ORIGINAL ARTICLE

Antioxidant-Treated Sperm Enhances Fertilization and Early Embryogenesis in Intracytoplasmic Sperm Injection (ICSI) Cycles

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ABSTRACT

Background: Oxidative stress (OS) is a major factor influencing sperm quality and has been implicated in 30–80% of male factor infertility cases, often resulting in compromised fertilization capacity and diminished embryo quality in assisted reproductive technology (ART). While antioxidant supplementation has demonstrated beneficial effects on sperm parameters, particularly in European and American populations, evidence from Asian cohorts remains scarce. Moreover, the role of antioxidants in modulating early embryogenesis following Intracytoplasmic Sperm Injection (ICSI) is not yet well established. This study aimed to evaluate the association between antioxidant supplementation, specifically PROfertil®**,** and early embryogenesis outcomes in Malaysian couples affected by Oligoasthenoteratozoospermia (OAT). **Methods:** A single-center retrospective analysis was conducted on 195 female partners of OAT-affected males who underwent ART treatment between 2016 and 2021 at the Reproductive Medicine Unit, Hospital Tunku Azizah. Male partners in the treatment group received PROfertil® (two capsules daily for three months) prior to ART, while those in the control group received no supplementation. A total of 783 oocytes were retrieved from the treatment group and 796 from the control group. Statistical anaylsis were done on the early embryogenesis including fertilization, cleavage and blastocyst rates as well as embryo quality. **Results:** Comparative analysis using Kruskal-Wallis and Chi-Square tests revealed significant differences. Demographic analysis showed statistical differences in husband's age and the etiology of infertility ($p<0.05$). The treatment group exhibited higher percentages of fertilization, cleavage on day 2, good cleavage on day 3, as well as quality embryo grading, including implantation potential on day 3 ($p < 0.05$). However, statistical analysis showed significant differences on day 5 in the treatment group compared to control group, with fewer good-quality blastocysts observed ($p = 0.03$). **Conclusion:** The findings suggest that antioxidant supplementation with PROfertil® improves the sperm quality and enhancing early embryogenesis outcomes in couples struggling with male factor infertility, particularly OAT-related cases. Further large-scale prospective studies are warranted to validate these results and to establish the clinical relevance of antioxidant supplementation in ART practice.

INTRODUCTION

A delicate balance of reactive oxygen species (ROS) is essential for proper sperm function. However, excessive ROS levels can detrimentally affect sperm quality and fertilization capacity. Oxidative stress (OS) has garnered significant attention due to ROS and their byproducts damaging DNA, lipids, proteins, and enzymatic systems, leading to cell death and deteriorating semen parameters associated with male infertility (Dutta et al., 2019). Antioxidant supplementation protects sperm from oxidative stress by neutralizing harmful free radicals, which helps to preserve sperm's structural integrity, DNA integrity, motility, and overall function. Commonly used antioxidants include vitamin E, vitamin C, carnitines, N-acetyl cysteine, CoQ10, zinc, selenium, folic acid, and lycopene, either as monotherapy or in combination.

A comprehensive review by Majzoub and Agarwal (2018) highlighted the positive impact of antioxidants on male fertility across 26 studies, covering basic semen parameters, advanced sperm function, and outcomes of assisted reproductive technology (ART). A Cochrane review by Smits et al. (2019) reported that antioxidants were effective in enhancing clinical pregnancy rates compared to placebo or no treatment. This conclusion was based on 105 clinical pregnancies from 786 couples across 11 small studies (Busetto et al., 2018; Barekat et al., 2016; Attallah et al., 2013). A study by Tremellen et al. (2007) stated that antioxidant supplement; Menevit (Bayer, Sydney, Australia), which contained Lycopene 6 mg, Vitamin E 400 IU, Vitamin C 100 mg, Zinc 25 mg, Selenium 26 µg, Folate 0.5 mg, and Garlic 1000 mg showed no significant effect on embryo quality and fertilization rates in IVF-ICSI cycles, but did demonstrate an improvement in pregnancy rates. These studies shown that antioxidant supplementation may positively influence pregnancy rates in assisted reproductive contexts.

