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Animal Experimentation

Cytogenetic Analysis of Embryos Generated from In Vitro Matured Mouse Oocytes Reveals an Increase in Micronuclei Due To Chromosome Fragmentation

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***Purpose*:** (i) To determine the prevalence of micronuclei in the cytoplasm of embryos generated from in vitro matured oocytes. (ii) Assess whether micronuclei presence are the result of chromosome fragmentation or the loss of whole chromosomes.
***Methods*:** In vitro fertilization was performed on mature oocytes generated from superovulated mice (control) and in vitro matured mouse oocytes. Fertilized oocytes were cultured to the two-cell stage and fixed to slides. Micronuclei assessment was performed after staining with Giemsa. Centromere assessment was made using immunofluorescent staining (CREST) of the centromeric kinetochores.
***Results*:** Micronuclei were observed in 2% (4/197) of control two-cell embryos and 36.2% (46/127) of two-cell embryos generated from in vitro matured oocytes ($P < 0.02$). Centromeres were not detected in micronuclei from either group.
***Conclusions*:** A significant increase in micronuclei was observed in embryos generated from in vitro matured oocytes. The lack of accompanying centromeres would suggest the micronuclei are the result of chromosome fragmentation.

KEY WORDS: Centromere; cytogenetics; embryos; in vitro maturation; micronuclei.

INTRODUCTION

In vitro maturation (IVM) has been reported in a number of mammalian species (1–4), including the human (5–7). There are potential benefits of performing IVM in immature oocytes for reproductive medicine and in agriculture (5–9). However, the embryo development of IVM oocytes is reduced compared to conventional in vitro fertilization (IVF) procedures where mature oocytes are retrieved after exogenous injections of gonadotrophins (5,7,10,11).

Nuclear maturation up to the Metaphase II stage of meiosis in IVM oocytes appears normal (12). However, the development of the embryo subsequent to fertilization is reduced (6,10,13), with the majority failing to develop further than the two- to four-cell stage. Problems associated with spindle formation and chromosome replication may account for this. A phenomenon that is often associated with reduced developmental capacity in embryos is the presence of micronuclei (14). Indeed, following exposure to toxins (15–18) or an increase in the oxygen tension of the culture environment (19), there is a significant increase in the presence of micronuclei in the cytoplasm, and a corresponding decrease in the developmental potential of the embryo (14,15). Micronuclei represent whole chromosomes or chromosome fragments lost from the normal nucleus as a result of chromosome lagging in the case of whole chromosomes or chromosome breakage in the case of fragments (20,21).

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The aim of this study was to compare the frequency of micronuclei in two-cell embryos generated from IVM oocytes, with that of normally matured embryos, using the mouse as a model. Additionally, immunofluorescent staining (CREST) (22) to centromeric kinetochores was used to determine whether the micronuclei were whole chromosomes or chromosome fragments.

MATERIALS AND METHODS

Three-week-old F1 (C57 × CBA) female mice were used for the experiment. Males used were CBA and of proven fertility, aged 2–4 months. All procedures with mice were authorized by the Animal Ethics Committee of Murdoch University (Murdoch, Western Australia).

There were two groups of female mice: (a) Control group, consisting of mature oocytes that were collected from superovulated mice. The superovulation consisted of an injection 7.5 IU Pregnant Mares Serum Gonadotrophin (PMSG; Sigma, MO), followed 54 h later with 10 IU Human Chorionic Gonadotrophin (HCG; Profasi, Serono, NSW, Australia). The mice were euthanased 15 h after the HCG injection and the cumulus masses containing the mature oocytes retrieved from the distal end of the fallopian tube by gently piercing with a 26-gauge needle. The cumulus was removed from the oocytes with 80 IU/mL hyaluronidase (Sigma, MO) and all those appearing normal and with a single polar body (Metaphase II) were placed into culture of MEM-α medium (Gibco, NY) supplemented with 0.05 mg/mL penicillin and 3 mg/mL Bovine Serum Albumin (BSA; Sigma, MO). (b) IVM group, IVM oocytes were retrieved 24 h prior to the control oocytes. In this group, the mice were euthanased, the ovaries removed, and the follicles gently dissected using 26-gauge needles at 37°C, as described previously (23). The follicles were placed in 20 μL droplets of MEM-α medium supplemented with 10% fetal calf serum (FCS), 0.1 IU/mL Follicle Stimulating Hormone (FSH; Metrodin HP, Serono, NSW, Australia), 0.5 IU/mL HCG (Profasi, Serono, NSW, Australia), and 0.05 mg/mL penicillin under sterile paraffin oil (BDH, Poole, U.K.). The follicles were cultured at 37°C for 24 h in 5% CO_2. The ovulated oocytes within their cumulus masses were placed in 80 IU hyaluronidase to remove the cumulus cells. All normal appearing Metaphase II oocytes were placed in fresh medium of MEM-α supplemented with 0.05 mg/mL penicillin and 3 mg/mL BSA.

