Role of zinc trafficking in male fertility: from germ to sperm

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STUDY QUESTION: What are the dynamics of zinc (Zn) trafficking in sperm, at the testicular, epididymal and ejaculate levels?

SUMMARY ANSWER: Zn transporters are peculiarly expressed in the cells of the germ line and Zn uptake is maximal at the post-epididymal phase, where Zn is involved in the regulation of sperm functions.

WHAT IS KNOWN ALREADY: Zn is known to influence several phases of sperm life, from germ cell development to spermiation. Zn trafficking across the membrane is allowed by specific families of transporters known as the ZnTs, which are involved in effluent release, and the Zips, which mediate uptake.

STUDY DESIGN, SIZE, DURATION: We enrolled 10 normozoospermic healthy participants in an infertility survey programme, as well as 5 patients affected by testicular germ cell cancer, and 18 patients presenting with obstructive azoospermia, without mutations of the CFTR gene, and undergoing assisted reproductive technologies.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The research study was performed at our University Clinic. Semen samples, or biopsies or fine needle aspirates from the testis or epididymis, were obtained from each of the participants. Protein expression of main members of the ZnT and Zip families of Zn transporters was examined in human testis and epididymis samples by immunofluorescence. Quantification of sperm Zn content was performed by flow cytometry, atomic absorption spectrometry (AA) and autometallography.

MAIN RESULTS AND THE ROLE OF CHANCE: Intratubular cells of the germ line displayed a high redundancy of Zip family members involved in Zn uptake, while ZnT transporters were more represented in epididymis. Testicular and epididymal spermatozoa contained less Zn than ejaculated spermatozoa (2.56 ± 0.51 and 12.58 ± 3.16 versus 40.48 ± 12.71 ng Zn/10⁶ cells, respectively). Gain of hypermotility and acrosomal reaction were significantly linked to the loss of Zn content in ejaculated spermatozoa.

LIMITATIONS, REASONS FOR CAUTION: This was an ancillary study performed on a small cohort of normozoospermic subjects. Although these results clarify the Zn trafficking during different phases of sperm life, no conclusive information can be drawn about the fertilizing potential of sperm, and the overall pregnancy outcomes, after Zn supplementation.

WIDER IMPLICATIONS OF THE FINDINGS: Our data disclose the dynamics of Zn trafficking during over the sperm lifespan.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was sought or obtained for this study. No conflict of interest is declared.

Key words: zinc / zinc transporters / male infertility / sperm motility / capacitation

Introduction

As an integral part of >200 biologically important enzymes, zinc (Zn) is one of the most common metal elements in the human body. Animal proteins are rich sources of Zn and may also enhance the absorption of dietary Zn (Sandström and Cederblad, 1980). Seminal studies have demonstrated the importance of Zn in several stages of sperm development following the discovery that Zn uptake is required to maintain optimal functioning of the testis, prostate and epididymis (Vera-Gil et al., 1991). Indeed, in animal models, an overall reduction of intracellular
Zn concentration impairs germin al cell proliferation (Yamaguchi et al., 2009), a phenomenon likely due to activation of caspase-3 and caspase-8 systems and triggering of germ cell apoptosis (Smith et al., 2012). In mammals, Zn also plays a role in spermiogenesis, when the DNA is packed in a highly condensed state inside the head of the sperm by protamine-1 and protamine-2. These two small proteins are rich in arginine, which allows them to bind in the major groove of DNA (Prieto et al., 1997), and cysteine residues, which are mostly involved in disulphide cross-links between adjacent protamine molecules. This extensive thiol-oxidation is mediated by the sperm nucleus GPX4 enzyme in the presence of H2O2 (Schneider et al., 2009) and it is believed that Zn acts as the regulator of disulphide cross-links in the sperm nucleus by forming a precise number of SH-Zn-SH structures. The resulting tightly condensed chromatin is indeed a dynamic structure that provides significant protection from DNA damage that could arise in the male or female reproductive tract prior to fertilization (Aoki et al., 2006).

Besides the influence on germ cell differentiation, Zn ions appear to have a profound influence on other sperm functions. Indeed, it has been documented that Zn exerts a specific inhibitory effect on citrate oxidation by acting on mammalian mitochondrial aconitase (Costello et al., 2012). In addition, Zn has been documented that Zn exerts a specific inhibitory effect on citrate oxidation by acting on mammalian mitochondrial aconitase (Costello et al., 2012). It has been shown that Zn inhibits the citrate 3-koen reductase isoenzyme involved in the Krebs cycle (Adinolfi et al., 1969). As observed in somatic cell systems, inhibition of the citrate 3-koen reductase isoenzyme minimizes citrate oxidation and provides an efficient metabolic step to optimize net citrate production (Costello et al., 1997). This would explain early observations by our group, demonstrating that high extracellular Zn concentrations result in higher Zn uptake, with decreased oxygen consumption and acrosome reaction rates by early ejaculated spermatozoa (Forest et al., 1990).

