Improved detection of mineral oil toxicity using an extended mouse embryo assay

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Abstract

Purpose Successful in vitro fertilization (IVF) relies on sound laboratory methods and culture conditions which depend on sensitive quality control (QC) testing. This study aimed to improve the sensitivity of mouse embryo assays (MEA) for detection of mineral oil toxicity.

Methods Five experiments were conducted to study modifications of the standard mouse embryo assay (MEA) in order to improve sensitivity using clinical grade mineral oil with known peroxide concentrations. Assessment of blastocyst development at either 96 h or in an extended MEA (eMEA) to 144 h was tested in each experiment. In experiment 1, ability to detect peroxides in oil was compared in the MEA, eMEA, and cell number at 96 h. In experiment 2, serial dilutions of peroxide in oil were used along with time-lapse imaging to compare sensitivity of the morphokinetic MEA to the eMEA. Culture conditions that may affect assay sensitivity were assessed in experiments 3–5, which examined the effect of group versus individual culture, oxygen concentration, and protein supplementation.

Results Extended MEA and cell counts identified toxicity not detected by the routine endpoint of blastocyst rate at 96 h. The eMEA was fourfold more sensitive than the standard MEA, and this sensitivity was similar to the morphokinetic MEA. Group culture had a protective effect against toxicity, while oxygen concentration did not affect blastocyst development. Protein supplementation with HSA had a protective effect on blastocyst development in eMEA.

Conclusions The standard MEA used by manufacturers does not detect potentially lethal toxicity of peroxides in mineral oil. While group culture may mask toxicity, protein supplementation and oxygen concentration have minimal effect on assay sensitivity. The eMEA and time-lapse morphokinetic assessment are equally effective in detection of peroxide toxicity and thus provide manufacturers and end-users a simple process modification that can be readily adopted into an existing QC program.

Keywords Mineral oil · Embryo culture · Quality control · Peroxides

Introduction

In vitro fertilization (IVF) success depends on the quality of laboratory methods, conditions, and supplies. Extended culture to the blastocyst stage, which is routine for freeze-all cycles and preimplantation genetic testing, lies at the center of this success. Thus, the embryo culture environment must be monitored, maintained, and improved through quality control (QC) testing [1, 2]. Current laboratory QC relies on alternatives for human embryos, most commonly the mouse embryo assay (MEA) [3, 4], which detects toxicity via effects on development to the blastocyst stage. Previous studies have shown that an MEA using time-lapse imaging [5] or embryos from outbred mice [6] are more sensitive than the standard MEA, neither method is a practical solution for IVF laboratories performing their own QC testing.

Mineral oil, an essential component of embryo culture, has been previously recalled due to toxicity [5]. Derived from crude oil, mineral oil contains aromatic and unsaturated hydrocarbons [7, 8] that are susceptible to peroxidation and free radical formation, known embryo toxins that can

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develop during storage and thus adversely affect embryo development and IVF outcomes even after passing testing by the manufacturer [7, 9, 10]. The chemical nature of mineral oil makes it the product in the IVF lab with the highest risk for adversely affecting clinical outcomes and warrants additional, end-user scrutiny.

The MEA is the industry standard for QC, as it is reported on nearly all certificates of analysis, yet neither the assay's endpoint nor the conditions are standardized. The most common endpoint reported with the MEA is blastocyst formation at 96 h of culture. This routine assay has been criticized for a lack of standardization in the number and type of mouse embryos used and for the consistency of the mouse model to represent human outcomes [11]. Additionally, the end-point of expanded blastocysts is not reliably associated with viability [12]. A recently described method of embryo evaluation is morphokinetic assessment of cell division, captured by time-lapse imaging [5]. Unlike the traditional MEA which uses a qualitative endpoint of blastocyst development, a morphokinetic MEA has increased sensitivity by incorporating multiple quantitative measures. However, morphokinetic assessment requires costly equipment which may not be universally available or realistically used for QC testing.

In contrast to assay endpoints, there is little evidence regarding the effect of assay conditions on MEA sensitivity. For human IVF, decreased oxygen concentration (5%) is both physiologic in the reproductive tract and associated with improved outcomes [13–16], yet many IVF clinics throughout the world still use atmospheric oxygen for culture and for QC testing [17]. The type of protein also varies in practice, thus, representing another variable that could influence assay sensitivity [18, 19]. A third variation in embryo culture methods, group versus individual culture, influences QC sensitivity [10], and evidence suggests that group culture improves IVF outcomes, either through diffusion of unknown paracrine factors or maintenance of a stable microenvironment [20].

