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Original Article

Effects of reactive oxygen species levels in prepared culture media on embryo development: A comparison of two media

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ABSTRACT

Objective: This study determined the correlation between the levels of reactive oxygen species (ROS) in prepared culture media and the early development of human embryos.
Materials and methods: This was an autocontrolled comparison study. A total of 159 patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection treatment were recruited in this study. The pH values, osmolarity pressures, and ROS levels of 15 batches of two culture media were measured. Sibling oocytes or embryos from individual patients were randomly assigned to two culture groups with Quinn's Advantage Cleavage and Blastocyst media (QAC/QAB) or GIII series cleavage and blastocyst media (G1.3/G2.3). The difference between the two culture groups was analyzed using one-sample *t* test.
Results: The QAC/QAB and G1.3/G2.3 media exhibited similar pH values and osmolarity pressures. However, the prepared QAC/QAB media were characterized to contain lower amounts of ROS than the G1.3/G2.3 media. Furthermore, the blastocysts that developed under the QAC/QAB media were morphologically superior to those that developed under the G1.3/G2.3 media.
Conclusion: The elevated ROS levels in culture media were associated with poor development of blastocyst-stage embryos. Measurement of ROS levels may be a valuable process for medium selection or modification.

Introduction

Assisted reproductive technology (ART) cycles comprise controlled ovarian hyperstimulation, oocyte retrieval, *in vitro* fertilization (IVF), *in vitro* embryo culture, and embryo transfer. Among these complex processes, *in vitro* embryo cultures can directly provide information regarding the implantation potential of human embryos [1]. The success of *in vitro* embryo culture considerably affects the chances of achieving pregnancy in ART cycles. The culture media and incubation settings used are essential for enhancing the embryo quality during *in vitro* development.

Simple culture media are bicarbonate-buffered systems with pyruvate, lactate, and glucose. Complex media contain amino acids or other additional elements found in serum. Among these various culture media, the human tubal fluid (HTF) medium simulates the fallopian tube microenvironment and is viewed as a physiologic medium for human embryo development at the cleavage stage [2]. Currently, the introduction of commercial media to culture systems has improved the consistency of culture system quality [1] and increased blastocyst formation and embryo implantation rates. Although various commercial media are available and employed worldwide [3], the optimal or superior culture media for IVF/intracytoplasmic sperm injection (ICSI) cycles must be confirmed [4,5].

Most commercial media used for cleavage-stage embryos today are developed based on modifications of the HTF or Eagle's medium. However, the relative deficiency of antioxidative enzymes or antioxidants in oviductal epithelia and fluid renders embryos in *in vitro* culture systems susceptible to high levels of reactive oxygen species (ROS) in culture media [6]. Furthermore, ROS are generated during *in vitro* culture and are detrimental to embryo development

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[7]. A previous report indicated that preimplantation embryos were extremely sensitive to conditions that induce oxidative stress [8].

The physiological process of oxygen consumption inevitably generates ROS in cellular respiration for energy production. Although ROS, in limited amounts, are considered to mediate inter- and intracellular signaling [9], it has been reported that elevated ROS levels and the resulting oxidative stress are associated with poor or arrested embryo development [6,10]. Furthermore, oxidative stress has recently been determined to be one of the major factors detrimental to ART outcomes [10,11]. Such oxidative stress can be aggravated because of suboptimally formulated culture media. By contrast, minimization of intracellular trauma or stress results in successful culturing of mammalian embryos [12].

In this paper, we designed an autocontrolled study by randomly assigning cultures of sibling embryos from individual patients to compare the efficacy of two commercial media. The common quality control measurements and ROS levels of these two media were compared before use. Embryo development at the cleavage and blastocyst stages was compared to elucidate the effect of ROS levels in prepared culture media on the early development of human embryos. The results of this study may indicate an efficient process for selecting superior culture media and the direction for improving the efficacy of culture media.

Methods

Study participants and controlled ovarian stimulation

This study analyzed *in vitro* embryo development after IVF/ICSI treatments using two sequential culture media. Only cycles from female participants younger than 38 years of age that contained at least six mature oocytes available for randomly splitting cultures were included. Cycles from oocyte and sperm donation programs were excluded. In this study, 159 IVF/ICSI cycles performed at Lee Women's Hospital, located in Taichung, Taiwan, between February 2010 and May 2010 were analyzed. The Institutional Review Board of Chung Shan Medical University Hospital, Taichung, Taiwan approved this research.

