Effect of a new injectable male contraceptive on the seminal plasma amino acids studied by proton NMR spectroscopy

Koel Chaudhury, Uma Sharma, N.R. Jagannathan, Sujoy K. Guha

Abstract

Effect of RISUG™, a newly developed male contraceptive, on various amino acids of seminal plasma ejaculates was studied by proton magnetic resonance spectroscopy at 400 MHz. Levels of amino acids were compared with the seminal plasma of obstructive azoospermia and controls. Glutamic acid, glutamine, and arginine were found to be high in concentration in human seminal plasma. The concentration of aromatic amino acids such as tyrosine, histidine, and phenylalanine in RISUG-injected subjects showed no significant difference compared to controls (p > 0.1); however, there was a statistically significant decrease in the concentration of these amino acids in obstructive azoospermia. The concentration of some prominent amino acids that showed overlapping resonances, such as isoleucine+leucine+valine (p < 0.01), alanine+isoleucine+lysine (p < 0.01), arginine+lysine+leucine (p < 0.01), and glutamic acid+glutamine (p < 0.01), showed a statistically significant decrease in RISUG-injected subjects compared to controls. Overlay of these amino acid resonances were noticed even at 600 MHz. In general, the total amino acids concentration in RISUG-injected subjects was found to be higher than in azoospermic subjects, confirming the occurrence of 'partial' obstructive azoospermmia in subjects injected with this contraceptive.

Keywords: Male contraceptive; RISUG™; Seminal plasma; Azoospermia; Amino acids; Proton NMR

1. Introduction

The search for a non-toxic, reversible male contraceptive with long-term effectiveness prompted us to carry out research in the field of male contraception. Investigations over the past 25 years have led to the successful development of a new contraceptive injected into the lumen of the vas deferens. The drug, a biologically active polymer, styrene maleic anhydride (SMA) dissolved in dimethyl sulphoxide (DMSO) in a 1:2 ratio has been given the name RISUG (an acronym for Reversible Inhibition of Sperm Under Guidance). When the sperm come in contact with RISUG™, their membranes rupture, enzymes such as acrosin and hyaluronidase are released, and they are rendered incapable of fertilization [1]. Phase I and Phase II clinical trials proved the safety [2] and efficacy [3] of a specific dose of RISUG, respectively. Duration of the contraceptive action can be predicted based upon the dose selection. Dose-dependent contraceptive action with changing dose of RISUG in monkeys (ranging from 5-400 mg of SMA dissolved in DMSO as 1:2 ratio) [4] and in humans (dosage ranging from 40-70 mg of SMA in 80-140 μL of DMSO) [5] has been well demonstrated. Normal semenology picture returned within 3 months of injection in monkeys treated with a low dose of 5 mg, and there was no definite period of contraception. At a higher dose of 16.6 mg in some monkeys, there was a short period of azoospermmia following which spermatozoa were seen to be present in the ejaculate. Administration of SMA over the threshold level of 60 mg produced azoospermia for more than 5 years. In humans, the study shows that 40 mg SMA in 80 μL of DMSO may be taken as the lower limit for contraceptive action. A dose higher than the lower limit of 40 mg, that is, 60 mg of SMA in 120 μL of DMSO, was adopted so that even if some loss occurred because of evacuation by sexual activity in the initial stages after injection, effective contraceptive action was ensured. Further,
close monitoring of Phase II clinical trial subjects (injected with 60 mg of SMA dissolved in 120 μL of DMSO bilaterally in 1994, i.e., about 8 years ago) until now indicates continuing azoospermia and effective contraception (unpublished data, follow-up results).

