

## Effects of Multivitamin/Mineral Supplementation on Trace Element Levels in Serum and Follicular Fluid of Women Undergoing in Vitro Fertilization (IVF)

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**Abstract** We investigated effects of multivitamin/mineral supplementation on element levels in serum and follicular fluid of women undergoing IVF. We used three groups in this study. The first group was used as an age-matched and nonpregnant control ( $n=13$ ). Group 2 ( $n=30$ ) constituted the IVF group and women in the third group who were undergoing IVF also received a multivitamin/mineral tablet daily for 45 days. Follicular fluid and serum selenium and zinc levels and follicular fluid copper levels were lower in IVF patients than in controls although follicular fluid aluminum and iron levels were higher in IVF patients than in controls. However, follicular fluid and serum aluminum, copper, zinc and selenium levels, and serum magnesium levels were higher in the multivitamin/mineral group than in the IVF group although follicular fluid iron levels were lower in the multivitamin/mineral group than in the IVF group. In conclusion, we observed that copper, zinc, and selenium in serum and follicular fluid decreased in women undergoing IVF. Multivitamin/mineral supplementation in serum and follicular fluid of women undergoing IVF normalized the trace element levels.

**Keywords** Zinc · Selenium · Copper · IVF · Human fertilization · Follicular fluid

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## Abbreviations

|        |                               |
|--------|-------------------------------|
| FSH    | Follicle stimulating hormone  |
| GSH-Px | Glutathione peroxidase        |
| hCG    | Human chorionic gonadotrophin |
| IVF    | In vitro fertilization        |
| ROS    | Reactive oxygen species       |
| SOD    | Superoxide dismutase          |

## Introduction

There has been special interest in effects of dietary trace element deficiencies on physiological functions including reproduction particularly [1, 2]. Severe dietary deficiencies of trace elements including copper, selenium, and zinc are commonly seen in patients with IVF [1–3].

A significant, but largely neglected, factor, causing infertility is poor nutrition. Good nutrition is particularly important for DNA synthesis, because most of its essential components are derived from the diet. Moreover, several enzymes involved in DNA synthesis are zinc or vitamin B dependent [2, 3]. DNA synthesis is important for the development of oocytes. Animal studies have demonstrated that deficiencies of vitamin A, C, and D result in diminished fertility [4, 5]. Despite their suspected impact on female reproduction and environmental prevalence, concentrations of these elements within the follicular fluid have not been investigated thoroughly [2].

Furthermore, the diet is the source of exogenous antioxidant vitamins (vitamin A, C, and E) and trace elements (selenium, zinc, and copper) and they are also essential for oocyte maturation and rupture. These antioxidants protect DNA and other important molecules from oxidative damage, which would otherwise induce apoptosis. For example, zinc plays critical roles in biological membrane stabilization, protein synthesis, and nucleic acid metabolism and growth of normal tissue and as a cofactor of several enzyme systems [6]. The antioxidant role of zinc and copper is mainly due to the presence of Cu–Zn superoxide dismutase (SOD) [6]. Selenium is an essential trace element for human health. The function of selenium as an essential element in animals and humans are attributed to about 12 known mammalian selenoproteins, thioredoxin reductases, selenoproteins P and W, and phospholipid hydroperoxide, contain selenocysteine, that is specifically incorporated through a unique cotranslational mechanisms [7]. Selenium is also a cofactor for glutathione peroxidase (GSH-Px), an important antioxidant enzyme for removing lipid hydroperoxides and hydrogen peroxide [7]. Catalase contains zinc and copper as cofactors. It also catalyzes the reduction of hydrogen peroxide to water [8]. Magnesium provides of stabilization of DNA, RNA, and ribosomes and also activates approximately 300 enzymes including those in energy metabolism and reactive oxygen species (ROS) production. In addition, magnesium plays a protective role in the developing fetus brain [9]. Calcium has a basic function in neurotransmitter secretion and magnesium blocks the entrance of calcium into cells [10]. Enzymatic and nonenzymatic antioxidants are also essential for inhibition of free radical production related to oocyte maturation and rupture [11–13]. However, whether a multivitamin/mineral mixture supplementation modulates infertility-induced element changes in the serum and follicular fluid of patients with IVF is currently unknown and is therefore the basis of this study.

We investigated the effects of multivitamin/mineral supplementation on the concentrations of various minerals and vitamins in serum and follicular fluid of patients with IVF.

