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The Impact of a Standardized Oral Multinutrient Supplementation on Embryo Quality in in vitro Fertilization/ Intracytoplasmic Sperm Injection: A Prospective Randomized Trial

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The Impact of a Standardized Oral Multinutrient Supplementation on Embryo Quality in in vitro Fertilization/Intracytoplasmic Sperm Injection: A Prospective Randomized Trial

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Established Facts

- The relationship between healthy nutrition and fertility has been demonstrated repeatedly and recently.
- Multinutrient supplementation products are used widely, but evidence for their clinical value is scarce.

Novel Insights

- A multinutrient supplementation that includes folic acid, selenium, vitamin E, catechins, glycyrrhizin, diosgenin, damiana and omega-3-fatty acids seems beneficial for embryo quality.

Key Words
Micronutrients · Infertility · In vitro fertilization · Intracytoplasmic sperm injection · Embryo quality · Antioxidants

Abstract
The role of micronutrients in fertility has recently gained increased attention. We aimed to test the impact of a standardized, multinutrient supplementation on outcomes after in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) in a pilot study. One hundred women undergoing IVF/ICSI were prospectively included and randomized to receive either a multinutrient supplementation named PROfertil® female that included folic acid, selenium, vitamin E, catechins, glycyrrhizin, diosgenin, damiana and omega-3-fatty acids (study group; n = 50), or 400 µg folic acid (control group; n = 50). Outcome parameters were embryo quality on day 3 after oocyte retrieval (good quality vs. poor quality) and the clinical pregnancy rate. In an intention-to-treat anal-

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yses, a higher rate of women with at least one good quality embryo (with at least 6 cells and a fragmentation rate <20%) were found for the study (29/50, 58.0%) compared to the control group (18/50, 36.0%; p = 0.045 in chi-square test; relative risk 1.611, 95% CI 1.009–2.597). In conclusion, a multinutrient supplementation that includes folic acid, selenium, vitamin E, catechins, glycyrrhizin, diosgenin, damiana and omega-3-fatty acids seems beneficial in terms of embryo quality.

Background

As a result of recent scientific discussions, the role of micronutrients in fertility has recently gained increased attention. The relationship between healthy nutrition and fertility has been demonstrated repeatedly and recently [1, 2]. Micronutrients include essential vitamins and minerals and are required in small quantities as dietary components. Despite the fact that these micronutrients do not provide energy to the human body, they are essential for catabolic and anabolic processes and need to be supplied externally [3]. In a prospective study, adhering to the Dutch dietary recommendations led to increasing chances of ongoing pregnancy in women undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [4]. The evaluation of the influence of a micronutrient shortage on female infertility is difficult due to the large number of essential cofactors [1].

In addition, in a recent review, some investigators have pointed out the possible effects of micronutrients on female fertility without being able to provide general recommendations [1]. This underlines the urgent need for further research on this topic. To determine the effects of possible dietary supplementation on female infertility, we needed a homogeneous, subfertile patient population and an outcome parameter that is influenced by only a few possible other cosfactors. We, thus, chose to conduct a prospective study including only women undergoing IVF/ICSI to test the impact of a standardized, multinutrient supplementation on embryo quality. Recent studies support the role of micronutrients in female fertility [5, 6]. Since antioxidative mechanisms are essential for follicular growth and the role of antioxidants in female fertility has been addressed in many studies without a sound final conclusion [7], we chose a standardized, multinutrient supplementation preparation that included a high amount of omega-3 unsaturated acids and antioxidants.

