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Cryopreservation of Mouse Oocytes: Mutagenic Effects in the Embryo?[1]

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ABSTRACT

We have shown in previous studies that the complete cycle of cryopreservation and prefreezing manipulations increases the degeneration and decreases the fecundability of mouse oocytes. The present study confirms these results. Moreover, we show that the increase of polyploidy previously observed in one-cell zygotes derived from frozen-thawed oocytes persists during the early stages of embryonic development. Furthermore, embryos obtained from frozen oocytes or oocytes exposed to prefreezing manipulations show an increase in the frequency of sister chromatid exchanges. Since the estimation of sister chromatid exchange is a sensitive test of mutagenicity, this suggests that the complete cycle of cryopreservation might alter the oocyte and, more particularly, induce DNA damage.

INTRODUCTION

Cryopreservation of unfertilized human oocytes would seem to be an attractive alternative to cryopreservation of human embryos in light of medical and ethical problems concerning the latter. Moreover, this procedure offers the possibility of establishing oocyte banks similar to those already used for spermatozoa. Although this procedure seems to have had only limited success so far in humans [1, 2], its feasability has been demonstrated in mammals such as mice [3–5], hamsters [6, 7], rats [8], and rabbits [9, 10]. However, the use of oocyte cryopreservation for conceptuses obtained after in vitro fertilization (IVF) must be further evaluated. The present study was undertaken specifically to investigate the possible mutagenic effects of the complete cycle of freezing-thawing of mouse oocytes and/or of prefreezing manipulations on subsequently derived pre-embryos. We used the sister chromatid exchange (SCE) test in early mouse embryos before implantation. In fact, an increase in SCE is considered to be a very sensitive test of the exposure of an individual, or cells, to potentially mutagenic and/or carcinogenic agents [11–14]. Because SCE reflects an interchange between DNA molecules at homologous loci within a replicating chromosome, this procedure allows us to determine presistent DNA lesions during the early stages of embryonic development.

MATERIALS AND METHODS

Source of Oocytes

Mice (C57 BL/6J × CBA/ca F1 hybrids) were induced to superovulate at 4–6 wk of age by the injection of 5 IU of eCG (Folligon, Intervet, Angers, France) followed 48–52 h later by 5 IU of HCG (Chorulon, Intervet). The mice were killed 13.5–14.0 h after the last injection, and the oviducts were collected in M2 medium [15] supplemented with 5 mg/ml BSA (Fraction V, Sigma Chemical Co., St. Louis, MO). The cumulus mass-oocyte complexes were released in the same medium and randomly assigned to cryopreservation or to IVF (control group and oocytes exposed to cooling and/or to dimethylsulfoxide (DMSO). On the same day, other cumulus mass-oocyte complexes were thawed, examined, and submitted to IVF. Thus the same group (control 1) was used for the two procedures of thawing (control of the quality of IVF) and freezing (control of the quality of the oocytes before any manipulation).

Freezing

The methods used for freezing were similar to those previously described [5]. Briefly, the cumulus mass-oocyte complexes were equilibrated for 15 min at 0°C in M2 medium supplemented with 1.5 M DMSO (Sigma Chemical Co.). During this equilibration period, the cumulus mass-oocyte complexes were placed in plastic straws (6–10 masses/straw). The straws, placed in a Minicool Cell Freezer (Minicool LC40, CFPO, Paris, France), were cooled at −2°C/min to −6°C. Ice formation was induced by seeding, and the straws were cooled at −0.3°C/min to −80°C and then at −35°C/min to −150°C. The samples were then transferred to liquid nitrogen for storage at −196°C.

Thawing and Dilution

The straws were thawed by use of a slow protocol with three different steps as previously described [5]. Briefly, the cumulus mass-oocyte complexes were thawed slowly in 20-min steps at −80°C, at −20°C, and for a few minutes at room temperature. The DMSO was removed from the cumulus mass-oocyte complexes by adding 1 ml of M2 medium at room temperature. After 1 min, the cumulus mass-oocyte complexes were recovered and washed through three changes of M2 medium (2 ml/wash) and one change of

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M16 medium [3] supplemented with Fraction V BSA (15 mg/ml). They were then placed in M16 medium at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 h before IVF.

Exposure to Cooling and/or DMSO

Since the prefreezing step in the procedure of oocyte cryopreservation corresponds to the exposure of the cumulus mass-oocyte complexes to 1.5 M DMSO at 0°C for 15 min, freshly recovered cumulus mass-oocyte complexes were randomly divided into 4 groups and treated before IVF as described below.

