Building a better mouse embryo assay: effects of mouse strain and in vitro maturation on sensitivity to contaminants of the culture environment

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Abstract

Purpose The aim of this study is to compare the sensitivity of the standard one-cell mouse embryo assay (MEA) to that using in vitro-matured oocytes from hybrid and outbred mice. **Methods** The study was done by culturing embryos in the presence or absence of two concentrations (0.0005 or 0.001 %v/v) of Triton X-100 (TX100). Embryonic development, blastocyst cell numbers (total and allocation to the trophectoderm [TE] and inner cell mass [ICM]), and blastocyst gene expression were evaluated. **Results** Neither concentration of TX100 affected ($P>0.05$) cleavage, blastocyst development, or hatching in one-cell embryos from BDF1 mice. However, all cell number endpoints were reduced ($P<0.05$) by the high concentration of TX100 and the number of ICM cells was reduced ($P<0.05$) by the low concentration of TX100. Inhibitory ($P<0.05$) effects of the high concentration of TX100 were observed in in vitro maturation (IVM) embryos from BDF1, CF1, and SW, but not ICR, mice. Cell number and allocation were negatively affected by the high concentration of TX100 in CF1 and SW embryos, but not

in BDF1 or ICR embryos. The only developmental endpoints affected by the low concentration of TX100 were cleavage of BDF1 oocytes, blastocyst development of SW embryos, and cell numbers (total and inner cell mass (ICM)) of SW blastocysts. **Conclusions** The sensitivity of the MEA to TX100 is improved by using embryos from in vitro-matured oocytes, using oocytes from some outbred (SW or CF1, not ICR) strains of mice, and evaluating blastocyst cell number and allocation.

Keywords Quality control · In vitro maturation (IVM) · Mouse embryo assay (MEA)

Introduction

The effects of culture conditions on embryo viability are widely acknowledged, but controlling these conditions is not always straightforward. Even when the highest-quality reagents and plasticware are used, the introduction of some unknown substances (contaminants) into the culture environment is unavoidable [1–3]. As a result, all supplies that will come into contact with the embryo and/or the culture medium should be subjected to strict quality control (QC) testing, including the ability to support the growth of murine embryos [4, 5].

Although the mouse embryo assay (MEA) is used to screen many products before use in human assisted reproductive technologies (ART), the clinical relevance of its results must be interpreted cautiously. The use of the murine embryo in quality control assays requires one to assume that the sensitivity of the murine embryo to various contaminants is similar to, or even greater than, that of human embryos. However, we are not aware of any experimental data comparing the minimal inhibitory concentrations of various contaminants on murine and human embryos. If a given product inhibits the development of murine embryos, there is little question whether that

Capsule The sensitivity of the MEA to TX100 is improved by using embryos from in vitro-matured oocytes, using oocytes from some outbred (SW or CF1, not ICR) strains of mice, and evaluating blastocyst cell number and allocation.

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product should be used for clinical ART. The more troubling scenario is the possibility that a product could support the development of murine embryos but still be unsuitable for clinical use [3, 6]. The key to preventing such products from entering the clinic is to improve the sensitivity of MEAs and ensure clinically relevant results.

There are multiple ways to alter the sensitivity of a MEA, including changing the developmental stage of the embryo at the initiation of culture, the conditions used for culture, or the strain of mouse [4, 6–8]. All of these manipulations effectively alter the amount of stress placed on the embryo during culture. By increasing the amount of cellular stress the embryo must cope with under control conditions, the additional stress of contaminants should have a more pronounced effect on development, allowing detection in the MEA. One-cell embryos have not developed all of the necessary mechanisms to maintain intracellular homeostasis [9] and have not yet activated the embryonic genome [10, 11], so they are very sensitive to the culture environment [4, 12]. As a result, several studies have demonstrated that MEAs using one-cell embryos are more sensitive than MEAs utilizing two-cell embryos [7, 13]. Alternatively, the gas atmosphere, culture volume, embryo density, and concentration of protein during the MEA can be manipulated. Embryos will develop better when they are cultured in reduced oxygen, in groups, and with protein, so the use of atmospheric oxygen, individual culture, or culture in low protein or protein-free media will increase the sensitivity of the MEA [4, 7, 14]. Finally, it is possible to alter the sensitivity of the MEA by using embryos from different strains of mice [15, 16]. Although most MEAs use embryos from hybrid mice, these embryos are relatively insensitive and will develop well in a variety of media [12, 16, 17]. In contrast, embryos from inbred or outbred strains are more sensitive to culture conditions, which may greatly improve the sensitivity of the MEA to detect contaminants in the culture environment [4, 8, 18].

