human reproduction open

ORIGINAL ARTICLE

Antioxidant pretreatment for male partner before ART for male factor subfertility: a randomized controlled trial

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STUDY QUESTION: Does oral antioxidant pretreatment for the male partner improve clinical pregnancy rate in couples undergoing ART for male factor subfertility?

SUMMARY ANSWER: There was no significant difference in clinical pregnancy rate following oral antioxidant pretreatment for male partner in couples undergoing ART for male factor subfertility compared to no pretreatment.

WHAT IS KNOWN ALREADY: Damage to sperm mediated by reactive oxygen species (ROS) contributes significantly to male factor infertility. The ROS-related injury reduces fertilization potential and adversely affects the sperm DNA integrity. Antioxidants act as free radical scavengers to protect spermatozoa against ROS induced damage. During ART, use of sperms which have been exposed to ROS-mediated damage may affect the treatment outcome. Pretreatment with antioxidants may reduce the ROS-mediated sperm DNA damage. Currently, antioxidants are commonly prescribed to men who require ART for male factor subfertility but there is ambiguity regarding their role.

STUDY DESIGN, SIZE, DURATION: This was an open label, randomized controlled trial conducted at a tertiary level infertility clinic between February 2013 and October 2019. The trial included 200 subfertile couples who were undergoing ART treatment for male factor subfertility.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Couples were randomized into treatment arm (n = 100) and control arm (n = 100). In the treatment arm, the male partner received oral antioxidants (Vitamin C, Vitamin E and Zinc) for 3 months just prior to the ART cycle. In the control arm, no antioxidant was given to the male partner. The primary outcome was clinical pregnancy rate, while live birth rate (LBR), miscarriage rate and changes in semen parameters were the secondary outcomes.

MAIN RESULTS AND THE ROLE OF CHANCE: Out of 200 women randomized, 135 underwent embryo transfer as per protocol. Following intention to treat analysis, no significant difference was noted in clinical pregnancy (36/100, 36% vs 26/100, 26%; odds ratio (OR) 1.60, 95% CI 0.87 to 2.93) and LBR (25/100, 25% vs 22/100, 22%; OR 1.18, 95% CI 0.61 to 2.27) between antioxidant and no pretreatment arms. The clinical pregnancy rate per embryo transfer was significantly higher following antioxidant pretreatment (35/64, 54.7% vs 26/71, 36.6%; OR 2.09, 95% CI 1.05 to 4.16) compared to no pretreatment. There was no significant difference in LBR per embryo transfer (25/64, 39.1%, vs 22/71, 31.0%; OR 1.43, 95% CI 0.70 to 2.91) after antioxidant pretreatment versus no pretreatment. The semen parameters of sperm concentration (median, interquartile range, IQR) (18.2, 8.6 to 37.5 vs 20.5, 8.0 to 52.5, million/ml; $P = 0.97$), motility (median, IQR) (34, 20 to 45 vs 31, 18 to 45%; $P = 0.38$) and morphology (mean ± SD) (2.0 ± 1.4 vs 2.2 ± 1.5%; $P = 0.69$) did not show any significant improvement after intake of antioxidant compared to no treatment, respectively.

LIMITATIONS, REASONS FOR CAUTION: The objective assessment of sperm DNA damage was not carried out before and after the antioxidant pretreatment. Since the clinicians were aware of the group allotment, performance bias cannot be ruled out.

WHAT DOES THIS MEAN FOR PATIENTS?

Sperm can be injured by unstable molecules (free radicals) and this is termed oxidative injury. Using sperm affected by oxidative injury might affect the success rates of fertility treatment such as ART. As antioxidants are thought to reduce oxidative injury, they are commonly prescribed to men who require ART due to male factor subfertility. However, there is no clear evidence that such use of antioxidants actually improves the success rates of fertility treatment. Therefore, we invited 200 couples who were having ART due to male factor subfertility to be randomly allocated to treatment and control arms and they were aware of which arm they were allocated into. Men in the treatment arm received oral antioxidants (Vitamin C, Vitamin E and Zinc) for 3 months prior to ART cycle. Men in the control arm did not receive any antioxidants. We found no significant improvement in clinical pregnancy rate (36% compared to 26%) with the use of antioxidants. These findings need to be rechecked in a larger trial using similar pills which contain either antioxidant or no active ingredient (dummy pills) so that men taking them are unaware which arm of the trial they fall into.

Introduction

It is estimated that approximately 45–52 million couples are affected by subfertility worldwide (Mascarenhas *et al.*, 2012). Male factor subfertility contributes to approximately half of subfertility cases, either alone or in combination with female factors (Irvine, 1998; Winters and Walsh, 2014). Over the past few decades, a steady decline in semen quality among men has been observed across the world (Levine *et al.*, 2017). Increasingly, it is being suggested that the environmental and lifestyle factors, such as pollution, use of pesticides, smoking, unhealthy diet and exposure to radiation from modern gadgets, may have a role in the reported decline in semen quality (Rubes *et al.*, 2005; Sikka and Wang, 2008; Agarwal *et al.*, 2009).

