

Original Article

Follicular fluid concentrations of zinc and copper are positively associated with *in vitro* fertilization outcomes

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Abstract: Objective: To determine follicular fluid concentrations of zinc (Zn) and copper (Cu) and to assess whether they are associated with *in vitro* fertilization (IVF) outcomes. Methods: A retrospective study was conducted based on 53 patients recruited from June 2013 to February 2014 in Fujian Provincial Maternity and Children's Hospital. These women with normal ovulatory cycles but with tubal infertility underwent IVF using the GnRH-agonist long protocol. Out of these 53 patients, 23 had successfully conceived (pregnant group) and the other 30 failed (non-pregnant group). Follicular fluid samples were collected from these women on the oocyte retrieval day. Concentrations of Zn and Cu in these follicular fluid samples were measured. The correlations between follicular fluid concentrations of Zn and Cu and the number (No.) of MII oocyte retrieved, fertilization rate, and cleavage rate were determined. Results: In all subjects studied, the mean follicular fluid concentrations of Zn and Cu were 1.16 ± 0.19 mg/L and 0.96 ± 0.23 mg/L, respectively. However, no difference was observed in follicular fluid concentrations of Zn and Cu on the oocyte retrieval day between pregnant and non-pregnant groups. However, for both pregnant and non-pregnant groups combined, follicular fluid concentrations of Zn were positively correlated with fertilization rate ($r = 0.2904$, $P = 0.0349$) and cleavage rate ($r = 0.2942$, $P = 0.0325$). Follicular fluid concentrations of Cu were positively correlated with the No. of MII oocyte retrieved ($r = 0.5010$, $P = 0.0001$) and fertilization rate ($r = 0.2894$, $P = 0.0356$). Conclusions: These data suggest that in infertile patients who undergo IVF using the GnRH-agonist long protocol, optimal follicular fluid concentrations of Zn and/or Cu may be beneficial to oocyte growth, fertilization, and early embryonic development.

Keywords: Zinc (Zn), copper (Cu), follicular fluid, *in vitro* fertilization (IVF), embryo transfer (ET)

Introduction

The trace elements in human tissues are essential for cell growth, maturity, and physiological functions, the latter of which include regulation of hormone production and secretion of hypothalamus, pituitary, and gonad, thereby critically affecting the oocyte maturation and embryo quality and ultimately significantly impacting pregnant outcomes [1-3]. Excessive trace elements may also be harmful to cells [4]. Trace elements such as zinc (Zn) and copper (Cu) are present in oocytes and follicular fluid. Indeed, Popescu et al. found that Zn and Cu were asymmetrically distributed in the cytoplasm of *Xenopus* oocytes with high Zn and Cu present in the animal pole, while Cu was more abundant in the nucleus [5]. Both Zn and Cu have also been reported to be present in human follicular fluid [3, 6, 7].

It is well known that *in vitro* fertilization (IVF) is one of critical treatments for infertility, but the clinical pregnancy rate of IVF is affected by multiple factors including Zn and Cu [3]. To date, however, there are only a few reports determining the trace element levels in serum and follicular fluid of patients who undergo IVF, and the data are not consistent. For example, it was reported that the levels of Zn in serum and follicular fluid as well as the levels of Cu in serum were lower in women who underwent IVF than in the healthy control subjects, suggesting important roles of both Zn and Cu in the normal development of oocytes [3]. In contrast, Ng et al. investigated human follicular fluid levels of Zn and Cu in 33 patients stimulated with clomiphene citrate and human menopausal gonadotropin in the IVF program and showed no significant difference in Zn and Cu in those collected follicles of different sizes and no association

Association of follicular fluid zinc and copper with *in vitro* fertilization outcomes

between the follicular fluid concentrations of Zn and Cu with the status and maturity of oocytes [7]. Thus, a clear association between the follicular fluid concentrations of Zn and Cu with IVF outcomes is not yet defined.