The primary aim of this study was to optimize the standard MEA for detecting mineral oil peroxide toxicity using two commercially supplied oils with detectable peroxides at low and high concentrations. In the first study, traditional MEA outcomes such as cell number and blastocyst formation at 96 h were compared to a new method: blastocyst rate at 144 h in an extended MEA (eMEA). Based on results from experiment one, we proceeded with a dose-sensitivity study to compare the eMEA with our previously described time-lapse-based morphokinetic MEA [5]. In the final series of experiments, we evaluated the impact of altering culture conditions on eMEA sensitivity by studying the effect of group versus individual culture, oxygen concentration, and protein supplementation.

Materials and methods

Embryo collection and culture

The Mayo Clinic Institutional Animal Care and Use Committee (IACUC) approved all procedures involving animals. The mouse strains included inbred FVB females (in-house breeding colony) crossed with outbred CF1 males (Charles River Laboratories). Female mice were superovulated at 5-to-7 weeks old with 5 IU intraperitoneal pregnant mare serum (PMS;NHPP). Ovulation was triggered with 5 IU intraperitoneal human chorionic gonadotropin (hCG; APP Pharmaceuticals) 48 h later. Females were individually caged with CF1 males for breeding. Copulation was confirmed by the presence of a vaginal plug. One-cell mouse embryos were collected from the oviducts 18–20 h after hCG administration following removal of cumulus cells with hyaluronidase (Sigma). Embryos were rinsed in HTF-HEPES containing 0.1 mg/mL poly-vinyl alcohol (PVA; Sigma) before allocation to treatment groups.

Culture plates (60-well, ThermoFisher Scientific) were prepared the day prior to embryo culture and allowed to equilibrate overnight in an incubator at 37°C with atmospheric oxygen in 6.5% CO₂ to maintain standard pH, 7.28–7.32. Embryos in 60-well dishes were cultured individually in microdrops of 10 µL HTF + PVA (0.01 mg/mL) covered with 9 mL of treatment or control oil. All experiments were completed in triplicate.

Mineral oil preparation

Three individual lots of oil were used for the studies: a control (non-detectable peroxides), a previously studied oil that was recalled from the market that routinely passed the standard MEA (low peroxides, [5]), and a high peroxide oil. Peroxide concentrations were determined with SafeTest kit as previously described [10]; all three oils were tested in triplicate for each study replicate at the time oil dilutions were prepared for experiment 2. This peroxide assay was not sensitive enough to reliably detect peroxide concentrations less than 20 µEq/kg and did not detect other breakdown products of oil which were likely present (e.g., malonaldehydes and alkenals). The studies performed thus tested oil that contained peroxides and other undefined products of oil breakdown. Since oil composition is highly heterogeneous and peroxide is the most common quantitative measure for oil quality, these studies use the actual peroxide concentration, either as tested or based on serial dilutions, as the treatment. This limitation combined with the unknown chemical nature of the two oils means a comparison of the effects of the two oils relative to peroxide levels (i.e., experiment 2 vs. experiment 1) is not possible.

For experiments 1 and 3–5, the low peroxide oil (20 $\mu\text{Eq/mL}$) that was previously shown to not affect blastocyst development at 96 h [5] was used to assess the effects of culture conditions on the eMEA. For experiment 2, treatments consisted of dilutions of the high peroxide containing mineral oil (52 $\mu\text{Eq/kg}$). A control oil with undetectable peroxides was used to prepare dilutions of the affected oil to obtain oil that reduced blastocyst formation at 96 h for at least two treatments in order to test serial dilutions of putative sublethal peroxides. The prepared dilutions gave theoretical peroxide concentrations of 13.1, 6.5, 3.3, 1.6, and 0.8 $\mu\text{Eq/kg}$ that were below the detection limit of the assay.

Experiment one: cell counts versus extended MEA culture

Embryos were cultured individually ($n = 30$) in a 60-well dish in control oil or undiluted mineral oil containing peroxide (20 $\mu\text{Eq/mL}$). At 96 h of culture, development was assessed and half of the embryos ($n = 15$) were removed from culture and fixed in 4% paraformaldehyde for 20 min. After rinsing in Dulbecco's phosphate-buffered saline containing 0.1% PVA, the embryos were permeabilized in 70% ethanol for 5 min and stored at -80°C . Nuclear staining was completed with DAPI in Vectashield (ThermoFisher Scientific), and the stained embryos were stored at 4°C until counting. Embryo analysis with cell counting at 96 h was completed by one individual, blinded to the treatment groups, using three-dimensional imagery. Cell counts reflected total cell number, without differential. For the eMEA, embryos were assessed at 144 h of culture to determine the percentage of embryos at the expanded blastocyst stage. All studies were performed in triplicate.