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PROfertil® is a formulated antioxidant supplement developed to enhance sperm quality. It comprises eight essential micronutrients: L-carnitine (440 mg), L-arginine (250 mg), zinc (40 mg), vitamin E (120 mg), glutathione (80 mg), selenium (60 µg), coenzyme Q10 (15 mg), and folic acid (800 µg). Previous clinical studies have reported that PROfertil® supplementation significantly improves semen parameters, including concentration, motility, and morphology (Lipovac et al., 2016; Lipovac et al., 2014; Imhof et al., 2012; Schauer, 2011). Reported spontaneous pregnancy rates following six months of supplementation ranged from 25.8% to 27.8% (Imhof et al., 2012; Lipovac et al., 2021). Likewise, Schauer et al. (2011) demonstrated that PROfertil® enhanced sperm morphology and was associated with a 41.18% spontaneous pregnancy rate.

To date, no study has specifically investigated the effects of PROfertil®-treated sperm on embryo development following intracytoplasmic sperm injection (ICSI) cycles. Therefore, the present study aimed to explore the role of PROfertil® in supporting embryonic development after ICSI. Specifically, we evaluated the impact of PROfertil®-treated sperm on early embryogenesis, including fertilization, cleavage, and blastocyst formation rates, as well as overall embryo quality. Following the documented improvements in sperm quality, the subsequent phase of this study involved performing ICSI using oocytes retrieved from the female partners of the same couples undergoing assisted reproductive treatment (ART).

MATERIALS AND METHODS

Study recruitment

This retrospective study analyzed 1,579 oocytes collected from 195 female partners who underwent ICSI at the Reproductive Medicine Unit, Hospital Tunku Azizah (Hospital Wanita dan Kanak-Kanak Kuala Lumpur) between 1 January 2016 and 19 April 2021. Their corresponding male partners, all diagnosed with oligoasthenoteratozoospermia (OAT), were divided into two groups: (i) the treatment group, in which men received two daily capsules of the antioxidant supplement PROfertil® (LenusPharma, Vienna, Austria) for three months as prescribed by a clinician, and (ii) the control group, in which men received no micronutrient supplementation.

The inclusion criteria for male partners were subfertility for at least one year, age between 20 and 50 years, and at least one prior abnormal semen analysis consistent with OAT, defined as low sperm concentration (<15 million/mL), reduced motility (<32%), and normal morphology <4% (Cooper et al., 2010; WHO, 2010). Exclusion criteria included infertile male partners with azoospermia, aspermia, varicocele, or recent urogenital infections. Patients with diabetes, inflammatory disease, chronic kidney or renal disease, or those receiving hormone therapy or antioxidant supplements were also excluded from the study. The inclusion criteria for female partners were couples who underwent an ICSI cycle with an infertile male partner and had been trying to conceive for at least one year. The exclusion criteria included female partners with diminished ovarian reserve or endometriosis.

Ethical approval

Ethical approval was obtained from the Medical Research and Ethics Committee (MREC) and the Clinical Research Centre (CRC), Hospital Tunku Azizah (NMRR-20-2975-57354 [IIR]). All subject information was handled in accordance with the approved protocol, including justification for the inclusion criteria.

Sperm preparation for ICSI

An aliquot of 5-10 µL from the same sperm suspension was used for sperm analysis. Then, 0.5-2 ml of semen sample was prepared using sperm preparation (swim up, density gradient or simple wash) based on the quality of sperm such as concentration and motility. The final diluted sperm was placed at room temperature before use for ICSI.

Oocyte retrieval

The female partners underwent controlled ovarian stimulation with injectable follicle-stimulating hormone (FSH) for approximately 8–12 days until multiple mature follicles developed. In addition to FSH, gonadotropin-releasing hormone (GnRH) agonists or antagonists were administered to prevent premature ovulation. Follicular development was monitored by serial transvaginal ultrasound, and once a dominant follicle measuring ≥18 mm was observed, human chorionic gonadotropin (hCG) was administered to trigger final oocyte maturation. Oocyte retrieval was performed 34–36 hours after hCG administration via transvaginal ultrasound-guided aspiration. The collected follicular fluid was immediately examined by embryologists to identify and isolate oocytes. A total of 783 oocytes were retrieved from the treatment group and 796 from the control group.