Once the fertilizing spermatozoon enters the ooplasm, its nucleus undergoes a series of ultrastructural changes that eventually lead to pronuclear formation (Tesarik and Kopecny, 1989). Zn appears to have a key role also in this process since freshly ejaculated human sperm can be experimentally decondensed in vitro by exposure to extracellular chelating agents such as EDTA. In fact, metal depletions interrupts the SH-Zn-SH bridges interspersed between protamine macromolecules, inducing subsequent chromatin unpacking (Kvist et al., 1987). Thus, optimal integration of Zn into sperm chromatin contributes to rapidly reversible chromatin compaction leading to both chromatin condensation during spermiogenesis and rapid decondensation after fertilization (Kvist et al., 1988).

The movement of Zn across physiological membranes is mediated by two classes of Zn transport proteins that work together to maintain the proper intracellular Zn concentration. These are the SLC30 (ZnT) and SLC39 (Zip) protein families. ZnT transporters promote cellular Zn influx into the cell membranes, which consequently increases intracellular Zn concentration. In contrast, Zip transporters act as efflux transporters, facilitating the reverse transport of Zn out of the cell. The movement of Zn across physiological membranes is mediated by two classes of Zn transport proteins that work together to maintain the proper intracellular Zn concentration. These are the SLC30 (ZnT) and SLC39 (Zip) protein families. ZnT transporters promote cellular Zn influx into the cell membranes, which consequently increases intracellular Zn concentration. In contrast, Zip transporters act as efflux transporters, facilitating the reverse transport of Zn out of the cell.

Materials and Methods

All subjects provided informed consent for the study, which had been previously approved by the local Ethical Committee (protocol no. 2208P). The investigation was performed according to the principles of the Declaration of Helsinki.

Testicular samples

Biospies

A collection of 30 paraffin-embedded sections of testis biopsies, obtained by 5 patients affected by testicular germ cell tumours (kindly gifted by Dr Poletti, Histology and Pathological Anatomy Unit, Bassano del Grappa Hospital, Italy) and from 5 patients with idiopathic obstructive azoospermia but free from any mutation of the CFTR gene (Yu et al., 2012), were assessed by immunofluorescence (IF) as described below. Specimens from the cancer patients were derived from the healthy testicular tissue surrounding cancer, featuring tubules with normal spermatogenesis, as assessed by histological evaluation. The choice to use samples from testicular cancer patients depended on the availability of testicular and epididymal tissues. Characteristics of the cancer patients are reported in Supplementary data, Table S1.

Testis fine needle aspiration

Six patients presenting with idiopathic obstructive azoospermia but free from any mutation of the CFTR gene underwent testicular fine needle aspiration (FNA) with the aim of sperm retrieval and cryopreservation for subsequent assisted reproductive technologies (Bettella et al., 2005). Briefly, aspiration was performed with a 23-gauge (0.6 mm) butterfly needle attached to a 20-ml syringe. The retrieved material was in most part cryopreserved and an aliquot was resuspended in 1 ml of sperm washing medium (SWM, Irvine Scientific Newtown, United States). Three portions of 50 μl from each testis were smeared on SuperFrost® Plus microscope glass slides (Menzel GmbH & Co KG, Braunschweig, Germany), fixed in 4% paraformaldehyde/PBS solution for 15 min at room temperature and stored at −80°C until further evaluation. The samples then underwent further processing for flow cytometry analysis, atomic absorption (AA) spectrometry evaluation and cetochromatography as described below.

Epididymal samples

Biospies

Fifteen paraffin-embedded sections of caput epididymis were obtained from 5 patients affected by germ cell tumours with no sign of epididymal invasion as assessed by histological evaluation (Dr Poletti). Sections were assessed by IF as described elsewhere.

Epididymis fine needle aspiration

Seven patients, presenting with idiopathic obstructive azoospermia but free from any mutation of the CFTR gene, underwent epididymal FNA for sperm retrieval. Most of the retrieved material was cryopreserved for subsequent assisted reproductive technologies. A small aliquot of sperm cells were obtained, processed and evaluated as for testis cells obtained by FNA.

Semen samples

Semen was obtained from 10 normozoospermic healthy donors, attending the University Andrology Unit as participants in an infertility survey programme (mean age of 24.3 ± 6.1 years). None of the patients had a history of previous genital tract infections, cryptorchidism, testicular torsion or varicocele. Semen samples were obtained from each patient in two different sessions with a delay of 2 months. Patients underwent semen donation by masturbation into sterile containers after 2–5 days of sexual abstinence. Samples were allowed to liquefy for 30 min and were examined...
according to the WHO criteria (World Health Organization, 2010). For all samples, normal viscosity and leukocyte counts were recorded. All semen cultures were negative and antisperm antibodies were absent in all subjects. Specimens underwent processing for flow cytometry analysis, AA spectrometry evaluation and autometallography as described below.