The chances of detecting contaminants can also be increased by evaluating multiple developmental parameters. Most MEAs simply determine the proportion of embryos that have reached the blastocyst stage by the end of the culture period (blastocyst quantity), without addressing the possibility of inhibitory effects on blastocyst quality. Total cell number of the blastocyst, the number of cells in the trophectoderm (TE) and inner cell mass (ICM), outgrowth of hatched blastocysts, and embryo metabolism can all be affected by culture conditions, providing alternative endpoints for QC testing that may result in increased sensitivity [8, 14, 19–21]. For example, ammonium in the culture medium can alter cell number, apoptosis, cellular metabolism, gene expression, and fetal development with no effect on blastocyst development [22, 23]. Although assessing additional endpoints greatly increases the reliability of the MEA, it also adds significantly to the cost of the QC process, whether it is performed by the manufacturer or by the end user. Therefore, it is necessary to carefully

select the conditions in which the MEA is conducted and the endpoints that will be evaluated to balance the clinical relevance of the results with the costs associated with performing the MEA.

Since embryos derived from in vitro maturation (IVM) and in vitro fertilization (IVF) have reduced developmental competence compared to embryos produced by in vitro fertilization of in vivo matured oocytes or embryos recovered at the one-cell stage [16, 24–27], we hypothesized that utilization of embryos from IVM and IVF may improve the sensitivity of the MEA. Using two low concentrations of a known contaminant of culture products (Triton X-100) [2, 8], the objective of this series of experiments was to compare the sensitivities of one-cell embryos from hybrid mice and embryos produced by IVM and IVF from hybrid mice, as well as 3 strains of outbred mice using multiple developmental parameters (morphological development, blastocyst cell number and allocation, and blastocyst gene expression).

Materials and methods

Animals

All mouse protocols were approved by the National Foundation for Fertility Research Ethics in Research Committee and followed animal care and use guidelines, as described by the *Guide for the Care and Use of Laboratory Animals* [28]. Mice were maintained in a 12:12 h light/dark cycle in single (male) or group (female) housing with ad libitum access to food and water.

In vitro maturation and in vitro fertilization

Female mice B6D2F1 (BDF1) [Hsd hybrid], 22 to 30 days; CF1 non-Swiss albino [Hsd: NSA], 30 to 50 days; Swiss Webster (SW) [Hsd:ND4], 22 to 30 days; ICR [Hsd: ICR, CD-1®], 22 to 30 days; Harlan Laboratories, Indianapolis, IN) were treated with 5 IU pregnant mare serum gonadotropin (PMSG) (Calbiochem, Billerica, MA, USA) and cumulus-oocyte-complexes (COC) were collected 46 to 48 h post-PMSG into a MOPS-buffered medium containing 5 % fetal calf serum (FCS) (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA). COC with multiple layers of cumulus cells were selected and matured in 50 µl drops (10 COC/drop) of media under OvOil (Vitrolife, Englewood, CO, USA) for 17 to 18 h. The maturation medium [26] contained 0.5 mM glucose, 0.2 mM pyruvate, 6.0 mM lactate, 0.5 mg/mL insulin, 0.275 mg/mL transferrin, 0.25 ng/mL selenium, 10 ng/mL epidermal growth factor (EGF), 2 mg/mL fetuin, and 2.5 mg/mL recombinant human albumin (AlbIX; Novozymes, Bagsvaerd, Denmark). IVM and all subsequent cultures were conducted at 37 °C in a humidified atmosphere of 7.5 % CO₂

and 6.5 % O₂. Due to the elevation of Lone Tree, CO (~1830 m above sea level), gas concentrations were adjusted to approximate 6 % CO₂ and 5 % O₂ at sea level and maintain the pH of all media between 7.2 and 7.3.

