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Zinc concentrations in serum and follicular fluid during ovarian stimulation and expression of Zn^{2+} transporters in human oocytes and cumulus cells

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Abstract Serum and follicular fluid zinc concentrations were investigated in patients undergoing assisted reproductive treatment. No correlation was found between zinc and oestradiol concentrations in serum. At the time of oocyte retrieval, zinc concentrations in follicular fluid were significantly lower than serum concentrations ($P < 0.0001$). The expression of the two families of zinc transporters, ZnT and ZiP, as well as the metal regulatory transcription factors, MTF1 and 2, and metallothioneins, which are both involved in regulatory aspects of zinc transport, was assayed in cumulus cells and in germinal-vesicle oocytes. Most of the zinc transporters, metallothioneins and metal regulatory transcription factor are expressed in oocytes and not in cumulus cells. This may indicate an important role for zinc, in particular with potential linking to genome stability during early embryonic development. In contrast, cumulus cells seem to be at the end of their life's journey, with weak expression of transcriptional activity linked to cellular housekeeping. RBMOnline

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KEYWORDS: cumulus cells, follicular fluid, metallothioneins, oocyte, serum, zinc

Introduction

Zinc is an essential trace element that is required during mammalian developmental processes. Although literature regarding the exact number of enzymes is controversial, it acts as a co-factor for at least 200 enzymes, including carbonic anhydrase enzymes involved in methylation and prevention of oxidative stress (Zn superoxide dismutase;

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ZnSOD) and metallothioneins (MT) capturing superoxide and hydroxyl radicals. It is the second-most abundant transition metal after iron and binds strongly to MT (cystine-rich proteins), thereby protecting thiol groups and preventing their abnormal interactions with iron, which generate free radical groups. It also stabilizes DNA conformation through its involvement in numerous DNA repair enzymes, especially important during early embryogenesis (Jaroudi et al., 2009; Ménézo et al., 2007, 2010a). Epidemiological studies reveal associations between low circulating serum zinc concentrations and an increased risk of cancer (Ho, 2004), a consequence of its role in DNA repair activity (Ho et al., 2003; Song et al., 2009). Zinc-MT protects against DNA damage induced by radiation better than glutathione (Cai and Cherian, 2003). Through its association with zinc finger proteins, it is involved in the regulation of gene expression and control of apoptosis. In a similar manner, zinc seems to have a pivotal role in reproduction, and thus fertility, via mechanisms that control genome stability: it is an important co-factor in the folate cycle, involved in the recycling of homocysteine to methionine, both of which have a major influence on methylation reactions through *S*-adenosyl methionine (SAM). SAM is formed in the oocyte and homocysteine impairs both methionine incorporation and normal methylation reactions (Ménézo et al., 1989). Moreover, human oocytes have a very limited capacity for homocysteine recycling, because the cystathionine β synthase (CBS) pathway is absent and the betaine homocysteine methyltransferase (BHMT) pathway, which is zinc dependent, is poorly expressed. Zinc also has an important role in preventing generation of reactive oxygen species (ROS) during the first embryonic cleavage division. ZnSOD, located in the cytoplasm, is found in human oocytes and Fallopian tubes (El Mouatassim et al., 2000; Guérin et al., 2001) and in the environment of the pre-implantation embryo: it is present throughout the embryo's journey in the oviduct (Patek and Hagenfeldt, 1974). *In vitro*, non-physiologically high zinc concentrations may have negative effects on pre-implantation development (Stephenson and Brackett, 1999).

This study assayed serum and follicular fluid zinc concentrations in relation to oestradiol concentrations, i.e. in the environment of the follicle and of the female gamete. In order to understand whether zinc might have a physiological role during maturation and subsequently during early embryonic development, this work also studied the expression of genes for the zinc transporters ZnT (solute-linked carrier 30: SLC30) and Zip (Zrt and Irt-like proteins: SLC39A) in oocytes and in cumulus cells. The expression of the metal regulatory transcription factors (MTF) and MT, involved in regulatory aspects of zinc transport (Cousins et al., 2006; Liuzzi and Cousins, 2004), were also investigated.

