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Chelation of intracellular zinc ions affects human sperm cell motility

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The effects of two different zinc chelators, diethyldithiocarbamate (DEDTC) and calcium ethylenediaminetetra-acetic acid (EDTA), in full semen samples and 'swim-up' samples were investigated. DEDTC, which crosses cell membranes, and EDTA, which does not cross cell membranes, were added to semen samples in different concentrations. Sperm cell motility parameters were assessed by computer-assisted semen analysis (CASA). It was found that very small concentrations (0.01 mM) of DEDTC immobilized the sperm cells within 80 min, while EDTA had no depressing effect at the concentrations used. In full semen samples EDTA enhanced straight line velocity (VSL) at concentrations of 1.0 and 0.5 mM; this effect was not found at higher concentrations. It is suggested that intracellular mitochondrial zinc ions play a crucial role for sperm cell motility, while loosely bound or free zinc ions in the seminal plasma exert a secondary role on human sperm cell motility.

Key words: CASA/chelator/human/semen/spermatozoa/zinc

Introduction

Over the years there have been conflicting reports on the effect of seminal zinc on sperm motility. Some authors have reported high zinc concentration to be associated with enhanced sperm cell motility (Stankovic and Mikac-Devic, 1976; Caldamone *et al*., 1979), whereas Danscher *et al*. (1978) reported high zinc concentration to be associated with poor sperm motility. However, Lewis-Jones *et al*. (1996) were unable to find any association between total seminal zinc and sperm cell motility in 1178 patients referred for fertility treatment. It has been demonstrated that chelation of zinc ions in the ejaculate affects sperm motility in man (Danscher and Rebbe, 1974), rat, and dog (Saito *et al*., 1967; Stoltenberg *et al*., 1997a).

Carreras and Mendoza (1989) studied seminal zinc concentrations in normo-, oligo-, astheno-, oligoastheno- and azoospermic men. They observed statistically significant elevated zinc concentrations in asthenozoospermic men only, and for this group a significant correlation of zinc concentrations to sperm concentration was also observed. When all men with at least one abnormal spermiogram were grouped together and compared to the normal spermiogram group no difference in zinc concentrations was detected. Riffo *et al*. (1992) observed reduced grade 3 and 4 motility when 0.1 or 1 mM zinc was added to semen samples. These doses also caused a decrease in the number of sperm cells undergoing acrosome reaction. Both actions were partly reversible after removal of zinc and incubation in zinc-free medium.

The aim of the present study was to evaluate the role of chelatable zinc, i.e. free or loosely bound zinc ions in the ejaculate, on sperm cell motility. The zinc chelators diethyldithiocarbamate (DEDTC) and calcium ethylenediaminetetra-acetic acid (EDTA) were chosen in order to assess the significance of intracellular and extracellular zinc ion chelation in relation to sperm cell motility.

Materials and methods

Conventional semen characteristics

Semen samples from five healthy volunteers were obtained by masturbation into 50 ml sterile polystyrene jars after recommended 3 days of abstinence. Each donor delivered between three and five samples. The donors had proven fertility. Volume varied from 1.3 to 9.6 ml, and sperm concentration varied from 21 and 130×10^6/ml. Sperm motility was manually assessed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) on an Olympus BH-2 phase-contrast microscope (Olympus Denmark A/S, Glostrup, Denmark) at Ï400 magnification. Each spermatozoon encountered was graded according to the World Health Organization criteria (1992). The percentage motile was expressed as the percentage of sperm cells in category 'a + b + c' and ranged from 49–81%.

Washed semen preparations

Eight samples were subjected to 'swim-up'. SpermWash® (produced by Ciconia Products ApS, Denmark, distributed by Cryos, Vesterport 3, Aarhus C, Denmark) was used. SpermWash® medium contains Earle's balanced salt solution (EBS) with human serum albumin 2%, HEPES, and bicarbonate buffer stabilized with CO_2 to pH 7.4 in a sterile ampulla with teflon-coated rubber membrane.