For idiopathic severe male factor subfertility, ART remains an important treatment option for couples who are not keen on using donor gametes. Over the years, despite advances in ovarian stimulation protocols, *in vitro* laboratory techniques and cryopreservation methods, the live birth rate (LBR) per cycle following ART remains between 22% and 25% (De Geyter *et al.*, 2020). Many clinical adjuncts have been introduced in ART practice in order to improve the treatment outcomes despite a lack of conclusive evidence (Kamath *et al.*, 2019). Empirical use of antioxidants for treating male factor subfertility or its use as an adjunct prior to ART is common in contemporary practice (Eskenazi *et al.*, 2005; Agarwal and Majzoub, 2017).

An increased production of reactive oxygen species (ROS) can lead to cell damage and it is hypothesized that ROS-mediated damage to sperm plays a contributory role in up to 80% of male subfertility cases

(Agarwal *et al.*, 2006). The suggested mechanisms include ROS induced damage to the sperm membrane and sperm DNA, which affects sperm motility, fertilization, embryo development and may lead to early pregnancy loss (Shimura *et al.*, 2002; Robinson *et al.*, 2012; Simon *et al.*, 2014). Studies indicate that sperm exposed to high levels of ROS exhibit reduction in viability and motility as determined through both conventional assessment and the use of computer-assisted sperm motility analysis (Shi *et al.*, 2012; de Castro *et al.*, 2016). Antioxidants are substances which inhibit the oxidation of biological molecules either by free radical scavenging or chelation. Antioxidants protect the sperm against ROS-mediated damage by reducing the production of free radicals or inhibiting oxidation (Agarwal and Saleh, 2002). On the other hand, ROS are important in cell signalling pathways and play a vital role in sperm capacitation and maturation (Ford, 2004). Therefore, antioxidant therapy may not be always harmless and may upset the delicate balance between oxidation and reduction, and therefore be counterproductive (Tsunoda *et al.*, 2014; Henkel *et al.*, 2019).

Earlier studies have used multiple semen parameters as surrogate markers to indicate the effectiveness of antioxidants, such as improvement in sperm motility (Omu *et al.*, 2008; Balercia *et al.*, 2009) or DNA fragmentation index (Greco *et al.*, 2005). Owing to the perceived benefits, antioxidant use prior to ART for male factor subfertility has been promoted in order to improve LBR (Sigman *et al.*, 2006; Tremellen *et al.*, 2007; Kamath *et al.*, 2019). The results of studies evaluating the effectiveness of antioxidant pretreatment prior to ART are conflicting (Kessopoulou *et al.*, 1995; Sigman et al., 2006; Tremellen *et al.*, 2007). A recent update of the Cochrane review on the use of antioxidants for the male partner prior to ART

noted that LBR may increase following pretreatment with antioxidants (Smits et al., 2019). However, the quality of evidence was low as only two randomized controlled trials (RCTs) had reported LBR and further large RCTs were recommended to clarify the role of antioxidants in male subfertility prior to ART.

As highlighted by multiple Cochrane updates, the ambiguity about the role of antioxidants in male subfertility prior to ART continues (Showell et al., 2011, 2014; Smits et al., 2019). To fill in the knowledge gap, we planned an RCT to investigate the effectiveness of antioxidant pretreatment for the male partner prior to ART for male factor subfertility.

Materials and methods

The study was an open label, RCT and was conducted at Christian Medical College, Vellore, India, which is a tertiary level hospital, from February 2013 to October 2019. The institutional review board (IRB) approved the trial, and the trial was registered in the clinical trial registry of India (CTRI/2013/02/003431). This research was funded by an internal fund–Fluid research grant of Christian Medical College, Vellore. Neither participants nor the public were involved in the planning of the study.

Participants

Couples who were scheduled for ART owing to male factor subfertility were invited to participate. For the trial, abnormal semen analysis was defined as follows: mild oligozoospermia with a sperm concentration of more than 5 million/ml and less than 15 million/ml, and/or asthenozoospermia with sperm motility more than 25% and less than 32%, and/or teratozoospermia with sperm morphology of less than 4% (Cooper et al., 2010).

Couples in whom the female partner was over 37 years of age or those who were diagnosed with moderate or severe endometriosis were excluded from the trial. Couples with a male partner whose semen analysis was suggestive of severe male factor, defined as a sperm concentration <5 million/ml (World Health Organization criteria 2010) (Cooper et al., 2010), and those who had taken oral antioxidants in the past 3 months were also excluded.

The eligible couples, who were willing to participate in the trial, were recruited once they booked for ART. Written informed consent was obtained from all the participants. At the time of recruitment into the trial, a questionnaire was completed regarding demographic data, medical and surgical history of the subjects and investigation reports, including baseline semen analysis. Each couple underwent treatment only once during the study.

