Potential of inner cell mass outgrowth and amino acid turnover as markers of quality in the in vitro fertilization laboratory

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Objective: To compare sensitivity of inner cell mass (ICM) outgrowth assay and analysis of culture media amino acid turnover with the sensitivity of the human sperm motility assay (HSMA) and murine embryo assay (MEA) for detection of formaldehyde toxicity.
Design: Prospective in vitro study.
Setting: University hospital–based infertility center.
Animal(s): Murine embryos.
Intervention(s): The HSMA, MEA, and ICM outgrowth assays were performed with media containing 0–64-μM concentrations of formaldehyde. These assays were compared with dynamics of amino acid turnover in culture media.
Main Outcome Measure(s): The lowest concentration of formaldehyde in culture media detected by each quality control assay.
Result(s): Sperm forward progression, but not motility, detected formaldehyde at a concentration of 32 μM. Sperm motility index identified formaldehyde toxicity at 64 μM, whereas blastocyst rates in the MEA were affected at 32 μM formaldehyde. Evaluation of ICM using outgrowth and grade detected 16 μM formaldehyde. Leucine turnover in culture media detected 64 μM formaldehyde in the amino acid assay.
Conclusion(s): Inner cell mass outgrowth is a more sensitive bioassay than MEA and HSMA for the detection of formaldehyde in culture media. Amino acid metabolism may also provide a sensitive quality control measure for detection of formaldehyde. (Fertil Steril® 2012;98:863–9. ©2012 by American Society for Reproductive Medicine.)
Key Words: IVF quality control, inner cell mass (ICM), murine embryo assay (MEA), formaldehyde

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Quality control (QC) testing of materials used for IVF and embryo culture is a standard practice performed by both manufacturers and individual laboratories to minimize exposure of human embryos to potential exogenous toxins. The goal of QC is to ensure that embryos are cultured in optimal in vitro conditions. Currently, most IVF programs use the murine embryo assay (MEA) or the human sperm motility assay (HSMA) for laboratory QC testing (1–3). These tests help ensure that materials such as Petri dishes, mineral oil, media, incubators, and air systems are not toxic to sperm, oocytes or preimplantation embryos. Concerted efforts to improve IVF outcomes include development of more sensitive QC assays that are able to detect low levels of contaminants.

The industry lacks a consensus on which QC assay is best suited for testing in vitro conditions in the laboratory (4). Although the one-cell MEA is the standard for testing by the manufacturer, IVF laboratories commonly use the HSMA because human sperm are more affordable and readily available (5). However, relative sensitivities of the HSMA and the MEA to different toxins have been studied, and the results have not resulted in a firm consensus (2, 6, 7). Although the MEA

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and HSMA are the two most common assays currently used, new and improved assays are needed to effectively screen products and ensure minimal in vitro culture stress.

Traditional QC assays are limited by the endpoint studied: sperm motility or blastocyst development. Although these bioassays provide a measure of quality, assessing an embryo's potential beyond blastocyst may provide additional data on culture conditions. Current QC assays use gametes or blastocysts as a surrogate marker for implantation and live birth; however, formation of an inner cell mass (ICM) in culture provides an in vitro marker that more closely resembles implantation (8). In fact, outgrowth of the ICM has been described as a measure of embryo quality and as a better predictor of fetal developmental potential than blastocyst formation alone (9) and has also been used to test hydrosalpinx fluid for embryo toxicity (10). Therefore, ICM outgrowth as a QC assay may provide a better marker for in vitro stress, but its sensitivity compared with traditional MEA endpoints is unknown.

Recently, considerable attention has been given to the concept of embryo assessment using the products of embryo metabolism, an area termed "metabolomics" (11). Several investigators have studied conditioned culture media to determine whether the appearance or disappearance of metabolites can predict the quality of embryos and the success of human ET (11–14). Amino acid metabolism and turnover is one measure of metabolomics and seems to be related to developmental potential (15). Amino acid metabolism as a function of in vitro stress has not been studied but may be a useful tool for QC testing in IVF laboratories.

The aim of this study was to determine the relative sensitivities of standard QC assays, such as HSMA and MEA, and novel assays, such as ICM outgrowth and amino acid turnover. For this purpose we used formaldehyde, a volatile organic compound, as a model airborne toxin that has been implicated as a component of poor air quality with negative consequences on IVF (16).