Experiment two: comparison of sensitivity for peroxide in oil between embryo morphokinetics and the extended mouse embryo assay

Embryos were cultured individually ($n = 6$ –12) in an EmbryoSlide containing 20 μL HTF-PVA per well and covered with 1.4 mL diluted high-peroxide mineral oil serially diluted (calculated peroxide concentrations: 13.1, 6.5, 3.3, 1.6, 0.8, 0 $\mu\text{Eq/kg}$). Oil with undetectable peroxide was used for the control group. The EmbryoSlide was placed in an EmbryoScope for time-lapse analysis. Time-lapse microscopy with the EmbryoScope incubator captured division kinetics by collecting seven planes of images every 12 min for 144 h. Cell division timings were determined manually and included t2, t3, t4, t5, t6, t7, and t8 (time from fertilization to 2, 3, 4, 5, 6, 7, and 8 cells), time of compaction, tM, time of formation of a blastocoel, tB, and time to expanded blastocyst (tEB). Evaluation also included assessment of cc2 (time an embryo was at the 2-cell stage), cc3a (time an embryo was at the four cell stage), s2 (time an embryo was at the 3-cell stage), and s3

(time an embryo contained 5, 6, or 7 cells). All studies were performed in triplicate.

Experiment three: effect of group versus individual culture on sensitivity of the extended mouse embryo assay

Embryos were cultured individually ($n = 8$ –12) or in groups of four in an EmbryoSlide in 20 μL HTF-PVA (0.01 mg/mL) covered with undiluted peroxide mineral oil (20 $\mu\text{Eq/mL}$). Embryo development was assessed at 96 and 144 h. All studies were performed in triplicate.

Experiment four: effect of oxygen concentration on sensitivity of the extended mouse embryo assay

Embryos were cultured in individual wells ($n = 10$ –12) in a 60-well dish equilibrated and maintained at either 5% oxygen or 21% (atmospheric) oxygen. The dish was covered with 9 mL of undiluted peroxide mineral oil (20 $\mu\text{Eq/mL}$) and cultured in microdrops of 10 μL HTF + PVA (0.01 mg/mL). The control group was the 5% oxygen group. Embryo development was assessed at 96 and 144 h. All studies were performed in triplicate.

Experiment five: effect of protein supplementation on sensitivity of the extended mouse embryo assay

Embryos were cultured individually ($n = 36$ –39) in a 60-well dish. Embryos (12–13 per protein treatment) were cultured in 10 μL HTF containing 0.1 mg/mL PVA, 5 mg/mL HSA (LifeGlobal), or 10% SPS (Origio) covered with 9 mL of undiluted peroxide mineral oil (20 $\mu\text{Eq/mL}$). The control group was the HTF-PVA treatment. Embryo development was assessed at 96 and 144 h. All studies were performed in triplicate.

Statistical analysis

Statistical analysis was completed using the JMP statistical software (SAS Institute, Cary, NC, USA). Dunnett's t test was used to analyze data, which followed a normal distribution. ANOVA testing was used for multiple comparisons. Statistical significance was defined as $p < 0.05$.

Results

Experiment 1: extended culture and cell counts

Both eMEA and cell counts identified peroxide toxicity not detected by percentage of blastocysts at 96 h. Blastocyst development was similar at 96 h, 86 versus 90% (Fig. 1), but decreased at 120 h, 9 versus 13% ($p < 0.04$) and at 144 h, 30

Fig. 1 Blastocyst rate of individually cultured mouse embryos at 96, 120, and 144 h in control and peroxide mineral oil. Each bar represents data from three replicates of 10–12 embryos. (Asterisk) $p < 0.05$, (double asterisks) $p < 0.01$ peroxide oil (20 $\mu\text{Eq/mL}$) compared to control oil

versus 0% ($p < 0.01$), for embryos in control and peroxide oil, respectively. Blastocyst rates at 120 h did not provide significantly increased sensitivity and were not included in further experiments. Cell counts at 96 h also detected mineral oil toxicity with 64.3 ± 12.3 cells in control oil versus 52.7 ± 8.1 cells in peroxide oil ($p < 0.01$).