Materials and methods

The experiments were performed in three assisted reproduction centres, Clinique Pierre Cherest (Neuilly, France), Clinique de La Muette (Paris, France) for study of zinc in serum and follicular fluid and mRNA oocyte content and depending of the same analysis laboratory (Laboratoire d'Eylau, Paris). The work on cumulus cells (mRNA content and expression of zinc transporters, MT and zinc-dependent transcription factors) was performed in Gurgan clinic (Ankara, Turkey).

Zinc assay

An initial group of 129 patients were chosen at random on varying days during stimulation cycles for intrauterine insemination or IVF/intracytoplasmic sperm injection (ICSI), and zinc concentrations were measured in conjunction with oestradiol assays. All the assays were performed in the same laboratory (Laboratoire d'Eylau agreed for the two assisted reproduction technology clinics Clinique Pierre Cherest Neuilly and clinique de la Muette, Paris). In a second group of 24 patients, zinc concentrations were measured in serum and in follicular fluid at the time of oocyte retrieval. The samples were assayed per group, no longer than 48 h after retrieval and stored at −25 °C. Internal standards were used in order to control any shift in the reagents. Zinc assays were carried out via a colourimetric method by spectrophotometry at 560 nm, using (2,5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-sulpho-propylamino) phenol, at a slightly acidic pH (sodium citrate + sodium carbonate). For all the samples, protein precipitation was performed with trichloracetic acid. Dimethylglyoxime was included in the assay as transition metal chelator and salicylaldoxime was used for zinc extraction.

Various types of correlation between oestradiol and zinc in serum were investigated (linear, logarithmic, exponential and polynomial). A comparison of means was carried out to compare zinc concentrations in follicular fluid and in serum.

Expression of zinc transporters and metallothioneins in oocytes and cumulus cells

Analysis was carried out on five pools of 70 oocytes at the germinal-vesicle stage according to a protocol already described (Ménézo et al., 2007); cumulus cells were collected from 45 patients at the time of denudation for ICSI. The stimulation protocols were as classically described: long-term desensitization followed by ovarian stimulation with recombinant FSH or human menopausal gonadotrophin (Fostimon; Genevrier, Sophia Antipolis, France). Briefly, for oocytes, microarray analysis used Affimetrix HG U-133 plus 2.0 chips (Partner Chips, Evry, France). Amplification was performed via double in-vitro transcription (two cycles; cDNA synthesis kit; Affimetrix). For cumulus cells, microarray analysis was used (2.0 chips from Geno Splice (Paris, France)), and no amplification was necessary in the majority of samples. In cases where the amount of RNA was not sufficient, a single amplification was performed. Data were normalized using the algorithm MAS5, in order to generate a signal proportional to the level of expression for each of the 54,613 chip transcripts (probe set ID). A call signal indicated and specified if the gene is expressed at a level above the background. The background level is higher for cumulus cells as the amount of material is more important. Housekeeping and spike controls were used for each determination. The values given in **Tables 1–3** represent the intensity of the signal given for each mRNA (see also Benkhalifa et al., 2010)

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Table 1 Quality and specificity of the controls.

Controls	S-Oocytes	S-Cumulus cells
β actin	11480 (5210) positive	50201 (20415) positive
α tubulin	54463 (14009) positive	46613 (13208) positive
Amphiregulin	25 (17) negative	12427 (5222) positive
GDF9	15861 (5724) positive	402 (352) negative
BMP6	3637 (1416) positive	416 (280) negative

Values are mean signal (SD), background limit, unless otherwise stated. $S = 50$ for oocytes, $S = 100$ for cumulus cells.

Table 2 Metallothioneins and metal responsive transcription factors.