MATERIALS AND METHODS

Human Sperm Motility Assay

Fresh semen samples were obtained from a proven fertile donor and prepared on a single 90% layer of Isolate (Irvine Scientific). The sample was centrifuged, washed, and resuspended in G1 media (Vitrolife). Fifty microliters of the washed sperm suspension was added to 12 × 75-mm snap-cap tubes containing 450 μL of G1.5 test media to yield a final concentration of 5×10^6 motile sperm per milliliter. The G1 test media plus formaldehyde at 0 (control), 16, 32, and 64 μM was used to determine the effects on sperm. Tubes were loosely capped and cultured at 6.5% CO_2 and 37°C. Motility and forward progression (grade: scale of 1 to 4, with 4 having rapid forward progression) were determined at 48 hours. The control sample was required to have 75% of original motility at 48 hours for the assay to be considered valid. A sperm motility index (SMI) was calculated by dividing the percentage of motile sperm of the test media by the percentage progressive motile sperm of the control at the specified time intervals. For each human sperm motility assay, the control and formaldehyde concentrations were tested in triplicate.

Murine Embryo Assay

Cryopreserved two-cell mouse embryos were obtained from a commercial source (Embryotech Laboratories) and thawed according to the manufacturer's instructions; Institutional Animal Care and Use Committee was not required, per Mayo Clinic policy. Thawed embryos were rinsed in modified human tubal fluid (HTF-HEPES; Irvine Scientific) and supplemented with 5 mg/mL human serum albumin (Irvine Scientific) for 10 minutes at room temperature. Embryos (n = 20) were cultured at 37°C in 6.5% CO_2 in 50 μL of G1 (Vitrolife) media plus formaldehyde at 0 (control), 16, 32, and 64 μM in drops under mineral oil (Fisher Scientific). Twenty-four hours later, embryos were transferred to 50 μL of G2 (Vitrolife) test media plus the same concentrations of formaldehyde from the previous drop for an additional 48 hours of culture. Embryos were graded at 24-hour intervals, and the rate of expanded blastocyst formation was determined at 72 hours (Fig. 1A). Each experiment was performed in triplicate, with 20 embryos per treatment.

