Updating Quality Control Assays in the Assisted Reproductive Technologies Laboratory with a Cryopreserved Hamster Oocyte DNA Cytogenotoxic Assay

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Purpose: Despite advances in assisted reproduction, there is no progress in quality control bioassays. The objectives were to develop a comet assay to measure DNA fragmentation in thawed cryopreserved oocytes and compare this assay with one-cell mouse embryo bioassay.

Methods: Thawed hamster oocytes from a commercial source were incubated in culture media with either 0-, 50-, or 100- μ M hydrogen peroxide, or, in media exposed to different contact materials and unknown proficiency analytes. Incubation time was 1.5 h at 37°C. The oocytes were dried, fixed, stained with acridine orange, embedded in a mini-agarose layer and electrophoresis was carried out. Fluorescent images were analyzed. The results were compared with standard one-cell mouse assay data.

Results: The 100- μ M hydrogen peroxide treatment caused greatest DNA fragmentation in the hamster oocytes at Hours 1 and 2. A dose response was observed. Intrassay coefficient of variation was 5.7%. Only one of the five materials tested passed both assays. The data for the unknown proficiency analytes were similar for both assays.

Conclusions: The oocyte comet assay demonstrated DNA fragmentation in the presence of toxic substances. The detection of toxicity in two materials that passed the mouse bioassay suggested increased sensitivity in the new assay. The oocyte comet assay and the mouse bioassay results matched

in the proficiency test. However, more studies are still needed to determine optimal sensitivity.

KEY WORDS: Single-cell gel electrophoresis; comet assay; quality control; hamster ova; in vitro fertilization.

INTRODUCTION

Testing of water, culture media, protein supplements, and contact materials at the centers of assisted reproductive technologies are presently performed using either the one- or two-cell mouse embryo bioassay (1–8), the hamster epididymal sperm motility bioassay (9,10), the human sperm survival bioassay (11,12) or the hybridoma cell culture assay (13). Thus, the centers routinely perform bioassays and have become proficient in cytogenotoxic testing. Despite advances in assisted reproduction, there has been no progress in quality control bioassays since 1989. Present quality control bioassays are complicated, time-consuming (13), expensive, lack sensitivity (3,14,15) and require several days. Also, the bioassays do not directly measure DNA damage. The comet assay, or single-cell gel electrophoresis, is a simple, rapid, and inexpensive assay for the determination of DNA damage or fragmentation (16–18). The comet assay involves measuring the streaks of DNA strands (“comet” tail moment) unraveling from the individual nucleus of the white blood cell (19,20). The challenge was to adapt the assay for low numbers of oocytes, unlike the easily obtainable quantities of white blood cells.

The hypothesis was that toxic substances would cause DNA fragmentation in metaphase II oocytes. The objectives were (a) to develop a comet assay to measure DNA fragmentation based on oocytes,

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(b) to compare this new assay with the standard one-cell mouse embryo biological assay, and (c) to test this new assay on unknown proficiency analytes. Thawed cryopreserved oocytes at metaphase II were used because the chromosomal DNA were lined up in a vulnerable position; the frozen oocytes were commercially available, conveniently stored, and polar bodies were present to mark the oocyte position. It is hoped that the results will help in the development of a rapid, efficient, and inexpensive updated genetic assay to replace current quality control bioassays.

MATERIALS AND METHODS

Preparation of Hamster Oocytes

The cryopreserved hamster oocytes were obtained from a commercial source (Embryotech Laboratories, Wilmington, MA). The oocytes were thawed according to instructions provided. Briefly, the frozen straws containing the oocytes were warmed to room temperature (23°C) for 1–2 minutes, placed with the label-end down into an empty specimen container and incubated at 37°C for 3 min. After incubation, the straws were placed with the label-end up at room temperature for 1 min followed by expulsion of the contents into HEPES-buffered synthetic human tubal fluid culture medium (modified HTF, Irvine Scientific Co., Santa Ana, CA) containing 5% human albumin (Irvine Scientific Co., Santa Ana, CA). In our experience, the final 1-min room temperature equilibration period was critical and affected the cryosurvival of the oocytes.