**Semen processing**

The WHO sperm motility parameters were assessed using the sperm class analyser (SCA, Microptic S.L., Barcelona, Spain) as previously described (Zuccarello et al., 2011). Briefly, a 4-μl semen aliquot was placed in a Leja® analysis chamber (Leja Products B.V., Nieuw-Vennep, The Netherlands) (19.7 mm thickness). For the evaluation of sperm hyperactivation, the SCA cut-off points defined were: capture at 50 frames/s, curvilinear velocity (VCL) >150 μm/s, linearity <40% and amplitude of lateral head displacement >3.5. In accordance with WHO guidelines, 200 spermatozoa were analysed which is approximately equivalent to the capture of 5 or 6 randomly selected microscopic fields. After every scan, video sequences were assessed to validate whether all spermatozoa had been identified and that their trajectory had been correctly reconstructed by the SCA system.

Direct swim-up was performed as previously described (Garolla et al., 2012). Briefly, 1.0 ml of semen was centrifuged at 300 g for 10 min and the pellet was resuspended in 0.3 ml of SWM. Then 1.0 ml of SWM was gently layered over the samples and they were incubated for 1 h at 37°C with 5% CO₂. The uppermost 0.8 ml of the upper layer, containing motile spermatozoa that had swum up, was collected for further analysis.

Sperm samples were induced to capacitate by incubation in BWW medium supplemented with HEPES (10 mM, pH 7.4) and 3 mg/ml BSA at 5% CO₂, 37°C for 3 h, as previously described (Jaswal et al., 1998).

**Chemicals**

Zn chloride, 70% nitric acid for trace metal analysis, sodium sulphide powder, silver nitrate powder, hydroquinone, arabic gum, bovine serum albumin and JC-1 were all purchased from Sigma Aldrich (Milano, Italy). ZnAF-2DA (ZnAF), a fluorescent probe specific for intracellular Zn, was purchased from Santacruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal anti-ZnT1, ZnT2, Zip1, Zip5, Zip6, Zip8 and Zip14 antibodies were purchased from AbCam (Cambridge, UK) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was from Vector Laboratories ( Burlingame, CA, USA). Normal donkey serum was purchased from Millipore (Vimodrone, Milano, Italy). Mouse monoclonal FITC-conjugated anti-human CD46 antibody was purchased from BD Biosciences (Milano, Italy).

**Immunofluorescence and autometallography**

Expression of Zn transporters was evaluated by IF on both paraffin-embedded sections of testis and epididymis and smeared testicular FNA. Paraffin sections were previously deparaffinized, rehydrated and antigen retrieved by microwave in 15 min in 0.1 M citrate buffer (pH 7.9). Samples were then saturated with 5% BSA/5% normal donkey serum in PBS for 30 min at room temperature and then incubated for 1 h, at room temperature in the dark, with rabbit polyclonal anti-ZnT1, ZnT2, Zip1, Zip5, Zip6, Zip8 and Zip14 antibodies at their working concentrations following dilution in PBS. For negative controls, primary antibodies were omitted. Primary immunoreaction was detected by incubation with FITC-conjugated anti-rabbit IgG and finally, sections were counterstained with DAPI and mounted with anti-fade buffer. Each staining was performed in triplicate.

Intracellular Zn accumulation in testicular, epididymal and ejaculated spermatozoa was demonstrated by autometallography (Auto) as previously described (Stoltenberg et al., 1997). Briefly, sperm samples were mixed with 0.1% sodium sulphide in pH 7.3 phosphate buffer (1:1 v/v) for 30 min. Smears were allowed to air-dry and then fixed in 96, 70 and 50% alcohol for 15, 2 and 2 min, respectively, and then finally washed for 10 min with distilled water before development. Autometallographic development was performed at 26°C for 60 min in a dark box and the sections were post-fixed with 70% ethanol for 30 min. The developer consisted of 60 ml filtered gum arabic solution (1 kg dissolved in 2.1 distilled water), 10 ml sodium citrate buffer (1.21 M citric acid, 0.8 M sodium citrate) and 0.85 g hydroquinone dissolved in 15 ml distilled water. Immediately before use, 0.12 g silver nitrate in 15 ml distilled water was added and the solution was mixed thoroughly. The negative control was devoid of hydroquinone. Each sample was assessed in triplicate. All sample slides were analysed with a video-confocal fluorescence microscope (Nikon, Firenze, Italy).

