

Morphological alterations and DNA fragmentation in oocytes from primordial and primary follicles after freezing–thawing of ovarian cortex in sheep

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Objective: To evaluate DNA fragmentation in the oocyte of primordial and primary follicles and morphology of these follicles after freezing and thawing of ovarian cortex in sheep using two freezing protocols.

Design: Fragmentation of DNA was evaluated by the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL) technique.

Setting: Fertility clinic in a large university hospital.

Animals: Five- to 6-month-old lambs.

Intervention(s): Two-millimeter-thick slices of hemi-ovary cortex were prepared.

Main Outcome Measure(s): Histological structure and DNA fragmentation.

Result(s): In the frozen fragments, the percentage of morphologically normal follicles was significantly lower for both protocols compared with the case of the control group of fresh fragments. There was no significant difference between the two types of freezing protocols ($60.4\% \pm 13.2\%$ vs. $68.4\% \pm 13.7\%$). However, the distribution of abnormalities (nucleus, cytoplasm, and nucleus and cytoplasm) was dissimilar. The results of the TUNEL technique for the three groups showed no significant difference, but the percentage of the TUNEL-positive follicles was slightly lower for the frozen fragments for both protocols with respect to the control group.

Conclusion(s): The freezing and thawing process of the ovarian cortex does not induce fragmentation of the DNA on the oocyte of primary and primordial follicles. (Fertil Steril® 2002;77:595–600. ©2002 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, ovarian tissue, sheep, DNA fragmentation

At birth, the ovary contains a finite number of primordial follicles. Each primordial follicle consists of a single small oocyte surrounded by a few flattened somatic cells that are in a quiescent state. Cell proliferation will not resume until the primordial follicle begins to grow, and the oocyte of the quiescent primordial follicle is blocked at the first mitosis of meiotic division (1).

Primordial follicles can be considered the storage form and constitute a source of oocytes that could be used for clinical and zoological purposes like preservation of races in extinction. Many teams are working on the cryo-

preservation of ovarian cortex, where this reserve is found, to restore fertility. Animal studies have demonstrated that frozen and thawed ovarian tissue is able to restore cyclic secretion of ovarian steroids after an autograft (2, 3) or transplantation (4–6). Pregnancies after frozen ovarian autograph have been reported in rats, mice, and ewes (7–10). Recently, ovulation after transplantation of frozen ovarian tissue has been reported in humans (11). In this study, sheep ovaries were used because they are very similar to human ovaries in terms of size and structure.

Primordial and primary follicles cannot be

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regenerated, so once destroyed, permanent infertility can arise. Our previous study showed that a modified freezing protocol using 2 M of dimethyl sulfoxide (DMSO) with a more rapid cooling rate (2°C/min) was as effective as the standard freezing protocol (8) on primordial follicles' morphology and viability after thawing (12). The aim of the present study was to evaluate the DNA damage in the primordial follicle oocyte after freezing and thawing of the ovarian cortex with two different freezing protocols described by Gosden et al. (8) and by our group (12).

Fragmentation of DNA was used recently as a marker of DNA damage on frozen testicular sperm using comet assay (13). We used a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) in situ detection test to evaluate DNA fragmentation on histological sections.

MATERIALS AND METHODS

Ovaries

Ovaries from 5- to 6-month-old lambs were collected at the slaughterhouse. They were placed in serum-free organ transport medium, X-Vivo (Bio Whittaker, Walkersville, MD), and transported to the laboratory at 10°C. The ovaries were cut into two equal pieces at room temperature within 1.0–1.5 hours of collection. The ovarian medulla was removed so as to obtain a 2-mm-thick layer of hemiovary cortex. Each fragment measured approximately 1 cm², and the mean fragment weight was 212 ± 32.7 mg.

Freezing Procedures

Ovarian cortex fragments were placed in cryogenic vials (Nunc Brand Products, Roskilde, Denmark) containing 1 mL of BM1 (Ellios Bio Media, Paris, France) freezing medium supplemented with 10% fetal calf serum (Sigma, St. Louis, MO) and 1.5 M or 2 M of DMSO (Sigma), according to the freezing protocol. The vials were incubated at room temperature for 10 minutes before freezing. The vials were placed in a programmable freezer (Minicool 40PC; Air Liquide Santé, Paris, France).

