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ARTICLE

Prospective randomized trial of multiple micronutrients in subfertile women undergoing ovulation induction: a pilot study

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Abstract This study investigated whether subfertile women undergoing ovulation induction using standard treatment regimens with clomiphene citrate/gonadotrophins have higher pregnancy rates when on an adjuvant multiple micronutrient (MMN) nutritional supplement compared with folic acid alone. A prospective randomized controlled trial was conducted in a teaching-hospital fertility clinic on 58 subfertile women, of which 56 women completed the study. Women undergoing ovulation induction were allocated to either receive adjuvant MMN supplementation or folic acid. Clinical pregnancy rates and blood nutrient concentrations were assessed after the third treatment attempt or as soon as the women achieved pregnancy. Using intention-to-treat analysis, it was observed that women on adjuvant MMN supplementation had a significantly higher cumulative clinical pregnancy rate (66.7%) compared with those on folic acid (39.3%; $P = 0.013$). The ongoing pregnancy rate in women on MMN supplementation was 60.0% versus 25.0% ($P < 0.02$) in the folic-acid group. Further, women who were on MMN supplementation had significantly fewer attempts to achieve pregnancy compared with women on folic acid ($P < 0.001$). The results of this pilot study suggest that women who take adjuvant MMN supplementation during ovulation induction have a higher chance of pregnancy compared with women on folic acid.

KEYWORDS: multiple micronutrient (MMN) supplementation, pregnancy rates, subfertility, vitamins

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Introduction

Several lifestyle factors affect fertility, such as bodyweight, stress, smoking, alcohol, caffeine, recreational drugs and nutrition (NICE, 2008). A significant lifestyle factor which is often neglected is nutrition. Lifestyle factors, however, can be subjected to preventive/curative measures. Of all couples, 11–20% experience subfertility during their reproductive life (Mosher and Pratt, 1990; Philippov et al., 1998; Snick et al., 1997; Templeton et al., 1990), of which 10% have unexplained infertility and 60% have anovulatory subfertility (Crosignani et al., 1999). Anovulatory polycystic ovary syndrome (PCOS) and unexplained subfertility together account for the commonest causes of subfertility and for most women undergoing ovulation induction (Tadokoro et al., 1997).

Micronutrient deficiencies are not uncommon in women of reproductive age and although conclusive evidence exists on periconceptual folate use in preventing neural-tube defects, evidence supports but does not conclude that the use of multiple micronutrient (MMN) supplementation beneficially affects fertility, embryogenesis/placentation and that prophylactic MMN use prevents adverse pregnancy outcomes (Cetin et al., 2010). Macronutrients are acquired easily from diet; micronutrients (vitamins, minerals, trace elements) however, are scarce in commonly consumed foods. Nutritional deficiencies are often not apparent and food scarcity does not cause MMN deficiencies; predisposing factors are the way the food is processed and preserved, soil depletion, overfarming, early picking of unripe food for transport and prolonged storage at low temperatures. A significant factor contributing to MMN deficiency is lifestyle: the consumption of convenience/pre-prepared food of low nutritional quality. MMN deficiencies have been associated with significant reproductive risks, ranging from infertility to miscarriage and fetal structural defects (Lim et al., 2007). There is evidence that administration of vitamins B_6, B_{12}, folates (Bennett, 2001; Mier-Cabrera et al., 2008; Westphal et al., 2006), vitamin E and multivitamins (Czeizel, 1998; Haggarty et al., 2006; Rumbold et al., 2008), iron (Rushton et al., 1991), zinc/copper/selenium (Bedwal and Bahuguna, 1994; Ebisch et al., 2007) may improve female fertility. Large longitudinal observational studies concluded that MMN supplementation improved pregnancy rates (Lim et al., 2007; Mier-Cabrera et al., 2008). Given the widespread growing role of assisted reproductive treatment in subfertile women, it is important to assess the role of MMN supplementation as an adjunct to standard ovulation-induction regimens used in routine clinical practice.