The long protocol for gonadotropin-releasing hormone agonist administration was used in this study. The ovarian stimulation procedures employed in our IVF program were published elsewhere [13]. In brief, participating women were administered the gonadotropin-releasing hormone agonist, leuprolide acetate (Lupron, Takeda Chemical Industries Ltd, Osaka, Japan) for the purpose of pituitary desensitization. A serum estradiol (E2) level of <50 pg/mL was used on Day 2 to confirm pituitary suppression, followed by treatment with recombinant follicular stimulation hormone (Gonal-F; Serono, Bar, Italy). The participants' ovarian responses were monitored using serial serum E2 levels and ultrasound examinations.

When more than two leading follicles reached approximately 18 mm in diameter with an appropriate serum E2 level, 250 μg of recombinant human chorionic gonadotropin (Ovidrel; Serono) was administered. All the women underwent transvaginal oocyte retrieval 34–36 hours after human chorionic gonadotropin injection. The retrieved oocytes were placed in a modified HTF medium in a triple-gas-phase (5% CO_2, 5% O_2, and 90% N_2) incubator at 37°C.

ICSI and IVF procedures

Motile spermatozoa were selected using a gradient separation technique accompanied by the application of PureSperm 80/40 (Nidacon International AB, Molndal, Sweden). The retrieved oocytes were inseminated 3–6 hours later, with an average of 10,000 motile sperms for one oocyte in a 10-μL droplet of a modified HTF medium supplemented with 2.5% human serum albumin. Intracytoplasmic sperm injection was performed 40–42 hours subsequent to human chorionic gonadotropin injection. Immediately prior to ICSI, cumulus cells were removed by pipetting the oocytes in modified HTF medium containing 80 IU/mL of hyaluronidase (H-3757; Sigma Chemical, St Louis, MO, USA). The denuded oocytes that demonstrated the first polar bodies were selected for ICSI, and the ICSI was performed 0–3 hours after oocyte denuding.

Approximately 2 hours subsequent to IVF insemination or immediately after ICSI, the oocytes from individual patients were randomly divided into two groups and were then further cultured in microdrops of the two commercial media: GIII series cleavage and blastocyst stage media (G1.3/G2.3; Vitrolife, Kungsbacka, Sweden) and Quinn's Advantage Cleavage and Blastocyst media (QAC/QAB; SAGE *In-Vitro* Fertilization, Trumbull, CT, USA). Before use, the media were warmed to incubator temperature (37°C) and equilibrated with the desired atmosphere, which contained 5% CO_2.

Quality assessment of commercial media for in vitro culture

To assess the quality of the commercial media before use, several parameters were analyzed weekly. We used a new batch of commercial media each week during the study period, to ensure that the quality of the media was not affected by the time in storage prior to usage. The tested parameters were the pH levels, osmolarity pressures, and ROS levels of the prepared culture media. From February 2010 to May 2010, 15 batches of G1.3/G2.3 and QAC/QAB media were assessed using this quality control process.

The osmolarity and pH levels were measured using a micro-osmometer (5004; Precision Systems Inc., Natick, MA, USA) and a pH meter (Orion 350; Thermo Fisher Scientific, Waltham, MA, USA), respectively. For testing, 5 mL of each medium was prepared in 15 mL test tubes with caps. Measurements were carried out according to the manufacturer's instructions.

ROS levels in the G1.3/G2.3 and QAC/QAB media were evaluated by performing a chemiluminescence assay [14] using luminal (5-amino-2,3-dihydro-1,4-phthalazinedione) as a probe. The medium (100 μL) was used in the 96-well white/opaque plate of a luminometer (Microplate Luminometer TR717; ABI, Bedford, MA, USA), and 2.5 μL of luminol (5 mM) in dimethyl sulfoxide was added to it. Horseradish peroxidase (2 μL; 12.4 U of horseradish peroxidase type VI, 310 U/mg; Sigma Chemical) was added to sensitize the assay for measuring extracellular H_2O_2. Each sample was scanned for 30 seconds. The ROS values were expressed in relative light units (RLUs). A negative or background control was prepared by adding 2.5 μL of 5 mM luminol to 100 μL of d2H_2O.

Quality assessment of the in vitro development of embryos

Subsequent to IVF or ICSI, embryo development, including the four-cell stage (45–46 hours), eight-cell stage (69–70 hours), and blastocyst stage (118–120 hours), was observed. The embryos at the cleavage stage were classified according to the criteria proposed by Steer et al in 1992 [15]. On Day 2 and Day 3, embryos were considered good when they exhibited fragmentation of <20% and equal-sized blastomeres. In addition, the number of blastomeres in good-quality embryos on Day 2 and Day 3 must be ≥4 and ≥7, respectively [16]. The good embryo rates on Day 2 and Day 3 were calculated as follows:

$$\text{rate}\ (\%) = (\text{number of good embryos/total embryos}) \times 100. \quad (1)$$

The blastocysts were graded according to the criteria proposed by Gardner and colleagues [17]. On Day 5, the good embryos must exhibit expanded blastocysts, wherein the blastocele volume is