After washing the oocytes free from cryoprotectant by passage through culture medium, the zona-intact oocytes were pipetted in groups of eight into either plain culture medium (negative control) or medium with 50- μ M or 100- μ M hydrogen peroxide (H_2O_2). Because this study was the first of its kind, the number of oocytes for each treatment was determined after pilot studies based on cost and assay efficiency and sensitivity. The oocytes were incubated for Hours 1, 2, 4, and 24 at 37°C in room air. Slides were prepared in the meantime. Self-adhesive sticker labels (Catalog No. 43-540, Preaply, Dennison Co., Framingham, MA) were cut into strips and pasted to form a rectangular frame around diamond-pen-etched circles made on each glass slide. The type of stickers was important and they served to anchor the mini-agarose gel layer. It was essential to make sure that the stickers were stuck to the glass slide by pressing down hard on

the stickers. The etched circles helped to locate the oocytes.

Two oocytes were pipetted out on to each glass slide and air-dried. The entire procedure was carried out with the room lights off or in diffuse lighting. As soon as the oocytes were dried on the glass slides, they were fixed by flooding with methanol solution (Fixative Solution, Diff-Quik, Dade Behring Inc., Newark, DE) for 15 s and air-dried again. At this point, the fixed oocytes could be batched and kept for a few days before continuing with the next step in the oocyte comet assay process as described later.

In a separate experiment involving the testing of contact material, zona-intact oocytes were pipetted into either control medium or media that had been exposed to new laboratory materials. The contact material media were prepared by placing aliquots of 1 ml of modified HTF medium for 1.5 h into either a new lot of Falcon 3037 petri dish (Becton Dickinson Labware, Franklin Lanes, NJ), Falcon 7575 sterile 3-ml transfer pipet, Henke-Sass Wolf 10-ml Fortuna syringe (Tutlingen, Germany), Pure Advantage powder free gloves (THC66TP-1, Tillotson Healthcare Corp., Nedford, NH) or Sempermed supreme gloves (Semperit Technische Produkte Ges.m.b.H., Vienna, Austria). The contact material media were also used for testing in the one-cell mouse biological assay system. A description of the mouse assay system is presented later.

Finally, a third section of this study involved using the oocyte comet assay on two unknown laboratory proficiency analytes supplied by the American Association of Bioanalysis. The assay was carried out alongside the standard mouse one-cell biological quality control assay system with the exception that this assay was completed the same day, whereas the mouse assay required several days to obtain the results. Sometimes the oocytes exposed to toxic media would appear darkened and lack the shiny “glass marble” structure, but this was inconsistent and we did not find a good correlation between appearance in culture and DNA fragmentation. Hence, the appearance parameter was not studied further.

Preparation of the Mouse Embryo Assay System

Mature female mice were stimulated with 5-IU pregnant mares serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO) followed by 5-IU human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO). Each female mouse was mated with a proven male 2 days after hCG treatment (21).

Pronuclear stage embryos were flushed from the oviducts 20 h after mating and pooled together for randomization. Groups of pronuclear stage or one-cell embryos (10 to 20 embryos) were incubated in test media (contact materials media) or plain medium (control) consisting of bicarbonate-based HTF medium containing 4 mg/ml bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO). The incubation conditions were 5% CO₂ in air mixture at 37°C. After 24 h of incubation, the groups of embryos were observed for growth, and one-cell stage embryos were discounted as unfertilized or damaged. After 4 days of incubation, the groups of embryos were scored according to stage of development. In general, in the mouse assay system, a passing grade was given when 80% or more of the embryos reached at least the blastocyst stage. The stage of embryonic development was visually confirmed using light microscopy.

Modification of the Comet Assay for the Zona-Intact Hamster Oocyte

The entire procedure was carried out with the room lights off or in diffuse lighting. The air-dried and methanol-fixed oocytes on each glass slide were stained for 5 min in freshly prepared acridine orange solution. This solution was prepared by dissolving 0.2-mg acridine orange (Catalog No. 10390, United States Biochemical Corp., Cleveland, Ohio) in 1.0-ml purified water.