Group 1: control 2. The cumulus mass-oocyte complexes were placed for 15 min at 37°C in a humidified atmosphere of 5% CO_2 in air.

Group 2: cooling alone. The cumulus mass-oocyte complexes were exposed to 0°C for 15 min.

Group 3: DMSO and cooling to 0°C. The cumulus mass-oocyte complexes were incubated for 15 min with 1.5 M DMSO at 0°C.

Group 4: DMSO at room temperature. The cumulus mass-oocyte complexes were exposed to 1.5 M DMSO at room temperature for 15 min.

After the incubation period, the cumulus mass-oocyte complexes of each group were recovered and washed through three changes of M2 medium (2 ml/wash) and one change of M16 medium, supplemented with Fraction V BSA (15 mg/ml).

IVF

IVF was set up in the same way as previously described [5]. Briefly, the cumulus mass-oocyte complexes were introduced to a fertilization drop (M16 medium) containing 3×10^6 spermatozoa/ml, 90 min after sperm collection. The spermatozoa were obtained by gently pressing two cauda epididymides taken from two F1 males (10–12 wk old). The cumulus mass-oocyte complexes and sperm were incubated for a further 5 h. The oocytes were then washed to remove excess spermatozoa and morphologically examined. Finally, the oocytes considered morphologically normal were placed in M16 medium containing crystallized BSA (5 mg/ml).

Morphological Abnormalities

Oocytes were classified as normal or abnormal through morphological examination (5 h after IVF). Abnormal oocytes included 1) Immature oocytes, oocytes which were surrounded with numerous follicular cells; 2) degenerated oocytes, oocytes for which the cell cytoplasm appeared dark, the perivitelline space was widened, and the cytoplasm was hypotrophied; 3) fragmented oocytes, oocytes with fragmented cell cytoplasm; and 4) oocytes with a fractured zona pellucida.

Rate of Cleavage

Twenty-four hours after the beginning of IVF, the eggs were observed to determine the cleavage rate. The estimation of this rate was obtained by the ratio: number of 2-cell eggs/total number of oocytes in culture.

The cleavage rate after 48 h was obtained by the ratio: number of 4-cell eggs/number of 2-cell eggs at 24 h.

Cell Culture and SCE Scoring

For the detection of SCE, the embryos were grown for the first 2-cell cycles in a medium containing 5-bromodeoxyuridine (BUdR), a thymidine analogue incorporated into nascent strands during the S phase. The method used was similar to that previously described by Perry and Evans [11]. The fertilized oocytes were transferred 28–30 h post-hCG injection (after the S phase) to M16 medium containing BUdR at a final concentration of 10 μM. The cultures were then kept in the dark to minimize the number of sister chromatid exchanges caused by photolysis of DNA containing BUdR [16]. After the first cleavage (44–47 h after hCG), 1×10^{-6} M of fluorodeoxyuridine (FUdR) was added. FUdR inhibits thymidylate synthetase and thus potentiates the incorporation of bromodeoxyuridine into DNA by the reduction of de novo synthesis of deoxythymidine-5′-monophosphate [17]. After the second cleavage (68–70 h post-hCG), colchicine was added, and 10–11 h afterwards, chromosome preparations were made according to the method of Tarkowski [18]. The embryos were placed in a hypotonic solution of sodium citrate (2%) for 8 min and then fixed individually. Freshly prepared acetic alcohol was used as the fixative (3:1, absolute ethyl alcohol and glacial acetic acid). The slides were stained with the Fluorescence-plus-Giemsa (FPG) technique [19]. The slides were incubated for 20 min in Hoechst 33258, rinsed with double-strength SSC buffer, mounted in the buffer, and exposed through coverslips to long-wave UV light for 2 h. The slides were then rinsed with water and stained with a 5% Giemsa solution for 5 min. The FPG technique, which differentially stains the sister chromatids, allowed the observation of SCE. For scoring purposes, a terminal exchange between two sister chromatids corresponded to one SCE and a symetric exchange to two SCE.

For each of the embryos, SCE scoring was done for all the analyzable metaphasic blastomeres, and then the observed SCE count for one randomly chosen blastomere was used for the global analysis. In each of the groups (control oocytes, frozen-thawed oocytes, and treated oocytes), the frequently of exchanges was expressed as the mean SCE/blastomere ± the standard error of the mean (SEM).

Statistics

The data were analyzed by means of chi-square analysis. Differences between numerical means of SCE per blastomere were compared using Student's *t*-test.

TABLE 1. The effects of freezing, cooling, and/or DMSO on in vitro cleavage and subsequent development in mouse oocytes. Cytogenetic analysis of the incidence of chromosomal abnormalities at the 4-cell stage.