Enzyme	S-Oocytes	S-Cumulus cells
Metallothionein		
MT1 E	4012 (1598)	11666 (3750)
MT1F	4067 (2580)	6144 (2405)
MT1G	4085 (2580)	7678 (3307)
MT1H	6494 (4661)	8549 (3267)
MT1M	2203 (829)	2081 (136)
MT1X	3345 (1009)	17954 (5954)
MTA2	11260 (5076)	42038 (18675)
Metal-responsive transcription factor		
MTF1	2267 (1315)	Not expressed
DMTF1	1424 (655)	Weakly present*
MTF2	17375 (9030)	Weakly present*

Values are mean signal (SD), background limit, unless otherwise stated. $S = 50$ for oocytes, $S = 100$ for cumulus cells.
*Weakly detected (1/3rd of the samples).

Table 1 shows the intensity signals for negative and positive controls, together with their specificity. β-Actin and α-tubulin are constitutive in all cells. BMP6 and GDF9 are specific to oocytes and are normally absent in cumulus cells. Amphiregulin is present in cumulus cells but not in oocytes.

Table 3 Zinc transporters in Oocytes and Cumulus cells.

Transporter	S-Oocytes	S-Cumulus cells
ZnT family (SLC30A)		
ZnT1	Weakly expressed*	Absent
ZnT2	Absent	Absent
ZnT3	937 (765)	Absent
ZnT4	Absent	Absent
ZnT5	383 (156)	Absent
ZnT6	696 (308)	Absent
ZnT7	586 (155)	1698 (363)
ZnT8	395 (257)	Absent
ZnT9	691 (351)	2560 (873)
Zip family (SLC39A)		
Zip1	Weakly expressed*	Absent
Zip2	Absent	Absent
Zip3	3072 (1791)	Absent
Zip4	596 (267)	Absent
Zip5	Absent	Absent
Zip6	14371 (3885)	1189 (541)
Zip7	Weakly expressed*	Absent
Zip8	Absent	1080 (200)
Zip9	274 (157)	Weakly expressed*
Zip10	4231 (2705)	Weakly expressed*
Zip12	500 (78)	Absent
Zip14	13524 (2435)	1159 (506)

Values are mean signal (SD), background limit, unless otherwise stated. $S = 50$ for oocytes, $S = 100$ for cumulus cells.
*Weakly expressed (1/3rd of the samples).

Results

The range of oestradiol was 15–2560 μg/l (mean 478 μg/l) and of zinc was 64–154 μg/l (mean 96.2 μg/l). No correlation was found between zinc and oestradiol concentrations (**Figure 1**; linear $R^2 = 0.034$, logarithmic $R^2 = 0.030$, exponential $R^2 = 0.055$, polynomial $R^2 = 0.10$).

In the 24 patients who had zinc concentrations assayed in follicular fluid and serum, the serum zinc concentrations were significantly higher (nearly double, 1.81) than in follicular fluid: (mean ± SD) 132.1 ± 26.9 μg/l versus 72.9 ± 30 μg/l ($P < 0.0001$, Fisher's least-significant-difference test and least-squares means).

Comparing the expression of zinc transporters and MT (**Tables 1–3**): The expression of transporters in oocytes was very high compared with cumulus cells, whereas MT expression had the same order of magnitude in both (but

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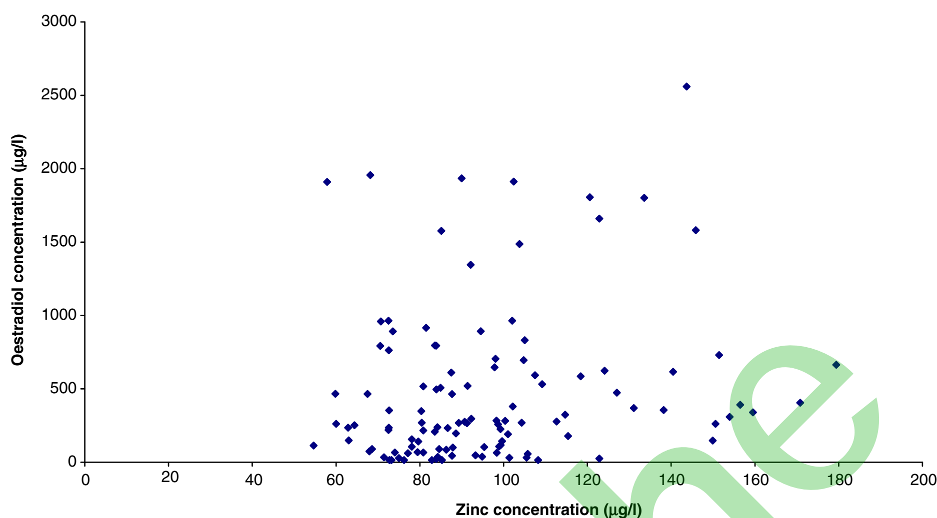