Follicular fluid is a type of blood plasma ultrafiltrate, which selectively excludes high weight molecular proteins [8]. Any change in the follicular fluid may reflect the alternations in micro-environment of oocyte, potentially affecting oocyte development and quality, fertilization, and early embryonic development [9]. It is believed that trace elements in follicular fluid may have more direct effects on oocyte than those in serum [8]. Therefore, to further explore the potential role of Zn and Cu in human oocytes, in the current study, we determined follicular fluid concentrations of Zn and Cu and also assessed if the concentrations of Zn and Cu were correlated to IVF outcomes in women who underwent the GnRH-agonist long protocol.

Materials and methods

Patients

This study was conducted in the Assisted Reproductive Technology Laboratory of Fujian Provincial Maternity and Children's Hospital of Fujian Medical University, Fujian, China. The protocol was approved by the Research Ethics Committee of Fujian Provincial Maternity and Children's Hospital of Fujian Medical University. Fifty-three women (23-35 years, $18 \text{ kg/m}^2 \leq$ body mass index $\leq 25 \text{ kg/m}^2$) who had normal ovulatory cycles were recruited from June 2013 to February 2014. These women were diagnosed with tubal infertility and underwent IVF treatment. Informed consent was obtained during pre-cycle preparation period from all patients recruited. As part of routine treatment, women who were recruited for IVF received an initial infertility evaluation which includes medical and reproductive histories.

Tubal infertility in this study was referred to women who had fallopian tube(s) removed for tubal pregnancy or tubal obstruction (identified by the uterus, fallopian tube iodine oil radiography, or by hysteroscope and laparoscope). All subjects were interested in becoming pregnant. Women with complications including but not limiting to history of ovarian surgery or radiotherapy and chemotherapy, basal follicle-

stimulating hormone (FSH) $> 10 \text{ mIU/mL}$, premature ovarian failure, poor ovarian response, adenomyosis, hyperprolactemia, thyroid disorders, pelvic tuberculosis, submucous myoma, intrauterine adhesions, diabetes mellitus or cardiovascular diseases were excluded. Women included had not received any medication other than the standard IVF protocol since the last 3 months and had not orally received a multivitamin/mineral tablet for 45 days before serum and follicular fluid collection.

Ovarian stimulation protocols

Ovarian stimulation was performed using the standard down-regulation protocol with a GnRH-agonist (Decapeptyl; Ferring Pharmaceuticals, Switzerland). Briefly, the patients were injected with the GnRH-agonist (0.1 mg/day), starting from the mid-luteal phase and onwards, which was reduced to 0.05 mg/day on Day 2 or 3 of the cycle. If the serum estradiol (E_2) levels were $< 50 \text{ pg/mL}$ and if there was no functional ovarian cyst, the patients were stimulated with 150-450 IU/day of recombinant FSH (Gonal-F; Merck Serono SA, Geneva, Switzerland). Follicular maturation and endometrial development were monitored by serum E_2 levels and transvaginal ultrasound. Subcutaneous human chorionic gonadotropin (hCG; Livzon Pharmaceutical Group Co., Ltd., Zhuhai, China), 5000-10000 IU, was administered when at least two follicles reached the average diameter of $\geq 18 \text{ mm}$. The oocytes were retrieved 36 h later under transvaginal ultrasound guidance and subsequently fertilized by conventional insemination. Embryo quality was assessed before embryo transfer (ET), and 1 to 2 embryos were transferred to patients approximately 48 h (6-8 cell stage) after fertilization. Luteal phase support with 90 mg of vaginal progesterone daily (Crinone 8% vaginal gel; Merck Serono SA, Geneva, Switzerland) was initiated on the oocyte retrieval day and continued for up to 9 weeks of pregnancy when available. Clinical pregnancy was defined as the presence of one or more gestational sac with cardiac activity as detected by transvaginal ultrasound at 7 weeks of gestation.

Determination of trace element concentrations

Follicular fluid samples (10 ml) were carefully collected from follicles $\geq 18 \text{ mm}$ in diameter during oocyte retrieval using the sterile oocyte collection tubes (Vitrolife; Göteborg, Sweden).

