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Original Article

Relationship between seminal plasma zinc concentration and spermatozoa–zona pellucida binding and the ZP-induced acrosome reaction in subfertile men

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Abstract

The aim of this study was to determine the relationship between seminal zinc concentration and spermatozoa–zona pellucida (ZP) binding and the ZP-induced acrosome reaction (ZPIAR) in subfertile men. Semen analyses and seminal zinc concentration assessments were carried out according to the World Health Organization manual for 458 subfertile men. A spermatozoa–ZP interaction test was carried out by incubating 2×10^6 motile spermatozoa with a group of four unfertilized oocytes obtained from a clinical *in vitro* fertilization programme. After 2 h of incubation, the number of spermatozoa bound per ZP and the ZPIAR of ZP-bound spermatozoa were examined. The effect of adding 0.5 mmol L^{-1} zinc to the media on the ZPIAR of spermatozoa from normozoospermic men was also tested *in vitro*. Seminal zinc concentration positively correlated with sperm count and duration of abstinence, but negatively correlated with semen volume. On analysis of data from all participants, both spermatozoa–ZP binding and the ZPIAR were significantly correlated with sperm motility and normal morphology, but not with seminal zinc concentration. However, in men with normozoospermic semen, the seminal zinc concentration was significantly higher in men with defective ZPIAR (< 16%) than in those with normal ZPIAR (≥ 16%) ($P < 0.01$). The addition of 0.5 mmol L^{-1} zinc to the culture media had no effect on spermatozoa–ZP binding, but significantly reduced the ZPIAR *in vitro* ($P < 0.001$). In conclusion, seminal zinc concentration is correlated with sperm count and the duration of abstinence in subfertile men. In men with normozoospermic semen, high seminal zinc concentration may have an adverse effect on the ZPIAR.

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1 Introduction

In current assisted reproductive technology treatment of human infertility, approximately 30%–60% of couples require treatment by intracytoplasmic sperm injection (ICSI) instead of conventional *in vitro* fertilization (IVF) [1]. This is because low fertilization rates in IVF are mostly attributed to sperm defects, which impair spermatozoa–zona pellucida (ZP) binding and penetration [2]. As conventional semen analysis cannot accurately predict the ability of spermatozoa to bind and penetrate the ZP, spermatozoa–ZP interaction tests using human oocytes are needed to diagnose these spe-

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cific sperm defects [3–6].

Our recent study of a large number of subfertile men showed that the most frequent sperm defect is a defective ZP-induced acrosome reaction (ZPIAR, defined as ZPIAR < 16%). Spermatozoa from these men can bind normally to the ZP, but are unable to undergo the ZPIAR at normal rates, thus impairing sperm penetration through the ZP [7, 8]. Although the frequency of defective ZPIAR was high in subfertile men with idiopathic oligozoospermia (65%) and severe teratozoospermia (62%, strict normal sperm morphology ≤ 5%), defective ZPIAR was found in 25% of normozoospermic subfertile men [8]. By contrast, defective spermatozoa–ZP binding was present in approximately 15% of subfertile men with a normal semen analysis and in approximately 25% with an abnormal semen analysis [7, 8]. It is clear that sperm from men with either defective spermatozoa–ZP binding or defective ZPIAR will have reduced or no ability to penetrate the ZP and fertilize oocytes either *in vivo* or *in vitro*. Patients with these conditions can now be treated by ICSI, which allows a single sperm to be injected into the cytoplasm of oocytes, bypassing the ZP barrier. However, it is very important to study and understand the underlying causes of defective spermatozoa–ZP binding and ZPIAR, as well as to develop a simpler test for routine clinical diagnosis of this condition.