**AA spectrometry**

Intracellular Zn content was measured with AA spectrometry with the graphite furnace technique under argon at a wavelength of 213.9 nm (Varian AA Duo Graphite Furnace Atomic Absorption Spectometer, Paloalto, CA, USA) (Henkel et al., 2005). Briefly, samples of testicular, epididymal and ejaculated spermatozoa were washed in PBS, evaluated for cell concentration in a Makler counting chamber and resuspended overnight in 70% nitric acid at room temperature. Calibration curves were prepared with Zn standard solution (Sigma) for standards, according to manufacturer’s instructions. Samples were washed properly with purified H₂O if necessary and Zn concentrations were evaluated in duplicate. Results were reported as ng Zn/millions of cells.

**Flow cytometry**

Samples of testicular, epididymal and ejaculated spermatozoa were washed and resuspended in SWM. Subsequently, incubation with ZnAF (final concentration 1 μM, for 45 min), JC-1 (final concentration 2 μM, for 20 min) or anti-human CD46 antibody (final concentration of 20 μg/ml, for 20 min) at 37°C in the dark was performed. After washing to eliminate excess reagent, propidium iodide (PI) (25 μg/ml) was added and samples were analysed on a FACScan flow cytometer (BD-Biosciences) as previously described (Zuccarello et al., 2011). As a gating strategy (Supplementary data, Fig. S1) 50 000 PI-negative viable cells, morphologically ascribable to spermatozoa, were evaluated for the percentage of ZnAF-positive cells and respective fluorescence intensity mean value, percentage of JC-1 red fluorescing cells (considered as cells with functional mitochondria) (Lachance et al., 2013) and percentage of CD46-positive cells (considered as acrosomal-reacted cells) (Grunewald et al., 2006).

**Statistical analysis**

All statistics were performed using the SPSS software (version 15.1; SPSS, Inc., Chicago, IL, USA). Comparisons between variables were made using Kruskal–Wallis analysis of variance by ranks. Relationships between continuous variables were assessed using non-parametric Spearman’s ρ correlation test. Student’s t-test was used to compare means. The significance level was set to P = 0.05.

**Results**

**Differential expression of Zn transporters at the testicular and epididymal levels**

Immunofluorescence data dealing with the expression pattern of Zn transporters in testicular and epididymal tissues (obtained from biopsies), testicular and epididymal cells (obtained by FNA) and in ejaculated sperm are summarized in Table I and shown in detail in Figures 1–3 (and negative controls for comparisons are reported in Supplementary data, Fig. S2). Within human seminiferous tubules (Fig. 1), a clear signal for ZnT1 was observed within tubule parenchyma, and in particular on.
basal membrane and on adjacent Sertoli cell membrane. No specific signal was detected for ZnT2. On the contrary, transporters involved in Zn uptake (Zip-family) were generally widely represented within the seminiferous epithelium.

To clarify the pattern of Zn transporters expressed by the distinct tubular cell populations, an IF assay was performed on testis FNA samples (Bettella et al., 2005) in a separate session (Fig. 2). Sertoli cells were confirmed to express ZnT1, while Zip5 was the main Zn uptake mediator in this cell type. With germ line cells, no evident signal for ZnT family members was detectable. On the other hand, Zip5, Zip6 and Zip8 were redundantly displayed by germ cells, from spermatogonia through spermatocytes and spermatids to spermatozoa. Moreover, Zip1 was present in post-meiotic phases of spermatogenesis, displayed in particular by spermatids and spermatozoa.

### Table 1
Expression pattern of Zn transporter proteins, assessed by immunofluorescence (IF), in human testis/epididymis biopsies and testis fine needle aspiration (FNA).

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<thead>
<tr>
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<th>Zn-efflux family</th>
<th>Zn-influx family</th>
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<tr>
<td></td>
<td>ZnT1</td>
<td>ZnT2</td>
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<tr>
<td><strong>Testis</strong></td>
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<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tubular cells</td>
<td>Sertoli</td>
<td>−</td>
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<tr>
<td><strong>Epididymis</strong></td>
<td></td>
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<tr>
<td>Tissue</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sperm cells</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ejaculated sperm</td>
<td>−</td>
<td>−</td>
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</table>

+, Positive expression; +/−, expression closed to the limit of detection of the assay; −, negative expression.

Figure 1  Expression analysis of Zn transporter proteins in human testis biopsies as assessed by immunofluorescence (IF). Primary immuno-reaction was detected by FITC-conjugated secondary antibody (green). Samples were counterstained with DAPI (blue).
Assessments of Zn transporter expression in specimens of caput epididymis biopsies are shown in Figure 3. A thorough evaluation of IF staining showed that ZnT1 was expressed throughout the whole pseudo-stratified epithelium, thus likely by both basal and principal cells. For ZnT2, a clear signal for this protein was detected on the apical portion lining the duct, ascribable to principal cells, but a weaker signal for this Zn transporter appears also in myoid cells surrounding ducts. With respect to the Zip family, a clear signal for Zip6 was observed on principal cells in correspondence to the apical portion lining the duct, while staining for Zip5 was very close to the limit of detection. Zip1 and Zip8 were not expressed in this tissue.