Using the protocol described by Gosden et al. (8) (protocol A), five fragments were frozen with 1.5 M DMSO. The vials were cooled at 2°C/min to -7°C, then held at -7°C for 10 minutes for manual seeding. The temperature was then lowered by 0.3°C/min to -40°C and thereafter by 10°C/min to -140°C.

Using protocol B, five fragments were frozen in the freezing medium containing 2 M of DMSO. The vials were cooled at 2°C/min to -11°C, and then semiautomatic seeding was performed by the release of negative calories, then cooled at the same rate to -35°C, and thereafter by 25°C/min to -140°C according to the protocol perfected by our team, as described elsewhere (12).

For both of these protocols, the cooling curves were recorded throughout the procedure by computer. Tempera-

ture graphs were obtained for each freezing process. The cryogenic vials were transferred to liquid nitrogen and stored for 3 hours.

The fresh control fragments were incubated in BM1 medium at room temperature for 15 minutes and at 37°C for 15 minutes. They were then fixed in Bouin's solution.

Thawing Procedure

The ovarian tissue was thawed rapidly at 37°C and washed in BM1 at room temperature and then for 15 minutes in fresh BM1 medium. Vials were shaken gently to promote efflux of cryoprotectant from the tissue. Each fragment was then incubated at 37°C for 15 minutes in BM1 medium. After this, ovarian tissues were fixed in Bouin's solution for 24 hours. After fixation, ovarian fragments were embedded in paraffin and cut into 5-μm-thick sections every 100 μm and then stained with hematoxylin and eosin to examine the follicular morphology. Consecutive sections were mounted on silane-coated glass slides for in situ detection of DNA fragmentation.

Histology

Follicles were classified as described by Hirsfield (1), and primordial follicles, oocytes with one layer of flattened granulosa cells, and primary follicles with one layer of cuboid granulosa cells were counted. Follicles were counted in the section in which the nucleus of the oocyte was visible. The morphology of the follicles was recorded. Intact follicles had a complete basal lamina surrounding the pregranulosa cells, oocytes with cytoplasm, and a normal nucleus. Abnormal follicles were classified as follows: [1] oocytes with pyknotic nucleus; [2] oocytes with cytoplasm damage; and [3] oocytes with nucleus and cytoplasm damage combined. Follicles with an empty area between oocyte and the pregranulosa cells were included in the third group.

In Situ Detection of DNA Fragmentation

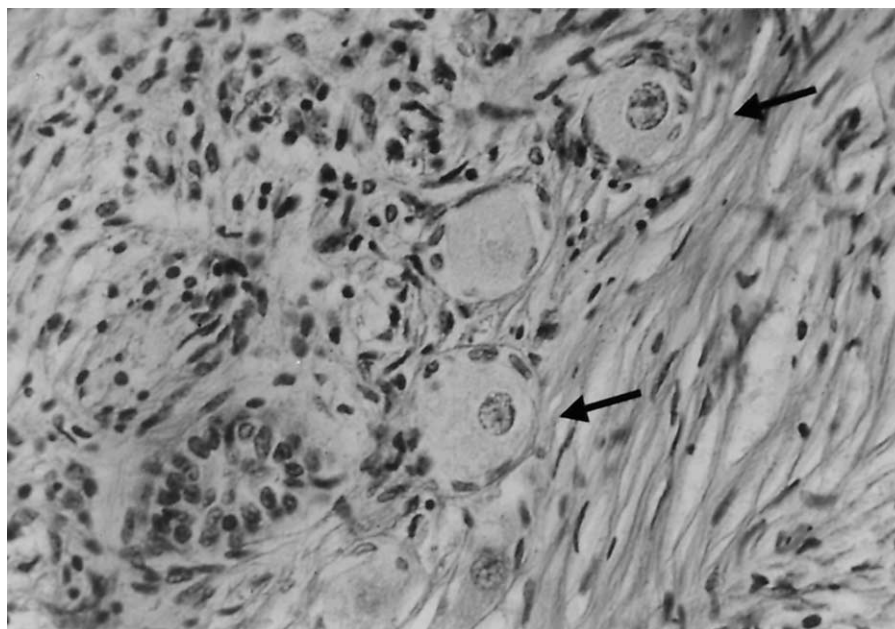
Fragmentation of DNA was evaluated by a recently developed 3-hydroxy nick end-labeling method. The method is based on the preferential binding of terminal deoxynucleotidyl transferase to the 3'-hydroxyl ends of DNA (14).