It is known that the zinc concentration of seminal plasma (SP) is very high, at over 30 times than that found in blood. Although it is known that SP zinc originates from the prostate, the physiological mechanism and implications for SP zinc in sperm function are not fully understood. Many studies have found that SP zinc concentration is positively correlated with sperm count [9–13]. Some have reported that a high SP zinc level has a negative effect on sperm motility [9–12]. After ejaculation, zinc in semen binds to the sperm plasma membrane, and uptake of zinc may further stabilize sperm nuclear chromatin DNA during sperm transportation in the female reproductive tract [14]. Zinc also has antioxidant properties that could counteract reactive oxygen species produced by the spermatozoa, affecting nuclear chromatin stability and sperm motility [15]. SP zinc may also affect spermatozoa–ZP interaction, particularly ZPIAR, as zinc has a role as a decapacitation factor by binding to the sperm plasma membrane. For instance, hamster spermatozoa–ZP penetration is completely inhibited when zinc (250 μmol L^{-1}) is present throughout the capacitation period [16]. Similarly, in an *in vitro* study using spermatozoa from fertile men, a high zinc concentration (1 mmol L^{-1}) in the culture medium impaired both sperm motility and sperm penetration of ZP-free hamster oocytes [17, 18]. Addition of zinc to the culture medium during capacitation of human spermatozoa inhibited spontaneous acrosome reaction (AR) and also the AR induced by the calcium ionophore, A23187 [18]. It is possible that zinc binding to the sperm plasma membrane affects calcium influx through ion competition during capacitation. It is known that SP contains decapacitation factors, as the addition of SP to the culture medium inhibits sperm capacitation and hyperactivated motility, as well as spermatozoa–ZP binding and penetration *in vitro* [19–22]. The overall, evidence suggests that a high SP zinc concentration may have a negative impact on the ZPIAR. The aim of this study was to determine the relationship between SP zinc concentration and spermatozoa–ZP binding and the ZPIAR in a large number of subfertile men (n = 458), and to examine the effects of zinc addition to the culture medium on spermatozoa–ZP binding and the ZPIAR *in vitro*.

2 Materials and methods

2.1 Semen analysis

Semen samples were collected by masturbation after the stipulated 2–5 days of abstinence from 458 subfertile men who attended the infertility clinic at the Royal Women's Hospital and Melbourne IVF between 1995 and 2005. Some of the patients in this study were also involved in our earlier study on the frequency of defective spermatozoa–ZP interaction, but the data on SP zinc for all patients in this study have never been reported [8]. All couples had tried to achieve natural conception for at least 12 months without success. Routine semen analysis was carried out within 1 h of semen liquefaction according to the World Health Organization (WHO) manual [23]. Both total motility (WHO criteria grades a + b + c) and progressive motility (a + b) were assessed manually by counting 200 spermatozoa. Viability of spermatozoa was assessed by eosin Y exclusion.

The morphology of spermatozoa in semen and motile spermatozoa, selected by either swim-up or colloidal silica gradient centrifugation (PureSperm, Nidacon International AB, Molndal, Sweden), was assessed on smears. These sperm smears were prepared

by washing spermatozoa with 10 mL 0.9% sodium chloride. This step was carried out to reduce the background staining by removing the SP or protein in the medium. Morphology slides were stained using the Shorr method after the smears were fixed in 90% ethanol for 30 min [23]. The percentage of normal sperm morphology was assessed according to the strict criteria [24]. For each sperm sample, 200 spermatozoa were scored from at least 10 individual fields using oil immersion at × 1 000 magnification with bright-field illumination.

2.2 Preparation of motile spermatozoa for the spermatozoa–ZP interaction test

Motile sperm were selected by the swim-up technique. Semen (~0.5 mL), or a sperm pellet obtained by centrifugation of semen was carefully added to the bottom of a test tube (12 × 75 mm) containing 0.7 mL human tubal fluid (HTF; Irvine Scientific, USA) supplemented with 10% heat-inactivated human serum (ICN Biomedicals, USA). Care was taken to avoid disturbing the interface between the semen and the medium. After 1 h of incubation, 0.5 mL of the top layer of the medium, which contained motile spermatozoa, was aspirated. The motile sperm suspension was then centrifuged at 1 000 × *g* for 5 min, and the sperm pellet was rewashed with 1 mL fresh HTF by centrifugation at 1 000 × *g* for 5 min. Subsequently, the washed sperm pellet was resuspended using a serum-supplemented HTF to sperm concentration of 2×10^6 mL^{-1} for the spermatozoa–ZP interaction tests.