Swelling of RISUG on hydration leads to the entry of the drug into the folds of the inner wall of the vas deferens for retention of the contraceptive which partially obstructs the smooth flow of spermatozoa from the epididymis. Non-invasive reversal can be achieved by dissolving the contraceptive in an appropriate solvent and flushing it out of the lumen [6] or by stimulating the vas deferens percutaneously for the expulsion of the contraceptive [7]. No evidence of any sclerosing action of RISUG on the tissues has been observed, and it was also found not to be tissue adherent. Toxicologic studies have also been established on removal of RISUG from the vas deferens [9]. The multicentric Phase III clinical trial is currently underway.

Mammalian seminal plasma has a high content of nitrogenous substances consisting of peptides, free amino acids, and various aliphatic and aromatic amines. We were interested in understanding the effect of RISUG on the free amino acid content of seminal plasma using nuclear magnetic resonance (NMR) spectroscopy. We had earlier used this technique to quantify citrate, lactate, glucose, GPC, and choline in seminal plasma ejaculates from RISUG-injected subjects and controls [10]. In recent years, high-resolution 1H NMR spectroscopy has emerged as a powerful tool in identifying and assessing the concentration of metabolites present in seminal fluids [11-15]. The main advantage that NMR spectroscopy offers over classic biochemical analysis is that it is not biased toward a particular compound; it can simultaneously detect metabolites which are expected or unexpected or which are difficult to assay using standard biochemical methods [16].

In the present study, proton NMR spectroscopy has been used to investigate the relative changes in the concentration of some of the free amino acids in seminal plasma ejaculates of RISUG-injected subjects compared to the levels in the seminal plasma of controls. Studies have also been carried out on obstructive azoospermia subjects for comparison.

2. Materials and methods

2.1. Preparation of seminal plasma samples

Semen samples were collected by masturbation after 4 days of sexual abstinence from: (1) 19 healthy volunteers with normal reproductive profiles who had fathered children (age group 32-40 years); (2) 17 subjects injected with the contraceptive RISUG (a typical dose of 60 mg of SMA dissolved in 120 μL of DMSO) bilaterally into the lumen of the vas deferens 6-8 years earlier (age group 36-40 years); and (3) 14 subjects diagnosed with obstructive azoospermia at the infertility clinic (age group 34-38 years). It was ensured that all subjects injected with RISUG had fathered children before the injection of the contraceptive. Further, to maintain uniformity in the present study, only those subjects who had been injected with RISUG during the same time frame, that is, 6-8 years prior, were included. Liquefaction of each sample was carried out at 37°C for 15 min to reduce viscosity. Since an increase in the amino acid content of human semen with time has been reported because of the breakdown of proteins [17], several precautionary measures were taken to keep the liquefaction time identical for each individual sample. An aliquot of 0.5 mL was removed to determine semen parameters using standard World Health Organization (WHO) procedures [18]. The remaining semen sample was centrifuged (1000 × g, 15 min) for removal of cells and spermatozoa. The supernatant was separated; 45 jμL of supernatant was diluted with 555 jμL of deuterium oxide (D$_2$O) (Aldrich Company, Inc., USA) to further reduce the viscosity of the sample and also for field frequency lock. Sodium trimethylsilyl-[2,2,3,3-$^2$H$_4$] - 1- propionate (TSP) (E-Merck, Germany) was added as a chemical shift reference (S 0.0) and internal quantitation standard. Ethylene di-amine tetra-acetic acid (EDTA) 0.1 mM was added to the sample to inhibit proteolysis [19]. As an additional precautionary measure, seminal plasma samples (600 jμL), after being transferred into NMR sample tubes (5 mm outer diameter), were immediately subjected to NMR experiment (within 1 min) to avoid progressive protein and peptide hydrolysis releasing individual amino acids.