The aim of the present study was to compare clinical pregnancy rates and cumulative conception rates in women taking MMN as an adjunct to standard ovulation induction with those who were not on MMN (on folic acid alone). This study also assessed whether circulating concentrations of vitamins B_{12}, vitamin D, folates, haemoglobin, haematocrit and ferritin differ within groups from treatment start (baseline) to 3–6 months later and between the 2 groups of women. The MMN used in the study is Pregnacare Conception (Vitabiotics, London, UK), which has a balanced combination of micronutrients and developed specifically for preconceptual women. **Table 1** compares the reference nutrient intake values of MMN for pregnant and nonpregnant women with those supplied by the supplement.

Materials and methods

A prospective randomized trial was conducted at a tertiary referral fertility centre, Royal Free/University College London Hospitals, UK. The study recruited 58 women, aged 32.3 years (range 19–40) with anovulatory infertility (mid-luteal progesterone $<$30 nmol/l) or at least 12 months unexplained infertility, between February and August 2009. All women were scheduled to undergo ovulation induction using standard treatment regimens with clomiphene citrate (Clomid) Clomiphene (Aventis Pharma Ltd. Guildford Surrey, UK) or human menopausal gonadotrophin (HMG, Menopur, Ferring Pharmaceutical, Langley, UK).

Suitable women were identified at the clinic and recruited if they fulfilled the inclusion criteria 4–6 weeks prior to having ovulation induction. Women whose partners had semen abnormalities (WHO criteria 1999) and those who had been on multivitamins (except folates) for 6 weeks prior to being recruited were also excluded; however, 4 weeks is a sufficient wash-out period for vitamins. In addition, women with tubal disease, moderate and severe endometriosis, medical disorders or haemoglobinopathies and those who were smokers or had excessive alcohol intake or body mass index $<$19 or $>$34 kg/m_2 were excluded. Informed written consent from participants and ethical approval was obtained from National Research Ethics Service, UK (reference no. 08/H0720/154). The researchers/sponsors audited the study in accordance with NHS Research Governance Framework. Participants were randomly allocated to receive either MMN supplementation (Pregnacare Conception) once daily, the recommended daily dose (n = 30), or folic acid (n = 28) as an adjunct to standard ovulation-induction regimens. Approximately 4 weeks after commencing MMN or folic acid, both groups of women received either clomiphene citrate 50–150 mg on days 2–6 or HMG 75 IU (Menopur; Ferring Pharmaceutical, Langley, UK) from cycle day 4. All women were monitored with pelvic ultrasound examination to confirm an optimum response to ovulation induction. Third-party randomization and allocation concealment were carried out through the research and development department of the University College London and The Royal Free Hospitals using stratification and numbered envelopes. Women, caregivers and investigators were blinded to the treatment allocation.

All women recruited had baseline measurements of hormones (FSH, LH, oestradiol, prolactin, testosterone, sex hormone-binding globulin, androstenedione, dehydroepiandrosterone, 17-α-hydroxyprogesterone and mid-luteal progesterone, thyroid function tests), glucose, full blood count, iron studies, haemoglobinopathies, vitamins B_{12} and D, folate, calcium, magnesium, urea/electrolytes and liver-function tests. A record of their dietary habits was maintained by patients themselves on a daily basis throughout the study period to ascertain that there were no dietary differences between the two groups of women which would affect their circulating micronutrient status.

Supplements were administered once daily until the study completion. Women who became pregnant were

Table 1 Nutritional Information for MMN (Pregnacare Conception) average per Tablet.