Figure 1 Relationship between zinc and oestradiol concentrations in serum.

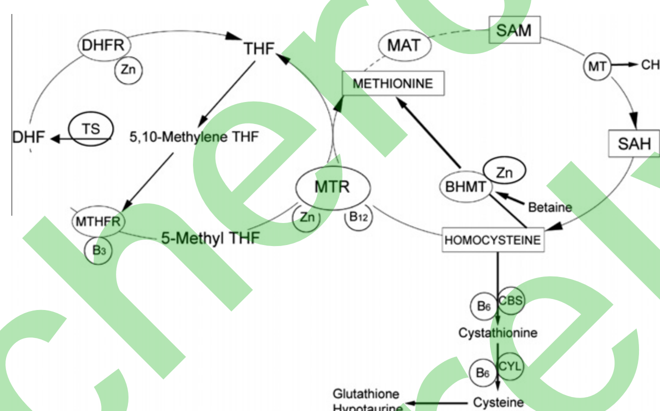

Figure 2 Implication of zinc in folic acid cycle and homocysteine recycling. BHMT = betaine-homocysteine methyl transferase; CBS = cysteine beta synthase; CYL = cystathionine-γ-lyase; DHF = dihydrofolate; DHFR = dihydrofolate reductase; MAT = methionine adenosyl transferase; MT = methyltransferase; MTHFR = 5,10 methylene tetrahydrofolate reductase; MTR = 5-methyl tetrahydrofolate-homocysteine transferase; SAH = *S*-adenosyl homocysteine; SAM = *S*-adenosyl methionine; THF = tetrahydrofolate; and TS = thymidylate synthase (forms dTMP from dUMP).

higher in cumulus cells especially for MT1E (×3), MTA2 (×4) and MT1X (×5)). **Table 1** shows that specific controls are correct.

All of the MTF (**Table 2**) were expressed; MTF2 appeared to be the most active, expressed at a level exceeding 300 times the background signal. These factors were not expressed in cumulus cells. The MT were highly expressed in oocytes, MTA2 being the most active, expressed at more than 200 times the background. They were all also expressed in cumulus cells, with a maximum for MTA2 (**Table 2**). Most of the zinc transporters (**Table 3**) of the ZnT family were expressed in oocytes, but were mainly absent in cumulus cells, apart from ZnT7 and 9. This situation is similar for the Zip family: Zip6 and 14 were strongly expressed in oocytes (more than 250 times the background level) and their expression in cumulus cells was less than twice that of background (**Table 3**).

Discussion

Zinc is a very important co-factor in genomic protection and the regulation of cellular housekeeping. Its role in male reproduction is well documented (Ebisch et al., 2006), but has not been documented in the female. Contrary to the findings of Michos et al. (2010), the present study found no positive correlation between serum oestradiol and zinc concentrations, although zinc concentrations seem to increase with very high concentrations of oestradiol. However, the current data are fully in accordance with the observations of Ng et al. (1987) in follicular fluid. The follicular wall appears to be a strong barrier to zinc permeation into follicular fluid, with serum zinc concentrations nearly twice (1.81) that of follicular fluid. If the homocysteine recycling pathway is considered, zinc is involved in folic

