Low seminal zinc bound to high molecular weight proteins in asthenozoospermic patients: evidence of increased sperm zinc content in oligoasthenozoospermic patients

A.Carpi no1,4, L.Siciliano1, M.F.Petrone1, C.De Stefano2, S.Aquila2 and S.Ando1

1 Dipartimento di Biologia Cellulare and 2 Centro Sanitario, Università degli Studi della Calabria, 87030 Arcavacata di Rende (Cs), and 3 Centro Trattamento della Sterilità, Diagnostica Medica, 86100 Avellino, Italy.

To whom correspondence should be addressed

Total seminal zinc concentration, seminal zinc fraction bound to high molecular weight proteins (HMW-Zn%) and zinc content in spermatozoa were assayed in the ejaculates of 90 asthenozoospermic patients subdivided into two study groups: normoasthenozoospermics (group I: n = 50) and oligoasthenozoospermics (group II: n = 40). The zinc concentrations of patients were compared with those of a control group of donors showing normal semen parameters. All samples were also investigated for their sperm membrane functional integrity by the hypo-osmotic swelling test (HOS). The results showed normal total zinc concentrations but very low HMW-Zn% values (P < 0.001) in seminal plasma of the two groups of asthenozoospermic patients compared to the controls. Furthermore higher zinc amounts (P < 0.001) were measured in spermatozoa of oligoasthenozoospermic patients compared to group I and to the control group. Oligoasthenozoospermics also displayed a lower HOS score (P < 0.001) compared to the other two groups. These data suggest that the increased unbound seminal zinc could contribute to the decrease of sperm motility in normoasthenozoospermic and oligoasthenozoospermic patients. A further impairment in sperm motility could occur in the oligoasthenozoospermic patients where the increase of seminal free zinc was followed by a major zinc uptake by spermatozoa. The higher intrasperm zinc content in these patients could be a reflection of their low sperm membrane functionality.

Key words: HMW-Zn%/human sperm motility/hypo-osmotic swelling test/intrasperm zinc/seminal zinc

Introduction

Human sperm properties may be influenced by interactions among the sex accessory gland secretions (Austin, 1985). One of the biochemical processes related to the genital fluid mixing is the regulation of the free seminal zinc fraction which can interact with spermatozoa (i.e. zinc bioavailability). Zinc is first secreted in prostatic fluid in two forms available for sperm cells (free zinc and zinc–citrate complex). During ejaculation, however, a partial redistribution of the ion from citrate to very high affinity vesicular ligands reduces the unbound zinc fraction (Arver, 1980; Arver and Eliasson, 1982; Björndahl and Kvist, 1990; Kvist et al., 1990). Therefore, the amount of zinc bound to vesicular high molecular weight proteins (HMW-Zn%) is considered to be an index of seminal plasma chelating capacity and it is a measure of zinc bioavailability (Björndahl et al., 1991). Consequently, despite the routine measurement of total seminal zinc in the assessment of sperm activity, only HMW-Zn% values can be a suitable parameter with which to study the relationship between seminal zinc concentration and sperm function.

The aim of the present study was to evaluate the possible relationship between sperm motility on the one hand and total seminal zinc concentration and HMW-Zn% values on the other, by measuring these parameters in the ejaculates of two selected groups of asthenozoospermic patients (one normo- and one oligozoospermic). Furthermore the influence of zinc availability on the intracellular uptake of the ion was evaluated by measuring the intrasperm zinc content in the same semen samples. The functional integrity of sperm membranes was also measured by the hypo-osmotic swelling test (HOS).

Materials and methods

Semen specimens

The ejaculates of 90 asthenozoospermic patients (between 20 and 36 years of age), consulting the Sterility Centre for couple infertility, were examined in the present study. The patients were considered infertile because they had failed to achieve a pregnancy for at least 2 years. They were submitted to clinical and laboratory examinations: they were healthy, they did not have a varicocele, they had normal testosterone (T), 5-α-dihydrotestosterone (DHT), follicle stimulating hormone (FSH), luteinizing hormone (LH) serum concentrations and had not received previous medication. Gynaecological evaluations of their partners excluded obstructive pathologies or ovulation disorders.

Asthenozoospermic subjects were subdivided into normoasthenozoospermics (group I: n = 50) and oligoasthenozoospermics (group II: n = 40) according to the World Health Organization criteria (WHO, 1992) (Table I). The ejaculates of 25 healthy donors (from 19 to 34 years of age) with normal clinical, hormonal and seminal parameters, served as the control group (Table I).

Informed consent was obtained from all the subjects examined.

Semen collection and analysis

Semen was collected from patients after 3–5 days of sexual abstinence. Following incubation at 37°C for 30 min to liquefy the semen, a small portion of each sample was used to carry out routine laboratory analysis (assessed by the same person throughout): volume, pH, viscosity and by phase-contrast microscopy, sperm morphology, sperm concentration, round cell determination and 2 h progressive sperm motility (WHO, 1992). In addition the HOS was also performed...
Centrifugation of semen onto isotonic Percoll solution. Spermatozoa intracellular assay was carried out on sperm cells obtained by was performed on a 1000-fold sample dilution. The intra- and interassay variation coefficients were respectively 3 and 5.5%.

