

## Original Article

# The Relationship between Zinc/Cadmium Ratio in Human Semen: Effect on Immune Response

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**ABSTRACT**

**Objective of study:** To investigate the relationship between seminal levels of zinc and cadmium and spermatozoal quality, and their effects on the immune response.

**Study Design and Methodology:** Forty-five infertile and 16 fertile males were included in this study. Investigations included semen analysis, a hypo-osmotic swelling test, antisperm antibodies by immunobeads, trace elements with atomic absorption spectroscopy and TNF- $\alpha$  and interleukin-4 with ELISA.

**Results:** A high zinc/cadmium (Zn/Cd) ratio of more than 200 was associated with a normal sperm count and

motility. There was an inverse relationship between the Zn/Cd ratio and impairment of spermatozoa motility. A high Zn/Cd ratio was associated with a low incidence of antisperm antibodies, especially IgA and IgM, a positive correlation with seminal IL-4 but inverse relationship with TNF- $\alpha$ .

**Conclusion:** The Zn/Cd ratio may be a better index of sperm quality than seminal zinc and cadmium, independently, through a differential reduction of the humoral immunity and enhancement of the T helper-2 cytokine Interleukin-4 as well as a down regulation of the T helper-1 cellular immune response.

KEYWORDS: semen, cadmium, zinc, ratio, immune response

**INTRODUCTION**

Many factors are known to affect the manifestation of humoral and cell mediated immunity in the pathogenesis of male reproductive failure<sup>[1,2]</sup>. Of the trace elements, the role of zinc in cellular immunity has been extensively studied. Zinc deficiency has been associated with several abnormalities of cellular immunity, like thymic atrophy, anergy, lymphopenia, and a reduced lymphocyte proliferation in response to mitogens. There is also a selective decrease of T4<sup>+</sup> helper cells and a decreased ratio of helper and suppressor T-lymphocytes as well as decreased natural killer (NK) cells activity. All these changes are reversed with Zn administration<sup>[3,4]</sup>. IL-2 is a cytokine produced primarily by T4<sup>+</sup> helper cells, and it plays a crucial role in T-lymphocyte proliferation. IL-2 production in zinc-deficient subjects is impaired<sup>[5]</sup>. Zinc is indispensable in normal testicular development, spermatogenesis, and spermatozoa motility and prevents degradation of spermatozoa as well as maintains viability by inhibiting DNAases<sup>[6]</sup>. Zinc, as a component of enzyme complexes, has a fundamental role in the antibacterial activity of seminal plasma<sup>[6]</sup>. Apart from the maintenance of immunocompetence, a very important function of zinc, in conjunction with copper, is to protect the body against free radicals

through superoxide dismutase (SOD), a cuprozinic enzyme which accelerates the dismutation of free radicals (O<sub>2</sub>)<sup>[7]</sup>. This enzyme plays a major role in protecting human spermatozoa against peroxidative damage of cellular enzymes and structures. The human spermatozoa membrane contains a large amount of polyunsaturated fatty acids, especially docosahexaminic acid (DHA), which are extremely sensitive to lipid peroxidation, with resultant permeabilization of the plasma membranes and subsequent cell damage. The production of reactive oxygen radicals by spermatozoa has been observed to be increased in infertile men and it is speculated that generation of reactive oxygen species might cause infertility in these men<sup>[7,8]</sup>. Zinc and copper are bound to a small cysteine-rich metal-binding protein, metallothionein (MT), which has been identified in the male mammalian reproductive organs such as testis, epididymis, prostate and seminal vesicles<sup>[9]</sup>. In general, MT is important in the homeostasis of essential metals, like zinc and copper, and provides protection from toxic metals such as cadmium.

Cadmium has a putative role on spermatozoa quality through testicular damage from ischemia. Tobacco is a major contributor to the cadmium intake in humans. There is strong evidence that, in heavy cigarette smokers, there is an association

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with a higher incidence of sperm abnormalities in terms of sperm counts, motility and increased anomalies in sperm morphology. A causal role for cadmium in male infertility has, therefore, been suggested<sup>[10]</sup>. Severe damage to the testes of rats, mice and toads has been demonstrated following cadmium injections with an increase in the size of the Leydig cell nuclei and the activity of 3B hydrosteroid dehydrogenase (3B-HSD). Cadmium decreases androgen biosynthesis, possibly by altering progesterone synthesis and metabolism<sup>[11]</sup>. Paradoxically, seminal levels of zinc and cadmium have not been authentically shown to be independently associated with sperm quality<sup>[12, 13, 14, 15]</sup>.