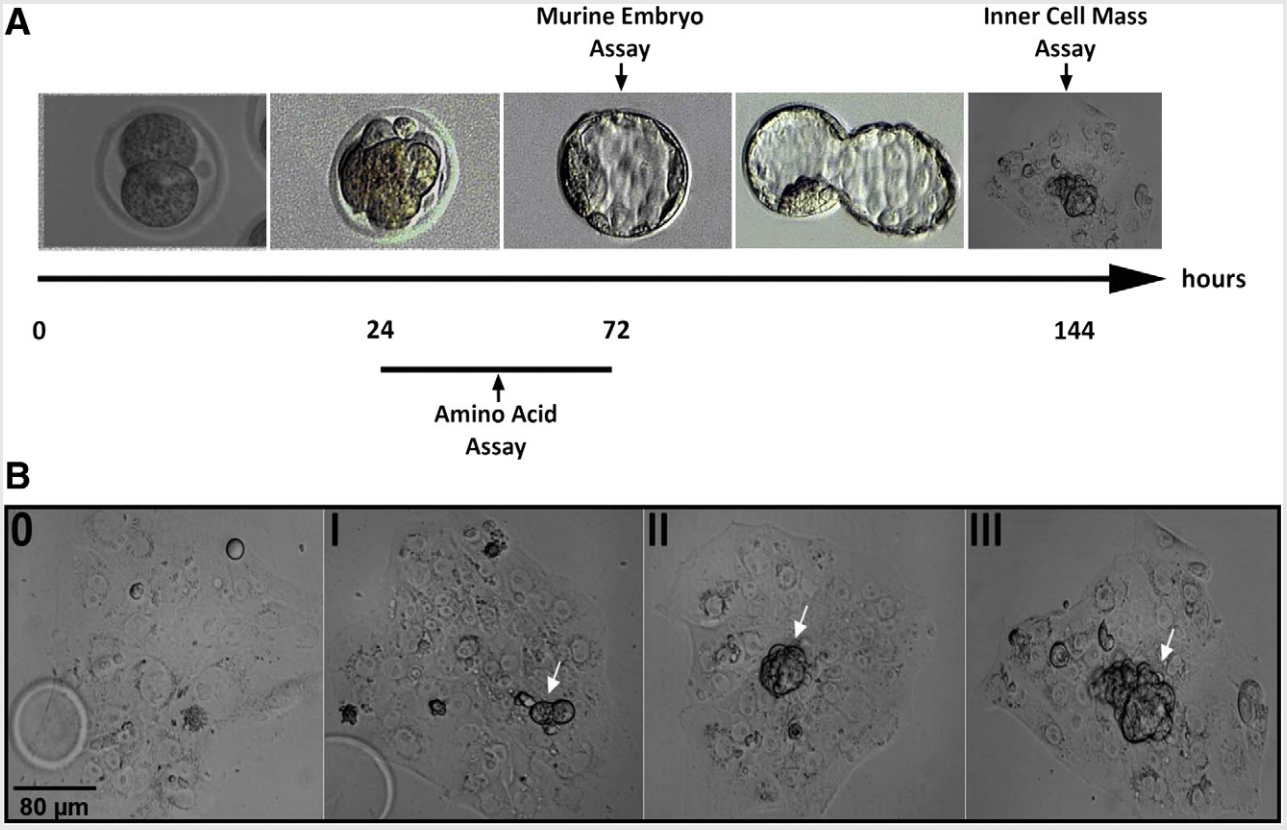
Inner Cell Mass Assay

After 72 hours of culture in media containing formaldehyde as described in the MEA methods, embryos that developed to blastocyst stage (n = 6–18) were transferred to 2 mL Glasgow minimal essential medium (Sigma Chemical) plus 20% fetal calf serum (Sigma) overlaid with 2 mL of mineral oil in a 35-mm Petri dish for the ICM outgrowth assay (9). The Petri dish was pretreated with 0.1% gelatin from bovine skin (Sigma) for 1 hour, and embryos were then cultured for an additional 96 hours. During this extended culture of 96 hours no formaldehyde was present. The gelatinous layer of the outgrowth plate allows the embryo to attach and continue growth once it has hatched from the zona pellucida. Once the embryos were removed from the original culture drops, 40 μL of test G2 media was collected and stored at −80°C for amino acid analysis. After the additional 96 hours of extended culture in the gelatin-coated dishes, the embryos were graded for ICM outgrowth (Fig. 1B). A Zeiss Axiovert microscope was used to obtain images at ×400 magnification. For each experiment three replicates were performed. The ICM rate was calculated by dividing the number of embryos that survived to ICM outgrowth by the original number of embryos placed in culture (n = 20).

Amino Acid Analysis

Culture media from the MEA described above was used for the amino acid assay. After the first 24 hours of culture in G1 media, the murine embryos were transferred to G2 media. The embryos were then cultured for another 48 hours, which was essentially the time of development from the morula to the blastocyst stage (Fig. 1A). The culture media during these 48 hours, referred to as conditioned media, was collected and stored at −80°C for amino acid analysis. Under the same conditions, G2 media containing no embryos was also incubated for 48 hours. This media was then collected and referred to as blank media. Once all experiments were performed, the media was thawed for amino acid analysis. Media was not stored more than 90 days at −80°C.

FIGURE 1



Model representation of the MEA (A), ICM outgrowth assay (B), and amino acid assay along a time line is illustrated. Both the MEA and amino acid assay can be completed simultaneously at the 72-hour mark. The ICM outgrowth assay requires an additional 96 hours of culture to be completed. Embryos were cultured for an additional 96 hours after blastocyst development, to evaluate the ICM. Inner cell mass outgrowth of murine embryos was graded using a four-category system (B, left to right, 0–III): grade 0 represents no ICM development; in grade 1 the arrow depicts a small area of ICM development; in grade 2 a larger ICM is present without development of depth; in grade 3 the ICM is completely developed with differentiation and depth. Images were obtained using a Zeis Axiovert microscope at ×400 magnification.

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Amino acids were analyzed with an aTRAQ reagent amino acid tagging system (AB Sciex). Culture media (40 μL) was mixed with 10 μL 10% sulfosalicylic acid, using an Apricot TPS-24 automated liquid handler and centrifuged to precipitate proteins. Then 10 N borate buffer (40 μL) was added to the supernatant and incubated with aTRAQ Reagent 121 (5 μL) for 30 minutes at room temperature. Five microliters of 1.2% hydroxylamine solution was added to each sample. Samples were dried and reconstituted with aTRAQ Reagent 113–labeled Standard Mix (32 μL). Amino acids were separated and detected by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS; API 3200) in positive multiple reaction monitoring mode (MRM). Chromatography was performed using a C18 (150 × 4.6 mm) column, with mobile phases 0.1% formic acid:0.01% HFBA:H_2O and 0.1% formic acid:0.01% HFBA:MeOH. Total analysis time was 18 minutes.