Sections were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of alcohol. After rehydration, the slides were incubated with Proteinase K (in 0.05 M of Tris-HCl containing NaN₃; DAKO Corporation, Carpinteria, CA). Endogenous peroxidase was inactivated by hydrogen peroxide 3%. Tissue sections were stained with an ApopDETEK system (Enzo Diagnostics, Farmingdale, NY) that identifies cells with internucleosomal fragmentation of DNA.

The procedure was performed according to the manufacturer's instructions. Briefly, residues of biotinylated deoxyuridine triphosphate were catalytically added to the ends of DNA fragments with the enzyme terminal deoxynucleotidyl transferase. For negative controls, deionized water was

FIGURE 1

Tissue frozen with protocol B. Oocytes have a diffuse pattern of nuclear chromatin, and they are surrounded by a layer of granulosa cells (arrows, primordial follicles; original magnification, $\times 400$).



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used instead of terminal deoxynucleotidyl transferase. After end labeling, the sections were incubated with avidin–horse-radish peroxidase and stained with diaminobenzidine to detect the biotin-labeled nuclei. Apoptotic bodies were stained in brown. Positive controls consisted of rat mammary glands. Primordial and primary follicles were counted and analyzed. Labeled cell analysis was based on oocytes and not on follicular cells.

Statistical Analysis

Kruskal-Wallis and Mann-Whitney nonparametric tests were used for statistical analysis. Differences were considered significant at $P \leq .05$. Minitab software was used for statistical analysis (Minitab SARL, Paris, France).

RESULTS

In this study, a total of 3,148 follicles were examined for morphological analysis, and 2,221 follicles were examined for labeling procedure.

In the control fragments, the percentage of morphologically normal follicles was $91.2\% \pm 1.0\%$, whereas in the frozen fragments, the percentage was significantly lower: $60.5\% \pm 13.2\%$ ($P < .005$) for protocol A and $68.5\% \pm 13.7\%$, ($P < .005$) for protocol B (Fig. 1). However, the P value between the two freezing groups was not significantly different.

The percentage of follicles in which only the nucleus of the oocyte was abnormal increased significantly ($P < .05$) when the fragments were frozen ($2.69\% \pm 0.8\%$, compared with $22.5\% \pm 5.4\%$ and $12.3\% \pm 4.2\%$, for freezing protocols A and B, respectively), and the difference was significant between the freezing protocols ($P < .05$). The difference of cytoplasm abnormalities was significant ($P < .05$) between the control group and protocol B ($2.51\% \pm .08\%$ and $9.8\% \pm 7.6\%$, respectively), and the percentage of oocytes with double abnormalities (nucleus and cytoplasm) was significantly different ($P < .05$) between the control group and the protocol A group ($3.7\% \pm 1.1\%$ and $11.6\% \pm 4.4\%$, respectively; Table 1 and Fig. 2).

For detection of DNA fragmentation, no labeling was observed on the negative control slices. The percentages of TUNEL-positive oocytes were $28\% \pm 10.7\%$, $16.5\% \pm 4.8\%$, and $20.8\% \pm 11.7\%$ for control group, protocol A, and protocol B, respectively; the differences were not significant (Figs. 3 and 4). In a few cases, we observed some TUNEL-positive oocytes showing no morphological abnormalities of the nucleus.

DISCUSSION

We have studied the morphology and DNA damage of the oocytes in immature follicles such as primordial and primary

TABLE 1

Comparisons of morphological abnormalities (nucleus, cytoplasm, and nucleus, and cytoplasm) between control group and two freezing protocols.

Abnormalities	Control	Protocol A	Protocol B
Nucleus (N)	2.69 ± 0.8 _a	22.5 ± 5.4 _b	12.3 ± 4.2 _c
Cytoplasm (C)	2.51 ± 0.8 _a	5.4 ± 4.02 _{ab}	9.8 ± 7.6 _{bc}
Both (N+C)	3.7 ± 1.1 _a	11.6 ± 4.4 _b	9.4 ± 5.22 _{ab}

Note: Values are means ± SD. The values assigned the same letter did not differ significantly (the comparisons made were within the same range among three groups: control, protocol A, protocol B).

