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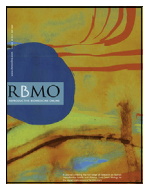

ARTICLE

Prospective randomized multicentre comparison on sibling oocytes comparing G-Series media system with antioxidants versus standard G-Series media system

BIOGRAPHY

David Gardner is a Distinguished Professor in the School of BioSciences, University of Melbourne, and the Scientific Director of Melbourne IVF. David has worked in embryology and IVF for over 35 years and maintains an active research programme in embryo physiology and culture, biomarkers, AI, metaboloepigenetics and blastocyst–endometrial signalling.

David K. Gardner[1,2,*], Takeshi Kuramoto[3], Miho Tanaka[4], Shigetoshi Mitzumoto[3], Markus Montag[5], Atsumi Yoshida[4]

KEY MESSAGE

In a prospective trial, antioxidants in the embryo culture media improved human embryo development in culture and improved transfer outcomes in patients 35 to 40 years old.

ABSTRACT

Research question: Does the inclusion of three antioxidants (A3), acetyl-L-carnitine (ALC), *N*-acetyl-L-cysteine (NAC) and alpha-lipoic acid (ALA) improve human embryo development and pregnancy potential?

Design: Prospective randomized multicentre comparison of sibling oocytes. A total of 1563 metaphase II oocytes from 133 patients in two IVF centres. Day 3 embryo and day 5/6 blastocyst quality were assessed. Good embryo quality on day 3 was defined as 8 to 10 cells with even cells and low fragmentation; good quality blastocysts as 3BB or greater. Clinical outcome was assessed on transfers of fresh or vitrified–warmed blastocyst on day 5.

Results: Of the two-pronuclei, 40.7% (G-Series) and 50.2% (G-Series with A3 group) resulted in good quality embryos on day 3 ($P < 0.05$). The implantation rate by fetal sac was 39.2% and 50.6%, and by fetal heartbeat was 37.8% and 47.1% for the G-Series and G-Series with A3 group, respectively. When stratified by female patient age, patients 35–40 years had an implantation rate by fetal sac and heart of 23.5% in the G-Series compared with 57.5% ($P < 0.05$) and 50.0% ($P < 0.05$) in the A3 group. The ongoing pregnancies in patients 35–40 years were significantly higher in the A3 group (50%) compared with the control (25.8%) ($P < 0.05$).

Conclusions: The presence of antioxidants during IVF and embryo culture for patients 35–40 years resulted in a significant increase in implantation and pregnancy rate. Supplementation of antioxidants to IVF and culture media may therefore improve the viability of human embryos in assisted reproductive technologies, plausibly through the reduction of oxidative stress.

[1] School of BioSciences, University of Melbourne, Parkville, Australia
[2] Melbourne IVF, East Melbourne, Australia
[3] Kuramoto Women's Clinic, Reproductive Medicine, Fukuoka, Japan
[4] Kiba Park Clinic, Reproductive Medicine, Tokyo, Japan
[5] ilabcomm GmbH, Eisenachstrasse 34, Sankt Augustin, Germany

*Corresponding author. E-mail address: david.gardner@unimelb.edu.au (D K.Gardner). https://doi.org/10.1016/j.rbmo.2020.01.026 1472-6483/
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KEYWORDS

acetyl-carnitine
acetyl-cysteine
alpha-lipoic acid
Embryo quality
Gametes, IVF

INTRODUCTION

Successful outcomes following human IVF depend significantly upon laboratory conditions used to support fertilization and embryo development (*Bavister, 1995*; *Gardner and Lane, 2003*; *Swain, 2015*). In support of this, improvements to embryo culture media over the past two decades have greatly improved transfer outcomes (*Gardner and Lane, 2018*; *Gardner et al., 1998*; *Pool, 2002*). However, with success rates of around 30% worldwide (*Wade et al., 2015*), it is evident that more research is required in order to increase live birth rates following IVF.