Sample size calculation

For power analysis, a clinical pregnancy rate of 52% with antioxidant and 30% with no antioxidant was assumed (Tremellen et al., 2007). With a power of 80% using a two-sided χ^2 test with $\alpha = 0.05$, the expected sample size was calculated to be 96 in each arm. The final sample size was calculated as 100 in each arm accounting for possible attrition.

Randomization and allocation concealment

Randomization was performed using computer-generated random numbers at the time of ART booking. Allocation concealment was achieved using opaque sealed envelopes which were sequentially numbered and contained the group code. Patients were allotted into the treatment (antioxidant) or the control (no treatment) arm, depending upon the group code by the principal investigator. In the antioxidant arm, the male partner was prescribed vitamin C, 500 mg (Tab. Celin, Glaxo Smithkline, India), vitamin E, 400 mg (Tab. Evion, Merck Consumer Healthcare, Ltd, India) and Zinc, 140 mg (Tab. Zincolak, Menarini Pharmaceuticals, India) to be taken once daily orally for 3 months prior to the ART treatment. Initiation of the ART cycle was planned within 1 month after completion of antioxidant pretreatment, with a maximum time lag of 3 months permitted to allow for unforeseen delays in the start of the ART cycle because of cycle programming issues. However, a delay of more than 3 months from the completion of antioxidant pretreatment to the initiation of the ART cycle was categorized as protocol deviation. In the control arm, the male partner was not given any antioxidant or placebo. The male partner underwent semen analysis during treatment booking and a repeat semen analysis was performed just prior to initiation of ART.

ART protocol

The ART treatment was carried out as per the institutional protocol. The downregulation was achieved using standard GnRH agonist, ultra long or GnRH antagonist protocols. For controlled ovarian stimulation, 100–300 IU of recombinant FSH (Gonal-f, follitropin alfa, Merck Serono, Inc. Rockland, USA or Recagon, follitropin beta, Scherring-Plough, USA) was used, and follicular monitoring was by serial ultrasound. Once three or more follicles with diameter greater than 17 mm were seen, ovulation trigger with recombinant hCG (250 μg) (Ovitrelle, Merck Serono, Inc. Rockland, USA) or GnRH agonist trigger (2 mg) (Leuprolide acetate) (Lupride, Sun Pharmaceuticals Industries, Ltd, India) was administered s.c. Transvaginal oocyte retrieval was performed under conscious sedation 35–36 h following the trigger.

Fertilization was achieved by ICSI. The embryo quality was assessed using morphological grading and between one and three embryo(s) were transferred either at cleavage stage (Day 2 or Day 3) or at blastocyst stage (Day 5). In case, there was no fresh embryo transfer, elective cryopreservation at the cleavage or blastocyst stage followed by a frozen embryo transfer was planned.

Following fresh embryo transfer, luteal support was continued until the pregnancy test. Women were advised micronized progesterone vaginally, 400 mg (Naturogest, Zydus Healthcare, Ltd, India) twice daily and i.m. progesterone 100 mg (Gestone, Ferring Pharmaceuticals, India) twice weekly.

In the group of women who underwent frozen embryo transfer the endometrium was prepared using escalating doses of oestrogen (oestradiol valerate 2 mg, Progynova, Bayer plc, Germany) which was started on day 1 of the menstrual cycle and the ultrasound was performed on Day 15 to measure endometrial thickness and confirm ovarian suppression. Micronized progesterone (400 mg) was administered vaginally when the endometrial thickness was ≥7 mm and embryo transfer was carried out on Day 3, 4 or 6 from the day of initiating progesterone, depending on the stage at which embryos were stored. The luteal support protocol for frozen cycles was similar

to fresh cycles except for the addition of oestrogen (oestradiol valerate, 2 mg three times daily).

Serum beta-hCG was assessed on the 18th day after the start of progesterone administration in both fresh and frozen embryo transfer cycles. For those women with positive results (serum beta-HCG > 5 mIU/l), luteal support was continued and a tranvaginal ultrasound was performed 2 weeks later to document clinical pregnancy. For women with an intrauterine clinical pregnancy, luteal support was given until 12 weeks of gestation, when they were referred to obstetric units for further antenatal care. Outcomes were followed up until childbirth through email and telephone.

Outcomes

The primary outcome was clinical pregnancy rate, which was defined as a pregnancy diagnosed by ultrasound evidence of one or more intrauterine gestational sacs, including a clinically documented ectopic pregnancy (Zegers-Hochschild et al., 2017). Secondary outcomes included miscarriage rate, fertilization rate, ongoing pregnancy, LBR per embryo transfer and changes in semen parameters.