## MATERIALS AND METHODS

A total of 61 men attending the combined infertility clinic (andrology) of the Maternity Hospital, Kuwait were enlisted into the study over a two-year period. They were divided into two groups:

Group 1: 45 infertile men undergoing evaluation and treatment. To be included in the study, the man must have had infertility for at least 12 months.

Group 2: 16 fertile men whose wives were being managed for ovarian dysfunction with induction of ovulation and who got pregnant. These 16 men acted as controls for this study. The protocol in the couple included full history and physical examinations of both spouses. In both the husband and wife, hormone profile LH, FSH, Prolactin, thyroid function, and testosterone assessments were routinely carried out. In the female spouse, ovarian function was confirmed with mid-luteal phase progesterone and presence of corpus luteum at laparoscopy. The male spouse underwent seminal fluid analysis in strict compliance with the WHO guidelines<sup>[16]</sup>, by the masturbation method of semen collection after three days of sexual abstinence. The fresh semen was used for the sperm count, motility, morphology, hypo-osmotic swelling test and antisperm antibodies. The seminal plasma was then separated after centrifugation and stored in aliquots at -20 °C until assayed for trace elements and the T-helper cytokines. A total of 5 ml of blood was taken from each man, and the serum separated and stored in 1 ml storage tubes and at -20 °C until assayed separately for circulating antisperm antibodies and estimation of the trace elements zinc and cadmium.

### Estimation of antisperm antibodies:

1. Immunofluorescence technique was used to detect circulating antisperm antibodies as previously described<sup>[17]</sup>.

Sperm antibody by immunobead test. This was used to determine the presence of specific IgM, IgG and IgA antibodies on the surface of the sperm using latex beads coated with anti-immunoglobulins that bind to human antibodies (Bioscreen Inc, distributed by Fertility Technologies, Inc, Natick, MA 0176, USA). Reagents:

- i. 0.8ml latex beads coated with anti-human immunoglobulin in a protein buffer with 0.1% Sodium azide
  - (a) Anti-IgM coated beads (green)
  - (b) Anti-IgG coated beads (blue)
  - (c) Anti-IgA coated beads (red)
- ii. Sperm washing medium containing 5% bovine serum albumin
- iii. Positive and negative serum controls

### Procedure

1. A total of .5 ml of the sperm suspension and 5 ml of the anti-immunoglobulin beads (anti-M, anti-G and anti-A, used separately) were mixed thoroughly on a glass slide and, after about 5 minutes, were examined under the microscope. At least 100 free-swimming sperm were then counted to determine the binding of any bead on the surface of the sperm.

$$\text{Percent total binding} = \frac{\text{No sperm with bound beads} \times 100}{\text{Total no of sperm counted}}$$

Any total binding of at least 50% was accepted as positive for antisperm antibodies.

2. T-helper Cytokines-TNF- and interleukin-4 estimated with ELISA.

### Materials

1. Human IL-4 and TNF- standard (recombinant IL-4 and TNF- )
2. Tetra Methyl benzieline (TMB) Chromogen
3. Streptavidin- Peroxidase (WHO HPR)
4. IL-4 and TNF- antibody coated wells, 96 wells per plate.

All reagents were supplied by Biosource International 820 Flynn Road. Camarillo, Ca 93012 USA.

### Methodology of estimation

A standard curve was first established by the doubling dilution of the 500 pg/ml to give an 8-point standard curve. The assay curve of the standard was plotted against absorbance at 450 nm, with a correlation coefficient of 0.99, an intra-assay coefficient of variation of 1.9 to 3.5% and an inter-assay coefficient of variation of 1.4 to 2.2%. Sera, standards and controls were incubated with a

polyclonal antibody, which had been previously coated into micro-litre wells, to bind IL-4 or TNF-antigens to the immobilised antibody.

After washing, a biotinylated monoclonal IL-4 or TNF- specific antibody was added. During the second incubation, this antibody got bound to the immobilised IL-4 or TNF- . After removing the excess biotinylated antibody by repeated washing, streptavidin peroxidase was added. Following a third incubation and washing, a substrate solution was added to produce a color measurable at 450 nm, the intensity of which is directly proportional to the concentration of IL-4 or TNF- in the sample. The concentration was read off from the reference curve. The lower limit of sensitivity for the assay was 4 pg/ml for TNF- , and 1 pg/ml for IL-4.

### 3. Analysis of trace elements: zinc and cadmium in semen.

The aliquoted semen samples were thawed at room temperature. Serum or seminal plasma was diluted 1:10 in deionized water. Standards were also prepared in deionized water. They were run in the range of 0.1 ppm to 0.5 ppm and 0.05 ppm to 0.2 ppm for Zn and Cd, respectively, and assayed with an atomic absorption spectrophotometry (AAS) (Varian model spectra AA 300/400, Australia). All the trace element stock standards (of concentration 1000 ppm) were obtained from Fluka Chemika Switzerland.