Gametes and the resultant preimplantation embryo are extremely sensitive to stress, of which there are several sources within the IVF laboratory (*Gardner and Kelley, 2017*; *Swain et al., 2016*; *Wale and Gardner, 2016*). Further, stress is cumulative, and stress from one source, such as oxygen concentration, can greatly amplify the negative effects of another (*Awonuga et al., 2013*), such as embryo density (*Kelley and Gardner, 2017*) or amino acid breakdown (*Wale and Gardner, 2013*). Oxidative stress represents a major source of trauma to gametes and embryos, generated from both oxidative metabolism and exposure to oxygen itself (*Agarwal et al., 2012*). Furthermore, the detrimental effects of oxygen increase with its concentration (*Truong et al., 2016*). Historically, atmospheric oxygen (~20% depending upon altitude) has been used for human IVF and embryo culture, which has been a major contributor to low embryo development and pregnancy rates (*Bontekoe et al., 2012*; *Meintjes et al., 2009*). Oxygen has been shown to impair gametes and preimplantation embryos at many molecular and cellular levels, including perturbed embryo morphokinetics (*Kirkegaard et al., 2013*; *Wale and Gardner, 2010*), gene transcription (*Gardner and Lane, 2005*; *Harvey et al., 2004*; *Kind et al., 2005*; *Rinaudo et al., 2006*), histone remodelling and methylation patterns, i.e. altered epigenetic programming (*Gaspar et al., 2015*; *Ghosh et al., 2017*; *Li et al., 2016*), the proteome (*Katz-Jaffe et al., 2005*) and metabolic state (*Belli et al., 2019*; *Khurana and Wales, 1989*; *Wale and Gardner, 2012*, *2013*).

Even at physiological levels of oxygen within the female reproductive tract of around 5% (*Gardner and Lane, 2017*; *Ng et al., 2018*), oxidative stress still occurs, albeit reduced compared with atmospheric levels of oxygen (*Truong et al., 2016*). Hence, in order to combat oxidative stress, there exists an array of antioxidants *in vivo*, designed to mitigate oxidative damage (*Agarwal et al., 2012*). However, with the exception of pyruvate, which has intrinsic antioxidant properties (*Andrae et al., 1985*), and can decrease intracellular hydrogen peroxide levels within the embryo (*Gardner and Lane, 2017*; *Kouridakis and Gardner, 1995*; *O'Fallon and Wright, 1995*), antioxidants are not typical components of human embryo culture media (*Morbeck et al., 2014*). Further, although more IVF laboratories are now employing a reduced oxygen concentration (~5%) for embryo culture, there are procedures during which gametes and embryos are exposed to atmospheric oxygen, such as gamete retrieval and preparation, intracytoplasmic sperm injection (ICSI), embryo biopsy, embryo assessment (in the absence of time-lapse technology), embryo transfer and embryo cryopreservation. It has been revealed that even a transient exposure of gametes and embryos to 20% oxygen can induce significant increases in intracellular hydrogen peroxide (*Truong and Gardner, 2017*), raising concerns given the cumulative nature of oxidative stress.

The impact of specific exogenous antioxidants on embryo development in culture has been considered for a number of years. Nearly 30 years ago, Noda and colleagues determined that the addition of superoxide dismutase (SOD), which dismutates superoxide radicals, increased the development of mouse zygotes beyond the 2-cell block to the blastocyst stage (*Noda et al., 1991*; *Umaoka et al., 1992*). However, these findings were not readily repeated in other studies (*Payne et al., 1992*), or in other species including rabbit (*Lindenau and Fischer, 1994*) or bovine (*Liu and Foote, 1995*). *Legge and Sellens (1991)* determined that exogenous glutathione stimulated development of mouse zygotes in culture. In contrast, *Nasr-Esfahani and Johnson (1992)* could not repeat these data. Significantly, glutathione is only protective as an antioxidant when in a reduced form (GSH), and GSH is not stable in solution. Therefore, reports that GSH has no benefit on embryo development may well be confounded by its breakdown in an aqueous solution. Of significance is that GSH is present in fluid of the reproductive tract, supporting a role in embryo development (*Gardiner et al., 1998*). Interestingly, the positive effects of cysteamine in media for bovine (*de Matos et al., 1996*, *2003*; *Lim et al., 1996*; *Takahashi et al., 1993*) and porcine (*Yoshida et al., 1993*) oocyte development have been ascribed to increases in intracellular GSH levels (*Caamano et al., 1998*). Consequently, it is plausible that maintenance of a high intracellular pool of GSH supported by the supplementation of its precursors such as cysteine (*Aliciguzel and Aslan, 2004*; *Parsanathan and Jain, 2018*; *Truong et al., 2016*), and/or other antioxidants such as lipoate (which can maintain GSH in its reduced form following oxidation) (*Bilska and Wlodek, 2005*), may be of great significance to embryo development.