2.2. NMR experiment

Proton NMR spectra were acquired at a frequency of 400.13 MHz using Bruker DRX-400 FT-NMR spectrometer (9.4 T) equipped with broad band inverse probe. Typical parameters for one-dimensional (1D) proton NMR experiments were: pulse width 90°, number of data points 16-32 K, spectral width 5000 Hz, number of scans 32-48, and relaxation delay of 14 s. A 0.3 Hz line broadening was applied before Fourier transformation. Intensity of amino acid resonances were measured with reference to TSP resonance. Two-dimensional (2D) double quantum filtered (DQF) correlation spectroscopy (COSY) and 2D J-resolved spectra were recorded for unambiguous assignment of resonances in the seminal plasma sample using standard parameters. The water resonance was presaturated during the relaxation delay of 14 s. A 0.3 Hz line broadening was applied before Fourier transformation. Intensity of amino acid resonances were measured with reference to TSP resonance. Two-dimensional (2D) double quantum filtered (DQF) correlation spectroscopy (COSY) and 2D J-resolved spectra were recorded for unambiguous assignment of resonances in the seminal plasma sample using standard parameters. The water resonance was presaturated during the relaxation delay of 2.5 s. 1D proton spectra were also recorded on Varian Unity Plus 600 MHz spectrometer at Tata Institute of Fundamental Research (TIFR), Mumbai, India, for a few samples. Typical parameters used for 600 MHz experiments were data points 32 K, number of scans 16, pulse width 60°, relaxation delay 5 s, and spectral width 6000 Hz.
2.3. Quantification of metabolites

Peaks or multiplets of prominent free amino acids, including that of internal standard TSP, were integrated to obtain signal intensity after baseline correction. The concentration of free amino acids present in seminal plasma was calculated following the methodology used earlier [10]. Extensive overlap of resonances (even at 600 MHz) and the nature of complexity of biofluid may act as limiting factors for accurate analysis of certain metabolites and, hence, their exact quantitation. However, the aim of the present study was to assess the relative differences in the amino acid levels of RISUG-injected subjects compared to controls and azoospermic subjects; therefore, the methodology used seemed appropriate.

2.4. Statistical analysis

Concentration of the various amino acids in the seminal plasma ejaculates of RISUG-injected subjects, obstructive azoospermic subjects, and controls were expressed as mean values ± SD. Data obtained from RISUG-injected subjects was compared to that of obstructive azoospermic subjects and controls using the Student’s t-test, with the level of significance set at p < 0.01.

3. Results

Fig. 1 (aliphatic region 0.7-2.5 ppm) and Fig. 2 (aromatic region 6.9-8.0 ppm) show the expanded region of a typical 1D proton NMR spectra of seminal plasma recorded at 400 MHz of (a) controls, (b) RISUG-injected subjects, and (c) obstructive azoospermic subjects, respectively. The assignment of resonances arising from various amino acids were made with the help of double quantum filtered COSY and J-resolved spectra (spectra not shown here). Methyl resonances of Ile, Leu and Val exhibited intense signals between 0.90 to 1.00 ppm in the spectra. Doublet corresponding to the methyl resonance of Ala was easily identifiable at 1.48 ppm (Fig. 1). Glu and Gln protons showed prominent signals in the spectra. The multiplet of β-CH2 of Glu and Gln at 2.04 ppm and 2.11 ppm, respectively, showed cross peaks with their γ-CH2 protons in COSY spectra (Fig. 1). Lys was easily assigned by tracing its cross peaks between e-CH2 (at 3.02 ppm) and S-CH2 (at 1.67 ppm). In a similar fashion, the proton resonances from Arg were assigned. Ring protons H3, H5, and H4, H6 of aromatic residue Tyr were assigned at 6.91 and 7.16 ppm, respectively, using DQF COSY. His and Phe also showed sharp peaks in the spectra. At higher concentration of seminal plasma, the aromatic protons from Trp were observed.