	Abnormal oocytes (%)	Cultured oocytes	2-Cell cleavage at 24 h (%)	4-Cell cleavage at 48 h (%)[a]	Analyzable embryos	Chromosomal abnormalities	
						Haploidy (%)	Polyploidy (%)
Control 1	301	1434	1093	892	296	7	21
(n = 1735)	(17.3)		(76.2)	(81.6)		(2.4)	(7.1)
Frozen-thawed	1382	2019	988	751	279	1	42
(n = 3401)	(40.6)*		(48.9)*	(76)**		(0.4)	(15.1)**
Control 2	98	415	271	215	101	3	2
(n = 513)	(19.1)		(65.3)	(79.3)		(3)	(2)
Cooled to 0°C	105	410	254	205	79	2	0
(n = 515)	(20.4)		(61.9)	(80.7)		(2.5)	(0)
Exposed to 1.5 M	213	723	362	284	121	3	1
DMSO at 0°C	(22.8)		(50.1)*	(78.4)		(2.5)	(0.8)
(n = 936)							
Exposed to DMSO at	300	666	169	116	42	1	0
room temperature	(31.1)*		(25.4)*	(68.6)***		(2.4)	(0)
(n = 966)							

*$p < 10^{-8}$ (chi-square test).
**$p < 0.01$ (chi-square test).
***$p < 0.02$ (chi-square test).
[a]Expressed as % of 2-cell eggs.

RESULTS

After 5 h of culture with sperm, a significantly higher number of frozen-thawed oocytes appeared to be morphologically abnormal compared to the fresh oocyte controls (Table 1, $p < 10^{-8}$). Among the abnormal oocytes, a significantly higher rate of degeneration was seen in the frozen-thawed series than in the control 1 group (91.1% of morphological abnormalities vs. 5.6% for the controls, $p < 10^{-8}$).

Only DMSO used at room temperature caused a similar increase in the morphological abnormality rate compared to that in the control 2 group (Table 1, $p < 10^{-8}$). However, the oocytes exposed to 0°C or DMSO were more often degenerated than were the controls: 8.2% for the controls vs. 21.9% for the oocytes exposed to 0°C ($p < 0.01$); 43.2% for the oocytes exposed to DMSO at 0°C ($p < 10^{-8}$) and 48.3% for the oocytes exposed to DMSO at room temperature ($p < 10^{-8}$).

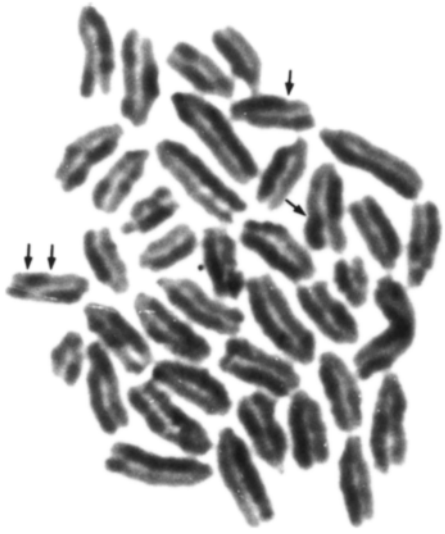

FIG. 1. Distribution of the number of chromosomes in analyzable SCE blastomeres. A total of 93, 77, 55, 36, 37, and 16 blastomeres were scored respectively in the control 1 group, the frozen-thawed group, the control 2 group, the group cooled to 0°C, the group exposed to DMSO at 0°C, and the group exposed to DMSO at room temperature (20°C).

The embryos derived from frozen oocytes cleaved significantly less frequently than the control 1 group (Table 1, $p < 10^{-8}$). This impairment of cleavage persisted during the second cellular division (Table 1, $p < 10^{-8}$).

Whereas cooling alone did not significantly alter the rate of cleavage to the 2-cell stage, DMSO caused a significant decrease (DMSO at 0°C or at room temperature: $p < 10^{-8}$, Table 1). However, only DMSO used at room temperature again altered significantly the 4-cell cleavage of embryos as compared to that in the control 2 group ($p < 0.02$).

Concerning polyploidy, the fertilization of frozen-thawed oocytes gave rise to more polyploid embryos than the control 1 group (Table 1, $p < 0.01$). In the control group, one embryo was mosaic, one blastomere was diploid, and another was triploid. In the frozen-thawed group, two mosaic embryos were observed. The two groups did not present any significant difference with regard to the rate of haploidy ($X^2 = 2.87$, NS).