The contradictory reports on the effects of antioxidants in embryo culture media can also be explained by their use in isolation, rather than a more complete antioxidant defence system. Recent work has revealed that using groups of, rather than individual, antioxidants can have a dramatic effect on the development and viability of mouse embryos cultured at both 5% and 20% oxygen. Truong and colleagues examined the individual and combined effects of three antioxidants (10 µmol/l acetyl-L-carnitine/10 µmol/l *N*-acetyl-L-cysteine/5 µmol/l alpha-lipoic acid; A3), and determined that while each antioxidant conferred a significant benefit, when the three antioxidants were present together there were even greater positive effects (*Truong et al., 2016*). *Truong et al. (2017)* went on to show that combined antioxidants were of significant benefit during the collection and preparation of gametes for IVF and subsequent embryo culture. Of great clinical relevance, the combined antioxidants conferred significant benefit post-transfer in the mouse, culminating in higher fetal size and weights closer to *in vivo* for embryos cultured in the presence of antioxidants (*Truong et al., 2016*).

Of further significance, when mouse embryos were cultured individually compared with those in groups, atmospheric stress had a greater effect on embryo development (*Kelley and Gardner, 2016*; *Truong et al., 2016*), reconfirming that one stress, i.e. oxidative stress, predisposes the embryo to greater

susceptibility to a second stress, i.e. individual culture (*Kelley and Gardner, 2019*; *Wale and Gardner, 2013*, *2016*). Given the move to single embryo culture in human IVF to facilitate embryo selection through, for example, either time-lapse analysis or preimplantation genetic testing (PGT), the significance of implementing an antioxidant defence system is therefore amplified.

This study reports the effects of a group of three antioxidants in a clinical sibling oocyte randomization study, whereby the effects of the use of a combination of these three antioxidants on human embryo development and subsequent pregnancy could be assessed.

MATERIALS AND METHODS

This was a blinded randomized controlled sibling oocyte study comparing the culture of oocytes and embryos in media with antioxidants (10 µmol/l acetyl-L-carnitine/10 µmol/l *N*-acetyl-L-cysteine/5 µmol/l alpha-lipoic acid; the G-Series with A3), with the standard G-Series group. Of note, some media from the standard G-Series (G-MOPS and G-1) already contain a single antioxidant (ALA). Spermatozoa were therefore not exposed to the antioxidants during their preparation. The study was performed in two centres in Japan: Kiba Park Clinic, Tokyo, and Kuramoto Women's Clinic, Fukuoka. Patients who participated in the study signed an informed consent form. Ethical approval was obtained from the Ethical Committee for Kuramoto Women's Clinic Medical Corporation on 25 November 2016 (approval no. 16002). The study was registered as a randomized controlled trial with ClinicalTrials.gov (NCT02999958).

The primary endpoint of the study was the number of good quality blastocysts per normally fertilized oocytes on day 5 and day 6. Secondary endpoints were embryo development and quality on day 3 and on day 5/day 6, total blastocyst formation and utilization rates (day 5 and day 6), clinical pregnancy rate and implantation rate. Randomization was performed at the oocyte level using a sibling approach. Random allocation of cumulus–oocyte complexes (COC) from each patient into the two culture systems was performed in a 1:1 ratio in blocks of two, meaning that from every two COC, one went to the G-Series and one to the G-Series A3 group. Randomization envelopes were generated using www.sealedenvelopes.com.