Methods

Study Population and Design

In our prospective pilot study, we included 100 women who were undergoing IVF/ICSI at the Department of Gynecologic Endocrinology and Reproductive Medicine of the Medical University of Vienna between March 2013 and September 2015 (n = 50 for both the study group and the control group). Due to the design as a pilot study, there was no power calculation. We chose to include 100 patients, which is the maximum sample size that is allowed for pilot studies by the local Ethics Committee of the Medical University of Vienna. Women had to be 19–42 years of age. Women who had taken any vitamin or multinutrient supplementation preparation, other than folic acid alone for the last 3 months, were excluded. Women were randomized according to the day of their first visit to our IVF clinic. Notably, this was done prior to the decision about whether the couple would undergo IVF or ICSI. This visit also served as the initial visit for randomization. On odd-numbered days, women received 'PROfertil® female (Lenus Pharma GesmbH, Vienna, Austria; assigned to the study group) in an amount dictated by the study. On even days, the control group was provided with capsules containing 400 µg folic acid only ('Folsäure Kapseln 400 µg', OTC Produktion und Forschung GmbH, Salzburg, Austria). Women had to use these preparations for a minimum of 28 days and a maximum of 56 days prior to ovarian hyperstimulation. Micronutrients and antioxidants can be measured in serum within hours after intake [8]. The effects of micronutrients (antioxidants) on oocyte selection (recruitment) and ovulation can be shown in the follicular phase after antioxidant intake for at least one cycle length [9, 10]. Thus, a minimum of 28 days, that is, the length of one female cycle, was warranted before a possible effect could be assumed. Since we believe that supplements that need to be given for many months in order to achieve a positive effect will not be easily applicable in the clinical routine, we limited the intake of the study medication to a maximum of 56 days. Patients in the study group were directed to use one tablet and one soft capsule of 'PROfertil® female' a day. The daily dose of 'PROfertil female' consists of one tablet and one soft capsule. One tablet contains 800 µg folic acid, 70 µg selenium, 30 mg vitamin E, 4 mg catechins, 12 mg glycyrrhizin, 32 mg diosgenin and 90 mg damiana; one soft capsule contains 500 mg omega-3-fatty acids. The multinutrient combination 'PROfertil® female' has a free sales certificate in Austria.

Notably, testing for vitamin D deficiency based on serum 25(OH) vitamin D levels is routine at our department for all infertile women. Vitamin D supplementation using Oleovit D3 Tropfen® (Fresenius Kabi Austria GmbH, Graz Austria; 1 ml contains 14.400 I.E. colecalciferol) is recommended until a 25(OH) vitamin D serum level of at least 75 nmol/l is reached.

In addition to the routine controls and examinations during the standard protocol for IVF/ICSI, women had to undergo 3 study-specific visits. At the initial visit, women were randomized to one of the treatment groups and received study-specific medication (visit 1). This was followed by an interim visit (visit 2) 4–6 weeks later, and a final visit directly before ovarian hyperstimulation (visit 3). During these visits, medication blisters were re-collected and women were asked about possible side effects. In some cases, visits 2 and 3 could be completed on the same day. Women were treated individually thereafter. The

study was approved by the local Ethics Committee of the Medical University of Vienna (IRB number 1659/2013, finally approved on September 24, 2013). All patients signed an informed, written consent.

Concerning conflicts of interest, M.I. and J.O. received remuneration for lecturing from Lenus Pharma GesmbH (Vienna, Austria).

Parameters Analyzed

The main outcome parameter was the embryo quality on day 3 after oocyte retrieval. One highly experienced biologist, who was blinded to the study medication that had been given to the patients, rated embryo quality. Embryos were divided into those with good quality (embryos with at least 6 cells and a fragmentation rate <20%) and those with poor quality (<6 cells and a fragmentation rate ≥20%) [11]. We subdivided women into those with at least one embryo of good quality and those without. We also analyzed the total number of embryos of good quality per patient. Notably, in women who underwent embryo transfer on day 5, embryo quality was also evaluated in the course of clinical routine on that day. However, this was not used as an outcome parameter. In addition, clinical pregnancy, defined as an intact pregnancy with a positive heartbeat on vaginal ultrasound, was set as the secondary outcome parameter. As patient- and treatment-specific parameters, we included age, body mass index, the indication for IVF (male factor defined by the fourth edition of the WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction [12], polycystic ovary syndrome defined by the revised Rotterdam criteria [13], histologically verified endometriosis, bilateral tubal factor and unexplained infertility), the duration of standardized multinutrient supplementation (study or control medication) until oocyte retrieval and the need for thyroid hormone supplementation (with the goal of maintaining the TSH level <2.5 IU/ml). Furthermore, the type of stimulation (antagonist protocol or long protocol) was registered.