2.3 Human oocytes

Oocytes that showed no evidence of two pronuclei or cleavage at 48–60 h after insemination in IVF were used for the spermatozoa–ZP binding test. If the oocytes had spermatozoa bound to the ZP from the IVF insemination, these were removed by aspiration using a fine glass pipette with an inner diameter of 120 μm, which is slightly smaller than the oocyte diameter [7]. Oocytes with > 10 spermatozoa penetrating the ZP, as well as degenerated, activated or morphologically abnormal oocytes, were not used. Oocytes were pooled from several patients and used for the test on the same day or kept in the incubator and used within 2–3 days.

All patients signed consent forms permitting the use of their unfertilized oocytes or sperm samples for research. The Royal Women's Hospital Research and Ethics Committees approved the project.

2.4 Spermatozoa–ZP binding test

For each sperm sample, motile spermatozoa (2×10^6) in 1 mL of medium were incubated with a group of four oocytes in a dish (four-well Nunc Multidishes, Thermo Fisher Scientific, Roskilde Site, Denmark) for 2 h at 37°C in 5% CO_2 in air. After incubation, the oocytes were transferred to a phosphate-buffered saline (PBS) solution (pH 7.4) containing 2 mg mL^{-1} bovine serum albumin (BSA) and washed by repeated aspiration with a glass pipette (inside diameter of ~250 μm) to dislodge the spermatozoa that are loosely bound to the surface of the ZP. The number of spermatozoa bound to each of the four oocytes was counted using an inverted phase contrast microscope with × 250 magnification. The average number of spermatozoa bound per ZP was used as the end point. Under these experimental conditions, with the sperm concentration in the insemination medium 20 times higher than that used for standard IVF insemination, the number of spermatozoa from fertile men that bound tightly to the ZP was usually >100 spermatozoa per ZP. Sperm samples with an average ≥ 40 spermatozoa per ZP were defined as having normal spermatozoa–ZP binding, according to our earlier studies [7, 8].

2.5 Assessment of the ZPIAR

For sperm samples with normal ZP-binding (average ≥ 40 spermatozoa per ZP), all spermatozoa that tightly bound to the surface of the four ZP were then removed by repeated vigorous aspiration using a narrow-gauge pipette with an inner diameter of ~120 μm [8]. This was carried out on a glass slide with approximately 3 μL PBS containing 0.2% BSA. The removed ZP-bound spermatozoa were smeared in a limited area (approximately 16 mm^2), which was marked on the back of the slides with a glass pen to help find spermatozoa under the microscope. This pipetting procedure for removing spermatozoa from the ZP surface does not affect sperm motility, morphology or acrosome status [25].

The AR of ZP-bound spermatozoa was assessed using fluorescin-labelled *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC, Sigma Chemical Company, St Louise, MO, USA), as described earlier [24]. Briefly, sperm smears were fixed in 95% ethanol for 30 min after air-drying and then stained using 25 μg mL^{-1} PSA-FITC in PBS for 2 h at 4°C. The slides were washed and mounted with distilled water. A total of 200 spermatozoa per sample were counted with a fluorescence microscope using excitation wavelengths

of 450– 490 nm and a magnification of × 400. The acrosome was considered intact when more than half of the sperm head was brightly and uniformly fluorescent. Spermatozoa with a fluorescent band at the equatorial segment or without fluorescence in the acrosome region were considered to have reacted. A ZPIAR 16% was defined as a defective ZPIAR. This was based on our earlier studies, which showed that men with 16% ZPIAR have very low (< 30% of oocytes) spermatozoa–ZP penetration and also very low fertilization rates with conventional IVF [7, 15, 26].

