J Assist Reprod Genet (2010) 27:87–92
DOI 10.1007/s10815-009-9383-x

TECHNICAL INNOVATION

Peroxides in mineral oil used for in vitro fertilization: defining limits of standard quality control assays

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Received: 13 November 2009 / Accepted: 22 December 2009 / Published online: 27 January 2010
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Abstract
Purpose To determine the relative sensitivities of the 1 and 2-cell mouse embryo assays (MEA) and the human sperm motility assay (HSMA) for peroxides in mineral oil. The effect of peroxide on blastocyst cell number and apoptosis was also studied.
Methods One and two-cell MEA and HSMA were performed using mineral oil containing cumene hydroperoxide (CH).
Results The 1-cell MEA was twice as sensitive as the 2-cell MEA and 20-times more sensitive than the HSMA for CH in mineral oil. The sensitivity of the 1-cell MEA doubled when embryos were cultured individually versus group culture. CH decreased blastocyst cell number in a dose dependent manner.
Conclusions Individually cultured 1-cell embryos had the highest sensitivity for peroxides in mineral oil. Current quality control assays, including group cultured murine embryos and human sperm motility, have limited sensitivity for peroxides in mineral oil and may not detect levels of peroxides that cause sub-lethal cellular damage.

Keywords Embryo culture · Human sperm motility assay · Mouse embryo assay · Peroxide · Mineral oil

Introduction

Mineral or silicone oil is often used as an overlay when embryos are cultured in microdrops of culture media during in vitro fertilization (IVF) [1]. Though oil is important for pH, temperature and osmolar stability, it is a petroleum product that can harbor embryo toxins, including zinc [2] and peroxides [3]. Recently, Otsuki and colleagues reported peroxide contamination of laboratory grade mineral oil and found the degree of peroxidation was dependent on exposure to heat, UV light, extended storage, manufacturer and lot number [3, 4]. Peroxide-contaminated oil was also shown to adversely affect murine embryo development and in vitro fertilization success [3].

Several quality control (QC) bioassays are used to screen for toxicity in embryology laboratories, but controversy exists whether they are sensitive enough to detect damaging but sublethal levels of toxins [5]. The 1-cell MEA is the assay used by most manufacturers to screen products intended for use with human in vitro embryo culture. IVF laboratories are not required to test items previously screened by the manufacturer; however, end users often retest oil, as peroxides can develop during transport or storage and become a potential hazard to

Presented at the 56th annual meeting of the Society for Gynecologic Investigation, Glasgow, Scotland, March 17–21, 2009.

Capsule The 1-cell MEA is 20-times as sensitive as the HSMA for peroxides in oil. The sensitivity doubles when embryos are cultured individually.

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assisted reproduction. While several different QC assays are used by IVF laboratories to retest materials, the relative sensitivities of QC assays for peroxides in mineral oil are unknown.

There is little consensus on the most effective and sensitive way to test laboratory materials used in IVF for toxicity. Although mouse embryo assays screen for toxins by simulating human embryo culture conditions the lack of standardization leads to variability in results [6]. Culture techniques have been employed to improve the sensitivity of the MEA, such as culturing fresh vs frozen embryos, using 1-cell vs 2-cell embryos or removing the zona pellucida prior to culture [7–9]. The 1-cell MEA, when compared to the 2-cell MEA, is ten times more sensitive for the toxin, Cidex, a common surgical sterilizing solution [7, 10]. Nonetheless, the 2-cell MEA is still commonly used by many in vitro fertilization laboratories. The human sperm motility assay (HSMA) is promoted as a viable, inexpensive alternative to the MEA [11, 12] and is used widely by IVF laboratories. However, sperm motility persists in conditions that do not favor fertilization or embryo development [6], indicating it may not be a suitable test for all toxins.

Cumene hydroperoxide (CH) is an oxidant compound that has been used to assess the effects of free radicals and reactive oxygen species in biological models [13–16]. Unlike hydrogen peroxide, CH is a relatively stable, lipid soluble and slightly water soluble chemical, making it a good candidate for studying peroxides in mineral oil.

The primary objective of these experiments was to compare sensitivities of routine QC assays for peroxide in mineral oil. In addition to comparison of murine and human sperm motility assays, the sensitivity of group versus individually cultured mouse embryos was tested. Blastocyst cell number and rate of apoptosis were measured to assess cellular effects of peroxides in oil.

