Oocyte Comet Assay of Luteal Phase Sera from Nonpregnant Patients After Assisted Reproductive Procedures

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Purpose: Serum factors in patients with recurrent spontaneous abortions (RSA) inhibit mouse embryo development in vitro. Serum factors affecting DNA integrity remain to be tested. The null hypothesis was that patient sera do not affect DNA integrity. The objectives were (a) to use the oocyte comet assay to assess DNA damage after exposure to patient sera and (b) to determine the effect of sera from gravidity 0 parity 0 patients to induce DNA apoptosis.

Methods: Luteal phase sera were drawn 1 week after embryo transfer following assisted reproductive procedures. Frozen-thawed hamster zona intact oocytes at metaphase II were incubated in groups of eight in either control medium or medium supplemented with 50% patient serum for 1.5 h at 37°C in room air. The oocytes were fixed, stained in acridine orange, embedded in agarose, lysed, and alkaline electrophoresis performed. The intensities of the digitized fluorescent images were analyzed.

Results: The sera of nonpregnant patients (64%) caused significant fragmentation of hamster oocyte DNA when compared with pregnant patient sera. This difference was also observed when adjusted for patient age. Sera of patients that had never been pregnant also resulted in oocyte DNA fragmentation.

Conclusions: The results suggested that sera from patients that did not conceive contained factors that did not support cell growth by causing DNA fragmentation and apoptosis. The level of the apoptotic factors varied from cycle to cycle.

However, more studies are needed to determine if the sera factors actually reach the uterine environment to cause the undesirable effects.

KEY WORDS: hamster ova; in vitro fertilization; maternal sera; single-cell gel electrophoresis.

INTRODUCTION

The presence of embryotoxic factors in the sera of patients with recurrent spontaneous abortions (RSA) or endometriosis has been reported (1–10). In those reports, RSA was defined as three or more miscarriages. The percentages of RSA patients with sera that inhibited murine embryo development in vitro ranged from 24 (8,10) to 70% (2) whereas the percentages in the endometriosis group ranged from 0 (9) to 100% (7). The wide ranges reported from the different centers and a lack of standardization make it more difficult to show cause and effect between the presence of sera embryotoxic factors and infertility.

While the sera of RSA and endometriosis patients have been investigated, there is a lack of information on the effect of the sera from patients that did not conceive after assisted reproductive technologies (ART) procedures on DNA integrity. A study of failed ART cycles is important as it provides information on how to optimize subsequent cycles. In this study, the null hypothesis was that luteal phase sera from nonpregnant and pregnant patients did not have an adverse effect on DNA. The objectives were (a) to use a novel oocyte comet assay to assess the DNA damaging effect of sera taken 1 week after assisted reproductive procedures and (b) to analyze the effect of sera from gravidity 0 parity 0 patients to induce DNA apoptosis. Unlike bioassays such as the

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laborious and time-consuming murine one-cell assay, the rapid comet assay or single-cell gel electrophoresis was used here to directly measure DNA damage (11–14).

MATERIALS AND METHODS

Preparation of Hamster Oocytes

Cryopreserved–thawed hamster oocytes (Embryotech Laboratories, Wilmington, MA) 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 (15). The frozen straws containing the oocytes were warmed to room temperature (23°C) for 1–2 min, 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, Santa Ana, CA) containing 5% human albumin (Irvine Scientific, Santa Ana, CA).

The oocytes were washed free from the cryoprotectant by passage through culture medium. An individual oocyte comet assay consisted of a group of eight zona intact oocytes pipetted into either plain culture medium (negative control), medium with 100 μ M hydrogen peroxide (positive control) or medium containing 50% patient serum. The serum of each patient was drawn 1 week after the embryo transfer procedure following the in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI) procedures. The methodology for these procedures have previously been reported (16). Negative control sera were obtained from left-over discarded sera of first trimester patients with normal glucose tolerance test results. Each serum specimen was stored frozen until it was used in the assay.