The quantitative estimation of various amino acids from the spectra in RISUG-injected subjects were compared with that of azoospermia and controls. Well resolved resonances were observed for Tyr, Phe, and His in all the groups, thereby facilitating accurate concentration estimation. In dilute samples, Trp protons were of very low intensity; hence, no attempt was made to quantify its concentration. Other amino acids, such as Leu, Ile, Val, etc., exhibited...
Table 1

Concentration of amino acids (mM/P\(^1\)) along with the chemical shift, assignment and multiplicity of human seminal plasma of (a) normal controls, (b) subjects injected with the contraceptive RISUG\(^\text{TM}\) into the vas deferens lumen, and (c) subjects with obstructive azoospermia

<table>
<thead>
<tr>
<th>Amino acids(^a)</th>
<th>Chemical shift (S)</th>
<th>Assignment</th>
<th>Multiplicity</th>
<th>Normal controls (a)</th>
<th>RISUG-injected subjects (b)</th>
<th>Obstructive azoospermia (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile+Leu+Val</td>
<td>0.92, 0.99</td>
<td>t, d</td>
<td>24.2 ± 6(^a)</td>
<td>12.5 ± 2.3</td>
<td>5.2 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.94, 0.95</td>
<td>d, d</td>
<td>p &lt;0.01(^c)</td>
<td>p* &lt;0.01(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98, 1.03</td>
<td>d, d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala+Lys+Ile</td>
<td>1.47</td>
<td>d</td>
<td>35.8 ± 8.5</td>
<td>19.2 ± 4.4</td>
<td>4.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.47</td>
<td>m</td>
<td>p &lt;0.01</td>
<td>p* &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.45</td>
<td>m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg+Lys+Leu</td>
<td>1.66</td>
<td>m</td>
<td>58.7 ± 5.5</td>
<td>31.9 ± 6.9</td>
<td>10.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.70</td>
<td>m</td>
<td>p &lt;0.01</td>
<td>p* &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>m</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>2.04</td>
<td>m</td>
<td>59.1 ± 9</td>
<td>27.8 ± 5.1</td>
<td>12.5 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.11</td>
<td>m</td>
<td>p &lt;0.01</td>
<td>p* &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>6.91</td>
<td>d</td>
<td>10.9 ± 2.9</td>
<td>9.7 ± 2.7</td>
<td>2.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.14</td>
<td>s</td>
<td>p &gt; 0.1</td>
<td>p* &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>7.33</td>
<td>d</td>
<td>2.4 ± 0.9</td>
<td>2.5 ± 0.7</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &gt; 0.1</td>
<td>p* &lt;0.01</td>
<td></td>
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</tbody>
</table>

\(^a\) Values are expressed as mean ± SD.

\(^b\) Abbreviations used: Ile: isoleucine; Leu: leucine; Val: valine; Ala: alanine; Lys: lysine; Arg: arginine; Glu: glutamic acid; Gln: glutamine; Tyr: tyrosine; Phe: phenylalanine; His: histidine.

\(^c\) p < 0.01: Student’s t-test for RISUG-injected subjects and controls.

\(^d\) p* < 0.01: Student’s t-test for RISUG-injected subjects and obstructive azoospermia.

overlapping resonances at 400 MHz, making accurate quantitation of individual amino acids difficult. Even the proton spectra recorded at 600 MHz did not show well resolved signals (spectra not shown). The concentration of the various amino acids in the seminal plasma of controls, RISUG-injected, and obstructive azoospermic subjects are presented in Table 1, along with their chemical shifts.

No significant difference was observed in the concentration of Tyr (p > 0.1), Phe (p > 0.1), and His (p > 0.1) in the seminal plasma of RISUG-injected subjects compared to controls. However, significant decrease was observed in the concentration of the overlapping peaks of Ile+Leu+Val (p < 0.01), Ala+Ile+Lys (p < 0.01), Arg+Lys+Leu (p < 0.01), and Glu+Gln (p < 0.01) in RISUG-injected subjects compared to controls. Interestingly, concentrations of all free amino acids were found to be significantly lower in obstructive azoospermic cases compared to RISUG-injected subjects (p* < 0.01).