## Subjects and Methods

### Control and Patients

A total of 56 paired samples of follicular fluid and serum were obtained from women who underwent IVF at the Antalya and Isparta IVF centers of Turkey. Also, 13 paired samples of follicular fluid and serum were obtained from healthy control subjects. The study protocol was approved by the local Ethics Committee of the Medical Faculty, Suleyman Demirel University, Isparta, Turkey, by protocol number June, 2009:17. Informed consent for experimental use of serum and follicular fluids was obtained from all patients and controls. The mean age $\pm$ SD of the untreated and treated patients was 28.8 $\pm$ 3.2 years (range of 22–43 years) and 30.7 $\pm$ 4.5 years (a range of 22–43 years), respectively whereas the mean age $\pm$ SD of healthy untreated subjects was 32.2 $\pm$ 5.2 years, with a range of 25–43 years. Healthy control subjects and treated and untreated patients were all nonsmokers and free from major medical illness including hypertension; all were interested in becoming pregnant. Patients were excluded if they had myoma, adenomyosis, a congenital uterine anomaly, ovarian tumors, or if they used estrogens, progesterone, androgens, or chronic use of any medications.

### Study Groups

We used three groups in this study. The first group was used as control ( $n=13$ ) who received a placebo (candy). Group 2 ( $n=30$ ) constituted the IVF group and they also received the placebo only. The third group ( $n=26$ ) daily received orally a multivitamin/mineral tablet (Megadyn Pronatal Film Tablet, Mecom Medical Health Product Inc, Istanbul, Turkey) for 45 days before serum and follicular fluid collection. The contents of the multivitamin/mineral tablet are shown in Table 1.

**Table 1** Vitamins and Mineral Content in a Multivitamin Tablet

|   |          |
|---|----------|
| Vitamin A (retinol)                       | 4,000 IU |
| Vitamin B1 (thiamin)                      | 1.6 mg   |
| Vitamin B2 (riboflavin)                   | 1.8 mg   |
| Vitamin B6 (pyridoxine)                   | 2.6 mg   |
| Vitamin B12 (cyanocobalamine)             | 4 mcg    |
| Vitamin C (ascorbic acid)                 | 100 mg   |
| Vitamin D2 (ergocalciferol)               | 500 IU   |
| Vitamin E ( $\alpha$ -tocopherol acetate) | 15 mg    |
| Vitamin H (D-biotin)                      | 0.2 mg   |
| Calcium D-pantothenate                    | 10 mg    |
| Folic acid                                | 0.8 mg   |
| Nicotinic acid                            | 19 mg    |
| Calcium                                   | 125 mg   |
| Iron                                      | 60 mg    |
| Magnesium                                 | 100 mg   |
| Phosphor                                  | 125 mg   |
| Copper                                    | 1 mg     |
| Manganese                                 | 1 mg     |
| Zinc                                      | 7.5 mg   |

## IVF Stimulation Protocol and Follicular Fluid Aspiration

A staff nurse randomized the patients at initiation of stimulation using a computer-generated list. All patients from both groups were instructed to take oral contraceptive pills (Ginera Schering, Germany) once daily for 21 days.

After the confirmation of pituitary down-regulation on day 3 of the cycle by sonographic detection of a linear endometrium and suppressed ovaries (no antral follicles 0.10 mm) and serum estradiol levels of 50 pg/ml, human chorionic gonadotropin (hCG) stimulation with recombinant follicle stimulating hormone (FSH; Gonal F, Serono, Turkey) was commenced. The initial FSH dose was 150 IU/day for high responders, 225–300 IU/day for intermediate responders and 450 IU/day for low responders. The initial dose for ovarian stimulation was based upon ovarian reserve indicators, which included the number of antral follicles on day 3 of a previous, basal spontaneous cycle. The dose was adjusted on day 6 if needed and thereafter in accordance with the patient's response as indicated by her estradiol level and number of developing follicles. The hCG administration continued until at least two follicles 0.17 mm in diameter were detected when 10,000 IU hCG was administered followed 35 h later by transvaginal ultrasound-guided oocyte retrieval.

## Blood and Follicular Fluid Collection and Preparation

Care was taken to completely aspirate each follicle to one tube. Each follicle was aspirated separately and follicular fluid containing the oocyte was collected. Immediately after removal of the oocyte, each follicular fluid was centrifuged at  $500\times g$  for 10 min at  $+4^{\circ}\text{C}$  to remove cellular components and the supernatant was kept frozen on ice.