Intracytoplasmic sperm injection (ICSI) technique

Mature oocytes at the metaphase II stage were selected for ICSI. The oocytes were stabilized with a holding pipette, and a single sperm was carefully injected into the cytoplasm using a micromanipulation system

(Eppendorf® Micromanipulator, US).

Embryo culture

Injected and fertilized oocytes were incubated in Global total LP medium (LifeGlobal, Belgium) at 37°C with 6% CO2 until day 5 in the same medium and conditions.

Embryo analysis

Fertilization check

The oocytes were examined under an inverted microscope 16–18 hours after injection. Fertilization was confirmed by the presence of two pronuclei (2PN). The fertilization rate (FR) was calculated as the percentage of fertilized oocytes relative to the number of injected oocytes.

Assessment of embryo quality

Embryo development was assessed daily from day 2 to day 5, except on day 4. Observations were performed under a microscope at predetermined time points, as shown in **Table 1**. Embryo cleavage quality was evaluated and recorded according to the criteria in **Table 2**. The grading system was based on the number of cells (blastomeres), cell size and shape, and the percentage of fragmentation (Balaban et al., 2011). On day 5, embryos were assessed for progression to the blastocyst stage and graded using Gardner's classification, as presented in **Table 3**, which includes blastocoele expansion, inner cell mass (ICM) size and trophectoderm (TE) cohesion. Blastocysts graded 3–5AA were considered excellent, those graded 3–4AB/BA/BB were considered good, those graded 3–4BC/CB/CA/AC were considered poor, and those graded 2–4CC/EB were considered very poor.

Embryo transfer

Two types of embryo transfer were performed: fresh embryo transfer (ET) and frozen embryo transfer (FET). ET was carried out when the embryo reached day 2–5, depending on embryo quality and the condition of the uterine cavity. Patients who were not suitable for fresh ET due to factors such as a high risk of ovarian hyperstimulation syndrome (OHSS) or an unfavorable uterine environment were advised to undergo FET. Embryo transfer, either fresh or frozen, was performed on day 2, day 3, or day 5.

Clinical outcomes following ICSI

Serum β-hCG was measured two weeks after ET. A β-hCG level >50 IU/L was considered positive for biochemical pregnancy, and clinical pregnancy was confirmed at the subsequent transvaginal scan (TVS) by the presence of a gestational sac or fetal heartbeat (fetal pole). Live birth was defined as the delivery of a baby born alive. Pregnancy, miscarriage, and live birth rates were recorded.

Statistical Analysis

Data on demographic and baseline characteristics were expressed as numbers and percentages and analyzed using the Kruskal–Wallis test. Early embryogenesis parameters (fertilization, cleavage, blastocyst rates, and embryo quality) were also presented as numbers and percentages and analyzed using Chi-square tests. A p-value <0.05 was considered statistically significant.

Table 1: Timing of observation of fertilized oocytes and embryos, and expected stage of development at each time point. Adapted from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology (2011).

Type of observation	Timing (hours post insemination)	Expected stage of development
Fertilization check	17±1	Pronuclear stage
Syngamy	23±1	Expect 50 % to be in syngamy (up to 20% may be at the 2-cell stage)
Early Cleavage Stage	26±1 (ICSI) 28±1 (IVF)	2 cell stage
Day-2 embryo assessment	44±1	4 cell stage
Day-3 embryo assessment	68±1	8 cell stage
Day-4 embryo assessment	92±2	Morula
Day-5 embryo assessment	116±2	Blastocyst

Table 2: Embryo cleavage scoring system. Adapted from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology (2011)

Grade	Rating	Description
1	Good	• <10% fragmentation • Stage-specific cell size • No multinucleation
2	Fair	• 10– 25% fragmentation • Stage-specific cell size for the majority of cells • No evidence of multinucleation
3	Poor	• Severe fragmentation (>25%) • Cell size not stage specific • Evidence of multinucleation

Table 3: Blastocyst scoring system based on Gardner's classification. Adapted from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology (2011).