Statistical Analysis

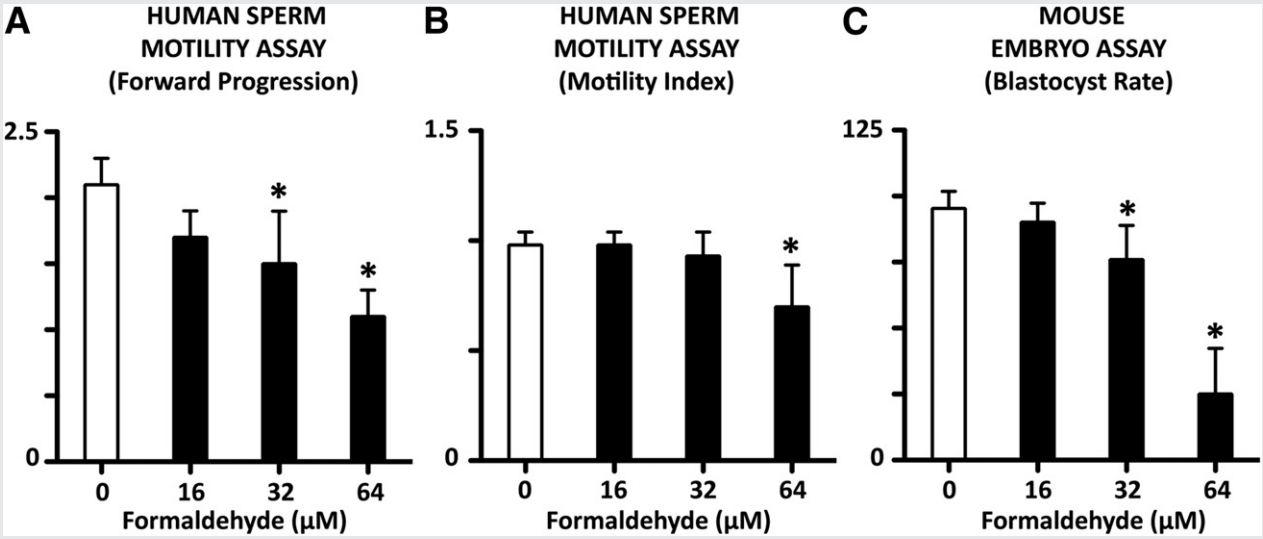
Results were analyzed by one-way ANOVA, and significance was determined by Student's *t* test, $P<.05$ for the three experiments using JMP statistical software (SAS Institute).

RESULTS

The HSMA and the MEA were performed at increasing concentrations of formaldehyde, to determine the lowest detectable concentration by each assay. In the HSMA, sperm toxicity was manifested by a decrease in forward progression (grade) of the sperm. The grade at 0 and 16 μM was 2.1 ± 0.02 and 1.7 ± 0.2, respectively, which was not statistically different. At formaldehyde concentrations of 32 and 64 μM, the HSMA grade decreased to 1.5 ± 0.4 and 1.1 ± 0.2 ($P<.05$), respectively (Fig. 2A). However, the SMI component of the HSMA remained unchanged at 32 μM compared with lower concentrations of formaldehyde. When the formaldehyde concentration was increased to 64 μM, the SMI was affected (0.70 ± 0.19) when compared with the control (0.98 ± 0.06; $P<.05$) (Fig. 2B).

The MEA was the second traditional assay evaluated, and blastocyst rates were 95.4% ± 6.4%, 90.2% ± 7.1%, 76.0% ± 12.9%, and 25.0% ± 17.3% for 0, 16, 32, and 64 μM of formaldehyde, respectively. The MEA, therefore, also detected formaldehyde effects at concentrations of 32 and 64 μM ($P<.05$) but not at the lowest concentration tested (Fig. 2C).