Spermatozoa were collected from the cauda epididymides and vas deferentia of BDF1 males (≥10 weeks). Spermatozoa were capacitated for 1 h in fertilization media (25.0 mM NaHCO₃, 3 mM glucose, 4.0 mg/mL BSA, 1.7 mM Ca²⁺ and 0.2 mM Mg²⁺) [26] and then co-cultured with COC (10 COC/50 µl drop, 1 × 10⁶ spermatozoa/ml) for 6 h.

One-cell embryos

Female mice (22 to 30 days BDF1) were treated with 5 IU PMSG (Calbiochem) and 5 IU human chorionic gonadotropin (48 h post-PMSG; Sigma, St. Louis, MO, USA) and paired with a male mouse of the same strain. Eighteen to 20 h post-hCG, embryos were recovered from the oviducts and placed into a MOPS-buffered medium containing hyaluronidase (~500 U/ml) to remove cumulus cells.

Embryo culture

Embryos were transferred (10 embryos/20 µl drop under OvOil) into the first medium of a sequential culture system (0.5 mM glucose, 0.3 mM pyruvate, 10 mM lactate) [29] containing Triton X-100 (TX100; 0 % control, 0.0005 % low, or 0.001 % high). After 48 h of culture, embryos were transferred (10 embryos/20 µl drop under OvOil) to the second medium (3 mM glucose, 0.1 mM pyruvate, 6 mM lactate) [29] containing the same concentration of TX100 used for the first culture period. Both culture media contained a reduced concentration (1 mg/ml) of recombinant human albumin (AlbIX). Cleavage was evaluated 24 h post insemination and uncleaved embryos were discarded at this time. On day 4 (D4) and day 5 (D5; 96 and 114 h of culture, respectively), blastocyst development was assessed.

Immunofluorescent staining of blastocysts

On D5, hatching and fully-hatched blastocysts were fixed in 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 to 20 min and stored in PBS with 0.5 % BSA (MP Biomedicals, Solon, OH, USA) until staining. Blastocysts were washed in PBS with 0.1 % TX100, permeabilized in PBS with 1.0 % TX100 (30 min), and blocked in PBS with 0.1 % TX100 and 0.5 % BSA (2 h), before incubation with primary antibodies. Anti-human SOX2 (Biogenex, Fremont, CA, USA, rabbit monoclonal) and CDX2 (Biogenex, mouse monoclonal) were used to detect the inner cell mass (ICM) and the trophectoderm (TE), respectively [30]. Secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse

IgG; Invitrogen, Thermo Fisher Scientific) were then used for SOX2 and CDX2, respectively. Blastocysts were mounted on a glass slide with ProLong Gold Antifade Reagent (Life Technologies, Thermo Fisher Scientific) and evaluated (×400) using a fluorescent microscope (Olympus BX52) and MetaMorph software.

Quantitative PCR

Primers for genes previously correlated with embryo viability [6, 32, 33] (bone morphogenic protein 15, *Bmp15*; caudal-type homeobox protein 2, *Cdx2*; cyclooxygenase 2, *Cox2*; DNA methyltransferase 3a, *Dnmt3a*; glutaredoxin2, *Glx2*; glucose transporter 1, *Glut1*; octamer-binding protein 4, *Oct4*), and the reference gene peptidylprolylisomerase A (*Ppia*) were designed for quantitative PCR (qPCR) using Primer3 [31], and qPCR was performed as previously described [6, 32, 33]. Accession number, primer sequence, and product length of target and reference genes are described in Supplemental Table 1. Blastocysts were collected in groups of four on D5, and frozen in 10-µl extraction buffer (Arcuturus Picopure, Thermo Fisher Scientific). The Picopure Isolation Kit (Applied Biosystems, Thermo Fisher Scientific) with an on-column DNase treatment (Qiagen; Valencia, CA, USA) was used to extract RNA from blastocysts. The complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Primer specificity was determined by performing melt curve analysis and gel electrophoresis. Resulting PCR products were cloned into pCR 2.1 TOPO vectors and transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen). The plasmids were sequenced by the DNA sequencing facility at Colorado State University (Fort Collins, CO) to confirm the identity of the transcript. They were also quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Quantitative PCR was performed in a threefold diluted sample cDNA run in duplicate. Transcript abundance of the reference gene (*Ppia*) remained stable between treatments. Target genes were analyzed using iQ SYBR Green Supermix (Bio-Rad; Hercules, CA, USA) and an Applied Biosystems 7300 Real Time PCR system. A standard curve was generated from serial dilutions of EcoRI digested plasmids and the efficiency of the primers was calculated.