Materials and methods

Mineral oil

Cumene hydroperoxide (CH; Sigma-Aldrich, St. Louis, MO) was added to embryo-tested laboratory grade mineral oil (Fisher Scientific, Pittsburgh, PA) to form a stock solution of 114.8 μM CH. This concentration was directly measured using the SafTest® Kit (SafTest®; MP Biomedicals, Irvine, CA) and was equivalent to 0.2 mEq/kg peroxide activity. The stock mineral oil solution was diluted using untreated oil that was confirmed negative for peroxide using the SafTest® Kit. Cumene hydroperoxide concentrations studied were 2.5 μM, 5 μM, 11.7 μM, 28.7 μM, and 57.4 μM.

Mouse embryo assay

Cryopreserved 1-cell and 2-cell embryos from F1 hybrid mice were obtained from a commercial source (Embryotech Laboratories, Haverhill, MA) and thawed according to the manufacturer's instructions. Thawed embryos were equilibrated in human tubal fluid (HTF) with HEPES (HTF-HEPES; Irvine Scientific, Irvine, CA) with 5% human serum albumin (HSA, Irvine Scientific, Irvine, CA) for 10 min at room temperature. Embryos were cultured at 37°C in 6.5% CO2 in 25 μL of HTF (In Vitro Care, Frederick, MD) without protein. Each experiment was performed in triplicate with ten to 11 embryos per treatment. Embryos were graded at 24 h intervals and the rate of blastocyst formation was determined at 72 h and 96 h post thaw for the 2-cell and 1-cell MEA, respectively. The blastocyst rate was defined as the percentage of embryos that developed to the expanded, hatching or hatched blastocyst stage.

Human sperm motility assay

Human sperm motility assay was performed using donated semen per IRB approval. The HSMA was performed as previously described [11]. A semen sample was obtained from a donor and processed on a single 90% layer of Isolate ™ (Irvine Scientific, Irvine, CA). The sample was centrifuged, washed and resuspended in protein free HTF. Fifty microliters of washed sperm suspension was added to 450 μL protein-free oil-conditioned HTF to yield a final concentration of 5×10^6 motile sperm/mL. Media exposed to a piece of latex surgical glove was used as the positive control and protein-free HTF as the negative control. All tubes were loosely capped and cultured at 6.5% CO_2 and 37°C. Motility and grade (forward progression) were determined at 24 h and 48 h. At the time of analysis, 50 μl HTF containing 100 mg/ml HSA were added to the solution and an aliquot of each sample was placed on a slide for evaluation. The control sample was required to have 75% of original motility at 48 h for the assay to be considered valid. Sperm motility index (SMI), the end point of this assay, was calculated by dividing the percentage of motile sperm of the test item by the percentage progressive motile sperm of the control at the specified time intervals. Three tubes were used per test item and the experiment was repeated in triplicate.

Apoptosis and cell number

Cell number and apoptotic index were determined by fixing and staining blastocysts with 4′,6-diamidino-2-phenylindole (DAPI; Abbott Abbot Park, IL) and TUNEL as described previously [17, 18]. Briefly, blastocysts were fixed and permeablized in a 4-well dish (Sigma-Aldrich St. Louis,

MO) and then washed in D-PBS containing a 1% solution of polyvinylalcohol (PVA; Sigma St. Louis, MO). To label fragmented 3′ DNA, fixed embryos were incubated in fluorescein labeled dUTP and terminal transferase (TUNEL, In Situ Cell Death Detection Kit, Fluorescein, Roche Indianapolis, IN) for 1 h at 37°C in the dark. Nuclear DNA was stained by mounting the embryos on shallow depression slides in 40 µl drops of Vectoshield (Vecto Laboratories Burlingame, CA) containing a 3% DAPI solution. Stained embryos were visualized with a 63× objective using confocal fluorescence microscopy (Bio-Rad MRC-600, LSM510 Confocal Laser Scanning Microscope with AxioVert 200M inverted platform, Carl Zeiss, Inc. Thornwood, NY). Blastocyst cell number and TUNEL positive nuclei were counted manually by a single evaluator who was blinded to the testing groups. Apoptotic index was expressed as percent TUNEL-positive nuclei per total nuclei per embryo. The experiment was repeated in triplicate.

Experiment One: HSMA vs. 2-cell MEA

HSMA was performed with HTF conditioned with mineral oil at 0, 5 µM, 11.5 µM, 29 µM, 57 µM, and 115 µM CH. Five parts oil were combined with one part protein-free HTF for each concentration and incubated for 24 h at 6.5% CO_2 and 37°C. After conditioning, the media were transferred to clean tubes and combined with the washed sperm suspensions. SMI and grade were calculated at 24 h and 48 h.