Similarly to testicular spermatozoa, both epididymal and ejaculated spermatozoa displayed expression of Zip1, Zip5, Zip6 and Zip8 but none of ZnT family members. No signal for Zip14 was detectable in any of the samples assessed (Table I).

Zn content of spermatozoa

The Zn content of human spermatozoa was assessed by three methodologies: flow cytometry using the specific probe ZnAF, (AA) spectroscopy and autometallography (Fig. 4). A progressive increase in ZnAF-positive viable sperm cells was observed in specimens obtained from testis, epididymis or ejaculate (7.85, 20.67 and 92.8%, respectively). The mean fluorescence value of ZnAF-positive cells, considered as an index of mean cellular Zn content, was observed to be significantly higher in epididymal and ejaculated spermatozoa, compared with testicular spermatozoa (P < 0.05). The absolute quantification by AA spectrometry of mean sperm-Zn content confirmed these observations. Assessment of intracellular Zn accumulation by autometallography demonstrated that in testicular and epididymal spermatozoa, Zn preferentially accumulated within the cytoplasmic residues of immature sperm cells. On the other hand, in ejaculated spermatozoa, Zn was essentially localized in the midpiece/neck and flagellar areas.

Zn uptake by epididymal spermatozoa

The intracellular variations of Zn content in epididymal spermatozoa, freshly obtained by FNA, and after extracellular loading with Zn (1 mM), were monitored by flow cytometry, AA spectrometry and autometallography (Fig. 5). After an incubation time of 30 s, data for intracellular Zn content showed very high dispersion with no significant difference from the basal condition [28.2 ± 35.3 ng Zn/10⁶ sperm (30 s) versus 12.0 ± 6.2 ng Zn/10⁶ sperm (basal)] for atomic absorbance spectrometry; 20.67 ± 7.17% positive cells (basal) versus 51.8 ± 52.6% positive cells (30 s) for flow cytometry; P > 0.05]. However, after 1 min of observation, the Zn content evaluated by both flow cytometry and AA increased reaching almost 80% of the maximal values observed after 60 min of incubation. No significant differences in terms of mean fluorescence values for ZnAF were evident among all time points evaluated (0 min: 1782.5 ± 215.1 AU; 1 min

![Figure 2](image-url)
Zn dynamics and swim-up

Assessment of intracellular Zn content was performed on ejaculated spermatozoa undergoing swim-up selection (Fig. 6). The mean fluorescence value for the Zn probe, an index of intracellular Zn content, was lower in motile sperm ($1547.3 \pm 197.7$ AU naive versus $678.14 \pm 203.6$ AU swim-up; $P < 0.05$). This observation was supported by AA spectrometry evaluation, showing an overall reduction of Zn content in motile sperm compared with both freshly ejaculated sperm ($9.65 \pm 6.32$ ngZn/10$^6$ cells versus $40.48 \pm 12.71$ ngZn/10$^6$ cells; $P < 0.05$). No significant difference in terms of percentage of ZnAF-positive cells was found between motile, non-motile and freshly ejaculated sperm population.

Autometallography showed that swim-up selected cells displayed an overall reduction of Zn staining in the whole cell body, while the immobile fraction showed strong Zn signals in the midpiece/neck area. Interestingly, the Zn content of recovered motile sperm, evaluated by AA after swim-up, was found to be inversely and significantly correlated with the percentage of recovered cells ($p = -0.5601; P < 0.05$; Supplementary data, Fig. S3).

Influence of Zn incubation on sperm function

The influence of Zn incubation during swim-up selection on sperm function was assessed (Fig. 7A). As reported above, highly motile sperm showed a significantly lower average Zn content when compared with slow or non-motile spermatozoa. When swim-up selection was performed in a medium containing Zn at the final concentration of 1 mM Zn (Lishko et al., 2010), motile spermatozoa retained higher levels of Zn, closer to naive samples. This was associated with a significantly lower recovery of motile spermatozoa ($21.3 \pm 9.8$ versus $43.1 \pm 10.6\%$, respectively; $P < 0.05$). No significant difference in the Zn content was noted in non-motile cells in both selection conditions. Interestingly, Zn incubation during swim-up did not affect sperm cell viability (Naive: $89.14 \pm 12.37\%$; Swim-up: $98.36 \pm 16.18\%$; Swim-up + Zn 1 mM: $97.92 \pm 13.85\%; P > 0.05$).