FIGURE 2



Traditional QC assays were evaluated using different concentrations of formaldehyde. (A) Human sperm motility grade was able to detect formaldehyde at 32 and 64 μM ($P<.05$). (B) Human sperm motility assay using SMI detected 64 μM formaldehyde ($P<.05$). (C) Blastocyst formation was reduced at formaldehyde concentrations of 32 and 64 μM ($P<.05$). Reported values are mean ± SEM. Experiments were done in triplicate. *$P<.05$.

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In summary, when the two traditional assays were used they yielded a similar sensitivity profile by detecting formaldehyde concentrations at 32 and 64 μM but did not detect formaldehyde toxicity at 16 μM.

We then used the ICM assay to determine whether the murine embryos were more sensitive to trace toxicity. The grade of the ICM was 1.78 ± 0.03, 1.14 ± 0.17, and 1.10 ± 0.44 at 0, 16, and 32 μM of formaldehyde, respectively, with no viable embryos or ICMs present at 64 μM (Fig. 3A). The formation and grade of an ICM was affected by formaldehyde at the lowest concentration (16 μM; $P<.05$). The ICM rate was also evaluated by calculating the percentage of embryos that had an ICM present or absent after 96 hours of culture. The ICM rate was 75.1% ± 6.7%, 56.5% ± 11.5%, 37.9% ± 6.3%, and 0.0 ± 0.0 at 0, 16, 32, and 64 μM of formaldehyde (Fig. 3B). Effects of formaldehyde on rate of ICM formation were similar to effects observed with ICM grade, with toxicity at 16 μM formaldehyde.

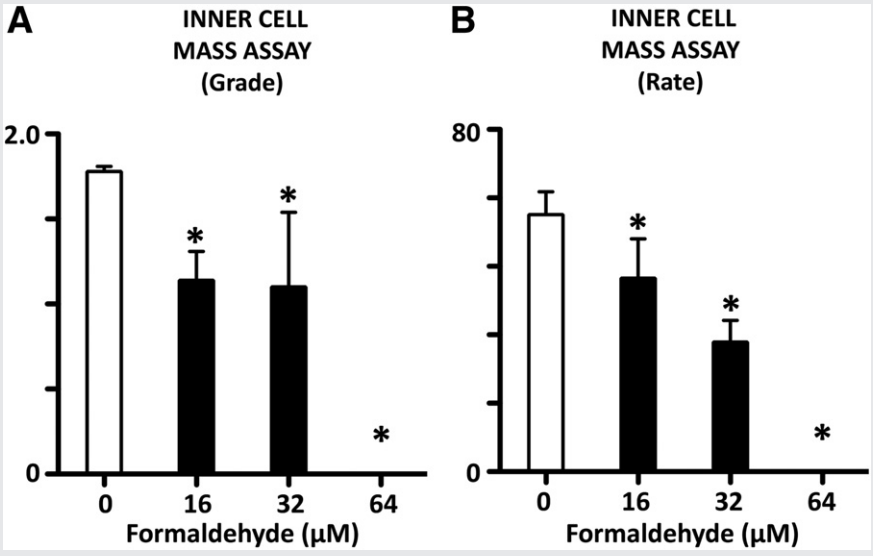
To determine whether changes in morphologic embryo development mirrored an underlying physiologic process, we examined amino acid turnover in the G2 culture media (Fig. 1A). We initially evaluated amino acid turnover by comparing amino acid concentrations in blank media with media conditioned with embryos (n = 20) cultured from the morula to the blastocyst stage without formaldehyde treatment. We found that alanine, aspartate, glutamine, leucine, and ornithine demonstrated a significant change, whereas the remaining amino acids remained unchanged (Supplemental Table 1, available online). To determine the sensitivity of amino acids as markers for toxicity, we further evaluated the effect of formaldehyde on these five amino acids in the media from the MEA experiments. Rather than using the absolute numbers in Supplemental Table 1, a net change in amino acid concentration was determined by subtracting the amino acid concentration in the blank media from the concentration in the conditioned media. A net production of alanine, glutamine, and ornithine was observed at all concentrations, whereas depletion of aspartate occurred (Fig. 4). Production of leucine occurred at lower concentrations of formaldehyde; however, it was depleted when formaldehyde concentration reached 64 μM ($P<.05$).