Association of follicular fluid zinc and copper with *in vitro* fertilization outcomes

Table 1. Parameters of PE800 atomic absorption spectrophotometer

Trace element	Lamp current (mA)	Wave lengths (nm)	Slit-width (nm)	Gas/airflow volume (L/min)	Detection limit (mg/L)
Zn	15	213.9	0.7	2.0/17.0	0.04
Cu	5	324.8	0.7	2.0/17.0	0.01

Table 2. Comparisons between the pregnant and non-pregnant groups

Variables	Pregnant (n = 23)	Non-pregnant (n = 30)	p Value	95% CI
Age (years)	30.13 ± 2.82	31.07 ± 3.30	0.2811	-0.7892 to 2.662
Duration of infertility (years)	5.26 ± 3.24	4.78 ± 3.27	0.5992	-2.290 to 1.335
Total dose of gonadotropins (IU)	2008.70 ± 409.52	2132.5 ± 502.03	0.3406	-134.57 to 382.18
Duration of stimulation (days)	10.26 ± 1.42	9.93 ± 1.31	0.3889	-1.084 to 0.4290
On Day 2-3				
FSH (mIU/mL)	3.75 ± 0.83	3.54 ± 0.67	0.3196	-0.6229 to 0.2073
LH (mIU/mL)	2.27 ± 1.36	2.89 ± 1.31	0.0973	-0.1176 to 1.365
E2 (pg/mL)	39.17 ± 12.61	34.6 ± 11.16	0.1681	-11.141 to 1.993
On the day of hCG administration				
LH (mIU/mL)	2.29 ± 1.85	3.38 ± 2.46	0.0812	-0.1405 to 2.327
E ₂ (pg/mL)	6598.17 ± 3829.50	5272.77 ± 2482.60	0.1334	-3069.90 to 419.08
P ₄ (ng/mL)	1.51 ± 0.66	1.72 ± 0.69	0.2815	-0.1713 to 0.5773
No. of MII oocyte retrieved (n)	12.87 ± 5.80	11.27 ± 5.82	0.3243	-4.836 to 1.631
Fertilization rate (%)	0.81 ± 0.11	0.77 ± 0.19	0.3416	-0.1352 to 0.04773
Cleavage rate (%)	0.78 ± 0.20	0.80 ± 0.16	0.7038	-0.08094 to 0.1190
No. of embryos transferred on Day 3 (n)	1.91 ± 0.29	1.90 ± 0.31	0.8751	-0.1788 to 0.1527
Endometrial thickness on the day of ET (mm)	11.34 ± 1.96	11.37 ± 2.93	0.9617	-1.387 to 1.456
Follicular fluid concentrations of Zn and Cu on the day of oocyte retrieved				
Zn (mg/L)	1.15 ± 0.20	1.18 ± 0.18	0.5629	-0.07541 to 0.1370
Cu (mg/L)	0.93 ± 0.20	0.99 ± 0.25	0.3802	-0.0720 to 0.1856

Data are expressed as means ± SD.

Follicular fluid contaminated with blood and/or washing media was excluded. Each sample was mixed with 1 ml of concentrated nitric acid (P = 1.42 g/mL, Electronic purity) in Falcon tube (BD Biosciences; Bedford, USA), and was centrifuged at 500×g for 10 min to remove cellular components. The supernatant was obtained and frozen at -20°C until further use.

The follicular fluid concentrations of Zn and Cu were determined using atomic absorption spectroscopy (PerkinElmer PE800; USA). Standard Zn (1000 mg/L) and Cu (500 mg/L) solutions containing 1% nitric acid were provided by the Chinese National Research Center for Certified Reference Materials. The standard curves were established using five concentrations for each trace element (0.00, 0.100, 0.500, 1.00, and 2.50 mg/L for Zn and 0.00, 0.100, 0.500, 1.00, and 1.50 mg/L for Cu) with parameters of PE800 atomic absorption spectrophotometer (Table 1).