Couples who underwent IVF or ICSI with the aim of blastocyst transfer were informed about the study. Couples were excluded from the study if one of the following criteria applied: female age >40 years, use of surgically retrieved spermatozoa, split IVF/ICSI cycles, PGT cycles, and fewer than eight COC. Couples who fulfilled the criteria above had to give written consent prior to treatment. For this study a superiority design with a power of 80% and a significance level of 0.05 was chosen. For this investigation, the number of fertilized oocytes to give a 50% blastocyst formation rate was used as the start point for the power calculation. For the superiority design 50% blastulation was used for both groups with a superiority limit of 8%. From retrospective data using simulation, the SD for the difference in percentage of utilizable blastocysts between the two media strategies was estimated to be 32%. Using www.sealedenvelopes.com, the sample size required per group using the parameters mentioned was calculated as 606, which meant 1212 oocytes for the total study, which were then to be randomly allocated to the two groups. Based on the minimum number of COC for inclusion being eight and with an average of 10 COC, the number of patients needed to reach the required sample size was calculated to be 122.

For each treatment cycle a unique study ID was used. Patient and study data for each cycle were summarized in an electronic case report form (eCRF). The eCRF was sent to the study coordinator as soon as an embryo culture was completed for a cycle. Following embryo transfer an updated version of the eCRF was sent when the pregnancy outcome was available and was further updated to reflect the course of pregnancy. Data in the eCRF were checked for inconsistencies and entry errors were updated with the help of a local study manager.

Patient stimulation and monitoring was performed according to the policy of the individual centre. G-series media (Vitrolife AB, Gothenburg, Sweden) were used for all steps of oocyte collection and embryo culture. Standard G-Series media (G-MOPS PLUS, G-IVF PLUS, G-1 PLUS, G-2 PLUS) were used according to protocol. For the test group, G-Series media were utilized with the addition of three antioxidants. Sperm preparation was performed for both groups in standard medium (G-IVF) without antioxidants. In case of ICSI, polyvinylpyrrolidone for sperm immobilization was also used without antioxidants.

Oocytes were collected after ovarian stimulation and immediately randomly allocated to the G-Series group (G-MOPS PLUS) or the G-Series with A3 group (G-MOPS PLUS with A3). Incubation until time of insemination (IVF) or time of denudation (ICSI) was performed in G-IVF PLUS with or without antioxidants. Insemination was performed either by standard IVF or by ICSI according to standard laboratory procedures. Further culture of normally fertilized oocytes was performed in G-1 PLUS with or without antioxidants until day 3. Embryos were transferred on day 3 into G-2 PLUS with or without antioxidants and cultured until day 5 or day 6. All procedures were performed in standard incubators using 6% CO_2 and 5% O_2. In general, embryo development was assessed on an inverted microscope. Classification on day 3 was performed using Alpha/ESHRE consensus criteria (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011*) and blastocyst scoring on day 5 and 6 followed the Gardner score (*Gardner et al., 2000*). Single blastocyst transfer was performed either in a fresh treatment cycle or in case of freeze-all, one vitrified–warmed embryo was transferred in a future cycle, with the exception of seven patients who requested two embryos transferred. Embryo selection for embryo transfer was based on morphology according to the policy and selection criteria of each individual study centre and was independent of whether an embryo was derived from the G-Series or the G-Series with A3 group.

Monitoring of implantation and presence of fetal heartbeat by ultrasound was performed around 7 to 8 weeks of pregnancy and confirmation of an ongoing clinical pregnancy at week 12 of gestation was taken as endpoint of the investigation.

Statistical analysis was performed with GraphPad Prism using a paired *t*-test for continuous variables by the central

TABLE 1 FERTILIZATION AND DAY 3 DEVELOPMENT BY MATERNAL AGE GROUPS

	G-Series	G-Series <35	G-Series 35–40	G-Series with A3*	G-Series with A3* <35	G-Series with A3* 35–40
COC allocated	1007	571	436	1005	576	429
MII oocytes	771	451	320	792	464	328
2PN	587	335	252	601	352	249
Fertilization rate (%)	76.1	74.3	78.8	75.9	75.9	75.9
Day 3 embryos with 6–10 cells (per 2PN)	422 (71.9%)	244 (72.8%)	178 (70.6%)	459 (76.4%)	271 (77.0%)	188 (75.5%)
Day 3 embryos with 8–10 cells Good/fair (per 2PN)	239 (40.7%)[a]	131 (39.1%)[b]	108 (42.9%)	302 (50.2%)[a]	181 (51.4%)[b]	121 (48.6%)

Number of patients = 133.