DISCUSSION

Minimizing exposure of gametes and embryos to environmental toxins is a critical step toward improving safety and outcomes of IVF. We have demonstrated that the murine ICM outgrowth assay is more sensitive than traditional QC assays for detecting formaldehyde, a common airborne volatile organic compound. Because effects of formaldehyde were detected with the ICM assay at concentrations below that shown with blastocyst development rates, these results highlight the limitation of the standard blastocyst endpoint in the MEA, indicating that it may be missing clinically relevant toxicity.

Quality control testing in IVF laboratories requires the use of surrogate bioassays because it is not ethical or feasible to use human embryos. Nonetheless, QC in the IVF setting is essential to minimize in vitro stress of human gametes or embryos due to exogenous toxins. Although human sperm and murine embryos are used as biomarkers, their utility is limited. Studies on the sensitivity of the two assays provide conflicting results: two studies demonstrated superiority of the

FIGURE 3



(A) Inner cell mass outgrowth grade was affected at 16, 32, and 64 μM formaldehyde ($P<.05$). (B) Inner cell mass outgrowth rate (%) was determined for embryos, with the rate reduced at 16, 32, and 64 μM formaldehyde ($P<.05$). Reported values are mean ± SEM. Experiments were done in triplicate. *$P<.05$.

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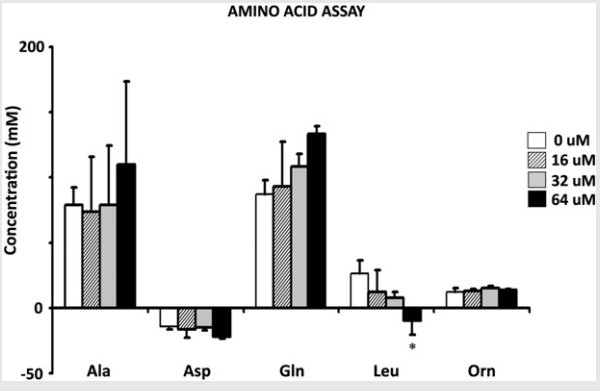
MEA (6, 7), and another showed that the HSMA is more informative (2). Furthermore, the two-cell MEA uses embryos from F1 hybrid mice that routinely develop to the blastocyst stage at rates >80% (6) even without protein. In contrast, only 30%–50% of human embryos cultured in optimal conditions develop to the blastocyst stage, and not all of them are high-quality blastocysts (17–19). The etiology of limited human blastocyst conversion in IVF is due to the embryo's innate quality and/or in vitro culture conditions. To determine whether in vitro conditions can be improved for human embryos, more sensitive QC testing of laboratory materials is necessary. We introduce ICM outgrowth as a novel and sensitive QC assay. In the present study, the two-cell MEA was used rather than the one-cell MEA. Although the two-cell MEA has an advantage because it requires less time, one-cell embryos may further improve the sensitivity of this new paradigm (20, 21).

FIGURE 4



Effect of formaldehyde on amino acid concentrations in culture media. Net amino acid turnover for embryos that were cultured in media for 48 hours from morula to blastocyst are shown. Leucine was produced at 0, 16, and 32 μM formaldehyde and was depleted at 64 μM ($P<.05$). *$P<.05$.

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In vitro culture conditions in an IVF laboratory can be affected by many factors. Air quality in IVF laboratories has been previously studied and noted to be worse than residential areas, such as homes and schools (16). Contaminants specific to IVF laboratories include volatile organic compounds, including aldehydes and benzenes (16, 22). For this reason, we used formaldehyde as a model toxin for comparing the sensitivity of different QC assays. In addition to being a known environmental contaminant, formaldehyde has been identified in cryoprotectant solutions (23, 24) and is used as a toxin in media for proficiency testing provided by the American Association of Bioanalysis (25). Formaldehyde is an environmental contaminant belonging to the aldehyde family, which has been shown to be present in IVF laboratories in concentrations tested in this study (16, 22). Further studies on assay sensitivities using known in vitro toxins similar to formaldehyde will improve our ability to detect both air pollutants as well as contaminants present in oil, protein, media and supplies.