	RNI for non-pregnant women		Increment in RNI for pregnant women			Micronutrients provided by supplement	% RDA	% RNI supplied by supplement during pregnancy	
	Age/years		Trimester					Age/years	
	15-18	19-50	1	2	3			15-18	19-50
Thiamin B1 mg/day	0.8	0.8			+0.1	8	727	889	889
Riboflavin B2 mg/day	1.1	1.1	+0.3	+0.3	+0.3	5	357	357	357
Niacin B3 (nicotinic acid equivalent) mg/d	14	13				20	125	143	154
Vitamin B6 mg/d	1.2	1.2				10	714	833	833
Vitamin B12 μg/d	1.5	1.5				20	800	1333	1333
Folate[b] μg/d	200	200	+100	+100	+100	400	200	100[c]	100[c]
Vitamin C mg/d	40	40	+10	+10	+10	90	113	180	180
Vitamin A (retinol equivalent) μg/d	600	600	+100	+100	+100	500 (3 mg β-carotene)		71	71
Vitamin D(as D3 600 I.U. μg/d			10[d]	10[d]	10[d]	15	300	150	150
Calcium mg/d	800	700							
Phosphorus mg/d	625	550							
Magnesium mg/day	300	270				60	16	20	22
Sodium mg/d	1600	1600							
Potassium mg/d	3500	3500							
Chloride mg/day	2500	2500							
Iron mg/day	14.8	14.8				14	100	95	95
Zinc mg/d	7.0	7.0				15	150	214	214
Copper mg/d	1.0	1.2				1.0	100	100	83
Selenium μg/d	60	60				50	91	83	83
Iodine μg/d	140	140				140	93	100	100
Vitamin E mg/d	>3	>3				30	250	>3	>3
Vitamin K μg/d	1 μg/kg/d	1 μg/kg/d							

L-Arginine 100 mg, Inositol 50 mg, N-Acetyl Cysteine 50 mg
Biotin 150 mcg 300
Pantothenic Acid 6 mg 100
Dietary Reference Values (DRV)[a] for micronutrients for pregnant and non-pregnant women together with quantity of micronutrients supplied by Pregnacare Conception.
RNI - Reference Nutrient Intake.
[a]DoH 1991. (Dietary Reference Values: A Guide. Department of Health, 1991(HMSO publications))[b]Women also recommended to take 400 μg/day supplement up to 12th week of pregnancy; women with a previous NTD affected pregnancy should take 5mg/day (DoH, 2000).
[c]100% of that recommended to be provided by supplementation.
[d]Recommended to provided by supplementation.

offered to continue MMN if they wished, whilst women who were not successful discontinued the MMN at the end of the study. Menstrual-cycle length changes and incidents of adverse effects were evaluated. The primary outcome measures were clinical pregnancy rate per treatment cycle, defined as the presence of a viable gestation with a presence of fetal heart and cumulative pregnancy rates after three treatment cycles, defined as the presence of a viable gestation with the presence of fetal heart after three attempts of ovulation induction. The secondary outcome measures were haemoglobin, haematocrit, ferritin, vitamin-B_{12}, folate and vitamin-D_3 concentrations prior to allocating women to the treatment groups and at end of the study (3—6 months).

Sample-size power calculation was performed using G*Power (Faul et al., 2007) software and tables determined by Campbell (2005). Since a nutritional supplement-based study has not been previously undertaken in women undergoing ovulation induction, a-priori sample-size calculation was performed using G*Power with an estimated difference

in pregnancy rate of 18% between the two groups of women. Chi-squared power analysis with 80% power and $\alpha = 0.05$ indicated that 52 participants were required in the study. To accommodate 25% attrition, a further 13 were recruited; therefore 65 participants were required. The Statistical Package for Social Sciences (version 17; SPSS, Chicago, USA) was used for data analysis. Normally distributed data were analysed using parametric independent *t*-test. Clinical pregnancy rates were tested using group × pregnancy) Pearson's chi-squared test. A *P*-value <0.05 was considered statistically significant.

Results

Fifty-eight women were recruited (30 in the MMN group and 28 in the folic acid group); 56 completed the study of which 29 were in the MMN group and 27 in the folic-acid group. One patient in the folic-acid group discontinued after 26 days because she wished to have MMN supplementation. One patient in the MMN group discontinued the study due to nausea associated with tablet use. The intended 65 women were not recruited since data on 52 participants who completed the study were required for statistical analysis to have an adequate power. Forty-two women were excluded who did not fulfil the inclusion criteria.