Mean (± SD) maternal and paternal age for <35 was 31.63 ± 1.88 and 35.11 ± 4.79, respectively.

Mean (± SD) maternal and paternal age for 35–40 was 36.98 ± 1.87 and 38.98 ± 4.64, respectively.

Like letters within a row indicate significant difference, a, b: $P < 0.05$.

* A3 denotes combined use of three antioxidants acetyl-L-carnitine (ALC), *N*-acetyl-L-cysteine (NAC) and alpha-lipoic acid (ALA).

2PN = two-pronuclei; COC = cumulus–oocyte complex; MII = metaphase II.

study coordinator and with support from an external, independent statistician. Dichotomous variables were compared using Fisher's exact test.

RESULTS

Fertilization rate in the G-Series and the G-Series with A3 group was similar (76.1% and 75.9%, respectively). Of the resultant pronucleate oocytes, 40.7% and 50.2% resulted in good quality embryos on day 3 ($P < 0.05$) (TABLE 1). The overall blastocyst development between the G-Series and the G-Series with A3 group was 57.1% and 61.2%, while good quality blastocyst rate was 20.1% versus 22.6% for day 5 and 26.9% versus 29.5% for day 5 and 6 combined, respectively (TABLE 2). The blastocyst utilization rate for cryopreservation ($n = 460$) or fresh transfer ($n = 1$), was 36.8% in the G-Series group, compared with 40.8% in the G-Series with A3 group (TABLE 2). This difference of 3.9% was not statistically different.

Seventy-one embryo transfer cycles were performed in the G-Series group and 81 in the G-Series with A3 group (TABLE 3). The overall implantation rate by fetal sac and fetal heartbeat (FHB) was 39.2% and 37.8% for the G-Series, and 50.6% and 47.1% for the G-Series with A3 group, respectively. When transfers were stratified by female patient age of <35 versus 35–40 years, an age-specific effect was revealed, with patients 35–40 years having an implantation rate by fetal sacs and heartbeats of 23.5% in the G-Series compared with 57.5% ($P < 0.05$) and 50.0% ($P < 0.05$), respectively. The ongoing pregnancies in patients 35–40 years were significantly higher in the A3 group (50.0%) compared with the control (25.8%).

DISCUSSION

Human embryo development during the cleavage stages was significantly improved in the presence of the three antioxidants, acetyl-L-carnitine, *N*-acetyl-L-cysteine and alpha-lipoic acid. All of these antioxidants have been shown to have effects individually in the mouse model, but their effects were greatest in combination (*Truong et al., 2016*). In the mouse model, the three antioxidants resulted in faster embryo development from the 5-cell stage, consistent with the observed significant increase in human embryo cell number/quality on day 3. The embryos in the present study were not analysed through time lapse, while in the animal studies, time lapse revealed distinct morphokinetic differences between embryos cultured with and without antioxidants. Hence further clinical studies are required to determine

TABLE 2 BLASTOCYST DEVELOPMENT AND UTILIZATION RATE BY MATERNAL AGE GROUPS

	G-Series	G-Series <35	G-Series 35–40	G-Series with A3*	G-Series with A3* <35	G-Series with A3* 35–40
Blastocyst day 5 1-2-3-4-5 (per 2PN)	250 (42.6%)	132 (39.4%)	118 (46.8%)	257 (42.8%)	143 (40.6%)	114 (45.8%)
Blastocysts day 5 3-4-5 (per 2PN)	173 (29.5%)	96 (28.7%)	77 (30.6%)	192 (31.9%)	106 (30.1%)	86 (34.5%)
GQB day 5 ≥3BB (3,4,5 AA-AB-BA-BB) (per 2PN)	118 (20.1%)	70 (20.9%)	48 (19.0%)	136 (22.6%)	83 (23.6%)	53 (21.3%)
Blastocyst day 5 + 6 1-2-3-4-5 (per 2PN)	335 (57.1%)	183 (54.6%)	152 (60.3%)	368 (61.2%)	208 (59.1%)	160 (64.3%)
GQB day 5 + 6 ≥3BB (per 2PN)	158 (26.9%)	91 (27.2%)	67 (26.6%)	177 (29.5%)	108 (30.7%)	69 (27.7%)
Blastocyst utilization rate day 5 + 6 (per 2PN)	216 (36.8%)	119 (35.5%)	97 (38.5%)	245 (40.8%)	139 (39.5%)	106 (42.6%)