Stimulation Protocol

All women were treated using an antagonist or agonist protocol. Ovarian stimulation was started with 125–300 IU of recombinant follicle-stimulating hormone (FSH; Puregon®, MSD Pharma, Austria or Gonal F®, Serono, Austria or Elonva®, MSD Pharma, Austria). Antagonists (Orgalutran®, MSD Pharma, Austria or Cetrotide®, Serono, Austria) were given when the leading follicle reached a diameter of 12 mm. Women treated with an agonist protocol underwent standard downregulation with a GnRH agonist (Decapeptyl®, Ferring, Austria) on day 21 of the preceding cycle. After downregulation, defined as estradiol (E2) less than 50 pg/ml, stimulation was started in a manner similar to that of the antagonist protocol. Follicle monitoring was performed by transvaginal sonography. When necessary, the FSH dosage was adjusted according to the follicle numbers and diameters. When adequate stimulation was achieved (≥3 follicles of at least 18 mm in diameter), 10,000 IU of human chorionic gonadotropin (hCG) (Pregnyl®, AESCA Pharma, Austria) were administered. Oocyte retrieval was performed 35 h after hCG injection. Conventional IVF/ICSI, following standard techniques, was used for fertilization. A maximum of 2 embryos were transferred on days 3 or 5 after oocyte retrieval. All women received luteal support with vaginal progesterone (Utrogestan®, Meda Pharma GmbH, Austria) of 600 mg per day.

Statistical Analysis

Variables are described by numbers (frequencies) and median (interquartile range, IQR). For numeric variables, statistical analysis was performed using the Welch test for normally distributed numeric variables and the Mann–Whitney U test in the case that there was no normal distribution. Data were tested for normal distribution using the Kolmogorov–Smirnov test. The chi-square or the Fisher's exact test was used for categorical variables. An intention-to-treat analysis was performed for both outcome measures. p values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS 17.0.1 for Windows.

Results

Basic patient characteristics are shown in table 1. None of the parameters differed between the study group and the control group. This was also true for the stimulation protocol: women in the study group were stimulated with an antagonist protocol in 46 cases (92.0%) versus 44 cases (88.0%) in the control group (p = 1.000). The remaining patients underwent hyperstimulation with a long protocol (n = 10). According to patients' statements and the re-collected medication blisters, all women had correctly adhered to the supplementation regimens.

There were no withdrawals or dropouts from the study. In 8 women, the IVF cycle had to be interrupted before oocyte retrieval due to ovarian hyperstimulation syndrome (study group: n = 3, 6.0%; control group: n = 5, 10.0%; p = 0.715). Focusing on the remaining patients, the number of retrieved oocytes did not differ between the groups (study group: 7, IQR 3–12 vs. control group: 7, IQR 3–9; p = 0.934). The same was true for the number of fertilized oocytes (study group: 3, IQR 1–5 vs. control group: 3, IQR 2–4; p = 0.580).

An intention-to-treat analysis was performed for the major outcome parameters and included all 100 women, as well as those who had not undergone oocyte retrieval (table 2): in the study group; in 29 women (58.0%), there was at least one embryo of good quality in contrast to 18 women (36.0%) in the control group (p = 0.045). In addition, the median number of embryos with good quality was higher in the study group (p = 0.048). There was no significant difference in pregnancy rates (study group: 20/50, 40.0% vs. control group: 13/50, 26.0%; p = 0.141).

Discussion

This prospective, randomized study demonstrated that the use of the multinutrient supplementation 'PROfertil® female' led to significantly higher fertiliza-

Table 1. Basic patient characteristics

	Study group (n = 50)	Control group (n = 50)	p value
Age, years	37.1 (33.6–40.2)	35.6 (32.4–39.1)	0.275[1]
Body mass index, kg/m^2	23.7 (21.5–27.7)	22.5 (20.7–26.0)	0.157[1]
IVF treatment cycle	2 (1–2)	1 (1–2)	0.143[1]
Male factor	28 (56.0)	32 (64.0)	0.541[2]
Polycystic ovary syndrome	9 (18.0)	8 (16.0)	1.000[3]
Bilateral tubal factor	18 (36.0)	11 (22.0)	0.186[2]
Endometriosis	7 (14.0)	7 (14.0)	1.000[3]
Unexplained infertility	0	3 (6.0)	1.000[2]
Duration of standardized micronutrient supplementation until ovarian hyperstimulation, days	35 (30–41)	34 (29–39)	0.728[1]
Thyroid hormone supplementation	8 (16.0)	5 (10.0)	0.554[3]

Data are presented as median and IQRs for numerical parameters or numbers and frequencies for categorical parameters; significances were tested using either.
[1] The Welch test, [2] the chi-square test, or [3] the Fisher's exact test.

