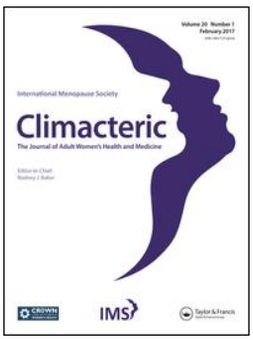

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ORIGINAL ARTICLE



# Ovarian tissue cryopreservation for patients with premature ovary insufficiency caused by cancer treatment: optimal protocol

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**ABSTRACT**

**Objective:** Premature ovary insufficiency is frequent after chemotherapy/radiotherapy in cancer patients. Ovarian tissue (OT) cryopreservation and later retransplantation, the routine method in Europe, has recently been implemented at the first center in China. We investigated the protective effect of the antioxidant *N*-acetyl-L-cysteine (NAC) during the decisive freezing–thawing steps.

**Methods:** Fifteen OT samples were obtained from each of 13 cancer patients prospectively and randomly assigned to a control group and four groups with different NAC concentrations (Group 1, 0 mM NAC; Group 2, 0.5 mM NAC; Group 3, 1 mM NAC; Group 4, 5 mM NAC; Group 5, 25 mM NAC). After thawing, the follicle viability, DNA fragmentation, levels of reactive oxygen species (ROS), and total antioxidant capacity (TAC) were evaluated.

**Results:** OT cryopreserved and thawed with 25 mM NAC (Group 5) has the lowest proportion of apoptotic stroma cells, but the worst follicle viability. The other four groups show similar anti-apoptosis and good follicle viability. Group 4 presented the lowest ROS and highest TAC levels.

**Conclusions:** OT cryopreserved and thawed in medium supplemented with 5 mM NAC shows the highest antioxidant and lowest ROS capability, good apoptotic parameters, and follicle viability. Our results need to be confirmed in larger patient cohorts prior to being accepted as a standard protocol.

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Ovarian tissue cryopreservation; *N*-acetyl-L-cysteine; follicle viability; DNA fragmentation; reactive oxygen species; total antioxidant capability

## Introduction

For women with malignant diseases, chemotherapy (especially exposure to a high dosage of alkylating agents) and abdominal/pelvic irradiation lead to a high risk of premature ovarian insufficiency and infertility[1–3]. Premature ovarian insufficiency is associated with serious risks such as menopausal symptoms, osteoporosis, cardiovascular diseases, and psychological disorders[4]. Hence, quality of life and fertility protection are important foci of attention.

Embryo cryopreservation and oocyte cryopreservation have been regarded as the main methods to preserve female fertility[5,6]. However, ovarian tissue cryopreservation (OTC) has some advantages as summarized in the recent guidelines of the Chinese Society of Gynecological Endocrinology[7]. OTC is the only option for women whose cancer treatment cannot be postponed and maintains the capability to restore long-term hormonal function after cancer recovery[8]. Recently, four publications concluded that OTC now is a valid technique rather than experimental[8–11].

The International Fertility Protection Center – Ovarian Tissue Cryobank of the Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, China is currently the first and only one in China. Other centers, especially in Asia, where the method is rare in contrast to Europe, will likely be established in the near future. Because cryopreservation and thawing are the decisive steps, it is important to continue the research on optimizing already existing protocols. This was the main aim of the present study.

Although OTC has some advantages, cryoinjury and ischemic injury seem to be inevitable[12,13]. Follicular loss and impairment of stroma cells occur due to intracellular generation of reactive oxygen species (ROS) during the freezing–thawing procedure[14]. Oxidative stress arises when excessive ROS production reacts with cellular lipids, proteins, and nucleic acids, resulting in DNA fragmentation, lipid peroxidation which alters the membrane structure and function, and protein damage[15,16].

There are two types of endogenous antioxidants: enzymatic (superoxide dismutase, catalase, glutathione peroxide and reductase) and non-enzymatic (vitamin C, vitamin E, β-carotene, selenium, zinc, taurine, glutathione) antioxidants[16]. Some studies have demonstrated the advantages of

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antioxidants after addition to transporting, freezing, and thawing solutions both in vitrification or slow-freezing of OT[17–19]. *N*-acetyl-L-cysteine (NAC) has been found to act as an effective non-enzymatic antioxidant in fertility preservation including sperm, embryo, and ovarian tissue (OT)[19–21].

NAC is a precursor of cysteine. It is involved in glutathione synthesis and can prevent apoptosis and promote cell survival. By scavenging of oxygen radicals or upregulating of antioxidant systems, NAC has been used during infertility treatment of patients with polycystic ovary syndrome, lung fibrosis, and psychiatry and neurology disorders[22–25].