* A3 denotes combined use of three antioxidants acetyl-L-carnitine (ALC), *N*-acetyl-L-cysteine (NAC) and alpha-lipoic acid (ALA).

2PN = two-pronuclei; COC = cumulus–oocyte complex; GQB = good quality blastocyst; MII = metaphase II.

TABLE 3 CLINICAL OUTCOME BY FEMALE AGE GROUPS

	G-Series			G-Series with A3*		
	All	**<35**	**35–40**	**All**	**<35**	**35–40**
Embryo Transfers (ET)	71	40	31	81	43	38
Number of embryos transferred	74	40	34	85	45	40
Beta-HCG positive	33 46.5%	23 57.5%	10 32.3%[a]	48 59.3%	24 55.8%	24 63.2%[a]
Implanted (sac)	29 39.2%	21 52.5%	8 23.5%[a]	43 50.6%	20 44.4%	23 57.5%[a]
Implanted (FHB)	28 37.8%	20 50.0%	8 23.5%[a]	40 47.1%	20 44.4%	20 50.0%[a]
OPR/ET	28 39.4%	20 50.0%	8 25.8%[a]	38 46.9%	19 44.2%	19 50.0%[a]

* A3 denotes combined use of three antioxidants acetyl-L-carnitine (ALC), *N*-acetyl-L-cysteine (NAC) and alpha-lipoic acid (ALA).

Like letters within a row indicate significant difference: a, $P < 0.05$.

FHB = fetal heartbeat; HCG = human chorionic gonadotrophin; OPR = ongoing pregnancy rate.

which cell cycle(s) and morphokinetic events the antioxidants are affecting.

In previous mouse studies, there were no observed increases in blastocyst formation, but there were significant increases in blastocyst cell numbers. Further, there were significant benefits to transfer outcomes, with no evidence of abnormal fetal development (*Truong et al., 2016*). In the present study, there was no capacity to ascertain blastocyst cell numbers, and so good quality blastocysts was used as a surrogate measure. Although there appeared to be differences in blastocyst formation and quality, these measures were not significantly different. Also, the apparently higher utilization rate among embryos that were cultured in the presence of antioxidants did not reach statistical significance. At the time of this analysis not all cryopreserved embryos were transferred. However, the combined effect of more utilizable embryos and an improved implantation in the G-Series with A3 group may have a potential cumulative effect on the clinical outcome, in favour of embryos grown in the presence of antioxidants.

With regards to transfer outcomes, it was determined that the presence of antioxidants conferred the greatest benefit to patients 35–40 years, with a significant increase in clinical pregnancy rate and ongoing pregnancy rate. These data are supported by animal data which also determined a positive effect of the A3 antioxidants on fetal development (*Truong et al., 2016*). Advanced maternal age is not only associated with a decrease in fertility but a concomitant increase in rates of aneuploidy, which increase significantly from the age of 35 (*Franasiak et al., 2014*; *Lewin and Wells, 2018*). The aetiology of this demise in female fertility can be attributed to an accumulation of oxidative stress over the lifetime of the oocyte, largely due to increasing mitochondrial dysfunction (*Eichenlaub-Ritter, 2012*; *Igarashi et al., 2015*; *Sasaki et al., 2019*). As the oocyte ages, its ability to protect itself from reactive oxygen species (ROS) declines, leaving it more vulnerable to the damaging effects of oxidative stress, culminating in compromised physiology and errors in chromosome segregation (*Eichenlaub-Ritter, 2012*; *Igarashi et al., 2015*). In Drosophila, experimental reduction in cytoplasmic or mitochondrial ROS scavenging ability, through the knockdown of SOD, leads to an increase in chromosomal segregation errors (*Perkins et al., 2016*). Conversely, the induction of SOD activity significantly decreases the rate of age-related non-disjunction, further supporting the hypothesis that aberrant mitochondrial function and oxidative damage, associated with advanced maternal age, are related to the induction of aneuploidy (*Perkins et al., 2019*). In support of this, Ben-Meir and colleagues (*Ben-Meir et al., 2015*) determined that exogenous Coenzyme Q10, which helps control cellular redox, and is in itself a powerful antioxidant, can help alleviate age-associated impaired mitochondrial function in aged oocytes. The beneficial effect of the three antioxidants on patients aged 35–40 years, presented here, are therefore consistent with the hypothesis that the ageing gamete has a reduced antioxidant capacity, and hence the presence of exogenous antioxidants in the IVF and embryo culture media help to alleviate this deficit, resulting in a higher developmental competence, as reflected by the higher pregnancy rates.