The slides were carefully rinsed with water to remove background stain and then air-dried. The 0.8% agarose was prepared by mixing 0.8-g agarose (Cata-

log No. 15510-019, Life Technologies, GIBCO BRL, Grand Island, NY) in 100 ml of the 1 × TBE solution (13.5-g Tris-HCl pH 7.5, 6.8-g Boric Acid, 5 ml of 0.5 M EDTA in 1250-ml purified water), heating until dissolved and cooling to 50°C. The agarose was pipetted on to each glass slide surface placed at a 20° angle and excess agarose allowed to run off the edges. Thinner agarose layers tended to be easier to work with under the microscope high powered objective. A cover slip was not used. Each slide was placed horizontal on the table. Once the agarose had solidified, the slides were placed in a plastic box and drops of 4°C alkaline lysis buffer (1% *N*-Lauroylsarcosine, 1.0-M Tris-HCl pH 7.5, 0.5-M EDTA, 0.3-M mercaptoethanol, pH adjusted to >10 with sodium hydroxide pellets) were pipetted on to the agar surface. The slides were kept in the dark for 30 min to release and unwind cell DNA. Then, the slides were rinsed with purified water and placed submerged (1–2 mm from the surface) in 1 × TBE solution in the electrophoresis chamber for 5–10 min to equilibrate. Following this, constant voltage at 50 V was applied for 40 min.

Excess solution was blotted from each slide and the slide was immediately examined using an ultraviolet (UV) fluorescent microscope at 500× magnification. The normal control oocyte generally has the appearance of a round bright green ball that resembled a planet with two moons (polar bodies) more than a comet (Fig. 1). Perhaps, calling it a “planet” assay would be more appropriate. An oocyte with a high degree of DNA fragmentation gave the appearance of an interstellar black hole (Fig. 1). For the novice, the position of each oocyte

Fig. 1. Modified alkaline comet assay of thawed cryopreserved hamster oocytes collected at the metaphase II (mature) stage. The images were converted to gray-scale for density analysis using a shareware image program. The oocyte colors were inverted using a software program. The images were (a) the horizontal electrophoresis tank with slides, (b) an example of a normal bright oocyte with low DNA fragmentation, and (c) an oocyte with a high degree of DNA fragmentation. The polar bodies appeared as small dense points of light. Lighter DNA density at the center represented increased fragmentation of nuclear DNA.

was rapidly determined by using phase contrast and low visible-light setting prior to switching over to fluorescent microscopy. It should be pointed out that artifacts such as indentations made in the agarose by displaced oocytes or trapped bubbles should be ignored. Generally, the bright green but small sized polar bodies should be present beside each oocyte.

The images were captured by placing an inexpensive QuickCam Pro camera (Logitech Inc., Fremont, CA) over the microscope eyepiece and saving the 640×480 pixels images to hard-disk. The area and the mean pixel intensity for each entire oocyte were measured using Paint Shop Pro 6 software (Jasc Software, Inc., Eden Prairie, MN). A 30-day evaluation copy of Paint Shop Pro is available for download at popular internet shareware web sites. For accuracy, the mean pixel intensity of each cell image was obtained after subtracting the background intensity. If the oocyte DNA was completely disintegrated and it appeared as a "black hole," then, the intensity was measured in the "black hole" area.

Statistical Analysis

The results were presented as mean pixel intensity $\pm$ standard error of the mean (SEM). Data on the mean pixel intensity for each group of oocytes were compared and tested using the Student's *t*-test statistic. A difference with $p < .05$ was considered significant.

RESULTS

The presence of 100- μ M hydrogen peroxide in the culture medium resulted in significant hamster oocyte

Table I. Oocyte Comet Assay Detection of Differences in DNA Fragmentation in the Metaphase Plate in Thawed Cryopreserved Hamster Oocytes After In Vitro Exposure to Hydrogen Peroxide for 1 h at 37°C^a

Time	Concentration of Hydrogen Peroxide ^b		
	0 μ M	50 μ M	100 μ M
Hour 1	81.0 $\pm$ 5.7*	32.5 $\pm$ 24.7	15.5 $\pm$ 9.2
Hour 2	73.5 $\pm$ 23.3*	25.5 $\pm$ 3.5*	6.0 $\pm$ 0.0
Hour 4	17.5 $\pm$ 3.5	28.5 $\pm$ 21.9	5.0 $\pm$ 1.4
Hour 24	24.5 $\pm$ 23.3	28.0 $\pm$ 1.4*	6.0 $\pm$ 2.8

^a Values are expressed as mean pixels $\pm$ SEM.