2.6 Seminal plasma collection and zinc assay

After semen analysis and sperm function tests were carried out, the remaining semen (0.5–1 mL) was centrifuged at 1 500 *g* × for 7 min to separate spermatozoa from SP. SP was stored at −20°C until zinc analysis was carried out. Zinc assessments were carried out in July and November 2007.

SP zinc concentration was assessed using a colorimetric method, according to the WHO manual [23]. A commercial kit was used, which contained substances that masked interference by other metals that may have been present. The frozen SP samples were thawed and mixed well on a vortex mixer. For zinc assays, 5 μL of SP was diluted with 300 μL of deionised water in a 1.5-mL tube and mixed by vortex for 5 s. A volume of 40 μL of the diluted sample was then mixed with 200 μL of colour reagent for 15 min in 96-well plates, before reading at 560 nm with a Vmax Kinetic Microplate Reader. A standard curve of known zinc concentrations, in the range of 10–100 μmol L^{-1}, was included in the assessment of each batch of samples. All samples were analysed in duplicates and the mean result was used for statistical analysis. Pooled SP from 19 normozoospermic men was used for the internal quality control of each assay, with a coefficient of variation of 7.8%. SP zinc measurements were calculated and expressed as zinc concentration in millimolars or the total amount of zinc in μmol per ejaculate.

2.7 Effect of zinc on ZPIAR in vitro

To determine the effect of zinc on ZPIAR, semen samples from some of the subfertile men were used. These samples were from men with a normal semen analysis and normal spermatozoa–ZP binding. HTF medium containing 0.5 mmol L^{-1} $ZnCl_2$ (test) or 0.5 mmol L^{-1} NaCl (control) was used for both swim-up sperm preparation and spermatozoa–ZP interaction tests. This zinc concentration had no adverse effects on sperm motility *in vitro*, as reported earlier [17]. Briefly, 0.5 mL semen and 1 mL of test or control medium were used for swim-up preparation. After 1 h of incubation, the upper layer of 0.7 mL of medium, which contained motile spermatozoa, was recovered. The number of spermatozoa recovered from the test and control media was similar. A spermatozoa–ZP interaction test was carried out by incubation of motile spermatozoa (2×10^6) in 1 mL of test or control medium, with a group of four oocytes. The ZPIAR of ZP-bound spermatozoa was assessed as described above. As zinc had no effect on spermatozoa–ZP binding, most of the oocytes had > 100 spermatozoa bound per ZP.

2.8 Statistical analysis

The Spearman (non-parametric) test was used to determine the significance of relationships between SP zinc and other semen test results. The significance of the difference in SP zinc content between men with defective ZPIAR (< 16%) and normal ZPIAR (≥ 16%) was determined by an analysis of variance (Wilcoxon rank-sum test) and a *t*-test. A paired *t*-test was used to determine the difference in the ZPIAR of spermatozoa prepared and incubated in medium with 0.5 mmol L^{-1} $ZnCl_2$ or with 0.5 mmol L^{-1} NaCl (control) for *in vitro* experiments.

3 Results

All sperm test results are summarized in Table 1. There were wide ranges in all results including SP zinc (0.1–11.2 mmol L^{-1}). The average SP zinc concentration was 2.8 mmol L^{-1} and 10.4 μmol per ejaculate. A total of 44 subfertile men had two semen tests carried out 2–10 weeks apart and the SP zinc concentration in the two ejaculates was significantly correlated (Spearman $r = 0.789$, $P < 0.0001$). The mean difference was 0.88 mmol L^{-1} , with a standard deviation of 0.87 mmol L^{-1}. The ZPIAR was only tested in men with normal spermatozoa–ZP binding (> 40 spermatozoa bound per ZP), as those with abnormal spermatozoa–ZP binding had insufficient ZP-bound spermatozoa for an accurate assessment of the AR.

