-injected subjects probably leads to a lowered epididymal activity. Lower seminal glycerophosphorylcholine concentrations have been reported in men with agenesis of the vas deferens (Calamera and Laveri, 1974) or after vasectomy (Frenkel et al., 1974; Naik et al., 1979; Giovenco et al., 1986). Jeyendran et al. (1989) reported a significant difference in glycerophosphorylcholine concentrations in fertile and infertile men, which indicates that glycerophosphorylcholine might influence the fertilizing ability of spermatozoa. 1H NMR studies on seminal plasma from fertile and infertile men also showed that glycerophosphorylcholine, citrate and lactate concentrations in seminal plasma were significantly lower for patients with azoospermia compared with normal control groups (Hamamah et al., 1993). In the present study, significant differences were observed in citrate:lactate and glycerophosphorylcholine:choline ratios ($P < 0.01$) between control and RISUG-injected men. The estimated value of glycerophosphorylcholine:choline reported in the present study for RISUG-injected subjects is in excellent agreement with the ratio calculated by Hamamah et al. (1998) in normal men with obstructive azoospermia. However, the term 'partial obstructive azoospermia' would be more appropriate in the present study as ejaculates from RISUG-injected subjects do contain sperm debris (Guha, 1996). Extensive overlapping of the resonances of metabolites and the nature of complexity of biofluid may act as limiting factors for accurate analysis of NMR spectra and, hence, the exact quantitation of metabolites. However, the aim of the present study was to study the relative differences between the two groups and, hence, the methodology used is appropriate.

In conclusion, the concentrations of prominent biochemical metabolites in seminal plasma, namely citrate, glucose, lactate, glycerophosphorylcholine and choline, were calculated using proton NMR spectroscopy and compared between RISUG-injected subjects and controls. Significantly lower concentrations of glucose, lactate, glycerophosphorylcholine and choline metabolites were observed in subjects injected with the contraceptive RISUG. The important outcome of the present study was the absence of a significant difference in the concentration of citrate in the seminal plasma of normal controls as well as in

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**Table 2.** Mean values of peak area ratios of metabolites present in seminal plasma of normal controls and men treated with the intravasal injectable contraceptive RISUG assessed using $^1$H nuclear magnetic resonance (NMR) spectroscopy.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Normal controls $(n=20)$</th>
<th>RISUG-injected subjects $(n=17)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline:citrate</td>
<td>4.4 ± 2.1</td>
<td>2.8 ± 1.7</td>
</tr>
<tr>
<td>Choline:lactate</td>
<td>5.9 ± 2.5</td>
<td>6.0 ± 2.6</td>
</tr>
<tr>
<td>Citrate:lactate</td>
<td>1.4 ± 0.5</td>
<td>2.4 ± 0.9*</td>
</tr>
<tr>
<td>GPC:choline</td>
<td>0.14 ± 0.05</td>
<td>0.08 ± 0.05*</td>
</tr>
</tbody>
</table>

GPC: glycerophosphorylcholine.
Values are mean ± sd.
*Value is significantly different from corresponding control value ($P < 0.01$).
RISUG-injected subjects, which clearly rules out the possibility of a necrotic effect of RISUG on the prostate. Peak area ratios of various metabolites were also calculated and compared for control cases and RISUG-injected subjects. The glycerophosphorylcholine:choline ratio, in particular, indicated the occurrence of partial obstructive azoospermia in RISUG-injected subjects.

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