^b Lower values are associated with more DNA fragmentation.

* $p < .05$, different from 100- μ M control.

DNA fragmentation when compared with the control treatment at Hours 1 and 2 (Table I). Most noteworthy was that at Hour 4 and beyond, the DNA in the oocytes had degenerated and differences in the treatments could not be determined using the oocyte comet assay. A dose response was observed between the concentration of hydrogen peroxide and amount of DNA fragmentation at Hours 1 and 2. The data at Hours 4 and 24 were similar and were not statistically different. The intrassay coefficient of variation was 5.7% for repeat analyses of images.

The results of the oocyte comet assay matched the results of the standard mouse one-cell embryo biological assay in only one of the five materials tested (Table II). The Fortuna syringe passed both assays. Measuring the percentage of hatched blastocysts instead of the percentage of blastocysts did not make a difference when compared with the oocyte comet assay results. The DNA fragmentation due to the two types of gloves were more severe when compared to the petri dish and Falcon transfer pipets. The high

Table II. Comparison of the Oocyte Comet Assay and the Mouse One-Cell Biological Assay for the Detection of Toxicity in Contact Materials and in Unknown Proficiency Analytes from the American Association of Bioanalysts^a

Materials	Mean pixel intensity (pixels)	No. of Blastocysts/total (%)	No. Hatched/total (%)
Control medium	95.8 $\pm$ 16.0	13/13 (100)	6/13 (46)
Pure Advantage gloves	34.1 $\pm$ 14.3*	0/8 (0)	0/8 (0)
Falcon 3037 petri dish	52.0 $\pm$ 20.1*	9/10 (90)	5/10 (50)
Semper gloves	13.7 $\pm$ 8.6*	0/10 (0)	0/10 (0)
Falcon 3-ml transfer pipet	57.4 $\pm$ 9.4*	9/9 (100)	3/9 (33)
10-ml Fortuna syringe	87.2 $\pm$ 13.4	8/8 (100)	5/8 (63)
AAB Analyte 1	64.3 $\pm$ 3.2	10/10 (100)	6/10 (60)
AAB Analyte 2	32.5 $\pm$ 19.1**	0/10 (0)	0/10 (0)
AAB Analyte 1 with serum	14.5 $\pm$ 14.8	10/10 (100)	7/10 (70)
AAB Analyte 2 with serum	0 $\pm$ 0**	0/10 (0)	0/10 (0)

^a Values are mean pixels $\pm$ SEM.

* $p < .05$, different from control; ** $p < .05$, different from corresponding Analyte 1.

degree of toxicity of the gloves was also mirrored in the mouse one-cell embryo assay as shown by 0% embryo development.

The results of the unknown analytes from the American Association of Bioanalysts were similar for both assays (Table II). Analyte 1 was acceptable, whereas, Analyte 2 was rejected. However, the addition of serum to the analytes resulted in increased DNA fragmentation although this did not affect the results. Interestingly, the mouse one-cell embryo assay did not show any differences with or without serum.

DISCUSSION

The oocyte comet assay presented in this study was based on a novel modification of the original comet assay for white blood cells (16–20). The results showed that the oocyte comet assay could be used to quality control test materials used in assisted reproductive technologies, except that it was considerably faster to perform when compared with the mouse one-cell embryo bioassay (1–8). The sensitivity in detecting toxicity appeared to be greater in the oocyte comet assay as suggested by the failing grade for two materials that had passed the mouse bioassay. Convincing support of the usefulness of the oocyte comet assay came from testing the proficiency analytes. The results were similar between the oocyte comet assay and the mouse one-cell embryo bioassay. The differences in DNA fragmentation were obvious in images of oocytes exposed to toxic and nontoxic proficiency analytes.