Experiment 2: morphokinetics versus eMEA

Diluted high peroxide oil containing low concentrations of peroxide had similar effects on cell division timings, the morphokinetic model and blastocyst development in the eMEA (Table 1). Data represents four replicates of 6–12 embryos each and represent Cc2 (duration of 2-cell stage), t8 (cleavage time of 8 cell embryo), tSB (time at start of blastocyst formation). An MK model, described in previously published work [5], was also applied. The duration of cell cycles was affected by peroxide oil at concentrations of 13.1 and 6.5 $\mu\text{Eq/kg}$ ($p \leq 0.001$). The duration of the 2-cell (cc2) stage was increased in response to higher peroxide concentrations,

resulting in a significant delay reaching the 8-cell stage and the start of blastocyst formation. This cumulative temporal effect was detected at a peroxide concentration of 3.3 $\mu\text{Eq/kg}$ as well. Percent embryos at the blastocyst stage at 144 h were also affected by peroxide concentrations of 13.1, 6.5, and 3.3 $\mu\text{Eq/kg}$ ($p < 0.001$), whereas the effect of 13.1 $\mu\text{Eq/kg}$ was detected at 96 h ($p < 0.001$) (Fig. 2), representing a fourfold increased sensitivity for the eMEA and the MK model relative to the 96 h MEA.

Experiment 3: effect of group versus individual embryo culture on eMEA

Blastocyst rates at 96 h were similar for embryos cultured individually versus in groups (Fig. 3a). More blastocysts remained at 144 h when embryos were cultured in groups (50.6%) than individually (3.7%), though this difference did not reach statistical significance ($p = 0.144$).

Experiment 4: effect of oxygen concentration on eMEA

Reduced (5%) versus atmospheric (21%) oxygen concentration was examined and found to have no significant effect on blastocyst rates (Fig. 3b). Blastocyst rates at 144 h post-fertilization at reduced and atmospheric oxygen in toxic mineral oil were 6 and 3%, respectively.

Experiment 5: effect of protein on eMEA

The presence and type of protein added to culture medium altered the detection of peroxide toxicity as assessed by blastocyst formation (Fig. 3c). A control macromolecule, PVA, was compared to HSA and SPS. While there was no difference in blastocyst rate at 144 h, SPS ($p < 0.001$) resulted in lower blastocyst rates at 96 h.

Table 1 Sensitivity to mineral oil peroxide: blastocyst rate in extended mouse embryo assay (eMEA) versus cell division timings (morphokinetics)

Peroxide concentration ($\mu\text{Eq/kg}$)	Blastocyst rates			Morphokinetics			
	96 (h)	120 (h)	144 (h)	cc2 (h)	t8 (h)	tSB (h)	MK model (%)
0	89.9%	85.7%	81.3%	23.0 (22.2–23.8)	49.7 (48.0–51.5)	77.1 (74.6–79.5)	80.0 (68.6–94.9)
0.8	100.0%	100.0%	86.1%	22.9 (21.9–23.8)	49.0 (48.2–49.8)	76.0 (74.6–77.4)	88.9 (76.2–101.5)
1.6	91.7%	85.4%	75.7%	22.9 (22.1–23.8)	51.0 (4.1–53.4)	78.4 (76.4–80.4)	57.6 (39.8–75.4)
3.3	82.6%	97.9%	24.8%***	23.6 (22.7–24.4)	53.3 (50.7–55.9)	83.7 (79.4–87.9)*	44.1 (26.5–61.7)***
6.5	57.3%	77.9%	3.1%***	24.7 (23.8–25.5)*	56.9 (53.3–60.6)***	90.1 (85.6–94.7)***	25.8 (9.5–42.1)***
13.1	21.9%**	16.7%**	0.0%***	27.0 (26.0–27.9)***	58.3 (52.7–63.9)***	89.5 (78.8–100.3)***	23.6 (1.0–46.0)***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control (0 $\mu\text{Eq/kg}$)

Fig. 2 Blastocyst rates of individually cultured mouse embryos at 96 and 144 h in mineral oil with increasing concentrations of peroxide toxicity. Data represents four replicates of 6–12 embryos each. (Double asterisks) $p < 0.01$ for peroxide concentration 13.1 $\mu\text{Eq/kg}$ at 96 h and (triple asterisks) $p < 0.001$ for peroxide concentration ≥ 3.3 $\mu\text{Eq/kg}$ at 144 h

Discussion

This study describes a practical, sensitive mouse embryo assay, the extended MEA, which can be applied in the clinical IVF laboratory for routine QC testing. Mineral oil, a standard component of embryo culture systems that is poorly defined, variable from batch to batch, and has the capacity to become toxic over time, remains the IVF laboratory's product with the most potential for variation in quality [7]. While previous data indicate that toxicity of manufacturer recalled oil can be detected with improved MEA methods [5, 6], neither method was suitable for routine use in clinical laboratories. This new method, the eMEA, provides a fourfold increase in sensitivity for toxic mineral oil, a sensitivity that is equivalent to our previously described morphokinetic model MEA.