Consistent with their role *in vivo*, the three antioxidants exhibited a beneficial effect when human embryos were cultured at 5% oxygen, for even at physiological levels of oxygen, ROS can form, especially generated through the metabolism of the embryo itself. The beneficial effects of the combined group of antioxidants used in this study have been shown to be attributed, in part, to their ability to maintain the levels of GSH within blastomeres, as well as their ability to reduce the intracellular levels of hydrogen peroxide (*Truong and Gardner, 2017*; *Truong et al., 2016*). Consequently, these antioxidants not only increase the inherent antioxidative capacity of the embryo, but at the same time they reduce a highly toxic reactive intermediate. It is important to acknowledge that the inclusion of the three antioxidants in the culture medium only reduces the levels of ROS by around 25%, and does not eliminate them (*Truong and Gardner, 2017*). This is important as ROS can also act as important signalling molecules (*Hamanaka and Chandel, 2010*; *Lees et al., 2017*). Consequently, optimal cellular function requires a balance between physiological versus toxic levels of ROS (*Patel et al., 2018*).

There has been much consideration regarding the effects of dietary antioxidants and orally administered supplements to improve the fertility

potential of patients (*Agarwal and Majzoub, 2017*; *Showell et al., 2014*, *2017*). To date clinical studies have reported marginal benefits, and further large prospective trials are required to fully evaluate their efficacy. However, their benefits to assisted human reproduction will be limited to the health of the gametes themselves prior to collection, and cannot necessarily impact the function of oocytes, spermatozoa and embryos within the IVF laboratory. Hence, we propose that it remains a valid approach to supplement IVF and culture media with antioxidants.

In previous IVF analyses in the mouse, it was determined that the greatest benefit of the three antioxidants was observed when they were present in the media employed for spermatozoa and for oocyte handling and IVF, prior to culture in the presence of antioxidants during the preimplantation period (*Truong and Gardner, 2017*). A shortfall of the current study was therefore the absence of the antioxidants from the sperm preparation. It is known that spermatozoa are very sensitive to oxidative stress (*Aitken, 2017*; *Aitken et al., 2003*; *Bui et al., 2018*), and that seminal fluid contains the antioxidant carnitine at high levels. Further, the inclusion of antioxidants has been shown to improve human sperm motility *in vitro* (*Banihani et al., 2012*), and in the mouse model embryo development following IVF (*Truong and Gardner, 2017*). Future prospective clinical studies should therefore include the presence of antioxidants in all media to which the spermatozoa are collected, processed and inseminated.

In summary, this study has determined that the presence of antioxidants during IVF and embryo culture imparts significant benefits on day 3 embryo quality. Implantation rates and ongoing pregnancy rates were significantly higher in media with antioxidants in patients with advanced maternal age (35–40 years) but not in patients below 35 years. Consequently, it appears that the inclusion of antioxidants in embryo culture media helps to ameliorate the age-related decline in IVF patients of advanced age. Such findings require verification in a larger prospective randomized trial including the treatment of spermatozoa with antioxidants. The data presented here are based on the inclusion of a limited number of antioxidants selected and proven by Truong and colleagues (*Truong et al., 2016*). Our continuing studies on the role of antioxidants could see this number increase from the initial three in due course.

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