The insemination of oocytes exposed to cooling and/or to DMSO did not give rise to more chromosomal abnormalities, haploidy, or polyploidy, than the control 2 group (Table 1, haploidy: $X^2 = 0.06$, 3 *df*, NS; polyploidy: $X^2 = 2.49$, *df* 3, NS).

A total of 64 embryos from control oocytes (with 93 analyzable blastomeres) and 56 embryos from frozen-thawed oocytes (with 77 analyzable blastomeres) were scored for SCE. The rate of SCE was not significantly different between

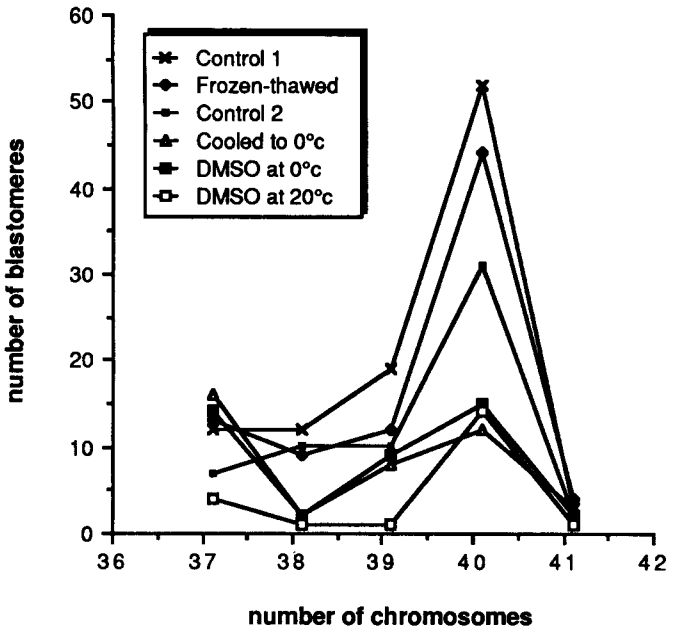

FIG. 2. Sister chromatid exchanges in one mouse embryo blastomere. Some SCEs are indicated by arrows. ×400.

the two groups (20.1% of karyotyped embryos had at least one blastomere where SCE were analyzable in the frozen group vs. 21.6% for the controls). The distribution of the number of chromosomes around the mean diploid number (40 chromosomes) in blastomeres of each group is shown in Figure 1. One blastomere derived from a 4-cell embryo is shown in Figure 2. Sister chromatid exchanges can be observed.

The SCE frequencies observed in 64 and 56 blastomeres, respectively, from the control 1 group and the frozen-thawed group are shown in Table 2 (one blastomere was randomly chosen in each of the 64 embryos). A significant increase in average SCE was observed in the embryos from frozen-thawed oocytes.

The rates of embryos scored for SCE were 39.1% (40 of 101), 27.8% (22 of 79), 23.9% (29 of 121), and 26.2% (11 of 42), respectively, in the control 2 group, the group cooled to 0°C, the group exposed to DMSO at 0°C, and the group exposed to DMSO at room temperature. The distribution of the number of chromosomes in the analyzable blastomeres of each group is shown in Figure 1. The frequencies of SCE observed in the randomly chosen blastomeres (one per embryo) for each of the four groups are shown in Table 2.

The SCE frequencies observed in the two control groups were not significantly different (t = 0.43, ddl 102, NS).

A significant increase in SCE frequency was observed for all the treatments compared to the control 2 group.

While there was no significant difference between the group cooled to 0°C and the group exposed to DMSO at 0°C, DMSO at room temperature caused a significant increase in SCE frequency compared to that in the cooled group.

A significant increase in SCE frequency was also observed for the DMSO group at 0°C compared to the DMSO group at room temperature.

TABLE 2. Cryopreservation of mouse oocytes and the frequency of SCE in embryos after IVF. Effects of cooling and/or DMSO.

Oocytes	Number of scored embryos	SCE/Blastomere /cellular cycle
Control 1	64	8.92 ± 0.4
Frozen-thawed	56	13.81 ± 0.51[a]
Control 2	40	8.67 ± 0.37
Cooled to 0°C	22	12.39 ± 0.82[b]
Exposed to DMSO at 0°C	29	13.96 ± 0.68[c]
Exposed to DMSO at room temperature	11	16.57 ± 0.77[d]

[a]Vs. control 1, $t = 7.56$, df 118, $p < 0.001$.
[b]Vs. control 2, $t = 4.72$, df 60, $p < 0.001$; vs. oocytes exposed to DMSO at 0°C, $t = 1.49$, df 49; NS vs. oocytes exposed to DMSO at room temperature, $t = 3.25$, df 31, $p < 0.01$.
[c]Vs. control 2, $t = 7.30$, df 67, $p < 0.001$; vs. oocytes exposed to DMSO at room temperature, $t = 2.16$, df 38, $p < 0.05$.
[d]Vs. control 2, $t = 9.68$, df 49, $p < 0.001$.