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acid recycling and regeneration of methionine from homocysteine at two levels, dihydrofolate reductase and 5-methyltetrahydrofolate-homocysteine methyltransferase or methionine synthase (**Figure 2**). A correlation between a low concentration of zinc and high concentration of homocysteine is logical. Impaired folate cycling increases homocysteine concentrations and alters the incorporation of uracil into DNA, thus affecting gene expression. Homocysteine enters the oocyte in competition with methionine (Ménézo et al., 1989) and can induce defective methylation, as well as oxidative stress, apoptosis and cellular dysfunction, by counteracting the action of S-adenosyl methionine (Ménézo et al., 1989, 2010b). As a consequence, homocysteine, which is increased during ovarian stimulation, is considered to be a negative indicator of oocyte quality (Berker et al., 2009; Boxmeer et al., 2008, 2009; Ebisch et al., 2006,2007) and that is why folic acid supplementation has been proposed before and during ovarian stimulation (Boxmeer et al., 2008). Moreover ovarian stimulation disturbs all trace element balances (Ozkaya et al., 2010). Considered together, these features may favour a negative impact of ovarian stimulation on oocyte quality and may explain the poor yield observed in terms of take-home baby rate per oocyte retrieved (Patrizio and Sakkas, 2009).

Zinc counteracts the effects of iron and copper on the Fenton reaction, which is involved in free radical formation. Low zinc concentrations are known to affect DNA repair capacity (Ho, 2004; Ho et al., 2003; Song et al., 2009), at the level of base excision repair, and so disable, in part, oxidative stress-related DNA repair processes. This feature is particularly important at the beginning of human life, during and immediately after fertilization, and at the onset of embryonic development. There is a high level of DNA repair enzyme gene expression in the early embryo (Jaroudi et al., 2009; Ménézo et al., 2007, 2010b) and it is important that tolerance towards DNA damage should be low during these critical stages of development. Analyses for level of transcription for some DNA repair steps have shown few or no differences (see also Jaroudi et al. (2009) and Wells et al. (2005)) between germinal-vesicle and metaphase-II oocytes. This confirms that the transcription is complete before ovulation and only post-transcriptional modifications (polyadenylation: El Mouatassim et al., 1999) will occur in order to regulate the timing of further translation: turnover of mRNA also starts before nuclear maturation is fully completed. Early human development depends on maternal transcripts until the 8-cell stage: the transcripts stored during oocyte maturation must control chromatin integrity, at least until the maternal/zygotic transition at the 4–8-cell stage in the human embryo. Expression of the genes involved in zinc transport is high in the oocyte and this suggests that there is active zinc transport during the first stages of pre-implantation development. Clearly several regulatory processes, in particular polyadenylation, must be involved in allowing the translation of stored mRNA. Indeed, some of the mRNA can be lost both pre- and post-translation. However, mRNA expression usually indicates that the corresponding proteins are being produced and further utilized, especially prior to genomic activation in the case of the early embryo (Wells et al., 2005). All of the MT are highly expressed: they are intracellular ligands of zinc and they can also counteract the negative impact of heavy metals such as cadmium. The importance of the input of MT in supporting early embryo development in human is emphasized by the transcriptomic profile of sperm leading to pregnancy after ICSI (García-Herrero et al., 2011): most of the MT (MT1A, B, F, G, H and X) transcripts are over-expressed in the pregnant group.

MT dysfunction may induce symptoms that resemble the neurological symptoms of autism, as do imprinting anomalies and oxidative stress: a correlation between these aspects of zinc physiology cannot be excluded. As MT play an important role in transcription factor regulation, anomalies in their expression may also lead to abnormal (malignant) transformation of cells. MTF, especially MTF2, are highly expressed in oocytes and are completely absent in cumulus cells. This probably means that the cumulus cells have reached the end of their life's journey. In comparing mRNA profiles of germinal-vesicle oocytes with metaphase-II cumulus cells, the possibility that some mRNA species in cumulus cells may be added, degraded or modified cannot be excluded, even if all the collected cumulus–oocyte–complexes had been submitted to the same hormonal stimulation. If this physiological aspect is considered, the tentative correlation between mRNA content of the cumulus cell and oocyte quality, as is sometimes proposed (Adriaenssens et al., 2010; Hamel et al., 2008), does not seem rational.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rbmo. 2011.03.015.

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