The follicular fluid samples were sonicated by an ultrasonic processor (AS7240B; Tianjin, China) for 2 h at the room temperature and mixed until crystal formation. Each sample (2.0 g) was transferred into a polytetrafluoroethylene digestion tank. Twenty ml of concentrated nitric acid and 5 ml of 30% hydrogen peroxide were added. After mixing overnight, the solution was dried in Pretreatment Heater (XT-9800; Shanghai, China). The dried sample was rehydrated in 10 ml of deionized water. The rehydrated sample (2 ml) was subjected to atomic absorption spectroscopy using deionized water as a reagent blank control. The concentrations of Zn and Cu were calculated based on the standard curve as described above.

Measurements of serum hormone levels

Serum FSH, luteinizing hormone (LH), and E₂ levels were determined in 3-4 ml morning blood samples on Day 2 or 3 of the stimulation cycle with gonadotropin after pituitary suppression.

Association of follicular fluid zinc and copper with *in vitro* fertilization outcomes

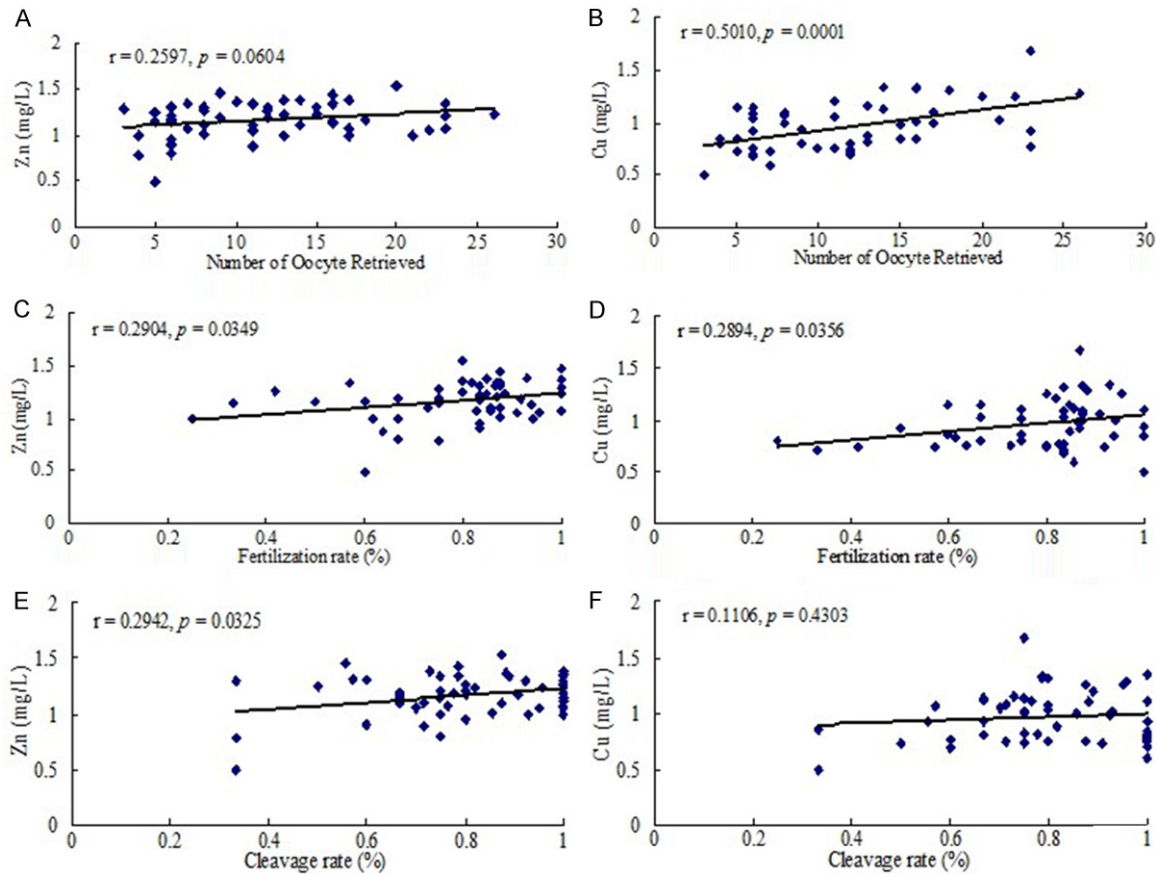


Figure 1. Scatter plots with regression lines showing correlations between follicular fluid concentrations of Zn and Cu with the No. of MII oocyte retrieved, fertilization rate, and cleavage rate (A. Zn and No. of MII oocyte retrieved; B. Cu and No. of MII oocyte retrieved; C. Zn and fertilization rate; D. Cu and fertilization rate; E. Zn and cleavage rate; F. Cu and cleavage rate).

Serum LH, E_2 , and progesterone (P_4) were also measured in blood samples collected on the day of hCG administration. All the hormones were measured using electrochemiluminescent method for the quantitative determination (Beckman Access analyzer; USA).

Statistical analysis

All data were presented as means \pm standard deviation (SD), wherever appropriate. Comparisons between groups were carried out using Student's t-test, Mann-Whitney U test, and Spearman correlation coefficient as applicable. Statistical significance was defined as $P \leq 0.05$ [10-12].

Results

Demographic values

Out of 53 patients studies, 23 had successfully conceived (pregnant group) and the other 30