Two ml of liquefied semen was deposited beneath 2 ml medium and the ampulla was kept at 37°C for 90 min in a 45° angle position. After incubation 1.5 ml of the upper layer containing the most vivid spermatozoa was harvested.

Chelators

Each sample was divided into smaller volumes, and the zinc chelators DEDTC (Merck, Darmstadt, Germany) and calcium EDTA (Sigma,

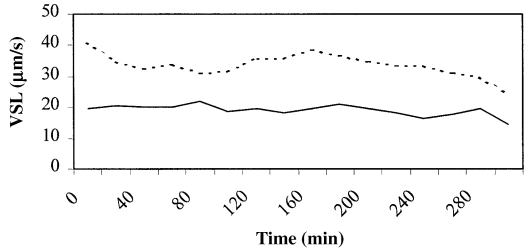

Figure 1. Straight line velocity (VSL) as a function of time for controls. The dotted line represents 'swim-up' controls ($n = 8$) and the solid line is full semen sample ($n = 8$). $P < 0.01$.

St Louis, MO, USA) added to the following concentrations: DEDTC: 5, 1, 0.5, 0.1 and 0.01 mM; EDTA: 5, 1, 0.5 and 0.1 mM.

$ZnCl_2$

To four 'swim-up' and four full semen samples was added 0.6 mM $ZnCl_2$.

Computer-assisted semen analysis (CASA)

Each semen sample was analysed every 20 min. 4.5 µl of fresh, well mixed sperm was transferred by pipette to a Makler counting chamber with a depth of 10 µm. The sample was placed in an Olympus BH-2 phase-contrast microscope (Olympus Denmark A/S, Glostrup, Denmark) with a heating plate (37°C) at Ï200 magnification, and a Sony video camera DXC-107 (Sony Corp., Tokyo, Japan) transferred the images to a Sony PVM-1440QM colour video monitor (Sony Corp., Tokyo, Japan). Recordings of the images were made on a JVC HR-D560EG/E video cassette recorder (JVC Victor Company of Japan, Tokyo, Japan). Recordings were later analysed on a Hobson Sperm Tracker (Hobson Tracking Systems Ltd, Sheffield, UK) at an acquisition frequency of 25 Hz, tracking time 2 s (total of 50 frames), and field of view 300Ï300 µm [allowing all straight line velocity values of up to 150 µm/s to be detected]. 100 spermatozoa were analysed per sample.

Controls

Full semen samples and 'swim-up' samples served as controls, and they were paired with the samples receiving either a chelator or $ZnCl_2$.

Statistics

Paired samples (case/control) were tested by a paired *t*-test for comparison of means. Non-Gaussian distribution medians were compared by Wilcoxon rank-sum test.

Results

'Swim-up' preparation of semen samples caused an enhancement in curvilinear velocity (VCL) and in straight line velocity (VSL) compared to full semen samples (Figure 1). Adding DEDTC to the ejaculate resulted in a dose-dependent immobilization of the sperm cells. At 1 mM the immobilization took place almost instantly while at 0.01 mM it took ~80 min (Figure 2). In 'swim-up' semen samples immobilization was also observed within 80 min (Figure 3). EDTA did not have any detectable harmful effect on motility assessed by CASA. VSL was unaffected at an EDTA concentration of 5 mM, whereas slightly raised VSL values were observed at 1.0 and 0.5 mM (Figures 4 and 5). This was statistically significant ($P < 0.01$). No reduction in the percentage of motile sperm cells in EDTA-treated samples compared to controls was

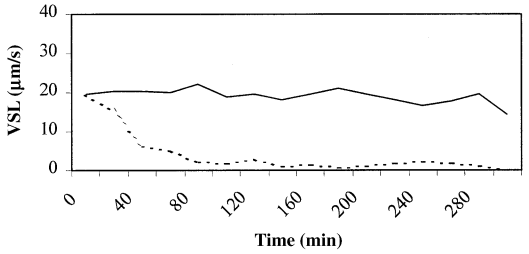

Figure 2. Straight line velocity (VSL) as a function of time for controls (solid line, $n = 8$) and semen samples with 0.01 mM DEDTC (dotted line, $n = 8$). $P < 0.01$.