Statistical analysis

For analysis of the effect of culture treatment on the percentage of embryonic cleavage, blastocyst development, blastocyst hatching, and blastocyst cell number, a one-way ANOVA was used with treatment (control, low TX100, high TX100) included in the model as a fixed factor. When significant differences were detected, treatment differences were determined using the Fisher LSD multiple comparison test.

For relative quantification of gene expression, data were analyzed using the relative expression tool REST 2009 version 2.0.13 [34], as described previously [35, 36]. Briefly, expression ratios were generated using PCR efficiencies of the target and reference genes and the Δ CT values of control blastocysts (0 % TX100) compared to blastocysts cultured in either low or high TX100. The levels of significance were calculated by pair-wise fixed reallocation randomization tests with 50,000 iterations. In all cases *P* values less than 0.05 were considered significant.

Results

One-cell embryos from hybrid (BDF1) mice

The proportions of recovered zygotes that cleaved (control, 91.6 ± 2.6 %), developed to the blastocyst stage on D4 (89.9 ± 2.8 %), or the hatching blastocyst stage on D5 (88.2 ± 3.0 %) were not affected ($P > 0.05$) by either the low (90.1 ± 2.7 %, 88.4 ± 2.9 %, 84.3 ± 3.3 %, respectively) or high (90.4 ± 2.8 %, 86.8 ± 3.2 %, 82.5 ± 3.6 %, respectively) concentration of TX100 (Fig. 1a). The proportions of cleaved embryos that developed to the blastocyst (control, 98.2 ± 1.3 %) or hatching blastocyst stage (96.3 ± 1.8 %) were also unaffected ($P > 0.05$) by the concentration of TX100 (Fig. 1a). When total cell number (control, 117.6 ± 6.0) and allocation of cells to the TE (102.5 ± 5.3) and ICM (15.1 ± 1.2) were evaluated, the high concentration of TX100 decreased ($P < 0.05$) in all three parameters (88.0 ± 5.5 total, 78.4 ± 5.0 TE, and 9.6 ± 0.7 ICM cells) compared to the control (Fig. 2a). In addition, the number of ICM cells was also decreased ($P < 0.05$) in the presence of the low concentration of TX100 (10.7 ± 0.9).

IVM embryos from hybrid (BDF1) mice

When BDF1 embryos were produced by IVM and IVF, exposure to both the low (79.2 ± 4.1 %) and high (69.6 ± 4.6 %) concentrations of TX100 reduced ($P < 0.05$) the proportion of oocytes that cleaved (control, 91.3 ± 2.8 %; Fig. 1b). However, the proportions of oocytes (control, 51.9 ± 4.9 %) and cleaved embryos (56.8 ± 5.1 %) that developed to the blastocyst stage on D4 were not affected ($P > 0.05$) by either concentration of TX100 (Fig. 1b). The proportion of hatching blastocysts on D5 was reduced ($P < 0.05$) by the high concentration of TX100 (15.7 ± 3.6 % of oocytes and 22.5 ± 5.0 % of cleaved embryos; Fig. 1b) compared to control embryos (36.5 ± 4.7 % and 40.0 ± 5.1 %, respectively), although the low concentration of TX100 had no effect ($P > 0.05$). In contrast, the total number of cells (control, 81.1 ± 4.3), the number of TE cells (71.8 ± 4.0), and the number of ICM cells (9.2 ± 0.7) per blastocyst were not affected ($P > 0.05$) by either concentration of TX100 (Fig. 2b).

IVM embryos from outbred mice: CFI

The proportions of oocytes that cleaved (control, 73.2 ± 4.2 %), developed to the blastocyst stage on D4 (35.7 ± 4.5 %) and the hatching blastocyst stage on D5 (20.5 ± 3.8 %) were all reduced ($P < 0.05$) in the presence of the high concentration of TX100 (38.5 ± 4.4 %, 11.5 ± 2.9 %, and 6.6 ± 2.3 %, respectively; Fig. 1c), but not by the low concentration of TX100. Neither concentration of TX100 affected ($P > 0.05$) the proportion of cleaved embryos developing to the blastocyst (control, 48.8 ± 5.6 %) or hatching blastocyst stages (control, 28.0 ± 5.0 %). Similarly, the high concentration of TX100 reduced ($P < 0.05$) the total number of cells (44 ± 7.8) and the number of TE cells (37.2 ± 6.7) compared to control embryos (77.4 ± 8.3 total, 68.2 ± 7.2 TE; Fig. 2c). The number of ICM cells (control, 9.2 ± 1.3) was not affected ($P > 0.05$) by either concentration of TX100.