failed (non-pregnant group), representing a clinical pregnancy rate of 43.40% in the GnRH-agonist long protocol. The demographic values and IVF outcome parameters are shown in **Table 2**. No significant difference was observed in mean ages, duration of infertility, total doses of gonadotropins, duration of stimulation, serum levels of FSH, LH, and E_2 on Day 2 or 3 of the cycle, serum levels of LH, E_2 and P_4 on the day of hCG administration, No. of MII oocyte retrieved, fertilization rate, cleavage rate, and endometrial thickness on the day of ET between pregnant and non-pregnant groups ($P > 0.05$) (**Table 2**).

Follicular fluid concentrations of Zn and Cu

The mean follicular fluid concentrations of Zn and Cu in 53 patients examined were 1.16 ± 0.19 mg/L, 0.96 ± 0.23 mg/L, respectively. Between pregnant and non-pregnant groups,

Association of follicular fluid zinc and copper with *in vitro* fertilization outcomes

no significant difference was detected in the follicular fluid concentrations of Zn and Cu on the day of oocyte retrieved ($P > 0.05$) (Table 2).

Correlations between follicular fluid concentrations of Zn and Cu and IVF outcomes

Follicular fluid concentrations of Zn had a trend, though not reaching a statistical difference ($P = 0.0604$), to positively correlate with the No. of MII oocyte retrieved and were positively correlated with fertilization rate and cleavage rate (Figure 1). In addition, positive correlations were also observed between follicular fluid concentrations of Cu and the No. of MII oocyte retrieved, and fertilization rate (Figure 1).

Discussion

In the current study, we determined the follicular fluid of Zn and Cu of patients who underwent IVF. We found that though follicular fluid concentrations of Zn and Cu were similar between pregnant and non-pregnant women, for both pregnant and non-pregnant groups combined, Zn and Cu levels were statistically positively correlated with the cleavage rate and No. of MII oocyte retrieved, respectively, while both Zn and Cu were positively associated with the fertilization rate. These data suggest that Zn and Cu may differentially promote oocyte development and early embryonic development.

In the current study, the follicular fluid concentrations of Zn and Cu determined in women studied were comparable to the median follicular fluid levels of Zn 0.77 mg/L and Cu 0.73 mg/L reported in women with unexplained infertility using GnRH-antagonist protocol [6].