Finally, the effect of Zn during sperm capacitation by the use of BWW as a capacitating medium was investigated (Fig. 7B). Standard incubation of spermatozoa in BWW medium for 3 h resulted in an overall decrease in intracellular Zn content, detected by reductions in both mean fluorescence intensity of the ZnAF probe and AA spectrometry. This was associated with an increased fraction of hypermotile cells. Capacitation performed in BWW medium containing Zn at the final concentration

Figure 3  Expression analysis of Zn transporter proteins in human epididymis biopsies as assessed by IF. Primary immuno-reaction was detected by FITC-conjugated secondary antibody (green). Samples were counterstained with DAPI (blue).
of 1 mM induced a strong increase in intracellular Zn and a significant re-
duction in the hypermotile fraction. Similar results were obtained when
sperm were capacitated for 3 h in standard BWW medium followed by
additional incubation for 1 h in BWW containing 1 mM Zn. Control
samples were obtained by incubation of spermatozoa for 3 h in SWM
as a non-capacitating medium, followed or not by additional incubation
with 1 mM Zn for 1 h. No significant variation in the intracellular Zn
content was documented in any control sample. Only a slight, not signifi-
cant, increase in the hypermotile sperm fraction was observed after incu-
bation in SWM. As shown in Table II, capacitation of spermatozoa in

<table>
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<tr>
<th></th>
<th>Testicular</th>
<th>Epididymal</th>
<th>Ejaculated</th>
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<tbody>
<tr>
<td>Flow Cytometry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZnAF+ (%)</td>
<td>7,85 ± 3,24</td>
<td>20,67 ± 7,17(^a)</td>
<td>92,8 ± 23,1(^a,b)</td>
</tr>
<tr>
<td>Fluorescence mean (A.U)</td>
<td>265,36 ± 85,85</td>
<td>1782,53 ± 215,1(^a)</td>
<td>1547,3 ± 197,7(^a)</td>
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<tr>
<td>AA</td>
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<tr>
<td>Zn content (ng Zn/10^6 cells)</td>
<td>2,56 ± 0,51</td>
<td>12,58 ± 3,16(^a)</td>
<td>40,48 ± 12,71(^a,b)</td>
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<td>Autometallography</td>
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Figure 4  Analysis of intracellular Zn content in testicular, epididymal and ejaculated spermatozoa. Staining with the Zn-specific probe ZnAF was assessed by flow cytometry and the relative results are reported as the percentage of ZnAF-positive spermatozoa (%) and as the mean fluorescence value of ZnAF-positive spermatozoa in arbitrary units (AU). The absolute Zn content of sperm was assessed by AA spectrometry and data are reported as ng Zn/10^6 cells. Zn localization within sperm cells was investigated by autometallography (Auto). Zn deposits develop as black staining. Significance: \(^a\)P < 0.05 versus testicular; \(^b\)P < 0.05 versus epididymal.
BWW medium for 3 h was associated with a significant increase in cells undergoing the acrosome reaction, as shown by exposure of sperm-CD46. This result was even more evident after 4 h of incubation in BWW. On the contrary, capacitation performed in the presence of 1 mM extracellular Zn did not affect the overall number of ZnAF$^+$ cells, sperm viability, mitochondrial integrity or the fraction of sperm cells undergoing the acrosome reaction compared with the naive samples.

**Discussion**

Zn influences several aspects of sperm life: from its formation and contribution to the ultrastructural stabilization of chromatin compaction to the modulation of mitochondria-dependent processes such as cell respiration and programmed cell death (Kvist et al., 1985; Foresta et al., 1990; Smith et al., 2012). Within the reproductive tract, there is a progressively increasing gradient of extracellular Zn concentrations that could represent a driving force for Zn uptake by spermatozoa. In fact, during the early stages of sperm development, spermatogenic cells reside within the seminiferous tubule which have a Zn content similar to or lower than that of other organs such as the liver or kidney. Subsequently, sperm encounter, in succession, the epididymis, vas deferens and seminal vesicles, which characterized by a progressively increased tissue Zn-content (Oldereid et al., 1993). Finally, spermatozoa are ejaculated in seminal plasma which is essentially formed by prostate

*Figure 5* Analysis of Zn uptake by epididymal spermatozoa incubated in 1 mM Zn-containing medium up to 60 min at 37°C. Staining with the Zn-specific probe ZnAF was assessed by flow cytometry and the results are reported as the percentage of ZnAF-positive spermatozoa (%). The absolute Zn content of sperm was assessed by AA spectrometry and data are reported as ng Zn/10$^6$ cells. Zn localizations within sperm cells were investigated by autoradiography (Auto). Zn deposits develop as black staining. Significance: *$P < 0.05$ versus 0 min.
secretion, where Zn appears nearly 100 times more concentrated than in blood serum (Riffo et al., 1992). However, data describing the dynamics of Zn trafficking in human sperm during their transit through the reproductive tract are currently scarce.