The relationship between SP zinc concentration, or the total amount of zinc per ejaculate, and other sperm test results was analysed by the Spearman test. SP zinc concentration was highly correlated with the sperm count (Spearman $r = 0.186$, $P < 0.001$) and the dura-

tion of abstinence (Spearman $r = 0.280$, $P < 0.001$), but negatively correlated with the semen volume (Spearman $r = -0.178$, $P < 0.001$). The total amount of zinc per ejaculate was strongly correlated with both duration of abstinence (Spearman $r = 0.426$, $P < 0.001$) and the total number of spermatozoa per ejaculate (Spearman $r = 0.262$, $P < 0.001$). However, there was no correlation (all $P > 0.05$) between SP zinc concentration and other sperm test results, such as sperm motility, viability, morphology, spermatozoa–ZP binding or ZPIAR (Figure 1). By contrast, most semen analysis variables were highly correlated with spermatozoa–ZP binding and ZPIAR. Sperm motility in semen (Spearman $r = 0.254$, $P < 0.001$) and sperm morphology, in both semen (Spearman $r = 0.169$, $P < 0.001$) and swim-up (Spearman $r = 0.143$, $P < 0.01$), were correlated with spermatozoa–ZP binding. Similarly, sperm concentration in semen (Spearman $r = 0.205$, $P < 0.001$) and sperm progressive motility in semen (Spearman $r = 0.188$, $P < 0.001$), as well as sperm morphology in both semen (Spearman $r = 0.286$, $P < 0.001$) and swim-up (Spearman $r = 0.278$, $P < 0.001$), were strongly correlated with the ZPIAR. As expected, most semen analysis variables were correlated with each other, such as

Table 1. Summary of all sperm test results in 458 subfertile men.

Tests	*n*	Mean ± SD	Range
Seminal zinc concentration (mmol L^{-1})	458	2.8 ± 1.6	0.1 – 11.2
Total seminal zinc (μmol per ejaculate)	451	10.4 ± 6.8	0.2 – 55.2
Abstinence (days)	428	3.7 ± 2.0	1.0 – 21.0
Semen volume (mL)	451	3.8 ± 1.7	1.0 – 15.0
Sperm concentration (10^6 mL^{-1})	458	78.7 ± 66.7	1.0 – 435.0
Sperm number per ejaculate	451	280 ± 262	9 – 2135
Motility (%)	454	52.1 ± 0.7	14.0 – 81.0
Progressive motility (%)	455	39.6 ± 12.0	2.0 – 75.0
Viability (%)	457	77.4 ± 8.7	43.0 – 96.0
Normal morphology (semen, %)	457	8.8 ± 6.9	0 – 35.0
Normal morphology (swim-up, %)	458	14.0 ± 9.3	0 – 52.0
Number of spermatozoa bound per ZP	458	60.0 ± 38.8	0 – 100
ZP-induced AR (%)[a]	307	20.9 ± 19.5	1.0 – 96.0

Abbreviations: ZP, spermatozoa–zona pellucida; AR, acrosome reaction.
[a]Only samples with normal spermatozoa–ZP binding (> 40 spermatozoa bound per ZP) were assessed.