**Table I. Ejaculate parameters**

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Sperm count ($\times 10^6$ ml$^{-1}$)</th>
<th>Progressive sperm motility (%)</th>
<th>Normal sperm forms (%)</th>
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<tr>
<td>Controls</td>
<td>3.9 ± 1.4</td>
<td>93 ± 38</td>
<td>55 ± 5</td>
<td>66 ± 10</td>
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<td>(n = 25)</td>
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<tr>
<td>Normoasthenozoospermics (I)</td>
<td>3.7 ± 1.6</td>
<td>83 ± 30</td>
<td>26 ± 6$^a$</td>
<td>60 ± 8</td>
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<td>Oligoasthenozoospermics (II)</td>
<td>4.03 ± 1.8</td>
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<td>57 ± 7</td>
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Values are means ± SD. $^a$Significantly different from group C ($P < 0.001$). $^b$Significantly different from group I ($P < 0.01$).

(See et al., 1984). An aliquot of the remaining semen was centrifuged at 2000 g for 15 min and the seminal plasma was collected and stored at −20°C until biochemical determination was made. Intracellular zinc analysis was performed on spermatozoa after having separated them from seminal plasma and other ejaculate cells by semen centrifugation on isotonic Percoll solution. Semen samples with increased viscosity or with round cell count $>10^6$ ml were excluded from the study. Collection and processing of donor and patient ejaculates were done over a period of 15 months (November 1995 to January 1997).

**Hypo-osmotic swelling test**

Soon after liquefaction, 0.1 ml of each semen sample was mixed with 1 ml of a hypo-osmotic solution (containing 0.04 M sodium citrate and 0.046 M fructose in water). Following an incubation period of 30 min at 37°C, the percentage of swollen spermatozoa observed under a phase-contrast microscope was calculated (Jeyendran et al., 1984).

**Semen centrifugation on Percoll**

Isotonic Percoll solution was prepared in H-KR buffer (121.3 mM NaCl; 4.8 mM KCl; 1.2 mM MgSO$_4$·7H$_2$O; 26.7 mM HEPES and 5.5 mM D (+) glucose; pH 7.4). Soon after liquefaction, a semen sample (1–2.5 ml) was layered slowly over 5 ml of pre-warmed isotonic Percoll (density $1.05$ g/ml) and then it was centrifuged at 2000 g for 10 min (Arcidiacono et al., 1983; Campana et al., 1989). Spermatozoa, collected in the pellet, were re-suspended in buffer for counting, then they were washed and lyophilized.

**Seminal plasma total protein assay**

Protein concentration in seminal plasma was determined by the Biuret method (Yatzidis, 1977) using human serum albumin for the standard curve. The intra- and interassay variation coefficients were respectively 3 and 5.5%.

**Zinc assay**

Zinc analysis was performed by atomic absorption spectrophotometry (Pabisch Hitachi, Atomic Absorption Spectrophotometer Model Z-1800) using the flame technique. An air-acetylene flame with an air flow rate of 15 litre/min and an acetylene flow rate of 1.5 litre/min was used. The standard curve ranged from 3 to $10^6$ µg/dl aqueous zinc standard.

Zinc analysis in seminal plasma and in spermatozoa was carried out according to Pleban and Mei (1983). The assay in seminal plasma was performed on a 1000-fold sample dilution. The intra- and interassay variation coefficients were respectively 4.5 and 6.2%. The intracellular assay was carried out on sperm cells obtained by centrifugation of semen onto isotonic Percoll solution. Spermatozoa were collected in the pellet, their concentration was adjusted to $20–50 \times 10^6$/ml (no significant interference signal was detected in this range) and they were lyophilized. Concentrated nitric acid was used to digest the cells (50 µl/mg of lyophilized spermatozoa, overnight) and a 100-fold cell digest dilution was analysed for zinc content. Intracellular zinc amount was expressed in µg/$10^6$ spermatozoa.

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Semen parameters of normoasthenozoospermic patients (group I), oligoasthenozoospermic patients (group II) and the control group (C) are shown in Table I.

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**Sephadex G-75 gel chromatography**

For determination of the amount of zinc bound to high molecular weight proteins (HMW-Zn%), the gel filtration of seminal plasma on Sephadex G-75 was performed (Arver, 1980). The gel was packed in a 2.5×40 cm glass column and equilibrated and eluted with 0.05 M Tris buffer containing 0.15 M NaCl, pH 7.4. The eluate was collected at 20°C at a flow rate of 10–16 ml cm$^{-2}$ h$^{-1}$ in 50 fractions. All the fractions were investigated for protein ($A_{280nm}$) and for zinc concentration by atomic absorption spectrophotometry. HMW-Zn% was calculated from the zinc concentration in all the fractions of the void volume and expressed as a percentage of the total zinc.