In this study, IF analysis demonstrated a peculiar localization of Zn-transporter proteins in the human testis. Indeed, within the seminiferous tubules, a notable redundancy of Zn transporters involved in the uptake of metal was observed, with multiple proteins expressed by

**Figure 6** Analysis of intracellular Zn content in ejaculated spermatozoa undergoing swim-up selection. Naive samples (ejaculated, dotted line) were compared with motile fraction (Sw Up, continuous thick line) and the non-motile fraction (Bottom, continuous line). Staining with the Zn-specific probe, Zn fluorescence was assessed by flow cytometry and the relative results are reported as the percentage of ZnAF-positive spermatozoa (%) and as the mean fluorescence value of ZnAF-positive spermatozoa in arbitrary units (AU). The absolute Zn content of sperm was assessed by AA spectrometry and data are reported as ng Zn/10^6 cells. Zn localization within sperm cells was investigated by autometallography. Zn depots develop as black staining. Significance: ^P < 0.05 versus ejaculated.
**Figure 7** (A) Analysis of Zn content of ejaculated spermatozoa undergoing swim-up selection in the absence or presence of 1 mM of Zn-containing medium. Staining with the Zn-specific probe ZnAF was assessed by flow cytometry and the results are reported as the mean fluorescence value in arbitrary units (AU). The absolute Zn content of sperm was assessed by atomic absorption (AA) spectrometry and data are reported as ng Zn/10⁶ cells. Significance: *P < 0.05 versus Naive. (B) Analysis of Zn content and cell motility of ejaculated spermatozoa undergoing capacitation, by incubation in BWW medium, in the absence or presence of 1 mM Zn. Staining with the Zn-specific probe ZnAF was assessed by flow cytometry and the results are reported as the mean fluorescence value in arbitrary units (AU). The absolute Zn content of sperm was assessed by AA spectrometry and data are reported as ng Zn/10⁶ cells. Hypermotile fraction, expressed as the percentage of total sperm population, was assessed by sperm class analyser. Significance: *P < 0.05 versus Naive; **P < 0.01 versus Naive.

**Table II** Variation of ejaculated sperm parameters during capacitation, in the absence or presence of extracellular 1 mM of Zn addition.

<table>
<thead>
<tr>
<th></th>
<th>Viable cells (%)</th>
<th>ZnAF+ (%)</th>
<th>JC1+ (%)</th>
<th>CD46+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>90.22 ± 12.10</td>
<td>92.03 ± 9.32</td>
<td>79.03 ± 21.18</td>
<td>2.73 ± 1.92</td>
</tr>
<tr>
<td>3 h BWW</td>
<td>85.02 ± 15.63</td>
<td>84.94 ± 10.10</td>
<td>76.23 ± 23.55</td>
<td>5.15 ± 1.83*</td>
</tr>
<tr>
<td>3 h BWW + Zn 1 mM</td>
<td>87.77 ± 14.98</td>
<td>80.62 ± 8.41</td>
<td>77.28 ± 17.37</td>
<td>3.62 ± 2.72</td>
</tr>
<tr>
<td>4 h BWW</td>
<td>75.70 ± 15.44</td>
<td>79.16 ± 11.84</td>
<td>70.06 ± 10.79</td>
<td>6.34 ± 2.05*</td>
</tr>
<tr>
<td>3 h BWW + 1 h Zn 1 mM</td>
<td>72.35 ± 11.61</td>
<td>81.27 ± 12.40</td>
<td>68.03 ± 18.16</td>
<td>3.59 ± 1.79</td>
</tr>
<tr>
<td>3 h SWM</td>
<td>85.34 ± 17.39</td>
<td>89.55 ± 7.88</td>
<td>76.84 ± 22.01</td>
<td>3.67 ± 2.13</td>
</tr>
<tr>
<td>3 h SWM + Zn 1 mM</td>
<td>78.86 ± 12.68</td>
<td>88.50 ± 8.13</td>
<td>66.28 ± 19.19</td>
<td>3.81 ± 1.84</td>
</tr>
</tbody>
</table>

