

Study protocol:
Impact of Repeated Antioxidant
Supplementation of Embryo Culture Media on
Blastocyst Utilization and Expansion Rate Under
Two Different O₂
Concentrations

October 31st, 2021

Title: The Role of Antioxidants in Mitigating Reactive Oxygen Species and Oxidative Stress in Assisted Reproduction Techniques: A Comparative Study

Introduction:

During assisted reproduction techniques (ARTs), monitoring the laboratory environment is essential for adequate embryo development and any deviation from optimal conditions could increase reactive oxygen species (ROS) (Kaskar, 2021). Excessive ROS is detrimental to early embryo development (Kaskar, 2021). Reactive oxygen species constitute highly reactive chemicals associated with oxidative stress (OS) and subsequent cellular damage to gametes and embryos (Lane & Lyons, 2000; Swain, 2010). Oxidative stress is observed when the accumulation of ROS molecules exceeds the capacity of antioxidants (AOXs) defense mechanisms to neutralize them, subsequently promoting lipid, protein, as well as DNA damage (Lane & Lyons, 2000; Swain, 2010).

In the past, embryo culture was performed in atmospheric O₂ concentrations of about 20% (Nastri et al., 2016). However, an O₂ concentration of 5% was found superior in terms of enhancing clinical pregnancy rate, live births, as well as implantation rate (Kaskar, 2021). Several exogenous factors affecting embryo development during in vitro culture, namely the type of infertility, as well as advanced maternal and paternal age, have been correlated with high levels of ROS production in gametes and embryos, which in turn result in decreased fertilization, implantation, and live birth rates, and increasing aneuploidy rates (Lane & Lyons, 2000; Edwards et al., 1998; Hong et al., 2014; Chui et al., 2008)

Therefore, it is conceivable that even minimal handling of gamete and embryos could induce OS. This assumption highlights the demand for strategies aiming to reduce the detrimental effects of OS. Implementation of AOX supplementation could help protect against OS damage attributed to alterations in laboratory conditions in the IVF setup. Antioxidants act as a counterpart of ROS, inhibiting their overproduction or neutralizing them when provided in sufficient concentrations (Agarwal et al., 2005; Carocho & Ferreira, 2013). The role of AOXs in enhancing the embryo's potential to successfully reach the blastocyst stage has been demonstrated (Guerin, 2001; Budani & Tiboni, 2020). The blastocyst quality of the expansion grade has been analyzed by the measurement of the blastocoel and has been associated with increased ongoing pregnancy rates (David Gardner et al., 1999)

Considering the pivotal role of AOXs *in vivo*, their implementation in embryo cultures *in vitro* is thought to help mimic the human reproductive tract's natural conditions. Previous studies have investigated the role of non-enzymatic AOX molecules namely vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, and carotene on embryo culture (Agarwal et al., 2005; Carocho & Ferreira, 2013).

Previously, our group determined the oxidation-reduction potential (ORP) values of follicular fluid in oocyte donors and established them as the "physiological" desirable ORP in embryo culture media to maintain a redox state during the entire embryo culture similar to what is observed *in vivo* (Truong, T., & Gardner, D.K., 2017)

However, the current literature is lacking studies to develop a physiological *in vitro* AOXs system that can effectively mitigate the OS phenomenon.

The present study aims to explore whether repeated supplementation of the embryo culture medium with AOXs every 12 hours to culture embryos in ORP physiological levels, could improve the cumulative rates of usable and expanded blastocysts of human embryos from infertile patients as assessed at two different O₂ concentrations (5% and 20% O₂) on days 5 and 6.

Research Design:

Study type: Interventional

Study Model: Parallel Assignment

Participants: 293 Infertile women aged 18 to 37 years at the time of oocyte collection, with tubal factor, polycystic ovary syndrome, uterine factor, or unexplained infertility.

Allocation

Zygotes will be randomly assigned to one of four groups.

- Group 1A: Antioxidants will be administered every 12 hours at 5% O₂ tension.
- Group 1B: Antioxidants will be administered every 12 hours at 20% O₂ tension.
- Group 2A: Antioxidants will be administered once at the beginning of embryo culture at 5% O₂ tension.
- Group 2B: Antioxidants will be administered once at the beginning of embryo culture at 5% O₂ tension.

Methods:

Study Location and Duration:

The study will be conducted at the Center for Technological Innovation and Reproductive Medicine (CITMER), Mexico City, Mexico, from October 2021 to May 2022.

Participants:

Eligible participants will include infertile women with tubal factor, polycystic ovary syndrome, uterine factor, and unexplained infertility, who have had fertilization of more than 2 oocytes by conventional in vitro fertilization (cIVF) and intracytoplasmic sperm injection (ICSI).