IVM embryos from outbred mice: SW

Although the proportion of oocytes that cleaved was not affected ($P > 0.05$) by either concentration of TX100, blastocyst development on D4 (control, 59.6 ± 4.7 % of oocytes and 87.8 ± 3.8 % of cleaved embryos) was inhibited by the high concentration of TX100 (35.2 ± 4.7 % of oocytes and 59.7 ± 6.3 % of cleaved embryos; Fig. 1d). The high concentration of TX100 also reduced ($P < 0.05$) the proportion of oocytes that developed to the hatching blastocyst stage on D5 (control, 48.6 ± 4.8 %; high TX100, 31.4 ± 4.6 %) and tended to reduce ($P = 0.08$) the proportion of cleaved embryos that reached the hatching blastocyst stage by D5. The proportion of cleaved embryos that developed to the blastocyst stage on D4 was also decreased ($P < 0.05$) by the low concentration of TX100 (74.0 ± 5.2 %). When blastocyst cell numbers were evaluated, the total number of cells (110.3 ± 5.0), the number of TE cells (97.3 ± 4.4), and the number of ICM cells (13.0 ± 0.9) in control embryos were all reduced ($P < 0.05$) in the presence of high TX100 (88.1 ± 4.4 , 77.7 ± 4.4 , and 10.1 ± 0.6 , respectively; Fig. 2d). In addition, the low concentration of TX100 decreased ($P < 0.05$) the total number of cells and the number of ICM cells in blastocysts compared to control (Fig. 2d).

IVM embryos from outbred mice: ICR

None of the developmental or cell number parameters evaluated were affected ($P > 0.05$) by either concentration of TX100 (Supplemental Figure 1).

Blastocyst gene expression

In blastocysts produced from one-cell embryos recovered from BDF1 mice, expression of genes correlated with embryo viability was affected ($P < 0.05$) by exposure to TX100.

Fig. 1 Development of one-cell embryos recovered from BDF1 females (a) or embryos produced by in vitro maturation (IVM) and fertilization from BDF1 (b), CF1 (c), and SW (d) oocytes that were cultured in the absence (control) or presence of low (0.0005 %) or high (0.001 %) concentrations of Triton X-100. Blastocyst formation was evaluated on

day 4 (d4BI) and hatching was evaluated on day 5 (d5Hg) and expressed as a percentage of cultured oocytes (/oo) or cleaved embryos (/cl). Different superscripts indicate a significant ($P < 0.05$) difference between treatments. If no superscripts are present, there were no significant differences ($P > 0.1$) between treatments

Transcript levels of only one gene, *Dnmt3a*, were reduced in blastocysts cultured with the low concentration of TX100, while transcript levels of four genes, *Dnmt3a*, *Glx2*, *Glut1*, and *Oct4* were reduced by culture of embryos with the high concentration of TX100, compared to control (Fig. 3). Gene expression in blastocysts from IVM oocytes was less affected by TX100 treatment during culture (data not shown). In BDF1 mice, transcript abundance of *Bmp15* was downregulated ($P < 0.05$) in IVM derived blastocysts after culture in the low concentration of TX100, but there were no effects on the expression of the examined genes following culture with the high concentration of TX100. In CF1 mice, expression of *Cox2* was elevated ($P < 0.05$) in IVM derived blastocysts following culture with the low concentration of TX100, while *Glx2* and *Glut1* transcript levels were elevated ($P < 0.05$) following culture with the high concentration of TX100.

Discussion

Selection of products for use in a clinical ART laboratory is often determined by their ability to support or inhibit the

development of mouse embryos in a mouse embryo assay, or MEA. In most cases, these embryos are collected from hybrid mice at the one-cell stage, cultured for 4 to 5 days, and the proportion of embryos reaching the blastocyst stage is determined [5]. In agreement with previous studies, we have shown that this type of MEA is relatively insensitive [8]. Our results demonstrate that a MEA using one-cell embryos from hybrid (BDF1) mice was unable to detect either of the tested concentrations of TX100 when embryonic development was the only endpoint evaluated. We also demonstrated that the sensitivity of the MEA could be increased by using embryos produced by IVM and IVF of oocytes from outbred strains of mice and/or assessing additional developmental endpoints (e.g., embryo cell number and allocation, gene expression, etc.). However, responses to the tested contaminant (TX100) varied between mouse strains, such that the most useful endpoint for detecting contamination was strain-dependent (Table 1).