Fertilization rate was defined as the proportion of injected oocytes with two pronuclei the day after ICSI (ESHRE Special Interest Group of Embryology; Alpha Scientists in Reproductive Medicine, 2017). Miscarriage was defined as the spontaneous loss of a clinical pregnancy before 22 completed weeks of gestational age (Zegers-Hochschild et al., 2017). Ongoing pregnancy was defined as a viable intrauterine gestation of at least 12 weeks (Braakhekke et al., 2014). Live birth was defined as birth of a live foetus after 22 completed weeks (Zegers-Hochschild et al., 2017). Changes in semen parameters were assessed in terms of volume, sperm concentration, progressive motility and morphology.

Statistical methods

For continuous data, the descriptive statistics mean, SD and, for non-normally distributed interval data and ordinal data, median, interquartile range (IQR) was reported. Frequency and percentage were reported for categorical data. The parametric Student's *t*-test, the non-parametric Mann–Whitney *U* test and the Pearson χ^2 test were applied to determine the difference between both groups for baseline parameters. The odds ratio (OR) and 95% CI were calculated to estimate the treatment effect on the primary and secondary outcomes. The change in semen parameters was calculated between baseline and post-intervention semen parameters. The Student's *t*-test and non-parametric Mann–Whitney *U* test was used to find out the difference in change between the two groups. All tests were two-sided at $\alpha = 0.05$ level of significance. All analyses were carried out using Statistical Package for Social Sciences (SPSS) software Version 21.0 (Armonk, NY, USA: IBM Corp).

Results

A total of 223 eligible couples were invited to participate in the trial, out of which 23 declined to participate. Finally, 200 couples who were willing to participate were randomly assigned into treatment (n = 100) and control arms (n = 100). A total of 65 couples (36 in antioxidant and 29 in the control arm) did not undergo ART, deviated from the protocol or had a cancellation of the treatment cycle before oocyte retrieval or embryo transfer. In the treatment arm, 25 couples did not return for commencing their ART treatment, six had cancellation before oocyte retrieval, two had no embryos for transfer and three deviated from the protocol. Amongst the 75 couples in the antioxidant arm, 69 had their ART treatment initiated within the month after the completion of antioxidant pretreatment, three had their treatment initiated within 2 or 3 months of ending the pretreatment and a remaining three couples deviated from the protocol. Similarly, in the control arm, 21 couples did not commence ART treatment and eight had the cancellation before oocyte retrieval. The reason for the cancellation included poor response, asynchrony and low oestradiol levels (Fig. 1). The overall attrition rate was high (32.5%, 65/200).

The baseline clinical characteristics, including age, BMI, duration of infertility and number of previous ART attempts were similar in both the groups (Table I). The baseline treatment characteristics were available for those couples who underwent ART (75 in antioxidant and 79 in the control arm) (Table II). There were no significant differences between the two arms in terms of treatment variables, such as protocols, total gonadotrophin usage, number of oocytes retrieved, day of transfer and fresh versus frozen cycles (Table II). In total, 135 couples (64 in the treatment arm and 71 in no treatment arm) underwent an embryo transfer with no deviation from the trial protocol. Thirty-five clinical pregnancies were recorded in the antioxidant arm with nine miscarriages and one ectopic pregnancy resulting in 25 live births among couples who did not deviate from the protocol. Among the three couples who had deviated from the protocol in the antioxidant arm, one clinical pregnancy was recorded which resulted in a miscarriage. Of the 26 clinical pregnancies reported in the no pretreatment arm, there were two miscarriages, one ectopic pregnancy and one still birth resulting in 22 live births (Table III).

Intention to treat analysis

We performed an intention to treat (ITT) analysis by including all the randomized couples (100 in each arm). We found no significant differences in clinical pregnancy (36/100, 36% vs 26/100, 26%; P = 0.13; OR 1.60, 95% CI 0.87 to 2.93), ongoing pregnancy (25/100, 25% vs 23/100, 23%; P = 0.74; OR 1.12, 95% CI 0.58 to 2.14) or LBR (25/100, 25% vs 22/100, 22%; P = 0.62; OR 1.18, 95% CI 0.61 to 2.27) per woman randomized after antioxidant pretreatment versus no pretreatment (Table III).