Two-cell mouse embryos were cultured in 25 µL of protein-free HTF under mineral oil in 35 mm Nunc petri dishes (Sigma-Aldrich, St. Louis, MO) with the same concentrations of CH as in the HSMA. Embryos were graded at 24-h intervals and blastocyst rates calculated at 72 h post thaw.

Experiment Two: 2-cell MEA vs. 1-cell MEA

Microdrops of media were covered with mineral oil containing CH at concentrations based on results obtained from experiment one. Cryopreserved one-cell and two-cell mouse embryos were thawed and cultured in 25 µL of protein-free HTF in 35 mm Nunc petri dishes (Sigma-Aldrich, St. Louis, MO) with oil containing 0, 2.5 µM, 5 µM and 11.5 µM CH. Blastocyst rates were calculated at 72 h and 96 h post thaw for 2-cell and 1-cell MEA, respectively.

Experiment Three: Group vs individual culture effects on the sensitivity of the 1-cell MEA

Embryos were cultured individually or in groups of ten using 60 well Nunc MiniTrays (Sigma-Aldrich St. Louis, MO). Ten µL of protein-free HTF media were added to each well in the 60 well trays. Media cultures were covered with oil containing 0, 2.5 µM or 5 µM CH. Blastocyst rates were calculated at 96 h post thaw. Blastocysts from the group and individual cultured embryos in the 10 µL wells were fixed and stained for DAPI and TUNEL to determine cell number and apoptotic index, respectively.

Statistical analysis

Results were analyzed by one way ANOVA and significance was determined by student's t test $p<0.05$ for the three experiments using JMP statistical software (SAS Institute; Cary, NC).

Results

Experiment One: HSMA vs 2-cell MEA

All sperm were immotile after 48 h of culture with media conditioned with oil containing 115 µM CH (Fig. 1a; $p<0.0001$). Sperm motility at 48 h was not affected by media conditioned with oil containing less than 115 µM CH. Two-cell mouse embryos lysed when cultured with oil containing ≥ 57 µM CH and developed no further than the multi-cell stage at the 29 µM CH dose. Two-cell embryos treated with

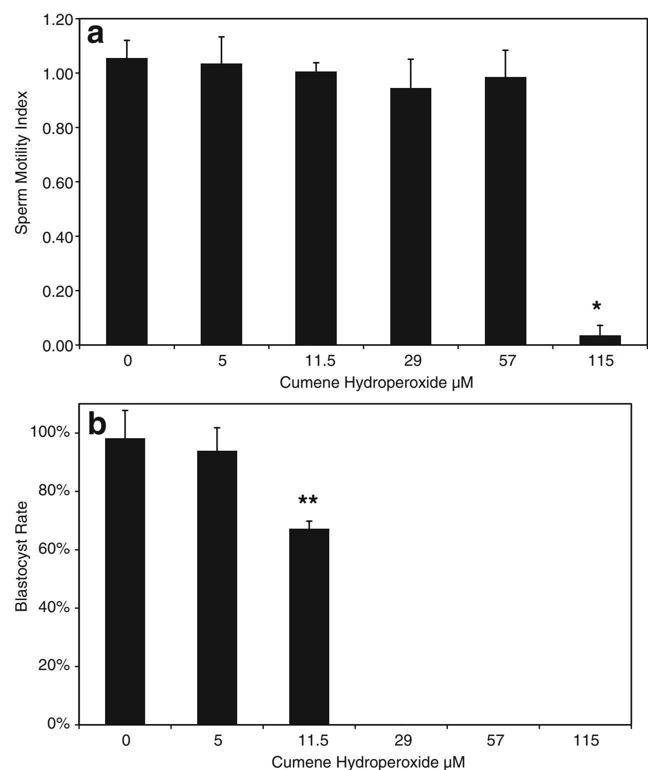

Fig. 1 The dose effect of CH on the HSMA and 2-cell MEA. **a** The SMI was statistically different from the control at 115 µM CH, $*p<0.0001$. **b** Expanded blastocyst rate for the 2-cell MEA was statistically different from the control at 11.5 µM CH, $**p<0.0001$

11.5 μM CH developed to blastocyst but at a rate significantly lower than for the control (Fig. 1b; $p<0.0001$).

Experiment Two: 2-cell MEA vs. 1-cell MEA

Two-cell embryos developed to blastocysts in 11.5 μM CH but at a lower rate than controls. Doses below 11.5 μM did not affect development of blastocysts from 2-cell embryos. One-cell embryos treated with 11.5 μM CH arrested at the morula stage, whereas one-cell embryos treated with 5 μM CH developed to blastocysts at a rate significantly lower than for the controls (Fig. 2; $p=0.0004$).