In general, the earlier an embryo is collected from the female reproductive tract, the more sensitive it is to culture conditions [9, 37]. As a result, MEAs utilizing one-cell embryos are more likely to detect contaminants in the culture

Fig. 2 Number of cells in the trophectoderm (TE, CDX2-positive) or inner cell mass (ICM, SOX2-positive) and the total cell number (TE+ICM) of hatching or hatched blastocysts from one-cell embryos recovered from BDF1 females (**a**) or embryos produced by in vitro maturation (IVM) and fertilization from BDF1 (**b**), CF1 (**c**), and SW (**d**) oocytes

that were cultured in the absence (control) or presence of low (0.0005 %) or high (0.001 %) concentrations of Triton X-100. Different superscripts indicate a significant ($P < 0.05$) difference between treatments. If no superscripts are present, there were no significant differences ($P > 0.1$) between treatments

environment than MEAs using two-cell embryos [7, 13, 15]. We hypothesized that IVM would be useful for MEAs because of their reduced developmental potential compared to

one-cell embryos, [25, 38, 39]. In the present study, IVM embryos from three of the four strains of mice tested were sensitive to TX100 when present at concentrations that did

Fig. 3 Expression of genes correlated with viability in blastocysts derived from one-cell embryos collected from BDF1 mice and cultured in the absence (control) or presence of low (0.0005 %) or high (0.001 %) concentrations of Triton X-100. Data show average fold change normalized against the housekeeping gene *Ppia*, and control gene expression is set to zero. Asterisks indicate a significant difference ($P < 0.05$) between the treatment and the control

Table 1 Developmental endpoints significantly ($P<0.05$) affected by the low (0.0005 %) or high (0.001 %) concentration of Triton X-100 (TX100) for each tested MEA

	One-cell embryos	IVM embryos			
	BDF1	BDF1	CF1	SW	ICR
Cleavage		Low and high	High		
Day 4 blastocyst					
/Oocyte			High	High	
/Cleaved embryo				Low and high	
Day 5 hatching					
/Oocyte		High	High	High	
/Cleaved embryo		High			
Blastocyst cell number					
Trophectoderm (TE)	High		High	High	
Inner cell mass (ICM)	Low and high			Low and high	
Total (TE+ICM)	High		High	Low and high	
Number Endpoints sensitive to:					
High TX100	3	3	5	6	0
Low TX100	1	1	0	3	0

not affect blastocyst formation or hatching of one-cell embryos from BDF1 mice. However, there did not appear to be a correlation between developmental potential and sensitivity to TX100 among the different strains. Embryos from one of the strains with the worst development (ICR, 58 % D4 blastocyst/cleaved embryo) were not affected by either concentration of TX100, while embryos from one of the strains with the best development (SW, 88 % D4 blastocyst/cleaved embryo) were sensitive to both concentrations of TX100. This unexpected finding illustrates the importance of selecting, and validating, the strain of mouse used for oocyte or embryo collections to determine the level of sensitivity of the MEA.

It is widely acknowledged that morphological evaluation of blastocyst development is an inadequate measure of embryo quality. Blastocysts at the same developmental stage with similar morphologies can have dramatically different cell numbers, metabolism, gene expression, and developmental potential post-transfer [19, 20, 23]. However, these additional endpoints are rarely, if ever, included in the MEAs conducted by manufacturers of products for human ART laboratories. In the present study, we demonstrated that evaluation of additional endpoints can make the difference between a product passing or failing and thus entering or being excluded from the clinical IVF laboratory. Using one-cell embryos from hybrid mice, media containing both concentrations of TX100 would have passed the MEA based on standard developmental parameters. However, if total cell number or gene expression was evaluated, the high concentration of TX100 would have been detected. The only way we could detect both concentrations of TX100 using one-cell embryos from BDF1 mice was by examining cell allocation to the ICM and/or the expression of a single gene (*Dnmt3a*). Thus, the evaluation of additional endpoints is essential to prevent potentially toxic products

from contacting human embryos when one-cell embryos from hybrid mice are used.