Modified ITT

We performed a modified ITT including those couples for whom ART treatment was initiated (75 in antioxidant and 79 in the control arm) while excluding those who did not turn up for treatment after randomization. There was a trend towards a higher clinical pregnancy rate in the antioxidant pretreatment arm compared with no pretreatment arm, but the difference was not statistically significant (36/75, 48.0% vs 26/79, 32.9%; P = 0.05; OR 1.88, 95% CI 0.98 to 3.61). There was no difference in the ongoing pregnancy rates (25/75, 33.3% vs 23/79, 29.1%; P = 0.57; OR 1.22, 95% CI 0.62 to 2.41). The modified ITT analysis also did not show any difference in LBR between the two arms (25/75, 33.3% vs 22/79, 27.8%; P = 0.46; OR 1.30, 95% CI 0.65 to 2.58) (Table IV).
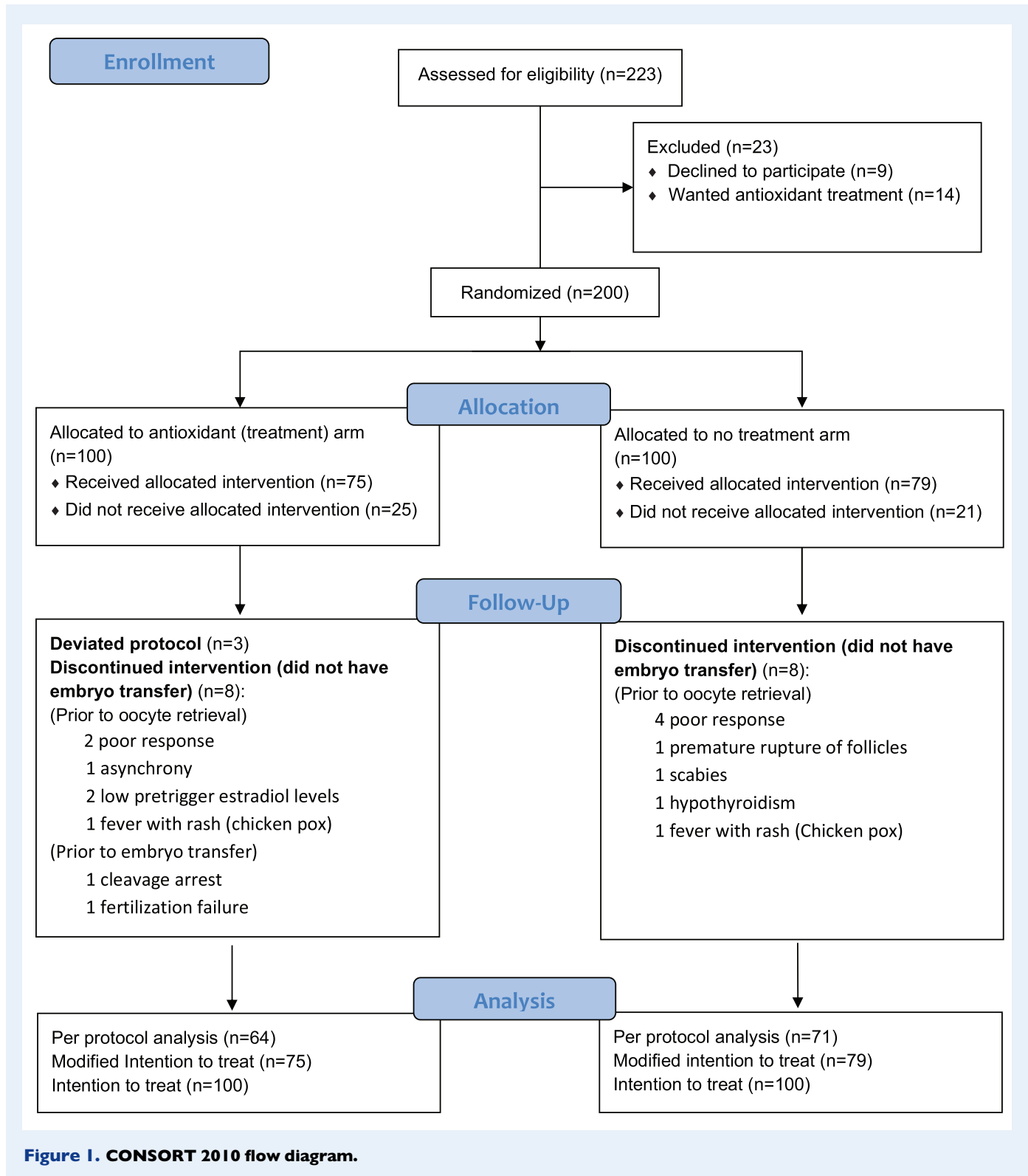
Figure 1. CONSORT 2010 flow diagram.

Per-protocol analysis

Per protocol analysis included all those couples who underwent embryo transfer except those who deviated from protocol (64 in antioxidant and 71 in the control arm). The clinical pregnancy per embryo transfer was significantly higher in the antioxidant arm (35/64, 54.7% vs 26/71, 36.6%; $P = 0.04$; OR 2.09, 95% CI 1.05 to 4.16) when compared with no pretreatment. The ongoing pregnancy rate per embryo transfer (25/64, 39.1% vs 23/71, 32.4%; $P = 0.42$; OR 1.34, 95% CI

Table I Baseline comparison of clinical characteristics between the groups.