Exclusion criteria will involve low ovarian response patients (fewer than 6 oocytes obtained after oocyte retrieval) and patients older than 37 years.

Sample Size and Group Allocation:

A total of 3603 zygotes obtained from 293 patients will be included in the study.

The zygotes will be divided into four groups based on the following conditions:

Group 1A: Antioxidants will be administered every 12 hours at 20% O₂ tension (793 zygotes).

Group 1B: Antioxidants will be administered only at the beginning of embryo culture at 20% O₂ tension (1286 zygotes).

Group 2A: Antioxidants will be administered every 12 hours at 5% O₂ tension (695 zygotes).

Group 2B: Antioxidants will be administered only at the beginning of embryo culture at (829 zygotes).

The procedures followed for IVF/ICSI involved the following steps:**Ovarian stimulation:**

Different ovarian stimulation protocols were used, including the gonadotropin-releasing hormone (GnRH) antagonist protocol with Cetrotide®, in combination with rFSH and rLH in Pergoveris®.

GnRH antagonist protocols were also used with follitropin alfa in Gonal-F® or with human menopausal gonadotropin in Merapur®

Gamete Retrieval:

Oocyte retrieval will be performed 36 hours after the administration of choriogonadotropin alfa (Ovidrell®, Merck Serono, Bari, Italy). Cumulus-oocyte complexes (COCs) will be transferred into dishes containing HTF HEPES® (InVitroCare®) supplemented with 10% human serum albumin (HSA) (InVitroCare®) and antioxidants (EmbryORP®). COCs will then be washed and placed in culture media (global® total®, Life global® Group) with stock antioxidants (EmbryORP®) until insemination.

Fertilization:

For intracytoplasmic sperm injection (ICSI) cases, mature oocytes (metaphase II) will be denuded using Hyaluronidase (10%) (InVitroCare®) approximately 40 hours after HCG administration. Only mature oocytes will be selected and injected. For conventional IVF insemination, oocytes will be co-incubated individually with capacitated sperm in embryo culture media for 17 to 20 hours.

Embryo Culture:

After the fertilization check, zygotes will be cultured under specific conditions in an incubator. The incubator settings will be either 37°C, 8% CO₂, 20% O₂, and 95% relative humidity (RH) or 37°C, 8% CO₂, 5% O₂, and 25% RH. Only 2 pronuclei (PN) zygotes will be considered for the study.

Antioxidant Supplementation:

The antioxidant supplementation protocol will involve the use of EmbryORP®, a novel antioxidant consisting of a mixture of four different compounds: reduced L- cysteine, L- glutathione, L- ascorbic acid, and L- carnitine hydrochloride (0.0124 M for carnitine, 0.0165 M for cysteine, 0.0065 M for glutathione, and 0.0227 M for ascorbic acid). Groups 1A and 2A, 6.5 µL of EmbryORP® antioxidants will be added at the beginning of the culture (in the zygote stage), followed by the addition of 3.5 µL of stock antioxidant media (EmbryORP®) every 12 hours. For groups 1B and 2B, 6.5 µL of EmbryORP® antioxidants will be added only at the beginning of the culture control of O₂ Tensions:

During embryo culture, two different O₂ tensions will be maintained. The first setting will be 20% O₂, and the second setting will be 5% O₂. These O₂ tensions will be controlled using the incubator, which will provide the desired atmospheric conditions.

Data Collection:**Variables to be Measured:**

The blastocyst expansion rate will be assessed by calculating the proportion of blastocysts that reach the expanded stage. Embryo evaluation will be performed based on the Gardner and Schoolcraft criteria from 1999, and the results will be documented in standardized databases.

Timeline and Frequency of Assessments:

Data collection will be performed at specific time points, specifically on day 5 and day 6 of the study.

Outcome Measures:

- Usable blastocysts on day 5.
- Expanded blastocysts on day 5.
- Usable blastocysts on day 6.
- Expanded blastocysts on day 6.
- Cumulative usable blastocysts.
- Cumulative expanded blastocysts.

Ethical Considerations:

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of CITMER CE-20-102.

Data analysis

The statistical analysis of the data will be performed using the R statistical software by the R Core Team.

Statistical Methods:

For statistical analysis, the data collected consisted of the continuous variable of age, which was expressed as the mean \pm standard deviation in each study group. In addition, the frequency and percentage of usable and expanded blastocysts were recorded as categorical variables.

Comparisons between usable and expanded blastocysts from each treatment were performed by calculating the odds ratio and chi-square test. A significance level of $p < 0.05$ was established to determine the existence of statistically significant differences between groups.

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