4. Discussion

Amino acids act as chelating agents and provide buffers which have a protective influence on sperm cells. They also serve as oxidizable substrates for spermatozoa and often undergo metabolic changes in the ejaculated semen under the influence of transaminases and deaminases [19]. A comparison of free amino acid concentration of human seminal plasma in four different andrological diagnoses has strongly indicated a connection between certain specific amino acids and spermatozoa [20]. Unfortunately, little is known about the role of individual amino acids present in seminal plasma. Arginine and glutamic acid present in semen have, however, drawn considerable attention of several researchers in the past years. A correlation between L-arginine deficiency and loss of spermatogenesis and a decrease in the motility of sperm cells have been reported [21-23]. Aydin et al. have shown that the administration of L-arginine to oligospermic and asthenospermic patients results in the im-
provement of both the sperm count and motility, without any side effects [24]. NMR has been used to elucidate the effect of L-arginine, L-lysine, and L-ornithine on the glycolysis of epididymal goat spermatozoa [25]. It has also been shown that arginine acts as a protective and reversal agent against glycolytic inhibitors in spermatozoa [26]. Glutamic acid is present in high concentration levels in testicular and seminal plasma. In human seminal plasma from normal ejaculates, a total amino acid content of 1.257 g/100 mL has been reported, 0.297 g of which is because of glutamic acid, whereas the total amino acid content in azoospermic semen is 0.752 g, of which 0.189 g comes from glutamic acid [19]. In the present study, the concentration of glutamic acid, glutamine, and arginine was observed to be higher than other free amino acids in the seminal plasma of RISUG-injected subjects, obstructive azoospermic cases, and controls. This is in agreement with the studies carried out by Brown-Woodman and White on the amino acid composition of semen using electrophoretic techniques, where they reported glutamic acid and arginine to be the predominant amino acids in the human seminal plasma [27].

The absence of significant changes in the aromatic amino acids such as Tyr, Phe, and His and significant decrease in other amino acids such as Arg, Lys, Glu, etc., in RISUG-injected subjects compared to controls requires further investigation. However, a probable hypothesis on the basis of interaction of epididymal amino acids with the charged groups of RISUG is suggested. When a typical dose of RISUG (60 mg of SMA dissolved in 120 pL of DMSO) is injected bilaterally into the vas deferens lumen, the SMA-DMSO complex becomes converted into hydride and swells up, accompanied by a lowering of pH at the site of action of the contraceptive [1]. The ionization state of an amino acid is known to vary with pH. Because the contraceptive has a pH-lowering effect, the amino acids reaching the vas deferens from the epididymis tend to become ionized. Subsequently, these ionized amino acids interact with the charged groups of RISUG. Tyr, His, and Phe, unlike other amino acids, have a hydrophobic character because of their aromatic side chains and, therefore, flow past RISUG, probably without any interaction. Therefore, no significant difference was observed in the concentration of aromatic amino acids on comparing ejaculates of RISUG-injected subjects to that of controls.

In conclusion, proton NMR spectroscopy was used to determine the concentration of free amino acids present in the seminal plasma ejaculates of RISUG-injected subjects, obstructive azoospermic cases, and controls. Glutamic acid, glutamine, and arginine were found to be high in concentration in human seminal plasma. The concentration of Tyr, His, and Phe in RISUG-injected subjects showed no significant difference when compared to controls. However, a significant decrease was observed in the concentration of Ile+Leu+Val, Ala+Ile+Lys, Arg+Lys+Leu, and Glu+Gln in the seminal plasma of RISUG-injected subjects compared to controls. The concentration of these amino acids, including Tyr, His, and Phe, was found to be significantly reduced in obstructive azoospermic cases compared to RISUG-injected subjects. This observation supports our earlier hypothesis of the occurrence of 'partial' obstructive azoospermia in subjects injected with the contraceptive RISUG. A simple theory based on the interaction between epididymal amino acids and RISUG in the vas deferens and their net effect on the amino acid content in the seminal fluid has been suggested to account for concentration differences in the seminal plasma of RISUG-injected subjects.

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