Mature oocytes from both groups were incubated with epididymal spermatozoa for 5 h. The oocytes were then washed twice with fresh medium and cultured overnight in Human Tubal Medium (made in our laboratory) supplemented with 3 mg/mL BSA (Sigma, MO). The number of two-cell stage embryos was noted for each group and then removed for cytogenetic analysis.

Micronuclei Assessment

The two-cell embryos were exposed to a hypotonic solution of 1% sodium citrate (Briemar, VIC, Australia) for 10 min. They were then fixed on slides by the addition of 2–3 drops of the fixative, consisting of 3 parts methanol (BDH Chemicals, VIC, Australia) and 1 part acetic acid (BDH Chemicals, VIC, Australia) (24). The slides were stained with 10% Giemsa and coded for analysis by the cytogeneticist in a double-blind manner. They were examined by oil immersion lens at 1000X microscopy and the presence or absence of micronuclei was recorded.

Centromere Assessment Using CREST Antibodies

The two-cell embryos were exposed to a hypotonic sodium citrate solution and fixed on slides in the same way as for micronuclei assessment (24). The slides were then prepared for centromere staining using CREST antibodies to the kinetochore regions, based on the methods by Afshari *et al.* (22). Briefly, this consisted of soaking the slides in 0.1% Tween (Sigma, MO) solution in Phosphate Buffered Saline (PBS, Gibco, NY) for 5 min at room temperature. Fifteen microliter of a 1:15 dilution of antikinetochore antibody (Chemicon International, Temucula, CA) solution in 0.1% Tween was applied to the slides and incubated at 37°C for 30 min. After washing three times in the 0.1% Tween solution, one drop of undiluted fluorescinated goat antihuman IgG (Chemicon International, Temucula, CA) was applied and incubated for a further 30 min at 37°C. The slides were then once again washed three times in the 0.1% Tween solution. Ten microliter of propidium iodide (Sigma, MO) at 1 μg/mL in antifade solution was applied prior to the cover slip. The slides were viewed at 1000X microscopy in oil immersion, using a Leica N 2.1 filter set. The protein in the cytoplasm appears green, nuclear DNA stains red, and centromeres appear yellow.

The significance of the incidence of micronuclei between the two-cell embryos of each group was tested by χ^2 analysis.

Table I. Incidence of Micronuclei for the Control and IVM Groups

Group	No. of two-cells analysed by Giemsa	No. of two-cells analysed by centromere staining	Total no. of two-cells analysed	No. of two-cells with micronuclei (%)	No. of two-cells with centromeres in micronuclei (%)
Control	112	85	197	4 (2)	0 (0)
IVM	62	65	127	46 (36.2)*	0 (0)

Note. Data represents amalgamation from both Giemsa and centromere staining procedures.
$^*P < 0.02$.

RESULTS

The fertilization rates for the control and IVM groups were 84.2% (223/265) and 67.1% (141/210), respectively. It was possible to accurately analyse 197 control and 127 IVM two-cell embryos. The incidence of micronuclei for both groups is given in Table I. There was a significant increase in the presence of micronuclei observed in the embryos of the IVM group ($P < 0.02$) (Fig. 1). The four embryos of the control group exhibiting micronuclei all had a single micronucleus. In the IVM group, the number of micronuclei ranged from one to five per embryo. Centromeres were not observed in the micronuclei of embryos from either group. Normal nuclei staining positive for antikinetochore antibody acted as a positive control for the CREST antibody reaction.