Table 2. Major results

	Study group (n = 50)	Control group (n = 50)	p value	Relative risk	95% CI
Total number of retrieved oocytes	7 (3–12)	7 (3–9)	0.934[1]	–	–
Fertilization rate, %	66.7 (30.4–96.4)	42.9 (25.0–57.8)	0.010[1]		
Number of fertilized oocytes	3 (1–5)	3 (2–4)	0.580[1]	–	–
Number of high quality embryos	1 (0–2)	0 (0–1)	0.048[2]	–	–
Number of patients with at least one high quality embryo, n (%)	29 (58)	18 (36)	0.045[3]	1.611	1.009–2.597
Number of transferred embryos	48	43	0.398[3]	–	–
Overall clinical pregnancy rate, n (%)	20 (40.0)	13 (26.0)	0.141[3]	1.538	0.824–2.949
Clinical pregnancy rate per embryo transferred, n (%)	20 (41.7)	30 (13.2)	0.257[3]	1.378	0.750–2.614

Data are presented as median and IQRs for numerical parameters (part of the number of transferred embryos, which is given as the total number per group) or numbers and frequencies for categorical parameters; significances were tested using either.
[1] The Welch test, [2] the Mann–Whitney U test or [3] the chi-square test.

tion rates and a higher chance for at least one embryo of good quality after routine IVF/ICSI, when compared to the use of 400 μg folic acid alone. The clinical pregnancy rate in the study group exceeded that of the control group, but did not reach significance. However, these data support the previous suggestion of a beneficial effect of multinutrient supplementation on female fertility [1]. In a recent review, investigators concluded that substitution with different micronutrients could also have a positive impact on female fertility in the case of the necessity for infertility treatment [14].

It is evident that the positive impact of multinutrient supplementation in our study cannot be attributed to one of the various ingredients. This has to be considered a study limitation. Moreover, the composition of the preparation used might be an issue. First and foremost, the preparation contains several micronutrients thought to act as antioxidants, that is, selenium, vitamin E and catechins [12, 15]. Notably, antioxidative mechanisms are essential for follicular growth, especially of the leading follicle [16]. Notably, the role of antioxidants in female fertility/infertility has been addressed in various reports, including a Cochrane analysis in 2013. The authors of that analysis concluded that the overall quality of the evidence was low; thus, no definitive recommendation could be made [7]. However, several studies have addressed the

possible value of the above-mentioned ingredients. In a recent overview, selenium has been reported to be an essential player in the undisturbed functioning of the human reproductive system, which is partly due to its antioxidative effects [17]. Vitamin E has been suggested to be among those supplements that likely have a positive impact on infertility treatment in women [14]. In women <35 years of age with unexplained infertility, increasing vitamin E levels were associated with a shorter time to pregnancy [18]. Interestingly, the majority of data about the beneficial, antioxidative effects of vitamin E in female fertility are derived from rodent trials [19]. Last, but not least, green tea catechins have been reported to exert antioxidative effects in humans. In addition, they seem to contribute to the regulation of vascular tone by activating endothelial nitric oxide, which seems also to be the case in female reproductive organs [20]. In addition, green tea extract has been shown to result in weight loss and improve both reproductive and metabolic features in polycystic ovary syndrome, with this effect possibly mediated through the glucose-insulin pathway [21, 22].