Venous blood (5 ml) was taken from the antecubital vein, using a monovette system of blood collection, into tubes without anticoagulant but protected against light, after an overnight fast. Serum samples were obtained from the blood samples by centrifugation at  $1,500\times g$  for 10 min at  $+4^{\circ}\text{C}$ .

Serum (2 ml) and follicular fluid (3 ml) samples were stored at  $-30^{\circ}\text{C}$  for  $<1$  month pending measurement of trace element. For each blood and follicular sample, two blank samples of highly purified water (element content  $<0.01$ ) were collected using the same tools/equipments (e.g., gloves, syringes etc.) in order to allow determination of background 'noise' (lower detection limit).

## Apparatus

The ICP-AES system used was a Perkin-Elmer Optima 3100 XL emission spectrometer equipped with the Perkin-Elmer AS 90 plus autosampler and was controlled by a computer. The plasma operating conditions used in the ICP system were 253.6 nm wavelength, 151/min plasma gas flow rate, 0.5 argon carrier flow rate, plus 21/min sample flow and rates. The peristaltic pump was a Watson Marlow 323 SD model. The microcolumn was a glass tube (0.7–10 cm, Aldrich C 3669) packed with modified anion-exchange resin (1 g). Transport lines consisted of 1.25 mm i.d. poly tetrafluoroethylene tubing.

## Reagents

All reagents were of analytical reagent grade and deionized water was used throughout. Stock solutions of copper, zinc, selenium aluminum, iron, magnesium, and calcium were prepared by taking appropriate amounts of standards in nitric acid solution. Working

solutions were prepared immediately before use. Adjustment of pH was made with buffer (acetic acid, boric acids, and their potassium salts). Double-distilled deionized water was used in the current study. All glassware used was washed with 10% nitric acid for 1 day and rinsed with deionized water before use.

### Copper, Zinc, Iron, Calcium, Magnesium, and Aluminum Analysis

Copper, zinc, iron, calcium, magnesium, and aluminum levels were estimated by atomic absorption spectrophotometry and the ICP-AES system after digestion with nitric acid and hydrogen peroxide as described in a previous study [14]. Serum samples (0.5 ml) were slowly digested with 3 ml nitric acid for 12 h. The samples were then digested in microwave oven for 5–10 min. Finally, they were allowed to cool at room temperature for 1 h. Before 0.5 ml hydrogen peroxide (30%) was added.

### Selenium Analysis by Hydride Technique

Temperature programs of flow injection mercury/hydride analyses for selenium determinations in serum and follicular fluid of patients with IVF are shown in Table 2. Palladium solution (200 mg/l) was used as a matrix modifier in selenium measurements in the frozen serum samples. Selenium in the serum samples measured directly without previous acid digestion and appropriate dilution (usually 1:1) was made only with acid (HCl). In selenium analysis we used 0.2% NaBH<sub>4</sub> in 0.05% NaOH as the reducing agent. Graphite furnace programs for selenium measurement in serum samples are described in Table 2. Samples and standards to be analyzed for selenium were treated with an equal volume of HCL followed by heating at 90°C for 20 min. After pre-reduction the solution may be diluted without the risk of re-oxidation from Se<sup>+4</sup> to Se<sup>+6</sup>. Each analysis was repeated three times.

### Statistical Analyses

All results are expressed as means±SD. Significance among values for control subjects, patients, and treatment groups was assessed with Student's *t* test. Data were analyzed using the SPSS statistical program (version 9.05 software, SPSS Inc. Chicago, IL, USA). *p* values of less than 0.05 were regarded as significant.

## Results

Follicular fluid element levels of the various groups of controls and patients with IVF are shown in Table 3. Follicular fluid selenium ( $p < 0.05$ ) and zinc ( $p < 0.01$ ) levels were

**Table 2** Temperature Programs of Flow Injection Mercury/Hydride Analyses for Selenium Determinations in Serum and Follicular Fluid of Patients with IVF

| Parameters           |                                      |
|----------------------|--------------------------------------|
| Integration time (s) | 15                                   |
| Lamp                 | HCL                                  |
| Slit (nm)            | 0.2                                  |
| Wavelength (nm)      | 196                                  |
| Cell temperature     | 900°C                                |
| Carrier solution     | 10% (v/v) HCl                        |
| Reducing solution    | 0.2% NaBH <sub>4</sub> in 0.05% NaOH |