Size and expansion	
Classification	**Description**
1	Early blastocyst: the blastocyst is less than half the volume of the embryo.
2	Blastocyst: the blastocele is greater than or equal to half of the volume of the embryo.
3	Full blastocyst: the blastocele completely fills the embryo.
4	Expanded blastocyst: the blastocele volume is larger than that of the early embryo and the zona pellucida is thinning.
5	Hatching blastocyst: the trophectoderm has started to herniate through the zona pellucida.
6	Hatched blastocyst: the blastocyst has completely escaped from the zona pellucida.
Inner cell mass (ICM)	
Classification	**Description**
A	Tightly packed, many cells
B	Loosely grouped, several cells
C	Very few cells
Trophectoderm (TE)	
Classification	**Description**
A	Many cells forming a tightly knit epithelium.
B	Few cells.
C	Very few cells forming a loose epithelium.

Notes: The scoring system for blastocysts is a combination of the size and expansion and of the grade of the ICM and of the TE (e.g., an expanded blastocyst with a good ICM and a good TE would be scored as 4AA).

RESULTS

Patient Demographic and Baseline Characteristics.

A total of 200 subfertile men whose female partners underwent ICSI and had at least one recent abnormal semen analysis at the Reproductive Medicine Unit, Hospital Tunku Azizah, were recruited for this study. Four participants withdrew, leaving 92 infertile men who completed three months of antioxidant supplementation (PROfertil®) and 103 men who did not take any supplements as the control group.

The mean (SD) age of men in the PROfertil® group was 37.04 (6.83), while the control group had a mean age of 35.30 (5.40). **Table 4** summarizes the demographic and baseline characteristics, showing significant differences (p<0.05) in the husbands' age and infertility etiology, while other parameters were similar between groups.

Interestingly, the treatment group was slightly younger, suggesting that younger men may be more proactive in seeking fertility treatment. Additionally, a higher proportion of men (70%) with male factor infertility received PROfertil® compared to those with combined infertility factors (30%) (p=0.02). This indicates that male partners with identified male factor infertility were more likely to undergo antioxidant therapy to improve sperm quality.

Embryo Development Rate

Fertilization and abnormal rate

The fertilization rate was calculated as the number of fertilized oocytes divided by the total number of oocytes observed per group after 16-18 hours of insemination through ICSI. Based on **Table 5**, there was a significant difference between both groups *(p*=0.003).

Abnormal fertilization rate was calculated as the number of fertilised oocytes in which consist of three or more pronuclei (>3PN) or only one pronuclei (1PN) and divided by the total number of oocytes observed per group after 16-18 hours of insemination through ICSI. Based on **Table 5,** there was no significant difference in the abnormal fertilisation rate between the PROfertil® and control groups (p=0.48).

Cleavage Rate

Cleavage rates were calculated by determining the number of embryos at the 4-cell stage on day 2 (44 ± 1 hours after ICSI) and the number of embryos at the 8-cell stage on day 3 (68 ± 1 hours after ICSI), divided by the total number of fertilized oocytes. Good cleavage rates were calculated based on the number of embryos graded as grade 1 or 2 on day 2 and day 3, according to the embryo cleavage scoring system by the Alpha Scientists in Reproductive Medicine and the ESHRE Special Interest Group of Embryology (2011).

Table 4: Demographic and baseline characteristics of the population by treatment group (i.e. PROfertil®) and control group**.**

CHARACTERISTICS	CONTROL (n=103)	PROfertil® (n=92)	*p*-VALUE
Age[a]			
Wife (mean, SD)	34.07 (4.45)	33.05 (3.67)	0.08
Husband (mean, SD)	37.04 (6.83)	35.30 (5.40)	**0.02***
Ethnicity[b]			
Malay (n,%)	64 (62)	59 (64)	0.96
Chinese (n,%)	15 (14.6)	13 (14)	
Indian (n,%)	24 (23.3)	20 (22)	
BMI of wife[b]			
Underweight (n,%)	2(2)	4(4)	0.65
Normal (n,%)	48 (47)	47(51)	
Overweight (n,%)	45 (44)	34 (37)	
Obese (n,%)	8 (8)	7 (8)	
BMI of husband[b]			
Underweight (n,%)	1 (1)	1 (1)	0.11
Normal (n,%)	34 (33)	19 (21)	
Overweight (n,%)	49 (48)	43 (47)	
Obese (n,%)	19 (18)	29 (32)	
Smoking[b]			
Yes (n,%)	12 (12)	16 (17)	0.25
No (n,%)	91 (88)	76 (83)	
Etiology of infertility[b]			
Male (n,%)	56 (54)	64 (70)	**0.02***
Combined (n,%)	47(46)	28 (30)	