The patient population for this study consisted of randomly selected 21 infertility patients scheduled for the IVF with ICSI procedures. The primary diagnosis for the patients varied (three endometriosis, seven tubal, seven male factor, four idiopathic) and so the data were not categorized by diagnosis due to the small sample size. Both, biochemical (three cases) and clinical pregnancies were grouped together because the sera used in the assays were obtained 1 week after embryo transfer and relevant for the context of

the study related to early embryo toxicity. Sera were collected and assays performed using contact materials such as syringes and tubes that had been quality control tested for nonembryotoxicity (15). The rapid oocyte comet assay measured changes in DNA and heat-inactivation of serum complements was not necessary. The assays were carried out either in duplicates or triplicates for a total of 74 assays. The study had previously been reviewed and approved by the Institutional Review Board.

A previous study (15) suggested that a 1.5-h incubation period at 37°C in room air was adequate and so this period was used here. During the incubation period, the hamster oocyte DNA, located in the chromosomes spread out in a vulnerable position at the metaphase plate were exposed to possible toxic factors in the sera of patients. In the meantime, self-adhesive sticker labels (Catalog No. 43-640, Presaply, Dennison, Framingham, MA) were cut into strips and pasted to form a rectangular frame around diamond-pen etched circles made on a glass slide. The etched circles helped to localize the oocytes.

About 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. The dried oocytes were fixed by flooding with methanol solution (Fixative Solution, Diff-Quik, Dade Behring, Newark, DE) for 15 s and air-dried again. The oocyte comet assay was performed as described below.

The Oocyte Comet Assay to Detect DNA Fragmenting Factors. 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 (15). The slides were carefully rinsed with water to remove background stain and then air-dried. A 0.8% agarose solution was prepared by mixing 0.8 g agarose (Catalog No. 15510-019, Life Technologies, GIBCO BRL, Grand Island, NY) in 100 mL of the 1 $\times$ 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 ultra-violet (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). An oocyte with a high degree of DNA fragmentation gave the appearance of a dark hole.

The images were captured by placing an inexpensive QuickCam Pro camera (Logitech, 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, Eden Prairie, MN). 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 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 ± standard error of the mean (S.E.M.). 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 sera from patients that did not conceive after in vitro fertilization with ICSI (ART cycles) were associated with significant fragmentation of hamster oocyte DNA when compared with pregnant patient sera. This was represented in the oocyte DNA assay as reduced or lower average intensity of pixels of the oocyte DNA. This association was also observed when the patients were separated by age into the 28–34 and 35–40 years age groups (see Table 1). As expected, the 100 μM hydrogen peroxide treated hamster oocytes

Table I. Oocyte Comet Assay Detection of Differences in Damage to Metaphase DNA in Cryopreserved–Thawed Hamster Oocytes After In Vitro Exposure to Sera from Pregnant Versus Nonpregnant Patients for 1 h at 37°C

Parameter	N	Mean DNA intensities (Pixels ± S.E.M.) ^a
Not pregnant after IVF and ICSI		
Age: 28–34 years	22	58.9 ± 0.1 ^b
Age: 35–40 years	18	84.5 ± 10.4 ^b
Combined	40	70.5 ± 9.6 ^b
Pregnant after IVF and ICSI		
Age: 28–34 years	22	140.8 ± 12.3
Age: 35–40 years	12	104.0 ± 15.1
Combined	34	129.7 ± 13.1
100 μM hydrogen peroxide control	2	28.0 ± 8.5 ^b
Negative control	5	167.0 ± 10.9

^a Lower values are associated with more DNA damage.

^b Different from corresponding groups, *P* < .05.

had significantly more DNA fragmentation when compared with the negative control.

When the patients were categorized into groups that either had 1–3 miscarriages, had never been pregnant, or had previous live deliveries, significant DNA fragmentation was observed in the sera of patients associated with failed ART cycles. The sera of patients with a history of 1–3 miscarriages and no children were associated with the greatest damage to the hamster oocyte DNA in comparison with patients that had children (see Table II).