ZnAF+, positive staining to ZnAF probe; JC1+, functional mitochondria assessed by JC1 staining; CD46+, presence of acrosomal reaction assessed by positive staining to anti-human-CD46 antibody; BWW, Biggers–Whitten–Whittingham capacitating medium; SWM, standard sperm washing medium. Significance: *P < 0.05 versus naive.
germ line cells. In parallel, poor representation of the Zn-effluent mediators was found, with the expression of the sole ZnT1 essentially limited to Sertoli cells. In the epididymis, the pattern appeared to be reversed with ZnTs being highly expressed on epithelial cells while only Zip6, and to a minor extent Zip5, were present. Moreover, data from autometallography also demonstrated a differential pattern of intracellular Zn localization between testicular or epididymal samples and ejaculated spermatozoa. The former showed essential staining within cytoplasmic residues, while the latter mainly displayed Zn in the midpiece/neck and flagellum. In addition, we observed an overall reduction of intracellular Zn content in ejaculated spermatozoa undergoing a gain in motility, as demonstrated by flow cytometry and AA Zn dosage in sperm cells recovered after swim-up selection. In this regard it is also possible that immotile spermatozoa show a higher Zn content due to larger number of dead cells. However, this is unlikely since the gating strategy in flow cytometry specifically employed propidium iodide in order to exclude non-viable cells. In parallel, the use of autometallography showed that the reduction in Zn staining mainly takes place at the midpiece/neck and flagellum levels, further suggesting a key role for this metal in sperm motility. Interestingly, this reduction in Zn was significantly compromised by the addition of 1 mM extracellular Zn, a concentration very similar to that found in seminal plasma (Colagar et al., 2009).

Based on these observations, it may be hypothesized that germ cells are highly adapted to take up Zn at each developmental stage. In particular for mature spermatozoa, this hypothesis is supported by our data showing the ability of epididymal spermatozoa to rapidly load extracellular Zn in experimental conditions. On the other hand, epithelial cells of both the testis and epididymis are programmed to supply Zn. In fact, during the transit through the epididymis (Franca et al., 2005), Zn effluent is enhanced in the luminal space. Consequently, intracellular Zn levels in epididymal spermatozoa are higher than those observed in the testis, but still much lower than that of ejaculated spermatozoa. In fact, this latter cell population reaches an even higher final accumulation of Zn by contact with Zn-rich prostate secretions (Behne et al., 1988). This evidence suggests that within the seminiferous tubule and during epididymal transit, Zn trafficking likely plays a functional role in sperm maturation, including events such as chromatin condensation and cytoplasmic re-arrangement (Garcia-Macias et al., 2006). Post-epididymal Zn trafficking seems likely to be linked to the modulation of sperm motility, which represent a key function supporting the fertilization potential of sperm cells (Henkel et al., 1999). Our data show that all conditions associated with sperm activation, such as hypermotility gain, capacitation and acrosome reaction are paralleled with a drastic decrease in intracellular Zn content. These effects appear to be reversed by exposure to physiological concentrations of extracellular Zn as those observed in seminal plasma. In this regard, a mechanistic model, viewing Zn as an inhibitor of sperm motility through intracellular alkalinization, has recently been proposed. This phenomenon represents a key event required for activation of CatSper (Kirichok et al., 2006), a sperm-specific Ca$^{2+}$ channel in the flagellum that is required for sperm hyperactivation and male fertility (Carlson et al., 2003). In this system, Zn would act as a potent inhibitor of Hv1, a voltage-sensor-only proton channel specifically localized within the principal piece of the sperm flagellum and exerting control over sperm intrflagellar pH, which, in turn, modulates CatSper activity (Lishko et al., 2010). After ejaculation, physiological events such as Zn chelation by high-molecular weight proteins of prostatic origin, known as prostasomes (Siciliano et al., 2000), together with high albumin concentrations and low Zn levels in the female genital tract (Hagenfeldt et al., 1973; Dickens et al., 1995), would induce a rapid drop in Zn concentration. The final results of this environmental change are the triggering of sperm hypermotility, capacitation and other events prodromal to acrosome reaction (Foresta et al., 1990).

Our findings suggest that along the whole genital tract, there is a prevalent expression of Zn transporters which supply Zn and that from the germ cells to mature sperm, there is an overall uptake of Zn. This is in agreement with the peculiar demand for Zn during the maturation steps. In particular, during spermatogenesis a low Zn content is required for germ cell survival and transition up through meiosis, spermiogenesis and histone–protamine substitution. During epididymal transit, the increase in Zn supply sustains chromatin compaction, which is fundamental to protect sperm nucleus from supervening pathogenic noxae (Golan et al., 1996). Finally, the prostate secretions concur to stabilize sperm before ejaculation. All of these mechanisms are a prerequisite for mature sperm to be able to undergo capacitation, motility hyperactivation and the acrosome reaction when the Zn levels fall during their transit in the female genital tract. To date, Zn supplementation has been empirically proposed for the treatment of male infertility. Our data outline new information about the roles of Zn in the male aspects of human reproduction. However, more studies are needed to clarify whether Zn supplementation might be able to improve sperm competence and the overall pregnancy outcome.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

C.F. developed the concept of the study and wrote the first manuscript draft; I.C., M.M., M.F. and V.G had a major contribution in the analytical methods, statistical analyses and results; A.G. and L.D.T. performed the design and the draft of the study; all authors contributed intellectually to the final version of the manuscript.

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**Conflict of interest**

None declared.

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