Experiment Three: Group vs Individual Embryo Culture

The blastocyst rate for individually cultured embryos differed significantly from the control at 2.5 μM CH (Fig. 3a; $p<0.0001$). However, blastocyst rates for the embryos cultured in groups were not affected by CH at the doses tested.

Cumene hydroperoxide had a dose dependent effect on blastocyst cell number in both the individual and group cultures. There was a statistically significant difference in cell number for group cultured embryos from the control at 5 μM CH (Fig. 3b; $p=0.02$). Apoptotic index was not statistically different between the groups but there was a dose dependent trend (Fig. 3c).

Discussion

This study examined the relative sensitivities of several QC assays to cumene hydroperoxide in mineral oil. Mineral oil used for IVF has been shown to undergo peroxidation under certain storage conditions [3]. In the present study, cumene hydroperoxide was used as a model for naturally occurring peroxides in mineral oil. Individually cultured 1-cell mouse embryos had the highest sensitivity for CH in mineral oil while the HSMA was the least sensitive assay. In addition to standard QC outcomes, we found that blastocyst cell number was affected in a dose-dependent manner. A decline in blastocyst cell number and increasing apoptosis were observed at concentrations of CH that were lower than levels affecting blastocyst development rates in the 1-cell or 2-cell MEA group culture assays. These results suggest that sublethal damage can occur with items that

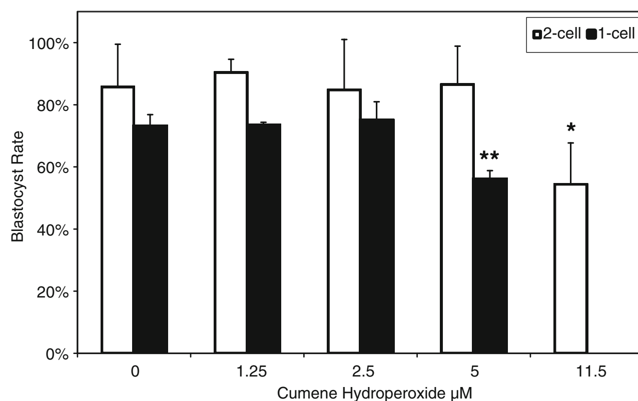

Fig. 2 The dose effect of CH on the 2-cell and 1-cell MEA. The 2-cell MEA blastocyst rate was statistically different from the control at 11.5 μM CH, $*p=0.05$, and the 1-cell MEA blastocyst rate was statistically different from the control at 5 μM CH, $**p=0.0004$