One study suggested that using a cryomedium based on 1,2-propanediol (PROH) and 25 mM NAC was efficient for OTC and thawing[19]. However, we used the same NAC concentration in a protocol with dimethylsulfoxide (DMSO) and achieved unsatisfactory results in preliminary studies. The aim of this prospective, randomized, five-arm study was to evaluate the protective effect of different concentrations of NAC in cryomedium for OTC.

## Methods

### Patients and study design

OT samples from 13 patients with malignant diseases were harvested during surgery before gonadotoxic treatment and were cryopreserved at our Ovarian Tissue Cryobank from August to December 2017. Fifteen OT samples from each patient were numbered sequentially. Using a randomized digital table, they were allocated to the control and four study groups containing NAC at increasing concentrations in the basic freezing and thawing medium (Group 1, control, 0 mM NAC; Group 2, 0.5 mM NAC; Group 3, 1 mM NAC; Group 4, 5 mM NAC; Group 5, 25 mM NAC). Each group contains three OT samples for each patient.

### Ethical approval

This study was approved by the ethics committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, China (Protocol number 2017-KY-020-01; Registration number ChiCTR1800014315). At least half an ovary was collected for each patient. More than 90% of OT from each patient was cryopreserved for future autotransplantation. Only up to 10% was used for research. All patients have been informed about this and signed their consent.

### Ovarian tissue collection and transportation

Patients underwent oophorectomy via laparoscopy or laparotomy at several Beijing hospitals. Tissue was resected without electrocoagulation and immediately placed into a sterile vial containing 25 ml of Custodiol® HTK solution (Dr. Franz Köhler Chemie GmbH) at 4–8 °C. Samples were transported to our cryobank within 2 h in a biological sample transport (temperature of 4–8 °C).

### Ovarian tissue preparation

OT samples were immediately transferred into a culture dish with fresh, cold Custodiol® HTK solution. Cortical slices (4 mm ×8 mm ×1 mm) were prepared for future autotransplantation on a surface plate (4 °C) by removing the medulla. The remaining part of the cortex was used for research by preparing equally-sized biopsies (3 mm in diameter,1 mm in thickness) using a biopsy punch. For each patient, 15 biopsies were used. All was performed under sterile conditions in a class A cabinet under clean room conditions.

### Basic protocol for freezing and thawing

The basic slow-freezing cryoprotectants in our cryobank contain Leibovitz's L-15 GlutaMAX medium (Gibco, USA) supplemented with 10% CryoSure-DMSO (WAK-Chemie Medical GmbH) and 1% human serum albumin (IrvineScientific, USA). The basic thawing medium contains Dulbecco's phosphate buffered saline (Gibco) with added 10% fetal bovine serum (Gibco). The controlled freezer was a PLANER Kryo 360-1.7.

### Assessment of follicle viability

Follicle viability was evaluated by Calcein-AM (Sigma) staining using a protocol published previously[13]. Calcein-AM is a non-fluorescent cell-permeable compound and is converted into fluorescent calcein only in live cells by intracellular esterase in the lysosomes, producing fluorescent green staining[13,26]. All viable follicles were observed and counted using fluorescence microscopy (excitation/emission ~495/515 nm).

### Analysis of apoptosis

When apoptosis or DNA fragmentation occurs, free 3′-hydroxyl termini on DNA were exposed via cleavage of chromatin into small fragments. The exposed termini could be labeled by terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, thereby enabling visualization of nuclei containing fragmented DNA[27].

To determine the DNA fragmentation, one biopsy from each group was fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin, and sliced into sections 5 μm thick using the TUNEL (Roche) assay. The slides were incubated with protease K solution at 37 °C for 25 min, washed with phosphate-buffered saline, and then incubated at 37 °C for 2 h with the TUNEL reaction mixture containing enzyme solution (terminal deoxyribonucleotidyl transferase) and dUTP at a ratio of 1:9 inside a humidified chamber. The slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI; Servicebio, China) and observed under a fluorescence microscope (Leica, Germany).

Green fluorescence was visible in apoptotic stroma cells, with excitation wavelengths ranging from 465 to 495 nm, and emission wavelengths ranging from 515 to 555 nm for fluorescein isothiocyanate-conjugated TUNEL. DAPI reached excitation from 330 to 380 nm, emitting blue fluorescence in all nuclei at ~420 nm. The total number of nuclei (DAPI+, blue stain) and the number of nuclei with fragmented DNA (TUNEL+, green stain) were counted. The percentage of

nuclei with DNA fragmentation was evaluated on three fields at ×400 magnification per section and calculated as the ratio between TUNEL + nuclei and the total number of cells (DAPI+) × 100%.