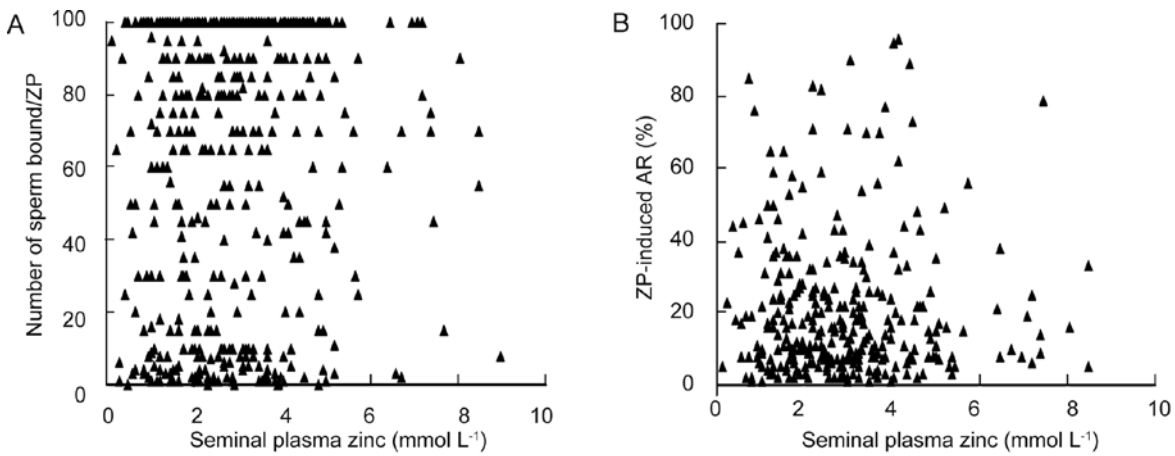

Figure 1. Correlation between seminal plasma zinc concentration and spermatozoa–ZP binding (A): $n = 458$, Spearman $r = 0.051$, $P > 0.05$, (B): ZP-induced AR, $n = 307$, Spearman $r = -0.034$, $P > 0.05$.
Abbreviation: ZP, spermatozoa–zona pellucida.

sperm concentration, motility and morphology.

However, when the subgroup of 120 men with a normal sperm count ($\geq 20 \times 10^6$ mL^{-1}) and normal sperm morphology ≥ 10% (normozoospermia) was analysed, SP zinc concentration was significantly ($P < 0.01$) higher in men with defective ZPIAR (< 16%) than in men with normal ZPIAR (≥ 16%). By contrast, in the subgroup of 149 men with a normal sperm morphology < 10% (teratozoospermia), SP zinc concentration was similar between men with defective ZPIAR and normal ZPIAR (Figure 2). The sperm count and ZPIAR were significantly higher in normozoospermic men than in teratozoospermic men (Table 2). Although SP zinc concentration was not significantly ($P < 0.05$) correlated with the ZPIAR in both subgroups, the P value was marginally close to a significant level of 0.05 in the normozoospermic group (Spearmen $r = -0.169$, $P = 0.06$). Sperm morphology was expected to be significantly ($P < 0.001$) different between the two subgroups, because they were defined by the sperm morphology results. The other 38 men with oligozoospermic semen were not analysed as a subgroup, because the numbers were too small.

Figure 3 shows the effect of adding 0.5 mmol L^{-1} zinc to a culture medium on the ZPIAR of spermatozoa from normal semen samples. ZPIAR was significantly reduced without any effects on spermatozoa–ZP binding.

4 Discussion

In this study, SP zinc concentration was highly correlated with the sperm count in subfertile men,

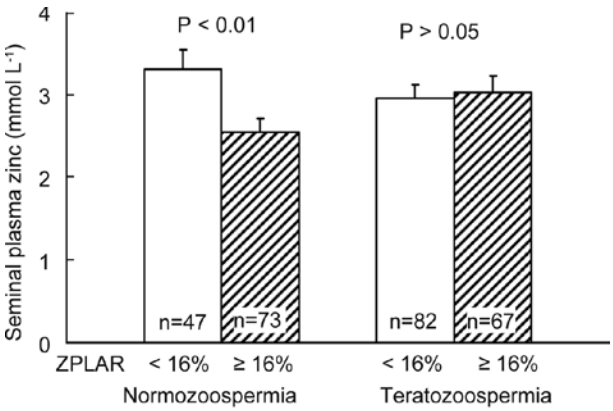

Figure 2. In the subgroup of 120 men with normal sperm concentration and normal sperm morphology (NSM) ≥ 10%, SP zinc concentration was significantly higher in men with defective ZPIAR (< 16%) than in those with normal ZPIAR (≥ 16%). By contrast, in the subgroup of 149 men with NSM < 10%, SP zinc was not significantly different between men with defective ZPIAR and normal ZPIAR. In addition, SP zinc was not significantly different between men with normal morphology >10% and 10%, either with normal or defective ZPIAR. The bar represents the mean and error bars represent the standard error of the means.