The experiment using two different concentrations of hydrogen peroxide showed a dose response which suggested that DNA unraveling or fragmentation involved several layers. We found 1.5 h to be optimal for our testing purposes. The DNA fragmentation observed was due to apoptosis rather than necrosis because the incubation time was only 1.5 h and, more importantly, the trail of fragmented DNA when present, appeared as a well-defined fan tail in contrast to the diffuse pieces of DNA trailing from necrotic cells (19,20).

Since the oocyte comet assay was based on the cytogenotoxic effects on the DNA, it was directly relevant to the assessment of factors that were detrimental to the genetics of the cell (sperm, oocyte, and embryos). Furthermore, the widespread use of intracytoplasmic sperm injection (ICSI), which bypasses the early steps of fertilization, makes it important

to use DNA effects as the end-point of quality control testing rather than cell cleavage mechanisms or motility. The mouse one- and two-cell embryo assays have been criticized for the lack of sensitivity (3,14,15). Furthermore, there are additional requirements for maintaining a mouse colony, sacrificing mice, unpredictable recovery of fertilized oocytes and slow turnaround time. In terms of error, the mouse assay here suggested that the 3-ml transfer pipets and petri dishes were safe to use. This was in contrast to the oocyte comet assay indicating some level of DNA toxicity associated with these two items. More work still needs to be carried out to refine the parameters of the oocyte comet assay for optimal sensitivity. For example, the exposure time to the media greatly affects the sensitivity.

An alternative assay, the hamster epididymal sperm motility assay (9,10), would require housing a colony of male hamsters and significant training and expertise to perform the cumbersome assay (13). Another alternative, the human sperm motility assay (11,12), lacked sensitivity and required at least one or more days of incubation time to detect differences. The sperm motility assay would be best suited for testing metabolic toxins and may not be suitable for detecting effects on cell DNA. In contrast, the oocyte comet assay circumvented these drawbacks and was rapid (1 day turnaround time), inexpensive (using glass slides), lacked cell cycle position artifacts, directly detected effects on DNA and was convenient (based on cryopreserved hamster oocytes and batched, fixed, and dried oocytes). Note that the cryopreserved hamster oocytes were the same type as those routinely used for the sperm penetration assay. The drawbacks of the oocyte comet assay included the need for a fluorescent microscope and a pixel computer program. Nevertheless, the oocyte comet assay is regarded as an upgrade of laboratory quality control assays that have languished over 10 years ending with hybridoma assays in 1989 (13) while assisted reproductive technologies have advanced forward.

The novel modification of the comet assay or single-cell gel electrophoresis was originally introduced in 1984 by Östling and Johanson (16) for the analysis of individual white blood cell DNA fragmentation. The broken pieces of DNA loops unwinding from the relaxed supercoiled nucleus gave the appearance of a comet tail; hence, the name “comet” assay. They used neutral electrophoresis buffer conditions to show that the migration of double-stranded DNA loops from the damaged cell nucleus was proportional to the extent of the damage inflicted on the cell. The comet

assay was modified by Singh and colleagues (1988) using alkaline electrophoresis buffers to expose alkali-labile sites (17,18) which increased assay sensitivity. The comet assay has been modified several times since its introduction (22,23). The original comet assay involved living cells suspended in a mini-agarose gel. Here, the assay was adapted for dried, fixed oocytes attached on a glass slide and overlaid with a mini-agarose gel layer. The comet assay usually involved the analysis of the comet tail, tail moment, or more recently, the intensity of the nuclear DNA such as the sperm head (24). In this study, the overall intensity of the oocyte DNA were measured and expressed as pixels.

In summary, the original comet assay was modified to analyze DNA fragmentation in oocytes that had been exposed to treated culture media. Greater concentrations of toxins caused greater DNA fragmentation, measured as decreased intensity of the fluorescent DNA material in each oocyte. The oocyte comet assay passed the proficiency test and had similar results as the mouse one-cell embryo biological assay. More studies are needed to refine the oocyte comet assay for optimal sensitivity.

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