DISCUSSION

Micronuclei represent whole chromosomes or chromosome fragments that have been lost from the cell nucleus during mitosis or meiosis (20,21). Heddle *et al.* (20) suggest that micronuclei may form by one of four basic mechanisms: 1) mitotic or meiotic loss of an acentric fragment; 2) a variety of mechanical consequences of chromosomal breakage and exchange; 3) mitotic or meiotic loss of whole chromosomes; 4) as a result of apoptosis. Micronuclei have been shown to be a sensitive measure of the chromosome damaging effect of radiation on sperm by scoring the micronuclei in embryos that develop from eggs fertilized by the irradiated sperm (25–27). Additionally, it has been shown that mitotic arrest and the presence of micronuclei may be associated with poor embryonic development in the mouse (28).

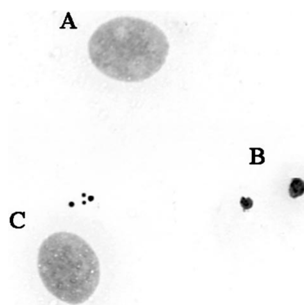

Fig. 1. A two-cell embryo generated from IVM. A – nucleus; B – polar bodies; C – micronuclei. Giemsa stain 1000X.

The embryos that developed from in vitro matured eggs showed a much higher frequency of micronuclei than those matured in vivo. Because micronuclei form as the result of chromosome damage or mitotic or meiotic error this finding suggests the in vivo maturation process exposes either the chromosomes or their spindle mechanisms to damage. Eichenlaub-Ritter *et al.* (29) have shown that there is increased damage to the microtubular cytoskeleton of human eggs aged in vitro and suggest that this damage might be responsible for increased aneuploidy and consequently the poor developmental potential of IVM oocytes.

Increased oxygen tension in vitro is a possible explanation for the increase in micronuclei in the IVM oocytes. In the current experiments, oocytes were matured and embryos cultured in medium under an atmosphere of 5% CO_2 in air (∼19–20% O_2). This results in a significantly higher oxygen tension in the medium than is physiological (30). There is evidence to suggest that mouse cells cultured under these conditions show more chromosome damage than those cultured under an atmosphere of 5% O_2, 5% CO_2, 90% N_2, which results in a medium with oxygen tension much closer to physiological levels (19). Higher oxygen tensions result in increased production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide that are potent damagers of not only chromosomes but also other cellular organelles, including the mitotic and meiotic spindles (31). Culturing embryos at lower oxygen tensions has also been shown to increase implantation rates in mice (32) and decrease in ROS has been implicated in this improvement (33).

This study found no evidence of centromeres in the micronuclei. It can therefore be concluded that the micronuclei represent chromosome fragments, as opposed to whole chromosomes. This finding suggests that the damage is more likely to be to the chromosomes themselves rather than spindle damage. Some embryos showed as many as five micronuclei, indicative of a high level of breakage and possibly representing the early stages of apoptosis.

In conclusion, this study has shown that there is a significant increase in the presence of micronuclei in two-cell embryos generated from IVM mouse oocytes, and that their presence may be attributed to chromosome fragmentation. We believe that the immature mouse model used in the current IVM study is highly relevant for the human situation. In particular it ensures that the studies are conducted on FSH-independent oocytes rather than the human reports that may be contaminated by the presence of some oocytes that have been recruited for further development, having been under the influence of FSH from either endogenous or exogenous sources. We have already shown cytoplasmic deficiencies underlie some cases of poor developmental competence in IVM and there may well be a useful role for ooplasmic transfer (23). The presence of micronuclei in a significant number of embryos generated from IVM matured oocytes is quite a different problem, possibly reflecting the initiation of apoptosis, commencing the process of atresia. It may be possible to reduce the problem and improve the developmental competence of IVM oocytes by adaptions to the culture system, such as exploring different oxygen tensions and perhaps the use of antioxidants. Studies in this direction are required.

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