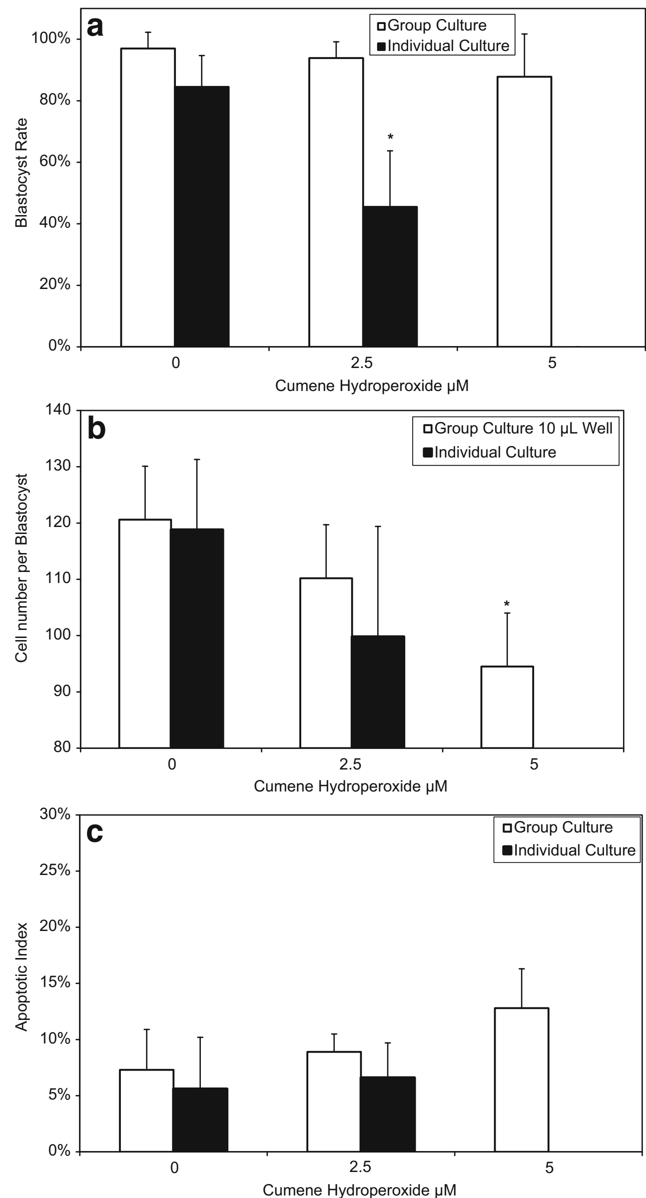

Fig. 3 The dose effect of CH on blastocyst rates for group and individually cultured embryos. **a** The blastocyst rate for individually cultured embryos differed significantly from the control at 2.5 μM CH, $*p<0.0001$. **b** Cell numbers were reduced for group cultured embryos at 5 μM CH $p=0.02$. **c** Effect of CH on apoptosis in blastocysts

pass the 1-cell MEA. Embryos exposed to concentrations of CH contaminated oil that passed the HSMA, 2-cell MEA or 1-cell MEA had fewer cells per blastocyst, a finding that is consistent with reduced developmental potential [19].

Development of better quality control of products used in the IVF laboratory is dependent on our understanding of the sensitivity, weaknesses and strengths of each bioassay. There is little consensus on the most effective and sensitive bioassay to test materials for use in IVF. While the mouse embryo assay is the most common QC method used by manufacturers, the human sperm motility assay is commonly used by IVF laboratories [11, 20]. The HSMA is an easy and inexpensive assay that detects toxicity of certain laboratory materials [12]. Sensitivity of the HSMA is comparable or superior to those of the mouse embryo assay for latex gloves and some laboratory supplies [11, 21]. The HSMA also detects endotoxins in contact supplies and culture media [22]. While the HSMA may be more sensitive to certain substances, human sperm may remain motile under conditions that do not favor fertilization or embryo development [6]. It has been suggested that the HSMA and MEA should be used in conjunction with each other to reliably screen for toxins present in the embryology laboratory [6].

Few studies have compared the sensitivities of commonly used QC assays. Our results show that the 1-cell MEA was twenty times more sensitive than the HSMA for peroxides in mineral oil, indicating that the HSMA should not be used for screening mineral oil for peroxides. Though sperm were not exposed directly to the treated oil, the objective of this experiment was to test the HSMA as it is commonly used for QC testing [11, 12]. The 1-cell MEA was the most sensitive assay for peroxide in oil of the three most commonly used QC assays. These results are consistent with previous reports that have shown the 1-cell MEA to be more sensitive (detecting toxins at lower levels) than the 2-cell MEA as a QC assay for toxins or laboratory conditions [7, 10].

Low levels of toxins present in materials used for IVF that go undetected by current QC methods may induce cellular stress and have adverse effects on IVF outcome. The current study measured indications of cellular stress using blastocyst cell number and apoptotic index. Blastocyst cell number has been shown to be superior to blastocyst rate for predicting IVF outcomes in mice [19]. The data in this study demonstrated a dose dependent effect of CH on blastocyst cell number at concentrations of peroxide that pass both the 1-cell and 2-cell group culture MEA. Since mice derived from in vitro blastocyst culture have decreased birth weight and weaning weight when compared with in vivo conceived offspring [23], this effect could be due to undetected sub-lethal doses of toxins present in the culture environment. These results highlight the need for more sensitive quality control assays.

This is the first study to evaluate individual culture of mouse embryos for quality control. The sensitivity of the 1-cell MEA for CH in oil doubled when embryos were cultured individually as opposed to when cultured as a group. The sensitivity of the MEA increases when the zona pellucida is removed [8, 24]. However, since zona-free embryos were cultured individually, further studies are needed to determine if the improved sensitivity was due to zona removal or culture density. Culturing embryos in groups results in increased blastocyst cell numbers and improved blastocyst rates due most likely to autocrine and paracrine factors that may play a role in embryo development [25, 26]. This study provides indirect evidence that paracrine/autocrine factors may also confer a protective advantage from stress caused by peroxides in mineral oil.

This study has important implications for quality control in IVF laboratories. We have demonstrated the sensitivity of current quality control assays for peroxides in oil and also techniques to improve assay sensitivity. These results suggest that undetectable levels of peroxides can be present in mineral oil that passes current laboratory QC assays. Unknown peroxide contamination may induce sub-lethal stress on human embryos and have undesirable effects on IVF outcome. Further investigations are warranted to define the sensitivity of quality control assays for potential laboratory toxins to insure best possible outcomes using assisted reproductive technologies.

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