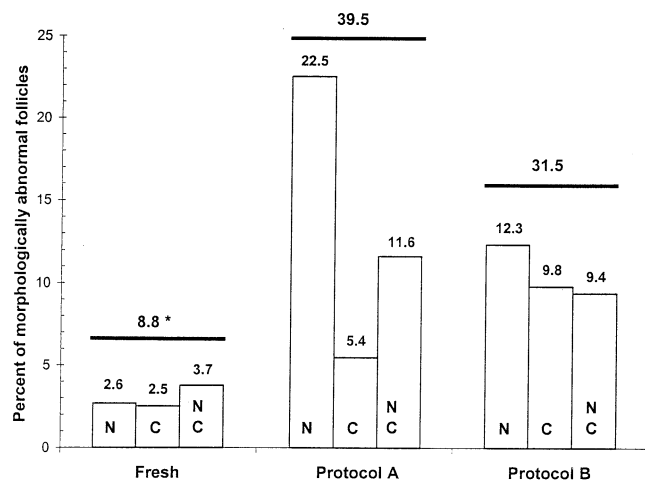
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follicles, cryopreserved in the ovarian cortex. We examined ovarian tissues fixed after freezing and thawing. The samples were incubated before fixing in the same conditions as before grafting.

We observed a decrease of normal follicles when the tissues were cryopreserved ($P < .005$): $91\% \pm 1\%$ compared with $60.4\% \pm 13.2\%$ (protocol A) and $68.4\% \pm 13.7\%$ (protocol B). It has been reported by many authors that some proportion of follicles is damaged during freezing and thawing (Hovatta et al. [15] in human; Candy et al. [4] in marmoset and in the mouse [16]), but a higher proportion of primordial follicles appeared morphologically normal after freezing and thawing.

FIGURE 2

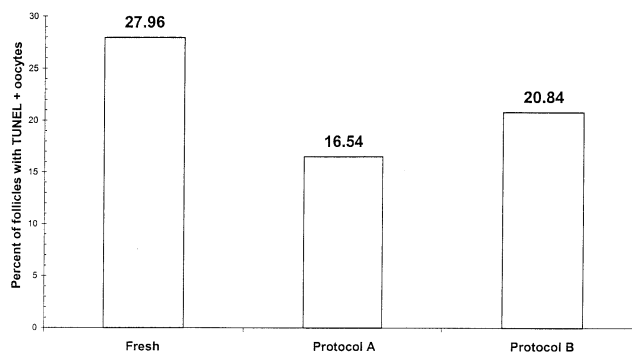
Percentage of morphologically abnormal follicles and distribution of abnormalities on fresh and frozen-thawed ovarian tissues. *Statistically significantly different from the two freezing protocols, $P < .05$. N, nucleus abnormalities; C, cytoplasm abnormalities; N C, nucleus and cytoplasm abnormalities combined.



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FIGURE 3

Percentage of follicles with TUNEL(+) oocytes on fresh and frozen-thawed ovarian tissues.



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All kinds of abnormalities were higher in frozen and thawed tissues for each freezing protocol; however, the distribution of abnormalities was dissimilar in fresh and frozen-thawed tissues and between the two freezing protocols: with protocol A, there was less cytoplasm (the difference was not statistically significant when compared with the control group) but significantly more nucleus anomalies (22.5%) compared with protocol B (12.3%). This could have been due to the freezing protocol used. Protocol B (13), which is now routinely used in our laboratory, is based on smaller temperature variations, which could lead to less damage to the cells nucleus when ice nucleation occurs.