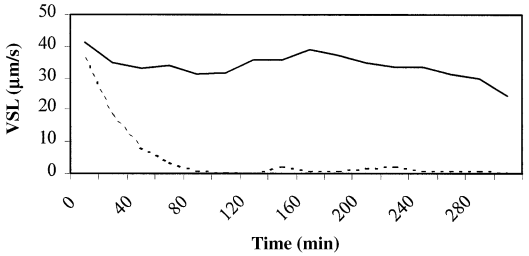

Figure 3. Straight line velocity (VSL) as a function of time for 'swim-up' controls (solid line, $n = 8$) and 'swim-up' semen samples with 0.01 mM DEDTC (dotted line, $n = 8$). $P < 0.001$.

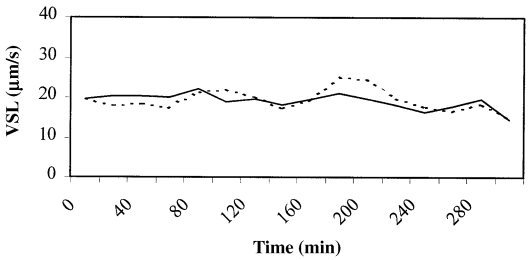

Figure 4. Straight line velocity (VSL) as a function of time for controls (solid line, $n = 8$) and semen samples with 5 mM EDTA (dotted line, $n = 8$). $P = 0.47$.

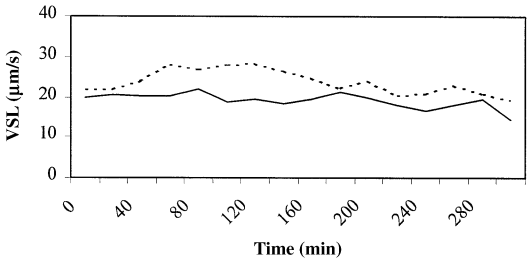

Figure 5. Straight line velocity (VSL) as a function of time for controls (solid line, $n = 8$) and semen samples with 1 mM EDTA (dotted line, $n = 8$). $P < 0.01$.

observed. In 'swim-up' preparations no effect was seen upon addition of EDTA in either concentration. Addition of 0.6 mM $ZnCl_2$ to either preparation did not change any parameter significantly.

Discussion

The present study shows that the membrane-penetrating zinc chelator DEDTC immobilizes sperm cells as previously reported by Danscher and Rebbe (1974). Even small concentra-

tions rapidly penetrate the membrane and influence sperm motility. No negative effects of the non-penetrating chelator EDTA were observed at any concentration. The middle piece of the sperm cell contains the mitochondrial sheath, and earlier studies have demonstrated the presence of zinc ions in these mitochondria (Stoltenberg *et al*., 1997b). Also it has been shown that DEDTC blocks these zinc ions and that when this block is completed the sperm cells stop moving. A previous study from this laboratory (Stoltenberg *et al*., 1997b) has demonstrated the presence of zinc ions in full semen samples in the midpiece, head and tail, while in 'swim-up' semen samples zinc ions were only detected in the helecine mitochondria of the midpiece. Following 'swim-up' the sperm cell lost the zinc ions that were associated with the cell membrane. DEDTC, which is the active metabolite of disulfiram [tetraethylthiuramdisulphide (Antabuse®)], irreversibly effects oxidation in liver mitochondria (Hassinen, 1966), and it is tempting to ascribe the effect of this compound on sperm motility to its effect on sperm mitochondria.