Table 2. Comparison of all sperm test results between the two subgroups of men with sperm concentrations ≥ 20×10^6 mL^{-1} and normal sperm morphology ≥ 10% (normozoospermia $n = 120$) and < 10% (teratozoospermia $n = 149$).

Tests	Normozoospermia	Teratozoospermia
Seminal zinc concentration (mmol L^{-1})	2.9 ± 1.7	3.0 ± 1.5
Total seminal zinc (μmol per ejaculate)	9.6 ± 5.5	10.9 ± 6.1
Abstinence (days)	3.7 ± 1.8	3.7 ± 2.2
Semen volume (mL)	3.6 ± 1.6	3.8 ± 1.4
Sperm concentration (10^6 mL^{-1})*	100 ± 76	75 ± 47
Sperm number per ejaculate**	345 ± 303	279 ± 204
Motility (%)	56.9 ± 9.6	53.1 ± 9.5
Progressive motility (%)	45.8 ± 10.7	40.1 ± 11.1
Viability (%)	78.2 ± 8.9	77.1 ± 7.9
Normal morphology (semen, %)***	16.2 ± 6.6	5.0 ± 2.6
Normal morphology (swim-up, %)***	23.3 ± 7.9	8.9 ± 4.4
Number of spermatozoa bound per ZP	85.6 ± 20.2	84.2 ± 8.7
ZP-induced AR (%)*	24.5 ± 19.3	19.4 ± 19.6

*$P < 0.01$. **$P < 0.05$. ***$P < 0.001$.

Abbreviations: ZP, spermatozoa–zona pellucida; AR, acrosome reaction.

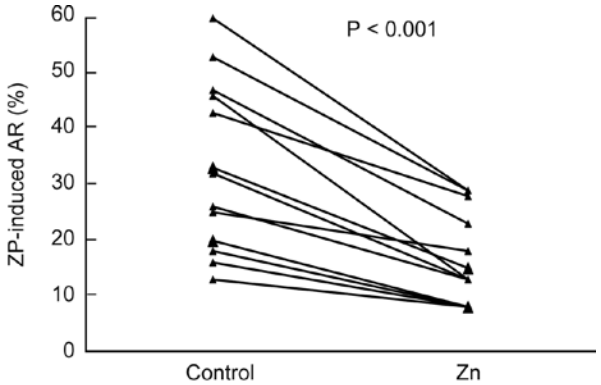

Figure 3. A comparison of ZP-induced AR (ZPIAR) in media containing 0.5 mmol L^{-1} $ZnCl_2$ (Zn) or 0.5 mmol L^{-1} NaCl (control) *in vitro*. Each line represents an individual sperm sample from 13 normozoospermic men. ZPIAR was significantly lower in media containing zinc than in the control (paired *t*-test, $t = 6.13$, $P < 0.001$).

which further supports the earlier reports [9–13, 27]. Currently, the fact that SP zinc concentrations are consistently correlated with sperm counts is poorly understood. It has been reported that zinc levels in the blood are important for spermatogenesis. This is because a zinc deficiency leads to gonadal dysfunction with decreased testicular weight and shrinkage of seminiferous tubules [28, 29]. Zinc deficiencies in men can also impair angiotensin-converting enzyme activity in Leydig cells, thus resulting in the depletion of testosterone and inhibition of spermatogenesis. However, despite the relationship between SP zinc concentration and sperm count, SP zinc concentration is still not a useful clinical marker to discriminate between fertile and infertile men [13, 27].

SP zinc concentration was positively correlated with abstinence duration and negatively correlated with semen volume. The duration of sexual abstinence is likely to have been an accumulating factor. On the other hand, semen volume is a diluting factor for SP zinc concentration [30]. Other semen analysis variables, such as motility, viability and morphology, were not significantly correlated with SP zinc concentration. The results were similar to most reports in the literature [9, 10, 31–33]. However, some studies have shown that a higher level of SP zinc may be associated with asthenozoospermia. Excessive binding of zinc to sperm tails has been postulated as the mechanism [10, 12, 32, 34]. Others have reported that the zinc concentration of the sperm tail is negatively correlated with sperm motility and velocity [35].