There were no differences in the demographic data between the two groups of women in terms of age, body mass index, parity, diagnosis, incidence of PCOS and ovulation induction (**Table 2**). None of the women on clomiphene citrate had previously undergone ovulation induction. Of the women treated with HMG, 16 in the MMN group and 15 in the folic-acid group had had between one and a maximum of three cycles of clomiphene citrate treatment at least 2 months prior to recruiting them to the study.

There was an equal distribution of patients with unexplained subfertility and PCOS (no statistical significant differences) in the two groups. Further there was no statistically significant difference in the number of women who had HMG or those who had clomiphene citrate (**Table 2**).

There were no differences in hormone parameters (FSH, LH, oestradiol, prolactin, testosterone, sex hormone-binding globulin, androstenedione, dehydroepiandrosterone, 17-α-hydroxyprogesterone, mid-luteal progesterone, thyroid function test) or in glucose, full blood count, iron studies, haemoglobinopathies, vitamin B_{12}, vitamin D_3, folate, calcium, magnesium concentrations, urea/electrolytes and liver-function test between the two groups of women in the baseline blood tests (data not shown).

The cumulative conception rate after three cycles of ovulation induction in women who completed the study ($n = 56$) was 69.0% (20/29) in the MMN group versus 40.7% (11/27) in the folic-acid group ($P < 0.01$). Using intention-to-treat analysis ($n = 58$), the pregnancy rate in women on MMN supplementation was 66.6% (20/30) versus 39.3% (11/28) ($P = 0.013$) in women on folic acid. The ongoing pregnancy rate in women on MMN supplementation was 60.0% (18/30) versus 25.0% (7/28) ($P < 0.02$) in the folic-acid group. There were no differences in the miscarriage or ectopic pregnancy rates between the two groups of women (**Table 2**).

Women who were on MMN took fewer attempts to become pregnant (15 women conceived in their first attempt, four in their second attempt and one in her third attempt) compared with those on folic acid (two in their first attempt, two in their second attempt and seven in their third attempt; $P < 0.001$).

Dietary records confirmed that both groups of women were on an adequate balanced diet and the dietary habits did not differ between the two groups of women. Both of the adjuvant treatment medications were well tolerated by patients, who reported no adverse side effects.

Haemoglobin (13.2 versus 12.5 g/dl), haematocrit (0.37 versus 0.35%), ferritin (34 versus 56 μg/l), vitamin B_{12} (536 versus 444 ng/l and vitamin D_3 (49.8 versus 35.2 μg/l)

Table 2 Demographic and primary outcome data on intention-to-treat women in the study (multiple-micronutrient) and the control (folic-acid) groups.

	MMN (n = *30*)	*Folic acid* (n = *28*)	P-*value*
Age (years)	32.2 ± 0.65	32.5 ± 0.83	NS
BMI (kg/m_2)	24.3 ± 0.91	25.1 ± 0.98	NS
Parity 0	28	25	NS
PCOS	19	16	NS
Unexplained	8	10	NS
Minimal endometriosis	3	1	NS
Hypothyroidism	0	1	NS
Ovulation induction			
Clomiphene citrate	6	7	NS
HMG	24	21	NS
Outcome			
Clinical pregnancy	20	11	0.013
Ongoing pregnancy	18	7	<0.02
Miscarriage	1	4	NS
Ectopic pregnancy	1	0	NS

Values are $n \pm$ SD or *n*. BMI = body mass index; HMG = human menopausal gonadotrophin; MMN = multiple micronutrient; NS = not statistically significant; PCOS = polycystic ovary syndrome.

showed a trend towards higher values in women on MMN compared with those on folic acid, although the values did not reach statistical significance. However, only 11 of the women recruited returned for their blood tests after study conclusion (3–6 months); therefore the differences between the groups were not adequately powered to draw conclusions on the secondary outcome measure.