	Antioxidant arm (n = 100)	No treatment arm (n = 100)	*P*-value
Male age (years)[a]	37.28 (3.9)	37.48 (4.9)	0.75
Female age (years)[a]	31.27 (3.8)	31.66 (3.8)	0.47
Male BMI[a]	26.92 (3.9)	26.42 (3.3)	0.34
Female BMI[a]	25.9 (4.1)	25.7 (4.5)	0.65
Duration of infertility (years)[b]	7 (5, 10)	8 (5, 11)	0.43
Type of infertility[c]			
Primary	69 (69)	74 (74)	0.57
Secondary	31 (31)	26 (26)	
ART cycle[c]			
1	80 (80)	80 (80)	0.57
2	19 (19)	17 (17)	
3	1 (1)	3 (3)	

[a]Presented as mean (SD).
[b]Presented as median (interquartile range, IQR); IQR (25th percentile, 75th percentile).
[c]Presented as frequency (percentage).

Table II Baseline comparison of ART treatment characteristics between the two groups.

	Antioxidant arm (n = 75)	No treatment arm (n = 79)	*P*-value
ART protocol[a]			
Antagonist	55 (73.3)	58 (73.4)	0.37
Long agonist GnRH	12 (16.0)	16 (20.3)	
Short GnRH	5 (6.7)	5 (6.3)	
Ultra long depot GnRH	3 (4.0)	0 (0)	
Total dose of gonadotrophins[b]	2086.99 (800.8)	2199.31 (873.9)	0.42
No. of oocytes[c]	8 (4, 13)	9 (5, 14)	0.34
Day of embryo transfer[a,d]			
Cleavage stage	46/67 (68.7)	41/71 (57.7)	0.18
Blastocyst stage	21/67 (31.3)	30/71 (42.3)	
Number of embryos transferred[b,d]	2.04 (0.6)	2.0 (0.6)	0.65
Type of embryo transfer[a,d]			
Fresh transfer	46/67 (68.7)	42/71 (59.2)	0.25
Frozen transfer	21/67 (31.3)	29/71 (40.8)	

[a]Presented as frequency (percentage).
[b]Presented as mean (SD).
[c]Presented as median (IQR); IQR (25th percentile, 75th percentile).
[d]Calculated per embryo transfer.

0.66 to 2.71) and the LBR per embryo transfer did not differ significantly between the two arms (25/64, 39.1% vs 22/71, 31.0%; *P* = 0.33; OR 1.43, 95% CI 0.70 to 2.91) (Table V).

No significant differences were noted in sperm concentration, progressive motility or morphology between semen analyses performed at baseline and after 3 months of antioxidant therapy (Supplementary Table SI). We performed a *post hoc* analysis within the antioxidant arm comparing ART outcomes between those who had an improvement in sperm motility with antioxidants with those who did not show an improvement. We found no significant difference in clinical pregnancy, ongoing pregnancy or LBR between those who had improved progressive motility as compared to those with no improvement following antioxidant pretreatment (Supplementary Table SII).

Discussion

The current trial found no statistically significant difference in clinical pregnancy rate in women undergoing ART owing to male factor subfertility following antioxidant pretreatment in the male partner compared to no pretreatment, as per ITT analysis. Furthermore, the ongoing pregnancy and LBR per woman randomized did not differ significantly following antioxidant pretreatment versus no pretreatment. Per protocol analysis revealed a significantly increased clinical pregnancy rate per embryo transfer in the antioxidant pretreatment arm compared to the control arm, but this did not translate into higher ongoing pregnancy and LBR. There was no significant improvement observed in semen parameters following 3 months of antioxidant pretreatment.

Earlier studies have compared different antioxidants, (Carnitine, Coenzyme Q and Zinc), either alone or in combination against placebo or no treatment for male factor subfertility (Omu *et al.*, 1998; Balercia *et al.*, 2005, 2009; Busetto *et al.*, 2017). Most of these studies have focused on the efficacy of antioxidant in improving semen parameters and increasing chances of natural conception, with only a few studies evaluating the role of antioxidants in male factor subfertility prior to ART (Kessopoulou *et al.*, 1995; Sigman *et al.*, 2006; Tremellen *et al.*, 2007; Exposito *et al.*, 2016). In a double blind RCT, investigators compared Menevit (Lycopene 6 mg, vitamin E 400 IU, vitamin C 100 mg, zinc 25 mg, Selenium 26 µg, Folate 0.5 mg, Garlic 1000 mg; n = 40) with identical placebo (n = 20) in severe male factor subfertility prior to ART (Tremellen *et al.*, 2007). Men who had evidence of significant oxidative stress (poor sperm morphology, motility or membrane stability) or increased DNA fragmentation were included and the primary outcome was the number of good quality embryos. No significant difference was found in the number of good quality embryos between the two trial arms. However, the authors calculated viable pregnancy rate (viable pregnancy at 13 weeks by number of transferred embryos) and reported a significantly higher rate following antioxidant compared to the placebo arm (20/52, 38.5% vs 4/25, 16%; *P* = 0.04). The study was included in the recent Cochrane update, and the clinical pregnancy rate per woman randomized did not differ between the two arms (21/40 vs 6/20; Peto OR 2.44, 95% CI 0.84 to 7.13) (Smits *et al.*, 2019). The current study also reports a significantly higher clinical pregnancy rate following antioxidant pretreatment when per protocol analysis (clinical pregnancy per embryo transfer) was performed, but the difference was no longer significant in the ITT analysis. A recent RCT, published as a conference abstract, evaluated the role of vitamin E prior to ART (Exposito *et al.*, 2016). The male partner was given oral vitamin E (n = 55), 300mgs once daily, 3 months prior

Table III Outcomes compared between groups calculated per intention to treat.