**Table 3** The effects of Multivitamin/Mineral Supplementation on Follicular Fluid Element Levels in Patients with IVF (Mean±SD)

| Parameters        | Control (n=13) | IVF (n=30)   | IVF+SUPPL (n=26) |
|-------------------|----------------|--------------|------------------|
| Copper (µg/l)     | 450.8±48.2     | 407.9±73.6   | 838.0±29.6***    |
| Zinc (µg/l)       | 545.8±49.3     | 307.6±11.5** | 464.9±78.6****   |
| Selenium (µg/dl)  | 6.02±0.2       | 5.03±0.1*    | 6.05±0.1****     |
| Aluminum (µg/dl)  | 21.58±1.5      | 33.61±5.2*   | 65.82±3.3***     |
| Iron (µg/l)       | 1208.3±68.3    | 1415.5±60.9* | 948.1±41.0*****  |
| Magnesium (mg/dl) | 9.51±0.6       | 9.32±0.5     | 9.58±0.5         |
| Calcium (mg/dl)   | 56.86±4.4      | 52.48±4.8    | 57.31±2.6        |

\* $p<0.05$  versus control; \*\* $p<0.01$  versus control; \*\*\* $p<0.01$  versus IVF group; \*\*\*\* $p<0.05$  versus IVF group; \*\*\*\*\* $p<0.001$  versus IVF group

significantly lower in the IVF patients than in controls although follicular fluid iron and aluminum levels were significantly ( $p<0.05$ ) higher in patients with IVF than in the controls. However, copper ( $p<0.01$ ), zinc ( $p<0.05$ ), selenium ( $p<0.05$ ), and aluminum levels ( $p<0.01$ ) in follicular fluid were significantly higher in the treatment group than in the untreated IVF group although iron levels were significantly ( $p<0.001$ ) lower in the treatment group than in the IVF group. Calcium and magnesium levels in patients with IVF were not increased by the multivitamin/mineral complex supplementation.

Serum element levels of the three groups are shown in Table 4. Serum copper, zinc, and selenium levels were significantly ( $p<0.05$ ) lower in patients with IVF than in controls, whereas serum copper, zinc, selenium, magnesium, and aluminum levels were significantly ( $p<0.05$  and  $p<0.01$ ) higher in the treatment group than in the untreated IVF group. Iron and calcium levels in patients with IVF were not increased by the multivitamin/mineral supplementation.

## Discussion

We detected the presence of copper, zinc, aluminum, iron, magnesium, calcium, and selenium in the follicular fluid of patients with IVF. There are few reports on metal levels in follicular fluid. Recently, Silberstain et al. [2] investigated the average concentrations of

**Table 4** The effects of Multivitamin/Mineral Supplementation on Serum Element Levels in Patients with IVF (Mean±SD)

| Parameters        | Control (n=13) | IVF (n=30)   | IVF+SUPPL (n=26) |
|-------------------|----------------|--------------|------------------|
| Copper (µg/l)     | 84.74±6.22     | 79.38±6.59*  | 85.96±4.33**     |
| Zinc (µg/l)       | 87.15±5.71     | 68.47±11.41* | 83.76±6.67***    |
| Selenium (µg/dl)  | 6.65±0.54      | 5.91±0.51*   | 6.74±0.85**      |
| Aluminum (µg/dl)  | 28.81±2.37     | 30.77±2.18   | 38.67±4.32**     |
| Iron (µg/l)       | 45.23±3.73     | 45.40±3.97   | 49.10±4.01       |
| Magnesium (mg/dl) | 1.57±0.13      | 1.59±0.21    | 2.12±0.34**      |
| Calcium (mg/dl)   | 8.30±0.57      | 8.31±1.07    | 8.96±0.65        |

\* $p<0.05$  versus control; \*\* $p<0.05$  versus IVF group; \*\*\* $p<0.01$  versus IVF group

elements in 33 follicular fluid samples and they found similar element levels in other human tissues and fluids. Also the Siberstein study reported that calcium and magnesium are the most abundant metals, followed by copper, zinc, and iron (present in concentrations of hundreds of ppb). The following metals namely lithium, selenium, aluminum, and manganese were found in trace amounts. Lithium, cadmium, barium, titanium, and bismuth were not detected (<0.2 ppb) in our studies of follicular fluid and blood of patients with IVF. Similarly, Zha et al. [15] reported the presence of zinc and manganese in follicular fluid of non-professionally exposed.

Serum copper levels were lower in patients with IVF than in controls. Copper is an essential element in biological systems. The biological functions of copper are intimately related to its redox properties as a transition metal. Redox cycling between  $\text{Cu}^{2+}$  and  $\text{Cu}^{1+}$  can catalyze the production of highly toxic hydroxyl radicals [16].