Notably, omega-3 fatty acids have already been reported to positively influence embryo morphology which is a surrogate parameter in IVF. In a study on humans, the increased intake of omega-3 was associated with higher estradiol levels, as well as with increased embryo quality [23]. The 3 other ingredients used in our preparation have also been reported to exert reproductive effects. According to in vitro studies, damiana seems to suppress aromatase activity and show estrogenic activity at the same time, which, however, seems of minor clinical relevance for ovarian hyperstimulation [24]. Glycyrrhizin might reduce serum testosterone levels in humans [25], and might, therefore, contribute to hormonal balance through the promotion of the production of estrone and estradiol. Moreover, it has been shown to inhibit over-reactive inflammation and activate multiple transcription factors to elevate the antioxidant system in vitro [26]. Diosgenin, which can be extracted from yams, serves as precursor of steroid hormones and is known to increase sex hormones, at least in postmenopausal women. The improved oxidative status seems to be related to the anti-inflammatory and antioxidant properties of the substance [27].

There is also the choice of PROfertil® female for the present study. We considered it interesting to test the mixture of antioxidative micronutrients in such a complex system as follicle maturation/embryo quality during IVF/ICSI. Moreover, it was our aim to evaluate the clinical value of a standardized, readily available preparation.

Notably, folic acid supplementation is usually recommended at a daily dose of 400 μg to reduce the risk of neural tube defects [28]. This standard supplementation was provided to the control group. However, a higher folic acid intake has also been reported to lead to increased live birth rates after assisted reproductive technology treatment. In that study, the median daily folic acid intake was 1,778 μg [29]. We, thus, consider that a daily dose of 800 μg, as received in the supplement for the study group, is not problematic with regard to possible upper limits of daily intake. Notably, we cannot completely rule out that the higher amount of folic acid in the PROfertil® female group might have contributed to the better outcomes in terms of embryo quality.

In this pilot study, we focused on embryo quality as the major outcome parameter. We are aware that this can serve as only a surrogate parameter and, thereby, limits the study. However, no supplements have been proven beyond doubt to increase conception rates in female infertility, as stated in a recent review [30]. However, significantly more women in the study group had at least one embryo of high quality compared to the control group. This might be of importance, especially in the era of single-embryo transfer. This phenomenon resulted in a higher pregnancy rate in the study group (40 vs. 26% in the control group), a result that did not reach statistical significance. Further studies are, therefore, warranted to confirm the clinical effect of the tested multinutrient supplementation.

Our results must be interpreted with care due to the unblinded study protocol, the design as a pilot study without an a priori sample size calculation and the pseudo-randomization based on the day of patient enrollment, which we consider to be the limitations of the study. However, we assume that some women of the control group who had been completely informed about the presumed beneficial effect of the supplementation might have bought the tested preparation or another micronutrient supplementation on their own. One would expect that such a bias would likely lead to a reduction of the beneficial effects observed in the study group. Nevertheless, the intention-to-treat analysis might be considered a strength of our study. But, we consider it another study limitation that we did not test the serum levels of the supplemented micronutrients. This was due to the fact that we aimed to evaluate the clinical value of the prefabricated multinutrient supplementation. One might argue that the 18 months needed for patient recruitment was long. This was due to the fact that some women did not want to postpone ovarian hyperstimulation for 1 month,

which would have been necessary to participate in the study. However, we feel that the participants still resemble our average patient population. Moreover, 2 factors might have had an impact on the clinical pregnancy rate: age and the number of transferred embryos. The latter is of special importance, because women with embryos of good quality, which is influenced by the use PROfertil® female, were more likely to undergo a single embryo transfer. Thus, we also calculated the clinical pregnancy rate per embryo transferred, which should have overcome this problem. However, the main outcome parameters, which deal with embryo quality, were seemingly not influenced by other factors, since the basic patient characteristics did not differ between the PROfertil® female and the control groups. Last but not least, the embryologist's intraobserver variability in the scoring was not evaluated, which might have introduced some bias.

In conclusion, our data suggest that a multinutrient supplementation that includes folic acid, selenium, vitamin E, catechins, glycyrrhizin, diosgenin, damiana and omega-3-fatty acids, given for a minimum of 28 days prior to ovarian hyperstimulation, is beneficial in terms of fertilization rate and embryo quality. These results should encourage future studies with the rate of pregnancy as the main outcome parameter.

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None.

Disclosure Statement

M.I. and J.O. received remuneration for lecturing from Lenus Pharma GesmbH (Vienna, Austria). All other authors declare that they have no commercial interest, financial interest and/or another relationship with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices, or with commercial providers of medically related services.

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