Zn, as a cofactor is involved in DNA transcription and protein synthesis which are essential for germ cell development [13, 14]. For example, Zn deficiency impairs oocyte maturation and cumulus expansion in mice during the peri-ovulatory period [15]. Acute dietary Zn deficiency in mice before ovulation also dramatically decreases oocyte quality and developmental potential [16]. Popescu et al. found that Zn in the *Xenopus* whole oocyte was asymmetrically distributed in the cytoplasm, suggesting the location of metal deposition may be important for normal development [5]. Zn is also involved in the synthesis of Vitamin A reductase as the

deficiency of Zn can decrease serum Vitamin A levels, possibly leading to ovulation failure [3]. On the other hand, high concentrations of Zn can inhibit production of cAMP and P_4 induced by FSH in chicken granulosa cell of dominant follicle, slowing down maturity of oocyte [16]. Thus, maintaining Zn at an optimal level is critical to oocyte development and ovulation. In the current study, beside statistically positive correlations observed between follicular fluid concentrations of Zn and the fertilization rate and cleavage rate in IVF patients, a positive correlation trend was found between follicular fluid concentrations of Zn and the No. of MII oocytes as reported by Tolunay et al. [6]. Collectively, these data imply that Zn may play important roles in fertilization and early embryonic growth.

Cu is another widely distributed essential trace element in human body involved in regulating normal reproduction of mammals as well as different metabolic processes and enzymatic reactions [2]. The metabolic disorder, deficiency, and excessive intake of Cu also result in various diseases [17]. For instance, the lower levels of serum Cu are associated with decreases in fecundity of buffalo [18]. These decreases could be partially contributed to the effects of Cu on secretions of hormone produced by pituitary gland, e.g., growth hormone (GH), thyroid-stimulating hormone (TSH), LH and adrenocorticotrophin (ACTH), all of which are critical to oocyte development and ovulation [19]. Recently, Babaei et al. found that antral follicles were the most susceptible to the Cu overexposure, leading to a significant decrease in the number of follicular cells, increased atresia, and decreased the number of corpora lutea [20]. Possible mechanisms for Cu-overexposure induced follicular damage include Cu-increased cell apoptosis, vacuolization of the cytoplasm organelles, and detachment of cell membrane from its basement membrane [20]. However, supplement of Cu (0.46 and 0.68 mg/L) in culture media accelerates successful formation of 8-cell embryos, morulae, and blastocysts in bovine, while a long-term lack of Cu increased the number of apoptotic blastomeres [21]. In this study, we confirmed that the follicular fluid concentration of Cu is positively correlated with the No. of MII oocyte retrieved ($P = 0.0001$), and fertilization rate. Together, these data suggest that an optimal concentration of Cu in follicular fluid may promote oocyte growth and improve the fertilization.

In conclusion, as optimal follicular concentrations of Zn and Cu in the infertile patients stimulated using the GnRH-agonist long protocol in IVF may be beneficial to oocyte and embryo growth as shown in the current study, we would also recommend supplement of Zn and Cu at the certain level, depending on the individual, for patients before IVF protocol, as suggested by the other investigators [3].

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Disclosure of conflict of interest

None.