Due to the increased sensitivity of IVM embryos to TX100, evaluation of morphological development alone was sufficient to detect the high concentration of TX100 on the development of embryos from BDF1, CF1, and SW mice. In fact, determination of blastocysts cell numbers was either ineffective for detection of TX100 (BDF1) or simply confirmed effects that were evident when morphological development was assessed (CF1 and SW). However, even with IVM embryos, detection of the low concentration of TX100 was difficult. Only in embryos from SW mice was evaluation of morphological development sufficient to detect the low concentration of TX100 and blastocyst cell numbers confirmed these effects. Aside from a small, but significant reduction in the proportion of BDF1 oocytes cleaving, evaluation of blastocyst gene expression was the only way to detect inhibitory effects of the low concentration of TX100 when IVM embryos from BDF1 (*Bmp15*) or CF1 (*Cox2*) mice were used.

Although we have demonstrated that the results of a MEA can be dramatically altered by the strain of the mouse used and the endpoints evaluated, it is unclear if the response of the embryo is dependent on the type of contaminant being tested. TX100 was chosen as a representative contaminant, since this chemical has been detected in culture supplies and used in other studies evaluating the suitability of MEA conditions [2, 8, 40]. In addition, two studies have shown that the effects of TX100 are similar to those of two other known contaminants [8, 40]. However, this is not the only potential contaminant that may be present in products used for embryo culture. A variety of other chemical contaminants have been detected in different culture supplies, including peroxides, alkenes, and octanoic acid [2, 3, 41]. Similarly, some components of the

culture environment, most notably protein supplements, can contain a wide variety of carbohydrates, amino acids, and other proteins in addition to serum albumin [6, 42, 43]. These other substances are not chemical contaminants, like TX100, but their concentration in protein supplements can vary between batches and can alter embryonic development. Additional studies are necessary to determine if there is an interaction between the type of embryo and/or strain of mouse used and the type of contaminant being tested.

Our results indicate that a MEA utilizing IVM-derived embryos from SW mice is highly sensitive to TX100, since multiple endpoints detected low levels of this contaminant. However, we do not know which type of embryo (or endpoint) provides the most clinically relevant results. Few products will ever be 100 % pure or contaminant-free. Therefore, for each contaminant there is a minimal inhibitory concentration, below which the contaminant can be considered safe for use with human embryos. Similarly, for every MEA, there will be a minimum detectable concentration of that same contaminant (the lowest concentration that significantly affects the endpoint being evaluated). Ideally, the minimum detectable concentration for the MEA should be equal to, or lower than, the minimal inhibitory concentration for human embryos. Since these concentrations have not been determined for either mouse or human embryos, the results of MEAs must be interpreted with caution. The only way to truly determine the clinical relevance of a MEA is by carefully monitoring clinical results following the introduction of a new product (or new lot of a product) into the laboratory, and correlating outcomes with MEA results. If clinical outcomes remain consistent when the new product is used, the MEA used to test that product is apparently suitable. However, if clinical results decline with the use of a new, MEA-tested product, the MEA used to test that product was not sensitive enough. We propose that the widespread use of the one-cell hybrid MEA, a relatively insensitive assay, results in products being approved for clinical use even though they contain clinically-relevant, inhibitory concentrations of contaminants.

Conclusions

With so many potential variables affecting the sensitivity of a MEA, it is concerning how little we know about the MEAs performed by the manufacturers of “embryo-tested” culture supplies. Many companies will specify whether they use one- or two-cell embryos and some will disclose the minimum development required for a product to pass (e.g., ≥ 80 % expanded blastocysts), but very few specify what strain of mouse is used, or if endpoints other than morphological development were evaluated. In addition, few, if any, companies report the sensitivity of their MEA to known contaminants. The results of the present study suggest that consumers should

demand more information regarding the rigor of pre-market testing of ART products in order to make informed choices for the clinical laboratory. Finally, we advocate that a more sensitive MEA should be adopted by manufacturers to more efficiently detect harmful components before they enter the IVF laboratory.

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Compliance with ethical standards

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Conflict of Interest The authors declare that they have no competing interests.

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