Outcomes	Treatment arm n = 100 (%)	No Treatment arm n = 100 (%)	P-value	Odds ratio	95% CI
Clinical pregnancy	36 (36)	26 (26)	0.13	1.60	0.87, 2.93
Ongoing pregnancy	25 (25)	23 (23)	0.74	1.12	0.58, 2.14
Live birth	25 (25)	22 (22)	0.62	1.18	0.61, 2.27

Table IV Outcomes compared between groups calculated per modified intention to treat.

Outcomes	Treatment arm n = 75 (%)	No Treatment arm n = 79 (%)	P-value	Odds ratio	95% CI
Clinical pregnancy	36 (48.0)	26 (32.9)	0.05	1.88	0.98, 3.61
Ongoing pregnancy	25 (33.3)	23 (29.1)	0.57	1.22	0.62, 2.41
Live birth	25 (33.3)	22 (27.8)	0.46	1.30	0.65, 2.58

Table V Outcomes compared between groups, calculated per embryo transfer (per protocol).

Outcomes	Antioxidant arm n = 64 (%)	No treatment arm n = 71 (%)	P-value	Odds ratio	95% CI
Clinical pregnancy	35 (54.7)	26 (36.6)	0.04	2.09	1.05, 4.16
Ongoing pregnancy	25 (39.1)	23 (32.4)	0.42	1.34	0.66, 2.71
Live birth	25 (39.1)	22 (31.0)	0.33	1.43	0.70, 2.91
Miscarriage[a]	9 (25.7)	2 (7.7)	0.09	4.15	0.81, 21.19
Fertilization rate[b]	76.93 (20.9)	78.07 (18.3)	0.74	−1.15[c]	−7.84, 5.55

[a]Calculated per clinical pregnancy.
[b]Presented as mean (SD).
[c]Mean difference.

to ART while in the control arm (n = 59), placebo was given. The study included a mixed population of men with normozoospermia, oligozoospermia and asthenozoospermia. The authors reported significantly higher pregnancy rates in the antioxidant arm (45.2% vs 25%; P = 0.04). The authors also reported a significant improvement in sperm concentration and proportion of progressive motile sperm following antioxidant pretreatment compared to placebo. The findings are in partial agreement with current study results. The possible reason for the disagreement could be the inclusion of men with normal and abnormal semen parameters which precluded its inclusion in the Cochrane update as well. It was also unclear whether ITT or per-protocol analysis was performed in the Exposito et al. (2016) study.

The recent Cochrane update evaluated the role of antioxidants in male subfertility and included 61 trials, of which four trials evaluated the role of antioxidants before ART (Smits et al., 2019). Pooled data from two trials (n = 90) showed a significantly higher LBR following antioxidant (Peto OR 3.61, 95% CI 1.27 to 10.29) compared to control arm. The clinical pregnancy rate did not differ significantly between the two arms when the results of two trials were pooled (Peto OR 2.64,

95% CI 0.94 to 7.41) (Smits et al., 2019). Even though a large knowledge gap exists regarding the role of antioxidants as a pretreatment prior to ART, as mentioned in the first Cochrane review and subsequent updates in 2014 and 2019, the paucity of trials still continues (Showell et al., 2011, 2014; Smits et al., 2019).

An earlier RCT (n = 45) evaluated antioxidants (zinc) alone or in combination (zinc with vitamin C and E) versus no treatment in men with asthenozoospermia with normal sperm concentration (Omu et al., 2008). The oxidative stress was assessed by estimating the serum and seminal plasma levels of Malone dialdehyde and tumour necrosis factor-alpha. The authors reported significantly improved semen parameters and a reduction in oxidative stress after 3 months of antioxidant therapy. The conflicting finding could be due to differences in the study population and variation in dosages of antioxidants. Another study evaluated the role of antioxidant in men (n = 38) with elevated DNA fragmentation levels (≥15% TUNEL test: Greco et al., 2005). These men were given vitamin C (1 g) and vitamin E (1 g) for 2 months after one failed ART attempt. Repeat semen analysis and a DNA fragmentation test were performed after 2 months of antioxidant therapy

and a second cycle of ART ICSI with ejaculate spermatozoa was offered in those cases where a reduction in sperm DNA damage was observed. The authors reported a significant reduction in percentage DNA fragmentation in 76% men and an improved clinical pregnancy rate (48.2% vs 6.9%) following the second ART cycle. However, no significant improvement was noted in any of the semen parameters following antioxidant therapy, which is in agreement with current study results. The recent high quality, double blinded, placebo-controlled trial (MOXI trial) which evaluated antioxidant therapy for male subfertility in a non-ART population (n = 174) also did not report any improvement in semen parameters following a combination of antioxidants, which is similar to current study results (Steiner *et al.*, 2020).