**Statistical Analysis:** Most of the data were expressed in mean and standard deviation, and analyzed by one-way analysis of variance (ANOVA) for multiple comparison and Pearson's regression coefficient of correlation (r) and student t-test for paired results with significance fixed at  $p=0.05$ .

## RESULTS

The spermatozoal parameters were significantly better in terms of sperm concentration ( $p<.02$ ), progressive motility ( $p<.01$ ) in the control group of men whose wives had ovarian dysfunction and got pregnant after induction (Table 1). Conversely, the index of impairment of sperm motility (asthenozoospermia), was more common in infertile than fertile men ( $p<.01$ ). The levels of seminal trace elements, T-helper cytokines and antisperm antibodies were assessed in both the study and control groups, as shown in the Table 2. There were no significant differences in the semen concentrations of zinc and cadmium between the two groups ( $p>.05$ ). However, the antisperm antibodies were significantly more common in the study group compared to the fertile controls, with 15.6 vs 6.3% ( $p<.03$ ) for immunofluorescence and

**Table 1**

Characteristics of patients and sperm parameters

	Study Group n= 45	Control Group n= 16	P value
Mean age (years $\pm$ )	38.6 $\pm$ 8.2	37.9 $\pm$ 7.8	NS
Duration of infertility (years)	6.8 $\pm$ 4.9	5.1 $\pm$ 3.2	NS
No. of living children	0.4 $\pm$ .2		NS
Semen parameters			
Volume (ml)	3.1 $\pm$ 1.8	2.9 $\pm$ 1.4	NS
Mean sperm count ( $10^5$ /ml)	51.8 $\pm$ 22.3	90.6 $\pm$ 40.9	0.02
Motility (%)			
*Progressive	32.4 $\pm$ 24.3	65.4 $\pm$ 18.4	00.1
*Weak	19.6 $\pm$ 12.8	14.6 $\pm$ 10.5	NS
*Non-motile	48.2 $\pm$ 24.4	22.7 $\pm$ 12.1	00.1
Morphology (%) (normal)	75.4 $\pm$ 24.1	76.3 $\pm$ 23.4	NS
Hypo Osmotic Swelling test (%)	38.3 $\pm$ 19.3	64.8 $\pm$ 20.6	0.04
Asthenozoospermia ( 40% non motile sperm)			
	53.3%	12.5%	0.01

NS - Not Significant

**Table 2**

Comparative values of seminal trace elements, circulating, antisperm antibodies and T helper cytokines

	Study Group n= 45	Control group n= 16	P value
a. Trace elements			
Mean in $\mu\text{g}/\text{ml}$			
i. Zn	166.2	170.6	NS
ii. Cu	165	169	NS
iii. Cd	1.78	1.73	NS
b. Incidence of circulating antisperm antibodies (%)			
	15.6	6.3	.03
c. T helper Cytokines in semen( pg/ml)			
TNF-	16.3 $\pm$ 7.7	1.1 $\pm$ 0.9	.01
Interleukin-4	27.5 $\pm$ 14.3	47.2 $\pm$ 8.6	.03

NS - Not Significant

11.1 vs 0% ( $p<.02$ ) with immunobead technique. Similarly, the T-helper1 Cytokine TNF- seminal concentration was higher in the infertile group than the fertile group (6.3 vs 1.1pg/ml,  $p<.05$ ). On the contrary, the main T-helper 2 Cytokine IL-4 had a higher semen concentration in the control group than the study infertile group ( $p<.01$ ). There was no direct relationship between the seminal plasma levels of the trace elements and the sperm parameters in either group.

As shown in Table 3, there was a definite relationship between zinc/cadmium ratio and sperm count ( $r = .65$ ,  $p<.01$ ). Of the 16 patients with normozoospermia, 93.8% had Zn/Cd ratio of 200 and above, compared to 55.6% in infertile men with normozoospermia and mild oligozoospermia, respectively, and 22.2% for severe oligozoospermia and 11.1% in azoospermia. Conversely, low sperm

**Table 3**

The relationship between semen zinc/cadmium ratio and spermatozoal parameters

Zn/Cd ratio	NZ n=16	INZ n=16	MOZ n=9	SOZ n=11	AZ n=9
0-99	0	1	0	2	2
100-149	0	1	1	5	3
150-199	1	5	3	2	3
200	15	9	5	2	1

NZ = Control group of men with normozoospermia  
 INZ= infertile men with normozoospermia  
 MOZ= infertile men with mild oligozoospermia  
 SOZ= infertile men with severe oligozoospermia  
 AZ= infertile men with azoospermia

count was associated with decreasing levels of zinc/cadmium ratios ( $r = -.54, p < .05$ ). In Fig. 1, the relationship between zinc/cadmium ratio and sperm motility is analyzed. Asthenozoospermia was defined as non-motile spermatozoa in a semen specimen of more than 40% and normal motility were those with non-motile spermatozoa less than 40%. There was a strong inverse correlation between zinc/cadmium ratio and impairment of spermatozoal motility (asthenozoospermia ( $r = -.72, p < .001$ ).