As EDTA does not cross sperm membranes it was to be expected that this chelator had no harmful effect on sperm motility. Huacuja *et al*. (1973) found an enhancing effect on sperm motility 15 and 30 min after addition of 6 mM EDTA to washed semen samples, but this effect on motility vanished after longer periods of time. Huacuja *et al*. used distance travelled by the spermatozoa assessed manually as sperm motility parameter, and the results were tested by an unpaired *t*-test. EDTA did not improve motility of 'swim-up' preparations in this study. However, addition of 1 and 0.5 mM EDTA was found to have an enhancing effect on VSL in full semen samples after 60 min, but this effect was absent at other concentrations of EDTA. We have no explanation for this observation. It might suggest that large amounts of free zinc in the seminal plasma can have a depressing effect on sperm motility, and that a certain lowering of this leads to improved motility. It should be noted that seminal zinc concentrations according to the literature are ~1–1.5 mM (Abou-Shakra *et al*., 1989; Riffo *et al*., 1992).

No changes in any of the parameters were observed when $ZnCl_2$ was added to the semen samples. The initial seminal zinc concentration in the full semen samples was not measured, but 'swim-up' samples must be expected to carry only zinc ions located intracellularly. The concentration of zinc added, 0.6 mM $ZnCl_2$, is only about half of the average total seminal zinc content, i.e. zinc ions and firmly bound zinc. In contrast to the results of this study, Saito *et al*. (1967) found 0.02 and 0.2 mM zinc chloride to enhance dog epididymal sperm cell motility, and 2 mM to slow down rat epididymal sperm cell motility. We have no explanation for these differences but that human sperm cells might behave differently from dog and rat sperm.

In the present study semen samples with different volumes and sperm concentration were used. Even though the range for volume was quite large (1.3–9.6), the SD was only 9.8% of the mean (mean 5.6, SD 0.55). For sperm concentration the range was 21–130$\times 10^6$/ml with a mean and SD of 57 $\pm$ 16.5, or 28.9%. In another study (Sørensen *et al*., 1998) no correlation between sperm volume, concentration or percentage motile to zinc concentration was found. This supports a study by Abou-Shakra *et al*. (1989) where no significant correlation between zinc concentration in seminal fluid and sperm density or motility was found. In this study the effect of DEDTC on sperm motility was profound even at small concentrations of the zinc chelator, and it is unlikely that different seminal zinc concentrations are responsible for the observations. Zinc concentrations in the samples were not known, and addition of 1 and 0.5 mM EDTA to full semen did have a positive effect on VSL. This was observed in all samples, which might suggest that lowering of seminal zinc concentration does in fact improve progressive movement. From this study it is not possible to tell if there is a lower limit. It has been suggested by Carpino *et al*. (1998) that an increase in the free zinc fraction could contribute to a decrease in progressive sperm motility in normoasthenozoospermic and oligoasthenozoospermic patients. In support of this it was observed that lowering of the free zinc fraction with EDTA caused improved VSL.

EDTA and DEDTC have pK-values of 16.5 and 15.3 for zinc, which are much higher than the pK values for calcium and magnesium. Neither calcium nor magnesium can create sulphide crystals that can be developed by autometallography (AMG). In a previous study (Stoltenberg *et al*., 1997b) it was observed that EDTA treatment of semen blocks the AMG staining of the plasma, while DEDTC blocks both intra- and extracellular staining. In a study by Bourinbaiar and Lee (1996) gramicidin and EDTA in combination was found to be very effective in arresting sperm motility. EDTA was also tested alone, and 16 mM EDTA was necessary to abolish all sperm movements. Such high doses were not used in this study, because that would lead to chelation not only of zinc ions, but also of other divalent cations such as calcium and magnesium in the seminal plasma, and it would therefore be difficult to ascribe the effect of EDTA to chelation of zinc only. It is interesting to note that there is a synergistic effect between gramicidin and EDTA. Further research might suggest a use of chelators such as EDTA and DEDTC in contraception.

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