**Statistical analysis**

Student’s $t$ and the Mann–Whitney $U$-tests were used for statistical analyses. A power study was performed to determine the sizes of the two groups in order to ensure the detection of significant differences at $P < 0.01$. Significance was assigned for $P < 0.01$.

**Results**

Semen parameters of normoasthenozoospermic patients (group I), oligoasthenozoospermic patients (group II) and the control group (C) are shown in Table I.

Similar total protein concentrations (mean ± SD) were detected in seminal plasma of group I, group II and C ($32 ± 12, 30 ± 10, 34 ± 11$ mg/ml respectively). Seminal zinc concentrations (mean ± SD) of group I and II were not significantly different with respect to the controls ($200 ± 108, 180 ± 93, 194 ± 92$ µg/ml respectively) (Figure 1). HMW-Zn (%) values (mean ± SD) in seminal plasma of group I and II were lower than in the controls ($5.9 ± 2.9$% group I versus $11.5 ± 4.6$% control, $t = 6.4$ ($P < 0.001$); $6 ± 3.2$% versus control, $t = 5.7$ ($P < 0.001$) (Figure 1). The intrasperm zinc amount (mean ± SD) in sperm cells of group I was not different from that of controls ($11 ± 5.6$ and $10.5 ± 4.8$ µg/$10^6$ spermatozoa respectively) while the intrasperm zinc concentration of group II was higher than both ($76 ± 33$ µg/$10^6$ spermatozoa, $t = – 9.8$,
Figure 1. Zinc concentration (A) and zinc bound to high molecular weight proteins (HMW-Zn%) (B) in seminal plasma of control (C), normoasthenozoospermic (group I) and oligoasthenozoospermic (group II) subjects.

Figure 2. Intraspem zinc amount (A) and percentage of swollen spermatozoa, under hypo-osmotic conditions (B) in semen of control (C), normoasthenozoospermic (group I) and oligoasthenozoospermic (group II) subjects.

Discussion

Conflicting clinical data concerning the influence of human seminal plasma zinc concentrations on sperm motility have been reported (Skandhan et al., 1978; Caldamone et al., 1979; Wood et al., 1982; Carreras and Mendoza, 1990; Ahlgren et al., 1995; Lewis-Jones et al., 1996). However, a clear inhibitory effect of free extracellular zinc on the motility of human spermatozoa was demonstrated by previous in-vitro studies at zinc concentrations lower than those in seminal plasma (Lindholmer, 1974; Riffo et al., 1992). Therefore, total seminal zinc concentration may not be a useful index of the zinc fraction interacting with sperm cells, and a more appropriate marker of the ion bioavailability should be used to evaluate its relationship to sperm function. In the present study free seminal zinc has been investigated by measuring the amount of zinc bound to vesicular high molecular weight proteins (HMW-Zn%) (Björndhal et al., 1991). In fact, the unbound zinc fraction depends on a post-ejaculatory redistribution of the ion from prostatic to higher affinity vesicular ligands.

As expected, total seminal zinc concentration was not a discriminating parameter among subjects with normal or low sperm motility, while HMW-Zn% was decreased in normo- and oligoasthenozoospermic subjects. This suggests an increase of the free extracellular zinc concentrations in their semen. So, the high unbound zinc fraction could contribute to the impairment of sperm motility in such patients; the zinc toxicity on cells might even be mediated by interference by the ion on plasma membrane permeability (Bettger and O’Dell, 1981).
Furthermore, as suggested by the reported inverse correlation between HMW-Zn% and zinc content in human sperm chromatin (Björndhal and Kvist, 1990), the amount of zinc in subjects’ spermatozoa was measured to evaluate how seminal ion availability can influence its intracellular uptake. Increased zinc concentrations were detected only in spermatozoa of oligoasthenozoospermic patients, in whom it is assured that the increase of zinc availability led to a major uptake of zinc by spermatozoa, with possible resultant ion interferences in metabolic energetic sperm processes (Huacuja et al., 1973). This might appear to be an additional cause of the very low sperm motility observed in these patients.

Oligoasthenozoospermic subjects also displayed a lower HOS score, indicating a reduced functional integrity of their sperm membrane. Therefore, in these patients, the higher intrasperm zinc concentrations could be a reflection of the impaired membrane permeability. This hypothesis is supported by the observation that zinc concentrations in a very small subgroup of oligoasthenozoospermic patients with normal HOS score were not significantly altered. Further investigations are needed to confirm this.

This study points out the importance of interactions within genital fluid on sperm properties. It also puts into perspective the relationship between the free (but not the total) seminal zinc concentrations and sperm motility. Therefore, in agreement with a recent study (Lewis-Jones et al., 1996), the evaluation of total seminal zinc concentrations is scarcely helpful for the assessment of sperm function. In fact, only further studies addressed to investigate zinc binding properties in semen will provide new therapeutic tools to improve sperm activity in infertile patients.

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References