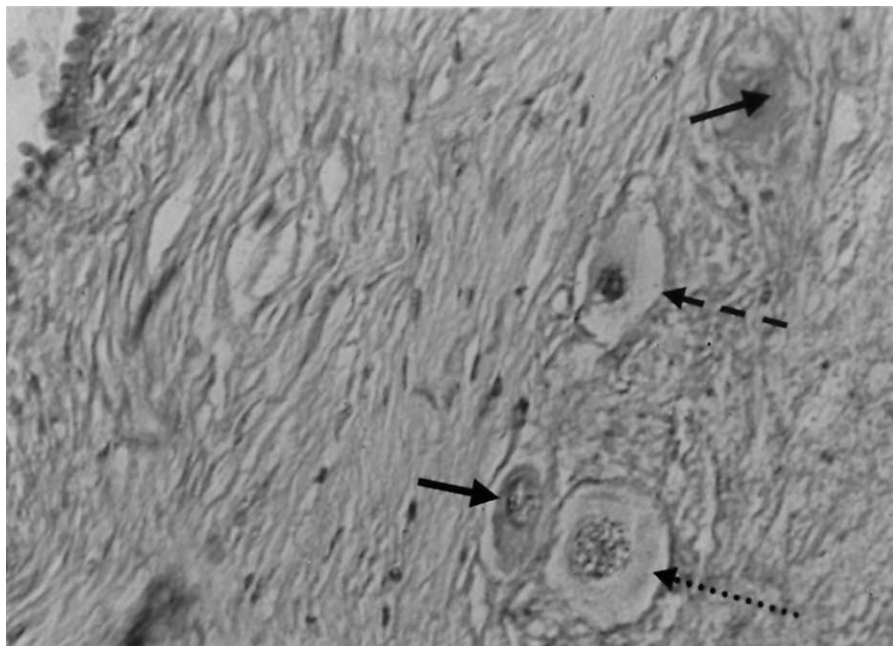
In this study, DNA fragmentation was detected by the *in situ* hybridization technique TUNEL; low molecular weight DNA fragmentation is the result of activation of specific endogenous endonucleases in apoptosis (17). A 3'-end labeling autoradiographic method to evaluate DNA cleavage was successfully used for labeling apoptosis in follicular atresia (18).

Labeled oocytes were present as follows in primordial and primary follicles: 27.96% in fresh tissues and 16.54% and 20.84% in frozen and thawed tissues with protocol A and protocol B, respectively ($P > .05$). Labeled oocytes in fresh ovarian tissue show the presence of primordial and primary follicles in atresia by apoptosis of oocyte. Certainly, the mammalian ovary contains a proportion of primordial and primary follicles in atresia. Driancourt et al. (19), in sheep, and Gougeon (20), in humans, reported that for each primordial follicle that began to grow, two to three primordial follicles underwent apoptosis. Recently, Lee et al. (21) reported a 32.5% and an 18.7% ratio of apoptosis for primordial and primary follicles, respectively, on fresh mouse ovarian tissues using morphological measures.

Our data are in agreement with those of Hovatta et al.

FIGURE 4

Tissue frozen with protocol B. *Solid right arrow*, primordial follicles with labeled oocyte; *dashed right arrow*, primordial follicles with nonlabeled oocytes; *dotted right arrow*, primary follicles with nonlabeled oocytes.



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(15), who observed 27% of primordial and primary follicles with morphological signs of atresia on fresh human tissues, whereas 19% of follicles in frozen and thawed tissues were atretic. The follicles in atresia could be more fragile and thereby have been destroyed during freezing procedures.

However, our results contrast with other reports in that positive-staining DNA fragmentation was not detected in primordial and primary follicles in rats (22) and in humans (23) by in situ hybridization. However, recently, the role of the Bax gene in atresia of primordial follicles has been proven in mice (24), which confirms our results.

Comparison of the protocols indicates a slight advantage of our freezing protocol compared with the standard freezing protocol: there was no statistically significant difference in terms of nuclear fragmentation, but nucleus abnormalities were significantly lower (12.3% vs. 22.5%). These findings are in agreement with our previous data concerning viability of isolated small follicles, measured by trypan blue staining (91.6% vs. 82.4%).

We can conclude that the freezing and thawing process of the ovarian cortex does not induce per se a fragmentation of DNA in an oocyte from primary or primordial follicles. Thus, immature follicles can be cryopreserved without subsequent irreversible DNA damage. Steele et al. (13) obtained similar results while comparing DNA integrity by comet

assay in fresh and frozen-thawed testicular sperm from azoospermic men.

To our knowledge, this is the first study to analyze DNA damage and the morphology of oocytes from frozen and thawed primordial follicles in comparison with fresh tissue. This technique can be used as a marker in the freezing and thawing protocol for evaluating ovarian cortex quality before using this tissue for grafting or in vitro culture.

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