Notes: *Significant differences as compared between different groups at $p<0.05$. [a]Data are expressed as mean (SD) and analyzed using Kruskal-Wallis test. [b]Data are expressed as frequency (%) and analyzed using the Chi-Square test. Independent variables were presented as demographic and baseline characteristics (i.e age of wife/husband, ethnicity, etiology of infertility, husband/wife BMI and primary/secondary fertility). Dependent variables were presented as the control or treatment (i.e outcomes).

As shown in **Table 5**, there was a significant difference in cleavage rate on day 2 (p = 0.004), with the treatment group showing a slightly higher percentage compared to the control group (99.8% vs. 98.2%). On day 3, there was also a significant difference in the percentage of good cleavage, with the PROfertil® group showing a higher rate compared to the control group (83.7% vs. 78.0%, p = 0.012). However, no significant differences were observed in good cleavage on day 2 (p = 0.15) or in overall cleavage on day 3 (p = 0.66) between the two groups, as presented in **Table 5**.

Multinucleated blastomeres rate (MNB)

The MNB rate was calculated as the number of embryos that present two, three, or more nuclei in their blastomeres and divided by the number total of fertilized oocytes observed per group on cleavage stage. Based on **Table 5,** there was no significant difference in the rate of MNB on cleavage stage between the PROfertil® and control groups (p=0.32).

Table 5: Comparison of the embryo development rate between the treatment (PROfertil®) and control group.

Fertilization rate	**Control group (n=796)**	**Treatment group (i.e.PROfertil®) (n=783)**	**Relative ratio**	***p* value**
Fertilisation noted 16-18H	568 (71%)	609 (77.8%)	1.09 (95%CI 1.03, 1.15)	**0.003***
Abnormal fertilization	25 (3.1%)	20 (2.6%)	0.81 (95%CI 0.46, 1.45)	0.48
Cleavage rate	**Control group (n=568)**	**Treatment group (i.e.PROfertil®) (n=609)**	**Relative ratio**	***p-value***
Cleavage on Day 2	558 (98.2%)	608 (99.8%)	1.02 (95%CI, 1.0 to 1.03)	**0.004***
Good cleavage Day 2	497 (87.5%)	549 (90.1%)	1.03 (95%CI, 0.99 to 1.07)	0.15
Cleavage on Day 3	563 (99.1%)	605 (99.3%)	1.00 (95%CI 0.99 to 1.01)	0.66
Good cleavage Day 3	443 (78.0%)	510 (83.7%)	1.07 (95%CI, 1.02 to 1.14)	**0.012***
Multinucleated blastomeres (MNB)	7 (1.2%)	12 (2.0%)	1.60 (95%CI 0.63, 4.03)	0.32
Blastocyst rate	**Control group (ᶻ=208)**	**Treatment group (i.e.PROfertil®) (ᶻ=222)**	**Relative ratio**	***p-value***
[1]Blastocyst	98 (47.1%)	85 (38.3%)	0.81 (95%CI 0.65 to 1.01)	0.06
[1]Good Blastocyst	35 (16.8%)	22 (9.9%)	0.59 (95%CI, 0.36 to 0.97)	**0.03***

Notes: Data was presented as numbers (%) and analysed using Chi-Square test; n is the number of injected oocytes following ICSI; ᶻ is the number of embryos pushed to the blastocyst stage; [1]Total of blastocysts formed out of the number of embryos pushed to blastocyst stage; *Significant difference between PROfertil® and control groups $p<0.05$.