Many earlier studies have shown an improvement in semen parameters and a reduction in oxidative stress after antioxidant therapy and have suggested its possible role in the treatment of male subfertility (Omu *et al.*, 1998; Balercia *et al.*, 2005; Sigman *et al.*, 2006; Smits *et al.*, 2019). It is suggested that antioxidant therapy may help to increase natural conceptions by increasing the proportion of genetically competent sperm with intact DNA. However, this hypothesis may not be applicable in the ART population undergoing ICSI treatment for male subfertility. Even if the proportion of genetically competent sperm increases following antioxidant pretreatment, the uncertainty about the genetic potential of the individual sperm used during ICSI remains. ICSI is an operator-dependent treatment, with sperm selection reliant primarily on morphological assessment before injection, with no information on actual DNA status: this may be one of the possible reasons for the apparent lack of benefit of antioxidant pretreatment before ART-ICSI cycles.

The current study is the largest trial evaluating the role of antioxidant pretreatment before ART in male subfertility. Clinically relevant outcomes, such as clinical pregnancy and LBR, were reported. The study was initiated in 2013 and the proposed duration was 2 years. As the recruitment was slower than anticipated, the study duration had to be extended by an additional 3 years to reach the planned sample size. However, there were no major protocol changes during the study period. One of the main reasons for slower recruitment was the reluctance of participants to join the trial once they had learned about possible benefits and the relative absence of side effects of antioxidants from the information sheet. While the planned sample size was large and adequately powered, the high dropout rate in both arms reduced the power of the study, as revealed by *post hoc* analysis (58%). We performed an ITT analysis to reduce attrition bias, but we have additionally reported a modified ITT analysis to include those who commenced their ART cycle. We were unable to confirm whether the included participants complied with the advice related to antioxidant intake (dosage and duration) in the intervention arm. There is a possibility that the men in control arm may have taken antioxidants over the counter, but concealed the information from the investigators. The proportion of blastocyst transfer was higher in the control arm. Even though the difference was not statistically significant, its impact on the ART treatment outcomes cannot be ruled out as blastocyst transfer is associated with a higher LBR (Glujovsky *et al.*, 2016). We did not directly assess the sperm DNA oxidative damage by tests such as oxidized deoxynucleoside, 8-oxo-7,8-dihydro 2′ deoxyguanosine (8-OHdG) owing to complex nature of the test and lack of standardization (Tremellen, 2008). Since sperm DNA damage can be caused by oxidative and non-oxidative stress (caused by incomplete sperm protamination or aberrant apoptosis), the use of DNA fragmentation tests may not be the perfect method for identifying men with high sperm DNA damage related to oxidative stress. Furthermore, we did not perform DNA fragmentation assay as the clinical utility of these tests prior to ART is very limited (Practice Committee of the American Society for Reproductive Medicine, 2013, 2015). The available tests for assessing DNA fragmentation lack standardized protocols or clinically relevant cut off limits for predicting pregnancy or choice of ART treatment, which limits their wider applicability (Barratt *et al.*, 2010; Cissen *et al.*, 2016). While the inclusion of DNA fragmentation test could have thrown more light on the impact of antioxidants on oxidative stress, we focused on capturing the clinically relevant outcomes of clinical pregnancy and live birth. Finally, this was an open label trial and both participants and clinicians were aware of group allotment. While this could introduce performance bias, the risk of detection bias would be low due to the objective nature of the outcomes assessed, such as clinical pregnancy and live birth.

Conclusion

The present study did not show a significant difference in clinical pregnancy and LBR in women undergoing ART for male subfertility following antioxidant pretreatment in the male partner versus no pretreatment. Furthermore, there was no improvement in semen parameters following antioxidant pretreatment compared to no pretreatment. There is a need to further investigate the current study findings with an adequately powered, multicentre, placebo-controlled randomized trial. Since the study population included infertile men with unknown DNA fragmentation status, the current findings may not be applicable to infertile men with significantly high DNA fragmentation. Future RCTs should explore the utility of antioxidants in the subset of infertile men with abnormal semen showing high levels of oxidative stress prior to ART.

Supplementary data

Supplementary data are available at https://hropen.oxfordjournals.org/.

Data availability

The data underlying this article cannot be shared publicly due to the privacy concerns of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author subject to local regulatory approvals.

Acknowledgements

Authors' roles

M.S.K. conceived the idea and designed the study along with M.M. and A.T.K. M.M., M.K. and T.J. were involved in recruitment and data

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