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References

- [1] Pathak P, Kapil U. Role of trace elements zinc, copper and magnesium during pregnancy and its outcome. *Indian J Pediatr* 2004; 71: 1003-5.
- [2] Michaluk A, Kochman K. Involvement of copper in female reproduction. *Reprod Biol* 2007; 7: 193-205.
- [3] Özkaya MO, Nazıroğlu M, Barak C, Berkkanoglu M. Effects of multivitamin/mineral supplementation on trace element levels in serum and follicular fluid of women undergoing *in vitro* fertilization (IVF). *Biol Trace Elem Res* 2011; 139: 1-9.
- [4] Yuyan L, Junqing W, Wei Y, Weijin Z, Ersheng G. Are serum zinc and copper levels related to semen quality? *Fertil Steril* 2008; 89: 1008-1011.
- [5] Popescu BF, Belak ZR, Ignatyev K, Ovsenek N, Nichol H. Asymmetric distribution of metals in the *Xenopus laevis* oocyte: a synchrotron X-ray fluorescence microprobe study. *Biochem Cell Biol* 2007; 85: 537-542.
- [6] Tolunay HE, Şükür YE, Ozkavukcu S, Seval MM, Ateş C, Türksöy VA, Ecemiş T, Atabekoğlu CS, Özmen B, Berker B, Sönmezer M. Heavy metal and trace element concentrations in blood and follicular fluid affect ART outcome. *Eur J Obstet Gynecol Reprod Biol* 2016; 198: 73-77.
- [7] Ng SC, Karunanithy R, Edirisinghe WR, Roy AC, Wong PC, Ratnam SS. Human follicular fluid levels of calcium, copper and zinc. *Gynecol Obstet Invest* 1987; 23: 129-132.
- [8] Bloom MS, Kim K, Kruger PC, Parsons PJ, Arnason JG, Steuerwald AJ, Fujimoto VY. Associations between toxic metals in follicular fluid and *in vitro* fertilization (IVF) outcomes. *J Assist Reprod Genet* 2012; 29: 1369-1379.
- [9] Oktem O, Urman B. Understanding follicle growth *in vivo*. *Hum Reprod* 2010; 25: 2944-2954.
- [10] Li Y, Wang K, Zou QY, Magness RR, Zheng J. 2,3,7,8-Tetrachlorodibenzo-p-dioxin differentially suppresses angiogenic responses in human placental vein and artery endothelial cells. *Toxicology* 2015; 336: 70-78.
- [11] Wang K, Li Y, Jiang YZ, Dai CF, Patankar MS, Song JS, Zheng J. An endogenous aryl hydrocarbon receptor ligand inhibits proliferation and migration of human ovarian cancer cells. *Cancer Lett* 2013; 340: 63-71.
- [12] Li Y, Wang K, Jiang YZ, Chang XW, Dai CF, Zheng J. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibits human ovarian cancer cell proliferation. *Cell Oncol (Dordr)* 2014; 37: 429-437.
- [13] Vasto S, Candore G, Listi F, Balistreri CR, Colonna-Romano G, Malavolta M, Lio D, Nuzzo D, Mocchegiani E, Di Bona D, Caruso C. Inflammation, genes and zinc in Alzheimer's disease. *Brain Res Rev* 2008; 58: 96-105.
- [14] Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Hum Reprod* 2007; 13: 163-174.
- [15] Tian X, Diaz FJ. Acute dietary zinc deficiency before conception compromises oocyte epigenetic programming and disrupts embryonic development. *Dev Biol* 2013; 376: 51-61.
- [16] Johnson AL, Brake J. Zinc-induced molt: evidence for a direct inhibitory effect on granulosa cell steroidogenesis. *Poult Sci* 1992; 71: 161-167.
- [17] Kedi Y. Trace element and health. Beijing: Science Publication House; 2003. pp. 1, 49.
- [18] Bhoopendra Singh, Rawal CVS, Swarup D. Soil-plant-animal relationship of Zn, Cu and Co in buffaloes of Rohilkhand region. *Indian J of Anim Sci* 2007; 77: 83-85.

Association of follicular fluid zinc and copper with *in vitro* fertilization outcomes

- [19] Chiba K, Kobayashi H, Wakabayashi K. Isolation and partial characterization of LH, FSH and TSH from canine pituitary gland. *Endocr J* 1997; 44: 205-218.
- [20] Babaei H, Roshangar L, Sakhaee E, Abshenas J, Kheirandish R, Dehghani R. Ultrastructural and morphometrical changes of mice ovaries following experimentally induced copper poisoning. *Iran Red Crescent Med J* 2012; 14: 558-568.
- [21] Gao G, Yi J, Zhang M, Xiong J, Geng L, Mu C, Yang L. Effects of iron and copper inculture medium on bovine oocyte maturation, pre implantation embryo development, and apoptosis of blastocysts in vitro. *J Reprod Dev* 2007; 53: 777-784.