Human sperm motility and mouse blastocyst development are the most commonly used endpoints for QC testing; however, ICM outgrowth in vitro is an important development that merits further consideration as a tool for QC (8, 26). In mammalian embryo development, a blastocyst is formed after the blastomeres have differentiated into the

trophectoderm and ICM, with the latter developing into the fetus. After blastocyst formation, in vivo development includes hatching from the zona pellucida, implantation, and embryonic outgrowth. Development to the blastocyst stage alone is a poor indication of potential viability (9), which suggests that a more robust endpoint is needed. The ICM outgrowth assay has been used to test effects of hydrosalpinx fluid (10) but has not been compared with other QC assays. Inner cell mass outgrowth culture allows for further downstream testing, and this study has demonstrated that it provides a more sensitive screening tool for IVF QC systems.

The "quiet embryo hypothesis" proposes that embryos with minimal metabolic activity are of higher quality and not undergoing apoptosis (27). Recently, many investigators have further described the use of noninvasive testing of culture media for prediction of human embryo quality (11, 13, 15, 28). We have demonstrated that similar profiling for murine embryos is possible, with significant metabolic activity of alanine, aspartate, glutamine, leucine, and ornithine using LC-MS/MS technology. Only leucine levels were significantly affected by formaldehyde treatment. There was a net increase of leucine in the control embryos and a trend to lower levels of leucine in the 16- and 32-μM treatments, with a significant decrease at the 64-μM concentration. However, it is important to note that this decrease occurred concomitantly with a decrease in the blastocyst rate at 64 μM formaldehyde, so it is unknown whether this change in leucine utilization was due to a change in metabolism or fewer blastocysts at the end of culture. Of note, all embryos were at the eight-cell to morula stage at the start of culture in G2, so a net decrease in leucine in the group receiving 64 μM formaldehyde compared with net production of leucine by the control embryos suggests that this was due to metabolic activity. Previous studies have shown that leucine is depleted in culture media during development from the morula to blastocyst stage in humans (13, 15, 28). This difference in results might be due to the lack of protein in the culture media in the present study, because protein may be a source of amino acids during embryo development. Given these methodologic differences, these results should not be viewed in the context of fundamental embryo metabolism but rather as evidence that metabolomics for QC testing should be investigated further. More objective measures such as amino acid levels for QC assays may prove to be more consistent compared with the relatively subjective nature of embryo morphology and sperm motility. We predict that QC testing incorporating both subjective and objective testing will ultimately prove to be the most useful and sensitive.

In summary, we confirmed the sensitivity of two traditional assays and compared them with two novel methods for IVF QC testing. Continued culture beyond the blastocyst stage and assessment of ICM development is a more sensitive assay for toxins present in IVF laboratories compared with traditional QC assays. Analysis of amino acids may be used to test murine embryo development as an adjunct QC assay. While "omic" technologies are being actively pursued as a means to predict human embryo viability, these same techniques have potential for use as new QC methods (29). Emerging data of time-lapse image analysis and gene expression profiling have also been described to predict human blastocyst development, which can similarly be applied in QC settings (30). To continuously improve IVF success rates, more sensitive QC testing should be developed to further reduce in vitro stressors that are present in IVF laboratories.

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SUPPLEMENTAL TABLE 1

Absolute amino acid concentrations.

Amino acid	Blank	SEM	Conditioned	SEM	*P* value
Ala	136	3.3	214	16.1	.009[a]
Arg	319	10.1	276	19.3	.125
Asn	104	4.1	106	3.0	.591
Asp	103	1.7	89	2.1	.007[a]
bAla	17	2.7	18	1.9	.749
Cys	30	2.0	33	4.2	.613
Gln	37	3.5	124	12.0	.002[a]
Glu	91	3.8	104	5.2	.113
Gly	109	1.2	119	6.0	.183
His	83	3.3	93	4.7	.177
Ile	194	6.6	211	6.2	.135
Leu	194	8.9	220	4.3	.055[a]
Lys	207	5.5	197	7.2	.350
Met	41	3.1	42	5.6	.898
Orn	2	0.1	13	2.9	.015[a]
Phe	99	1.0	109	6.4	.189
Pro	92	0.7	101	8.0	.324
Ser	106	3.8	117	5.6	.209
Thr	194	5.8	193	7.6	.896
Trp	23	0.2	26	1.8	.240
Tyr	104	3.6	103	9.7	.922
Val	189	6.5	220	20.5	.227

Note: Amino acid concentrations (mM) of culture media after 48 hours of culture with (conditioned) or without (blank) embryos from the early morula to the blastocyst stage. Experiment was performed in triplicate.

[a] Amino acids with a significant change ($P < .05$) in blank vs. conditioned media.

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