DISCUSSION

Factors have been detected in the sera of 20–100% of infertile patients with RSA or endometriosis and they have been shown to be embryotoxic and inhibit the development of mouse embryos in vitro (10). In this study, factor or factors that were damaging to DNA were detected in the sera of nonpregnant patients after ART procedures. The results were the same regardless of patient age. It is important to note that the sera was tested in the artificial condition of the comet assay based on hamster oocytes and it is not known if the uterine environment will detoxify sera factors. Using a threshold pixel intensity level of <90 pixels for damaged DNA, 7 of the 11 nonpregnant cases (64%) were positive for DNA damaging sera in contrast to 2 of the 10 pregnant cases (20% false positives).

Previously, work in this laboratory showed that the assessment of DNA material was more sensitive than the mouse embryo assay for the detection of toxins (15). The severity of DNA damage was measured

Table II. Oocyte Comet Assay Detection of Damage in Metaphase DNA of Cryopreserved–Thawed Hamster Oocytes After In Vitro Exposure for 1 h at 37°C to Sera from Patients that Had (1–3) Miscarriages

Group	No. of analyses	Mean DNA intensities (Pixels $\pm$ S.E.M.) ^a
Not pregnant after IVF and ICSI		
Never been pregnant (gravidity = 0, parity = 0)	17	48.1 $\pm$ 5.6 ^b
Previous deliveries (gravidity > 0, parity > 0)	20	94.9 $\pm$ 3.7 ^b
Miscarriages (gravidity > 0, parity = 0)	3	13.7 $\pm$ 3.9 ^b
Pregnant after IVF and ICSI		
Never been pregnant (gravidity = 0, parity = 0)	13	156.6 $\pm$ 5.1
Previous deliveries (gravidity > 0, parity > 0)	12	104.0 $\pm$ 8.7
Miscarriages (gravidity > 0, parity = 0)	9	119.7 $\pm$ 6.2

^a Lower values are associated with more DNA damage.

^b Different from corresponding pregnant group, $P < .05$.

using the hamster oocytes at metaphase II where the chromatin material was exposed at the metaphase plate. The oocyte assay was adapted from the single-cell gel electrophoresis procedure or comet assay for white blood cells (15) and was based on the integrity of the DNA measured in terms of brightness or intensity of the fluorescence after nuclei acid staining. A reduced intensity in fluorescent DNA suggested the presence of toxic factors that damaged the DNA.

Recent reports suggested that the toxic factor(s) responsible for arresting embryonic growth might either be a Th1-type cytokine, IFN- γ (5,17,18) or a cytokeratin inhibitor that would damage the cellular cytoskeleton (1,9). However, in the present study, the serum toxic factor had an effect on DNA material and appeared to be different from cytoskeleton disrupting factors. Furthermore, the damaged DNA material had comet tail-like features characteristic of cell apoptosis. The data supported a DNA apoptosis mechanism rather than necrosis because the incubation time was only 1.5 h and the trail of fragmented DNA appeared as a well-defined fan tail in contrast to the diffuse pieces of DNA trailing from necrotic cells (14,15,19). In addition, paraptosis characterized by vacuoles and mitochondria swelling were ruled out. Based on these observations, the DNA damaging factor might be peroxide or anoxide generating free radicals. The data support the concept of more than one type of toxic factor in the affected maternal serum. More studies are needed to determine the relationship between embryotoxic factors and DNA damaging factors in affected serum.

An analysis of nonpregnant patients after assisted reproductive procedures demonstrated that the patients with a history of 1–3 miscarriages and no children had the greatest amount of DNA damage when

compared with patients that did not have miscarriages or had prior live births. This is consistent with the observation of toxic factors in this group of patients as reported by others (1–10). Note that although DNA can be damaged by toxins, it is possible for DNA repair mechanisms to occur and restore DNA integrity. Furthermore, past RSA patients that became pregnant through ART procedures did not have the DNA damaging factor in their sera suggesting that there might be cycle to cycle variation in the sera factors. Certainly this aspect is very interesting and deserves to be further examined in future studies.

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