In the current study, follicular fluid iron levels were higher in patients with IVF than in controls. Iron levels were lower in multivitamin/mineral supplemented groups than in untreated IVF groups. It is well known that oxidative stress enhances non-heme iron absorption. Oxidative stress during oocyte maturation and rupture may influence biomarkers of iron status through its inflammatory effects on metabolism [11–13], or the continuous exposure to ischemia/reperfusion in IVF may produce a degree of hypoxia that leads to increased hemoglobin concentrations as an adaptive response [17].

Zinc is a micronutrient abundantly present in meat and seafood. Zinc serves as a cofactor for more than 80 metalloenzymes involved in DNA transcription and protein synthesis [6]. Because DNA transcription is a major part of germ cell development, zinc is likely to be important for reproduction [1]. Furthermore, zinc finger proteins are implicated in the genetic expression of steroid hormone receptors [3], and zinc also has antioxidant properties [18]. In the current study, zinc levels were lower in patients with IVF than in controls although zinc levels increased in multivitamin/mineral supplemented groups. Studies on the effects of zinc deficiency in women with IVF are scarce. Ng et al. [10] investigated zinc levels in follicular fluid of 33 women undergoing IVF in Singapore and did not observe any correlation between follicular fluid zinc concentration and follicle volume. Jameson [19] reported longstanding infertility in seven, normal sexually developed women with celiac disease. These women all had normal menstrual cycles, but low serum zinc levels. Soltan and Jenkins [20] measured plasma zinc levels in 48 infertile and 35 control women and found no differences between these two groups. Ronaghy and Halsted [21] reported two women aged 19 and 20 years, suffering from nutritional dwarfism with delayed sexual maturation. These women had no breast tissue or pubic hair and had infantile external genitalia with extremely low blood plasma and erythrocyte zinc levels. After zinc supplementation, these women experienced their first menstrual period and developed breast tissue as well as pubic hair growth.

Selenium levels were lower in IVF patients than in controls. The relationship between fertility and selenium status and Se-dependent GSH-Px enzyme activities has been examined in just a few studies that, in general, found lower serum and follicular fluid concentrations to be associated with increased infertility rate. Paszkowski et al. [22] found that 112 IVF patients had lower serum and follicular fluid selenium levels than controls, due mostly to a lower intake of selenium. Reduced levels of GSH-Px are reported in the follicular fluid of women with unexplained infertility [23]. Yang et al. [24] found higher levels of oxidant, hydrogen peroxide in fragmented embryos compared with nonfragmented embryos, while Paszkowski et al. [22] reported the elevated consumption of antioxidants, which suggests increased ROS levels, during incubation of poor quality of embryos. However, there is also evidence to suggest that antioxidant vitamins in follicular fluid act on the ovary to modify its function [12].

Follicular fluid zinc and selenium were significantly lower in IVF patients than in controls. Serum zinc levels were higher in the treatment group than in untreated IVF patients. To our knowledge, there is no publication on zinc levels in patients with IVF. However, our results are in agreement with studies conducted on genetically manipulated mice where decrease in the litter size and number of litters per month with deficiency of follicular fluid Zn–Cu–SOD activity has been reported [25, 26]. Intense metabolism of the granulosa cells together with high numbers of macrophages and neutrophil granulocytes in the follicle wall at ovulation may represent a site of active free radical generation in follicular fluid of IVF patients [27]. These free radicals can cause damage to biomolecules including DNA, and thus be mutagenic and carcinogenic. Aerobic cells have developed their own defense system in order to control the flux of free radicals. The Cu–Zn–SOD enzyme is the first line of defenses in oxidative stress. The Cu–Zn–SOD converts superoxide into hydrogen peroxide. Hydrogen peroxide can be transformed into water and oxygen by selenium-dependent GSH-Px enzyme. In a recent study, we observed that antioxidant vitamin were lower in patients with IVF than in control although lipid peroxidation levels were higher in patients with IVF than in control [28]. Hence, decreases of follicular fluid and serum zinc and selenium levels, and serum copper levels as a component of antioxidant enzymes attributed to increased levels of free radical production.

In conclusion, the results of the current study show that follicular fluid zinc and selenium, and serum zinc, copper, and selenium levels were lower in patients with IVF than in control. However, follicular fluid copper, selenium, and aluminum levels, and serum copper, zinc, selenium, aluminum, and magnesium levels were higher in IVF+vitamin+ mineral group when compared to the IVF group. Hence, a combination of these micronutrients provides a better effect on accumulation of the elements in follicular fluid.

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