Extending the MEA to 144 h is a simple and inexpensive improvement of the standard MEA. The manufacturer's stated endpoint, blastocyst development at 96 h, has poor correlation with pregnancy outcomes [21], suggesting that its sensitivity can be improved. This study found extended MEA to 144 h to be as sensitive as the morphokinetic MEA using time-lapse imaging [5]. In this study, both extended MEA culture and cell division timings were more sensitive to oil toxicity than the standard 96 h MEA. While the number of cells in blastocysts is also a sensitive measure of embryotoxicity, counting cells is technically challenging and time-consuming, making it impractical for clinical IVF laboratories. We observed an unexpected decrease in blastocyst rate at 120 and 144 h in the control group for experiment 1. This lower blastocyst rate can be explained by the experimental design, where half of the blastocysts were removed at 96 h, leaving non-blastocysts for extended culture. The extra handling at 96 h may also have contributed to the lower blastocyst rate in the eMEA for the controls.

Fig. 3 Effect of culture conditions on the sensitivity of the standard and eMEA to mineral oil peroxide (20 $\mu\text{Eq/mL}$) toxicity. **a** Blastocyst rates of embryos cultured individually and in groups of four in peroxide mineral oil. Data represents three replicates of 8–12 embryos each. **b** Blastocyst rates of individually cultured mouse embryos in 5 or 21% oxygen and peroxide mineral oil. Data represents three replicates of 10–12 embryos each. **c** Blastocyst rates of individually cultured mouse embryos in peroxide mineral oil with protein supplementation. Data represents three replicates of 12–13 embryos each. (Triple asterisks) $p < 0.001$ for blastocyst rate in SPS versus PVA at 96 h

While this study describes an improved QC testing method for toxicity of mineral oil, there are several limitations. First, in addition to peroxide, multiple compounds may contribute

to mineral oil toxicity [22, 23] and include malonaldehyde and alkenals as products of peroxide activity. Thus, the effects observed cannot be ascribed only to peroxide. The effect of toxicity on blastocyst rate is more objective than other endpoints, but still imperfect since blastocyst expansion and regression may alter interpretation.

In order to optimize the eMEA, this study assessed multiple variables that could confound results of QC testing by altering levels of reactive oxygen species or changing the stability of the culture microenvironment. These included group versus individual culture, oxygen concentration and effect of protein. Group culture was protective against sublethal toxicity as more blastocysts developed and persisted beyond 96 h when cultured in groups of four rather than when cultured individually, which is consistent with previous findings [10], that demonstrated cumene hydroperoxide toxicity is influenced by culture density, with reduced blastocyst formation, fewer cell numbers and increased apoptosis in individually cultured embryos. Taken together, the results indicate that group culture should be avoided for QC testing as it may stabilize the embryo microenvironment and mask product toxicity.

Oxygen concentration and protein supplementation are culture variables that are dependent on a laboratory's practice and thus could alter the sensitivity of the MEA. While reduced oxygen is known to yield better embryo development and outcomes in most species studied [13], it is not known if reduced oxygen affects MEA sensitivity. Therefore, experiment 4 investigated the effect of oxygen concentration on detection of mineral oil toxicity and found no effect on blastocyst development in the eMEA.

Since the type and amount of protein supplementation varies considerably in practice, its impact on assay sensitivity requires further study. Previous studies have demonstrated that both HSA and complex protein supplements are heterogeneous in nature [19] and thus represent variation that may impact MEA results. Specifically, complex proteins (SPS, LGPS) contain more transition metals than other protein supplements which may increase reactive-oxidant species and result in cell damage [18]. This study found SPS increased toxicity while HSA did not affect sensitivity. In contrast, Otsuki et al. found that peroxide toxicity is altered by the presence of albumin [22], though this was not observed in the present study. The impact of protein on assay sensitivity may be toxin-dependent and warrants further investigation.

Conclusions

This study investigated novel methods of QC testing and identified confounding factors in detection of mineral oil toxicity. Evaluation of blastocyst development in extended embryo culture to 144 h offers an inexpensive enhancement of the standard MEA that is equally sensitive to morphokinetic

assessment. Though manufacturers may incorporate more sensitive methods for screening mineral oil for toxicity, oil can become toxic during transport and/or storage. The eMEA, a simple modification of the standard MEA many labs currently use, provides a sensitive test for mineral oil peroxide toxicity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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