The semen concentration of each trace element (Zn and Cd) was analyzed with the antisperm antibodies and T-helper Cytokines to establish any relationship. There was no strong correlation between the trace elements and IL-4 and TNF-a ( $r = .11$  to  $.21, p > 0.05$ ) nor with the different antisperm antibodies (IgG, IgA and IgM). In Fig. 2, the T-helper Cytokines IL-4 and TNF-a were analyzed in relation to the sperm count: Normozoospermia, mild oligozoospermia, severe oligo-zoospermia and azoospermia. There was a strong positive association between high sperm count and Interleukin-4 ( $r = .61, p < .01$ ). On the other hand, there was inverse relationship between semen level of tumor necrosis factor and sperm count ( $r = -0.60, p < 0.01$ ). A similar pattern was also demonstrated with sperm motility. Progressive sperm motility was associated with high mean IL-4 level of 39.8 pg/ml versus 9.1 pg/ml ( $p < .001$ ), whereas TNF- was 2.3 versus 32.3 pg/ml ( $p < .001$ ).

In Fig. 3, Zn/Cd ratios were analyzed for any relationship with the T-helper cytokines. There was a positive correlation between IL-4 and Zn/Cd ratio ( $r = .59, p < .01$ ) but a negative correlation between TNF- and Zn/Cd ratio ( $r = -.58, p < .01$ ). Thus, a high Zn/Cd ratio was associated with high IL-4 levels in semen, while low Zn/Cd ratio was associated with high levels of TNF-. As shown in Fig. 4, zinc/cadmium ratio has significant effect on

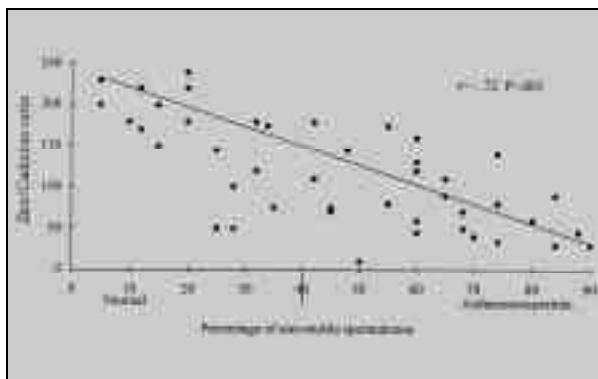


Fig. 1: Relationship between seminal zinc/cadmium ratio and asthenozoospermia

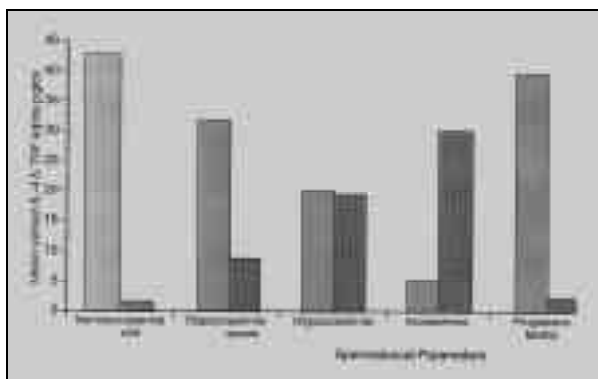


Fig. 2: Interleukin 4 and tumor necrosis factor seminal level according to spermatozoal parameters