Discussion

The current study demonstrated that in women undergoing ovulation induction for anovulatory or unexplained subfertility using standard ovulation-induction regimens and who were on MMN supplementation as an adjuvant therapy had significantly higher clinical pregnancy rates compared with those who were on folic acid alone. Further, women on MMN supplementation achieved pregnancy in significantly fewer attempts compared with women on folic acid, for whom the majority of pregnancies were achieved in their second or third attempt. Although haemoglobin, haematocrit, ferritin, vitamin-B_{12} and vitamin-D_3 concentrations showed a trend towards higher concentrations in women on MMN supplementation compared with those on folic acid, these values did not reach statistical significance as few women returned for their blood tests after the study conclusion.

Micronutrient intake and maternal dietary status affect the onset and developmental phases of pregnancy (Westphal et al., 2006) and micronutrient deficiencies are associated with significantly high reproductive risks, ranging from subfertility, miscarriages to fetal defects. There is a growing body of evidence establishing the relationship between placental development, fetal growth, pregnancy outcomes (Brough et al., 2010; Morris et al., 2001; National Research Council Committee on Diet and Health, 1989) and adequate nutrition, particularly vitamin intake. However, there is currently little information about the most appropriate vitamin or nutrient combination which may be beneficial (Bedwal and Bahuguna, 1994).

Lifestyle factors affect fertility, and inappropriate diet or insufficient dietary micronutrients may manifest itself in subfertility. Chavarro et al. (2008) demonstrated that a combination of lifestyle factors such as adequate diet, physical activity and weight control was associated with 69% lower risk of subfertility due to ovulatory disorder. They concluded that a healthy diet with added micronutrients may influence fertility favourably in healthy women.

Preconceptual nutritional status/health is therefore important since it affects critical steps of fertility, implantation, fetal organogenesis and placentation. Westphal et al. (2006) observed that women on a nutritional supplement containing vitamins, antioxidants (chasteberry, green-tea extract), L-arginine and minerals had a higher chance of becoming pregnant (32%) compared with women on placebo (10%). Mier-Cabrera et al. (2008) allocated women with endometriosis to receive vitamin C and vitamin E or placebo. Although pregnancy rates were higher in women on vitamins (19% versus 11%) the difference was not statistically significant possibly because the study had so few participants that it failed to statistically demonstrate the observed benefits of taking supplements (type-II error).

One of the strengths of the current study is that it has calculated the correct number of participants, which allows benefits of the supplement to be observed and statistically recognized. Women in the aforementioned studies, as in the current study, were from good socioeconomic background and therefore were presumed nutritionally adequate. This information was obtained from their dietary records. This is an important observation since it is known that micronutrient supplementation is beneficial in nutritionally deficient women (NICE, 2008). Therefore this study, as well of those of Westphal et al. (2006) and Mier-Cabrera et al. (2008), demonstrated beneficial effects of micronutrient supplementation in nutritionally adequate women.

Besides folic acid, the MMN supplementation in this study provides vitamin B, antioxidants, vitamin E (α-tocopherol), vitamin C (ascorbic acid), zinc, selenium, vitamin A (β-carotenoids), other multivitamins/minerals, inositol, *N*-acetylcysteine (a precursor of the antioxidant glutathione; Badawy et al., 2007; Cavalli et al., 2008) and L-arginine (which improves follicular/endometrial blood flow; Burnett, 1995).

In female reproduction, vitamin B and folates are important for oocyte quality, implantation, placentation and fetal growth (Kanakkaparambil et al., 2009). Women receiving vitamin B and folic acid have significantly better quality and more mature oocytes compared with women who do not (Steegers-Theunissen et al., 1993; Szymański and Kazdepka-Ziemińska, 2003). Haggarty et al. (2006) observed that poor vitamin-B status and raised homocysteine concentrations were associated with early pregnancy loss whilst high folate concentration was associated with increased twinning after IVF treatment.