In this study, after analysis of data from all participants using the Spearman test, SP zinc concentration was not significantly correlated with either spermatozoa–ZP binding or ZPIAR. However, in the subgroup of 120 subfertile men with a normal sperm count ($\geq 20 \times 10^6$ mL^{-1}) and strict normal sperm morphology ($\geq$ 10%), SP zinc concentrations were significantly ($P < 0.01$) higher in men with defective ZPIAR (< 16%) than in those with normal ZPIAR ($\geq$ 16%). By contrast, in the other 149 teratozoospermic men (strict normal sperm morphology < 10%), SP zinc was not significantly ($P > 0.05$) different between men with defective and normal ZPIAR. These results suggest that a relatively high SP zinc concentration might be associated with reduced ZPIAR in some subfertile men without severe sperm morphological defects. Thus, defective ZPIAR may be caused by different mechanisms in men with or without severe sperm morphological defects. In severe teratozoospermic subfertile men, defective ZPIAR is more likely to be related to major structural defects of the sperm head, such as small or abnormal acrosomes, or associated abnormalities in the overlying plasma membrane. By contrast, in men with normal sperm morphology, defective ZPIAR is most likely to be due to subtle biochemical or molecular defects in ZP receptors, signal transduction pathways, inefficient cholesterol or zinc removal from the plasma membrane during capacitation, actin polymerization or acrosomal enzyme activation [36–42]. Several *in vitro* studies have shown that zinc and SP inhibit sperm capacitation, including hyperactivation and AR, as well as spermatozoa–ZP binding and penetration [17, 20–22]. The hyperactivation of capacitated spermatozoa was highly correlated with the ZPIAR [43]. Results of this study suggest that there was an association between high SP zinc concentration and defective ZPIAR in subfertile men with normal sperm morphology. Our *in vitro* experiments clearly show that the addition of 0.5 mmol L^{-1} zinc to a culture medium significantly inhibits ZPIAR (Figure 3). Again this further suggests that zinc does have a negative impact on the ZPIAR. More studies are needed to determine whether the inhibitory effect of zinc on the ZPIAR is attributed to excessive binding of zinc to the plasma membrane, resulting in an interference with calcium influx, or to other effects. Furthermore, it is possible that other prostatic secretions (for example, citric acid) might affect zinc levels or even sperm function,

thus requiring further study.

In this study, sperm motility and morphology were strongly correlated with both spermatozoa–ZP binding and the ZPIAR. Sperm count was also highly correlated with the ZPIAR. Spermatozoa from subfertile men with defective ZPIAR cannot penetrate the ZP and fertilize oocytes under conventional IVF. Thus, patients diagnosed with this condition require treatment by ICSI. Spermatozoa–ZP interaction tests cannot be carried out routinely, because of a limited supply of human oocytes. Therefore, alternative tests are needed.

In summary, SP zinc concentration was highly correlated with sperm count and the duration of sexual abstinence. It was not correlated with other sperm test results, including spermatozoa–ZP binding and the ZPIAR, in all participants studied. However, in the subgroup of men with normozoospermic semen, SP zinc concentration was significantly ($P < 0.01$) higher in men with defective ZPIAR (< 16%) than in those with normal ZPIAR (≥ 16%). *In vitro* experiments confirmed that the addition of zinc to a culture medium significantly ($P < 0.001$) inhibited the ZPIAR. Thus, a relatively high SP zinc concentration may be associated with defective ZPIAR in certain subfertile men with normozoospermic semen. However, an assessment of SP zinc is unlikely to be a useful clinical marker for the prediction of defective ZPIAR, which currently can only be diagnosed by carrying out spermatozoa–ZP interaction tests using human oocytes.

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