DISCUSSION

In this study, and in line with percentages reported elsewhere [4, 5, 20, 21], the complete process of freezing and thawing produced an increase in the number of morphologically abnormal oocytes, and, more precisely, an increase in degenerated oocytes. Oocyte distortion and the formation of both intra- and extracellular gas bubbles during the freezing and thawing might explain this fragility [22]. Cytoskeletal disturbance of oocytes exposed to DMSO at 37°C or 4°C [23, 24] might also contribute to this negative morphological effect.

This study shows a decrease in the cleavage of morphologically normal frozen-thawed oocytes and oocytes exposed to DMSO, whereas the cleavage of oocytes did not alter after exposure to 0°C, in agreement with previous studies [4, 5, 20, 21]. This decrease might be explained by changes in the zona pellucida observed after exposure to DMSO and particularly by modification of the glycoprotein ZP2 by the premature exocytosis of cortical granules [25, 26]. The cleavage inhibition in fertilized oocytes due to damage to the cytoplasm such as an alteration of the organization of microfilaments [24] or a disruption of the meiotic spindle [23] might also explain this decreased cleavage.

In previous studies, an increased polyploidy rate at the 1-cell stage in embryos obtained from frozen oocytes was observed after IVF [4, 5, 20]. This increase seemed to be largely due to the presence of digynic embryos. In this study, we again found a similar increase in the incidence of polyploidy, but at the 4-cell stage of development. This suggests that there is no important regulation of this chromosomal abnormality in the subsequent development of the embryos. Such regulation has been observed after IVF in hu-

mans [27–29]. Indeed, these studies showed a possible return towards diploidy for human multipronuclear 1-cell eggs. The discrepancy between these observations might be explained by the different origins of the polyploidy. In the human, polyploidies observed after IVF are more often polyspermic, whereas in the mouse after cryopreservation, they tend to be of digynic origin [5, 20]. On the other hand, we confirm that prefreezing manipulations do not seem to influence the degree of ploidy in embryos derived from treated oocytes [21].

Although our understanding of the SCE process remains incomplete, a linear relationship between induced SCE and mutations has been shown [11, 13]. The common feature of agents that elevate SCE frequencies is an action on DNA, either directly or indirectly, via alterations in DNA replication or, possibly, chromatin structure [30]. In our experiment, the SCE rate at the 4-cell stage was increased for embryos derived from cryopreserved mouse oocytes. However, the freezing-thawing step did not seem to be responsible alone for this increase. Indeed, the prefreezing manipulations also caused an increase in SCE frequency. Although cooling to 0°C during this step seems primarily responsible for this increase, the involvement of DMSO cannot be completely excluded. Besides, it is known that BUdR can provoke chromosome aberrations in mammalian cells [31, 32]. Therefore, some SCE could be expected from the presence alone of this substance. However, since in our study the treated and the control groups were submitted to the same method of exposure and the same concentration of BUdR, the observed differences between the SCE frequencies cannot be due to the effects of BUdR alone. This suggests that cryopreservation and/or prefreezing manipulations might cause an alteration either in the DNA structure or of oocyte components implicated in repair mechanisms. On the other hand, it has been suggested that freezing could increase the production of free radicals [33], which are liable to injure DNA [34]. In any case, the exact target of the deleterious action of the complete cycle of freezing-thawing remains to be clarified.

It would seem necessary to study the development of embryos obtained from frozen mouse oocytes, particularly after embryo transfer and the production of descendants. In some cases, although no noticeable malformations had been observed, the use of behavioral tests revealed genetic alterations through the presence of functional heritable abnormalities [35]. The consequences of increased rates of SCE will also have to be studied and the possible negative effects on conceptuses induced by oocyte freezing considered. This seems all the more important since Hu et al. [36] showed in humans that an unequal sister chromatid exchange was involved in a mechanism of gene tandem duplication that occurred at the origin of some cases of Duchenne muscular dystrophy.

In conclusion, this preliminary study shows an increase in the SCE rate, suggesting DNA instability after mouse oocyte cryopreservation or exposure to DMSO at 0°C during the prefreezing step. This line of study should be continued in order to determine the possible consequences of increased SCE on conceptuses. Such study is necessary before the cryopreservation of human oocytes can be performed.

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