Blastocyst Rate

The blastocyst rate was calculated by dividing the number of blastocysts formed on day 5 of culture (116 ± 2 hours after ICSI) by the total number of embryos cultured until day 5 in each group. Good blastocysts were defined as those graded 3–5AA (excellent) or 3–5AB/BA/BB (good). As shown in **Table 5**, the percentage of blastocysts formed was slightly lower in the treatment group compared to the control group (38.3% vs. 47.1%), as was the percentage of good blastocysts (9.9% vs. 16.8%). Statistical analysis revealed a significant difference in the good blastocyst rate between the two groups (p = 0.03).

Grade of quality embryos and blastocyst

Cleavage stage

Embryo cleavage was assessed by grading based on the appearance of blastomeres, cytoplasmic defects, and the degree of fragmentation on day 2 or day 3 of culture. As shown in **Table 6**, the treatment group had a slightly higher number of grade 1 (good) and grade 2 (fair) embryos, and a slightly lower number of grade 3 (poor) embryos on day 2 compared to the control group; however, the difference was not statistically significant. Additionally, the PROfertil® group showed a slightly higher percentage of embryos graded as good and fair for potential implantation compared to the control group (90.3% vs. 89.1%), though this difference was also not significant.

In contrast, **Table 6** demonstrated a significant difference in embryo quality on day 3 (p = 0.039). The treatment group had a higher percentage of embryos graded as good and fair for potential implantation compared to the control group (p = 0.013). The proportion of poor-quality embryos was lower in the treatment group than in the control group on both day 2 and day 3, with a statistically significant difference observed only on day 3 (p = 0.013)

Blastocyst stage

In **Table 6**, no significant difference was observed on the fifth day between the treatment and control groups in terms of the distribution of excellent, good, poor, and very poor blastocysts. Additionally, there was also no significant difference in the percentage of embryos graded as good and poor quality for potential implantation between the two groups.

Table 6: The comparison of the quality of the embryos and blastocyst between the control and treatment groups (PROfertil®)

Grade of quality embryos (Day 2)	**Control group (n=558)**	**Treatment group (n=608)**	**Relative ratio**	***p-value***
Good	61 (10.9%)	65 (10.7%)	-	0.77
Fair	436 (78.2%)	484 (79.6%)	-	
Poor	61 (10.9%)	59 (9.7%)	-	
Considerable for implantation (Good and fair embryo)	497 (89.1%)	549 (90.3%)	1.014 (95% CI, 0.975 to 1.054)	0.49
Not considerable for implantation (Poor embryos)	61 (10.9%)	59 (9.7%)	0.888 (95%CI, 0.632 to 1.246)	
Grade of quality embryos (Day 3)	**Control group (n=563)**	**Treatment group (n=605)**	**Relative ratio**	***p-value***
Good	48 (8.5%)	49 (8.1%)	-	**0.039***
Fair	395 (70.2%)	461 (76.2%)	-	
Poor	120 (21.3%)	95 (15.7%)	-	
Considerable for implantation (Good and fair graded embryo)	443 (78.7%)	510 (84.3%)	1.071 (95% CI, 1.014,1.132)	**0.013***
Not considerable for implantation (Poor embryos)	120 (21.3%)	95 (15.7%)	0.737 (95%CI, 0.578 to 0.940)	
Grade of quality blastocyst (Day 5)	**Control group (ᶻ=98)**	**Treatment group (ᶻ=85)**	**Relative ratio**	***p-value***
[1]Excellent	9 (9.2%)	11 (12.9%)	-	0.12
[1]Good	26 (26.5%)	11 (12.9%)	-	
[1]Poor	18 (18.4%)	15 (17.6%)	-	
[1]Very poor	45 (45.9%)	48 (56.5%)	-	
[1]Considerable for implantation (Excellent and good blastocyst)	35 (35.7%)	22(25.9%)	0.72 (95%CI, 0.46, 1.13)	0.15
Not considerable for implantation (Poor and very poor blastocyst)	63 (74.1%)	63 (64.3%)	1.15 (95%CI 0.95 to 1.40)	

Notes: Data were presented as numbers (%) and analysed using Chi-Square test; n is the number of fertilised oocytes; ᶻ is the number of embryos pushed to the blastocyst stage; [1]Total of blastocysts formed out of the number of embryos pushed to blastocyst stage; *Significant difference between PROfertil® and control groups $p<0.05$