Secondary antioxidants such as vitamins A, C and E (Luck et al., 1995; Millar, 1992), zinc and glutathione are important in maintaining cellular oxidant–antioxidant balance (Ross et al., 2010). Despite a well-developed physiological system to counteract effects of reactive oxidative species (ROS), lifestyle factors, such as smoking, alcohol, environmental pollutants, food additives, diseases, stress, allergies and deficient diets, all contribute to high free-radical states (Riley and Behrman, 1991). The role of ROS in pathophysiology of reproductive functions is well established (de Lamirande and Gagnon, 1994; Riley and Behrman, 1991). ROS are involved in oocyte maturation, ovulation, progesterone production and luteolysis (Musicki et al., 1995; Sawada and Carlson, 1996; Shimamura et al., 1995; Sugino et al., 1993) and inhibition of ROS assists ovulation (Miyazaki et al., 1991). Antioxidants are acquired from diet and are also contained in the MMN supplementation used in this study. In addition, the MMN supplementation contains vitamin E, which improves glandular epithelial growth, blood-vessel development and endometrial vascular endothelial growth-factor expression (Mier-Cabrera et al., 2008).

The MMN supplementation in this study also provides inositol. Administration of the isoform (*myo*-inositol) helps support ovarian function and is involved in cell-signalling pathways which influence fertilization and cell-growth. Women with PCOS have insulin resistance and hyperinsulinaemia, possibly due to deficiency of D-chiro-inositol-containing phosphoglycan that mediates insulin action, (Gerli et al., 2007). Nestler et al. (1999) and Iuorno et al. (2002) demonstrated that D-chiro-inositol increased insulin

action, improved ovulatory function, decreased hyperandrogenism and improved the metabolic profile in PCOS women. Since several women in the current study had PCOS, the MMN supplement may have had a beneficial effect through inositol being one of its constituents.

The MMN supplementation provides L-arginine which is an essential amino acid and a precursor in nitric-oxide metabolism. L-Arginine improves follicular and endometrial blood flow and may ameliorate the process of embryo implantation (Battaglia et al., 1999).

N-Acetylcysteine is a precursor of glutathione, an antioxidant which aids healthy cervical secretions. In a study by Badawy et al. (2007) treatment with *N*-acetylcysteine and clomiphene citrate resulted in significantly better ovulation rates than with clomiphene citrate alone. The authors however, did not observe a difference in pregnancy rates between the two groups of women. The MMN also has essential trace minerals such as zinc, iron and magnesium, which are important in cell division and neural-tube development.

To summarize and conclude, nutrition affects biological processes in reproduction (Ebisch et al., 2007). Published studies provide evidence that micronutrients affect fertility, embryogenesis and placentation and that the prophylactic use of micronutrients may be useful in preventing several adverse pregnancy outcomes. Women should therefore be made more aware of lifestyle changes and a healthy diet, which are essential not only during pregnancy but also preconceptually. Those women susceptible to micronutrient deficiencies should receive micronutrient supplements to optimize their reproductive health. As far as is known, this study is the first to demonstrate that MMN supplementation as an adjuvant therapy to standard ovulation induction regimens in women undergoing treatment for anovulatory or unexplained infertility improves pregnancy rates. The beneficial effects of the MMN supplementation may have been mediated not only through the presence of multiple vitamins and minerals but through antioxidants, insulin sensitization with inositol and improvement of blood flow with the presence of L-arginine. MMN supplementation is a cost-effective remedy that is well tolerated with no adverse effects. Its use as an adjuvant in fertility treatment may benefit women preconceptually. The implications of this study are potentially far reaching as they suggest MMN supplementation in women undergoing ovulation induction improves pregnancy rates. Larger studies are now required to confirm these preliminary findings. A larger prospective randomized trial to test the beneficial effects of MMN supplementation on pregnancy rates and reproductive health of women preconceptually has been started.

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