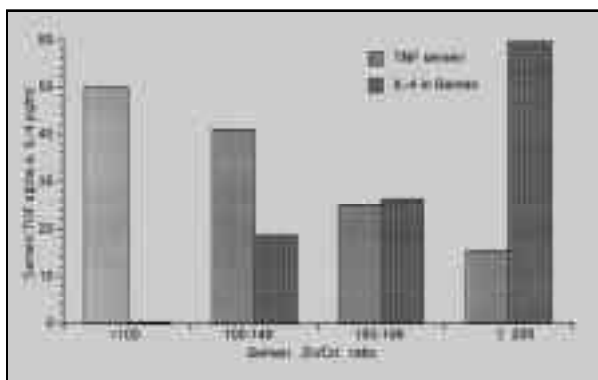


Fig. 3: Relation between semen Zn/Cd ratio and T helper cytokines

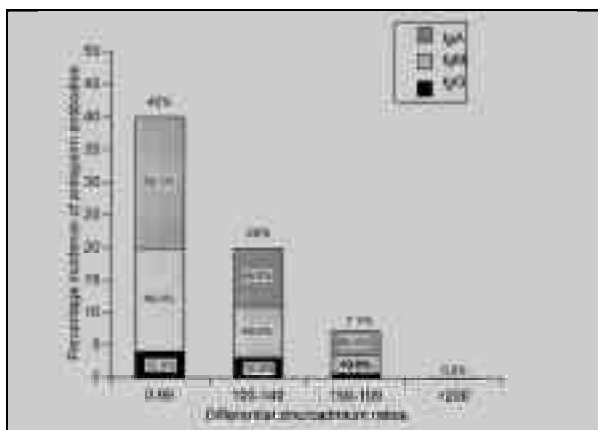


Fig. 4: Relationship between seminal zinc/cadmium ratio and seminal antisperm antibodies

seminal humoral immunity. A high Zn/Cd ratio was associated with a low incidence of seminal antisperm antibodies, especially IgA and IgM. There was no significant effect on IgG.

## DISCUSSION

The present study has demonstrated that Zn/Cd ratio may be a better index of the association between zinc and cadmium and sperm parameters. This is probably the effect of the mode of the transport and release of these metals in body fluids. In fluids, Zn is bound to a small cysteine-rich metal-binding protein, metallothionein, with the ability to bind class B metals such as cadmium, lead and copper. In general, metallothionein is important in the homeostasis of the essential metals and also provides protection from toxic metals such as cadmium<sup>[9, 18]</sup>. Since metallothionein has been identified in the testis, epididymis, prostate and seminal vesicles, it may act in concert with the trace elements in spermatogenesis, nutrition and movement of the spermatozoa through the male genital tract and their maturation or capacitation. These biological activities need an optimum balance between the trace elements, especially between zinc and cadmium. Cadmium usually follows the metabolic pathway of zinc, because of the physico-chemical similarities between the two.

An important finding in the present study is the association between the Zn/Cd ratio and the immune response. We have demonstrated that a high Zn/Cd ratio is associated with low seminal antisperm antibodies, especially IgA and IgM. Cadmium has been shown to have a differential effect on T- and B-lymphocytes. While enhancing T-cell independent antibody production, cadmium inhibits T-cell dependent antibody production, alters plasma membrane and disrupts APC and T-lymphocytes interaction<sup>[19]</sup>. This is in accordance with the findings of this study that a low Zn/Cd ratio (i.e high cadmium levels) is associated with high incidence of IgA and IgM antisperm antibodies.

The association between the Zn/Cd ratio and cellular immune response seems more diverse. This study has shown that there is an inverse relationship between the Zn/Cd ratio and the T-helper Cytokines. While a high Zn/Cd ratio is associated with production and expression of T-helper 2 cytokine IL-4, low levels have a strong correlation with the T-helper cytokine TNF- $\alpha$ . We have earlier reported that zinc therapy increases seminal IL-4 levels and improves sperm quality<sup>[20]</sup>. As modifiers of mRNA activity or stability, metal ions take part in the regulation of the production and expression of growth factors and cytokines<sup>[21]</sup>. Zinc may, therefore, stimulate the production of IL-

4 by activating the proliferation of T-helper 2 lymphocytes.

On the other hand, cadmium has been reported to induce apoptosis<sup>[22]</sup>. This could enhance TNF- $\alpha$  production. It is known that TNF- $\alpha$ , along with other cytokines, such as IL-1 and IL-6, induces an acute-phase response during infection or inflammation and cytosine induction of metallothionein in organs such as liver and testis and, thus, reduces the level of active zinc. Secondly, since TNF- $\alpha$  is a polypeptide, it could associate with zinc and diminish the latter's membrane stabilizing effects and antioxidant activity, especially on the endothelial function<sup>[23]</sup>. The role of Interleukin-4 in semen is probably protective of the spermatozoa. IL-4 may also function as a regulator of pro-inflammatory cytokine<sup>[24]</sup>. Zinc deficiency has also been associated with an impairment of IL-2 production, probably by decrease of mRNA of IL-2<sup>[25]</sup>.

In conclusion, in the direct assessment of the relationship between the common trace elements and sperm parameters, zinc/cadmium ratio may be a better index of sperm quality than the individual levels of trace elements.

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