DISCUSSION

The mean age of participants in the treatment group was slightly higher than that of the control group, 37.04 (6.83) versus 35.30 (5.40), respectively, with a statistically significant difference ($p < 0.05$). This finding aligns with demographic trends reported in other fertility studies, where age differences among male partners are often associated with variations in reproductive health–seeking behavior. Although sperm quality is generally believed to decline with advancing age, recent evidence by Pino *et al.* (2020) suggests that a significant reduction in sperm parameters may not occur until after the age of 50. Therefore, the age difference observed between the two

groups in the present study is unlikely to have had a substantial impact on sperm quality outcomes.

Our findings also revealed a significant difference in the etiology of infertility between groups. A larger proportion of men in the treatment group (70%) were diagnosed with male factor infertility compared to those classified under combined infertility factors (30%) (*p*=0.02). This pattern suggests that men with a confirmed male factor diagnosis are more inclined to pursue antioxidant supplementation, such as PROfertil®, in an effort to enhance sperm quality and improve their chances of conception through assisted reproductive technologies.

Various factors contribute to the complex process of fertilization, with sperm quality playing a crucial role. Sperm quality including concentration, motility, and morpholog is critical for successful fertilization (Vogiatzi et al., 2022; Bartolacci et al., 2018; Zhu et al., 2019). As shown in Table 5, fertilization rates were significantly higher in the PROfertil® group compared to the control group (p = 0.003), suggesting that supplementation may enhance sperm–oocyte interaction. This finding is consistent with Lipovac et al. (2014), who reported improved sperm-hyaluronan binding assay (SHBA) values following PROfertil® use, reflecting better fertilization potential. Similarly, Rashki Ghaleno et al. (2016) noted that SHBA outcomes depend on sperm concentration and motility. Supporting evidence from Gharagozloo et al. (2016) demonstrated that supplementation with antioxidants present in PROfertil® (vitamin C, vitamin E, carnitine, folic acid, lycopene, selenium, and zinc) in a mouse model led to a twofold increase in fertilization rates compared to non-supplemented animals (73.7% vs. 35.2%). Collectively, these findings highlight the potential benefits of PROfertil®, likely driven by its antioxidant components, in improving fertilization outcomes.

In our finding revealed no significant difference in terms of abnormal fertilization in both groups (p=0.48) as shown in Table 5. Some previous studies have looked into the effect of antioxidant oral supplements on IVF/ICSI cycle clinical outcomes (Scaruffi et al., 2021; Gambera et al., 2019; Karosi et al., 2017; Rago et al., 2017; Tremellen et al., 2007) but they did not report the abnormal fertilization result on their findings. However, Pantos et al. (2021) found that there was no connection between abnormalities in semen analysis and the rate of 1PN or 3PN formation.

The quality of cleavage embryos is the key determinant of success and reflects their potential to develop into robust blastocysts. As shown in Table 5, no significant difference was observed in cleavage rates on day 3 between the PROfertil® and control groups (p > 0.05). This is consistent with Greco et al. (2005) and Tremellen et al. (2021), who also reported no effect of antioxidant supplementation on day 2–3 cleavage rates. However, significant improvements were found in our study for good cleavage on day 3 (83.7% vs. 78.0%; p = 0.012) and overall cleavage on day 2 (99.8% vs. 98.2%; p < 0.05) in the PROfertil® group. These findings suggest that 3 months of PROfertil® treatment enhances embryo numbers post-ICSI. Supporting evidence comes from Gambera et al. (2019) and Scaruffi et al. (2021), who also observed increased cleavage rates and embryo numbers with different antioxidant supplement combinations.

The Alpha Scientists in Reproductive Medicine and the ESHRE Special Interest Group of Embryology (2011) defined multinucleated blastomeres (MNB) as blastomeres containing two or more interphase nuclei. MNB has been associated with poor embryo growth, aneuploidy, chromosomal abnormalities, and unfavorable IVF outcomes (Balakier et al., 2016; Desai et al., 2016). In our study, no significant difference in MNB incidence was observed between the PROfertil® and control groups (p = 0.32). Desch et al. (2017) reported lower implantation (27.7%) and live birth rates (22.7%) in embryos with MNB compared to those without (33.4% and 29.8%). Some MNB embryos, however, can self-correct during early cleavage, developing into euploid blastocysts and healthy offspring (Egashira et al., 2015; Zhu et al., 2014). Studies on antioxidant supplements and ART outcomes have not specifically reported on MNB (Scaruffi et al., 2021; Gambera et al., 2019; Karosi et al., 2017; Rago et al., 2017; Tremellen et al., 2007).

Fragmentation, cell debris, cell number, size, and symmetry are key markers of embryo quality in ART. In our study, the PROfertil® group showed higher favorable cleavage on day 3 (p = 0.012) and more good or fair-grade embryos suitable for implantation (p = 0.013) compared to controls (Tables 5 and 6). These findings suggest antioxidant supplementation improves embryo quality and increases embryo numbers in ICSI. Similar results were reported by Scaruffi et al. (2021), Rago et al. (2017), and Korosi et al. (2017), who found higher embryo quality with various antioxidant-based supplements. Collectively, this evidence supports the role of PROfertil® in enhancing cleavage and embryo quality. In clinical practice, embryos graded as good or fair are prioritized for transfer due to their higher implantation potential and lower miscarriage risk. The improvements in sperm quality observed in phase one of our study may explain the increased number of top quality embryos, contributing to better IVF outcomes.

Blastocyst transfer allows selection of the most viable embryo, which has high implantation potential and

improves outcomes. In our study, blastocyst development was lower in the treatment group, with fewer good-quality blastocysts compared to controls (Table 5). Statistical analysis showed no significant differences between groups, except for the rate of good blastocyst formation. Previous studies by Glujovsky et al. (2016) and Scaruffi et al. (2021) reported higher live birth rates in fresh blastocyst transfer compared to cleavage-stage transfer, which contrasts with our findings. This difference may be explained by the selection of good embryos at the cleavage stage for transfer, while remaining embryos were frozen for future use. As a result, fewer embryos were available for culture to day 5, and those that continued often had lower quality. Embryos with minimal fragmentation are more likely to reach the blastocyst stage, while those with high fragmentation usually arrest after day 3 (Tan et al., 2019). This indicates that research on the effect of oral supplements at the blastocyst stage remains limited, particularly in Malaysia. Further studies are needed to evaluate the efficacy of PROfertil® on blastocyst outcomes.

Additionally, our study revealed fewer poor-quality embryos in the treatment group compared to the control group on both Day 2 and Day 3 (Table 6). A significant difference was observed in the proportion of good- and poor-quality embryos on Day 3 between the treatment and control groups ($p = 0.013$). This finding is important, as embryos graded as 1 (excellent) or 2 (good) have been shown to exhibit higher implantation success rates than poor-quality embryos (Kirillova *et al.*, 2020). The transfer of poor-quality embryos has also been associated with an increased risk of miscarriage and pregnancy loss (Kirillova *et al.*, 2020; Zhu *et al.*, 2014). These results suggest that the improvement in sperm quality achieved after three months of PROfertil® antioxidant treatment may contribute to enhanced early embryonic development, including better fertilization rates, improved cleavage on Day 2, more favorable cleavage on Day 3, and a higher proportion of good-quality embryos on Day 3. Nevertheless, this study has several limitations, including variations in stimulation dosage among female patients and differences in sperm and oocyte quality.

CONCLUSION

Our study demonstrated that three months of PROfertil® supplementation led to measurable improvements in sperm quality, which translated into enhanced early embryonic development. The treatment group exhibited higher fertilization rates, increased day 2 cleavage, enhanced cleavage on day 3, and a greater number of good- to fair-quality embryos compared to controls. These findings highlight the role of antioxidant supplementation in reducing poor-quality embryos and supporting the development of embryos with higher implantation potential. Although improvements in blastocyst formation were limited, the overall outcomes underscore the potential clinical benefits of PROfertil® in assisted reproductive technology. Further studies with larger cohorts and extended follow-up are warranted to confirm its efficacy and impact on live birth outcomes.

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