### Reactive oxygen species levels

The intracellular ROS levels were evaluated by a spectrofluorimetric method using 2′,7′-dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Biopsies in each group were cultured separately in a 24-well plate (Thermo Scientific, China) at 37 °C in humidified air with 5% $CO_2$ after thawing. Each well contained 1 ml of Minimum Essential Medium Alpha (Gibco) and 10% fetal bovine serum (Gibco). The cortex biopsies were cultured for 4 days and then were homogenized on ice in 50 mM Tris–HCl (Sigma) at pH 7.5. The supernatant was separated by centrifuging at 3000 × *g* for 10 min at 4 °C and mixed with DCHF-DA (0.5mM) in a 96-well plate for 30 min at room temperature in the dark. The DCHF-DA was oxidized to fluorescent dichlorofluorescein by intracellular oxidative species. Dichlorofluorescein fluorescence intensity emission was assessed at 520 nm (with 480 nm excitation) using a spectrofluorimeter[28], and the final intensity was normalized by protein concentration. All experiments were performed within three independent experiments.

### Total antioxidant capacity assay

The total antioxidant capacity (TAC) assay was conducted based on the Trolox equivalent antioxidant capacity method described by Re at al.[17,29]. In brief, this assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable 2,2-azinobis-3-ethylbenzthiazoline-6-sulphonic acid diammonium radical within 3 min. The Trolox equivalent antioxidant capacity of antioxidant was calculated using Trolox solution (5–20 μM Trolox; Sigma) as the standard curve[30]. For each sample, TAC levels were repeated within at least three independent experiments.

### Statistical analysis

All data were performed using Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, IL, USA). Normality of distribution was assessed with the one-sample Kolmogorov–Smirnov test. Viable follicle counting among groups was compared with the Kruskal–Wallis test, and Dunn's multiple comparison test for post-hoc comparisons. Apoptotic analysis, ROS levels, and TAC levels were performed with one-way analysis of variance (ANOVA) for continuous variables. Post-hoc tests were used for further comparisons, which showed significant difference by ANOVA. All tests were adopted using a two-sided method. $p < 0.05$ was considered statistically significant.

## Results

### Follicle viability

After thawing with NAC, the follicle viability in each group was as presented in Figure 1(a). The viable follicle count in each group showed an abnormal distribution. There was no significant difference between Group 1 (control group) (median 24, IQR 5–31.5), Group 2 (median 12, IQR 9.5–30.5), Group 3 (median 16, IQR 11–23), and Group 4 (median 19, IQR 10.5–49.5). However, compared with other groups, Group 5 (median 0, IQR 0–1) showed minimum follicle viability ($p < 0.05$). Group 5 (i.e. the group with the highest NAC concentration, 25 mM) had almost no live follicles or stroma cells (Figure 1(j, k)).

### Apoptosis

The proportion of apoptotic stroma cells is shown in Figure 2(p). No significant difference was observed among the five groups by ANOVA ($p = 0.141$). In the post-hoc comparisons the proportion of apoptotic stroma cells in Group 2 was significantly higher than that in Group 5 (6.24 ± 6.51% vs. 1.67 ± 3.40%, $p = 0.01$). There was no significant difference among other groups (Group 1 vs. Group 3 vs. Group 4, 4.09 ± 4.58% vs. 4.02 ± 3.56% vs. 3.59 ± 2.87%, $p > 0.05$). As shown in Figure 2(a–o), TUNEL + cells were found in the stroma, while follicles were TUNEL negative (all groups).

### Reactive oxygen species and total antioxidant capacity levels

As shown in Figure 3(a), compared with Group 1 (control group without NAC) both Group 4 (4725.69 ± 1939.81 vs. 2676.06 ± 1001.52, $p = 0.001$) and Group 5 (4725.69 ± 1939.81 vs. 3272.36 ± 1500.10, $p = 0.016$) showed lower ROS levels with significant difference; especially, Group 4 showed the lowest ROS level among all of the groups. No significant difference was observed between Group 1 vs. Group 2 and Group 3.

Comparisons of TAC among the five groups performed with one-way ANOVA did not show significant difference ($p = 0.073$) (Figure 3(b)). However, the TAC level in Group 4 (0.18 ± 0.06) was higher than that in Group 1 (0.13 ± 0.04), Group 3 (0.14 ± 0.03), and Group 5 (0.14 ± 0.03), respectively ($p < 0.05$).

## Discussion

In this study, we compared follicle viability, apoptosis, ROS production, and total antioxidant capability in four groups with increasing concentrations of NAC vs. a control group without NAC. All groups except Group 5 (highest NAC concentration) showed good viability both in follicles and stroma cells. Addition of NAC resulted in anti-apoptotic capability that was higher compared to an absence of NAC. Because of the lowest ROS and highest TAC levels, we assess Group 4 as the group with best results.

### Follicle viability and apoptosis

Interestingly, Group 5 with a very high concentration of NAC (25 mM NAC) had the least apoptotic stroma cells (Figure 2) while almost all cells and follicles were not visible with

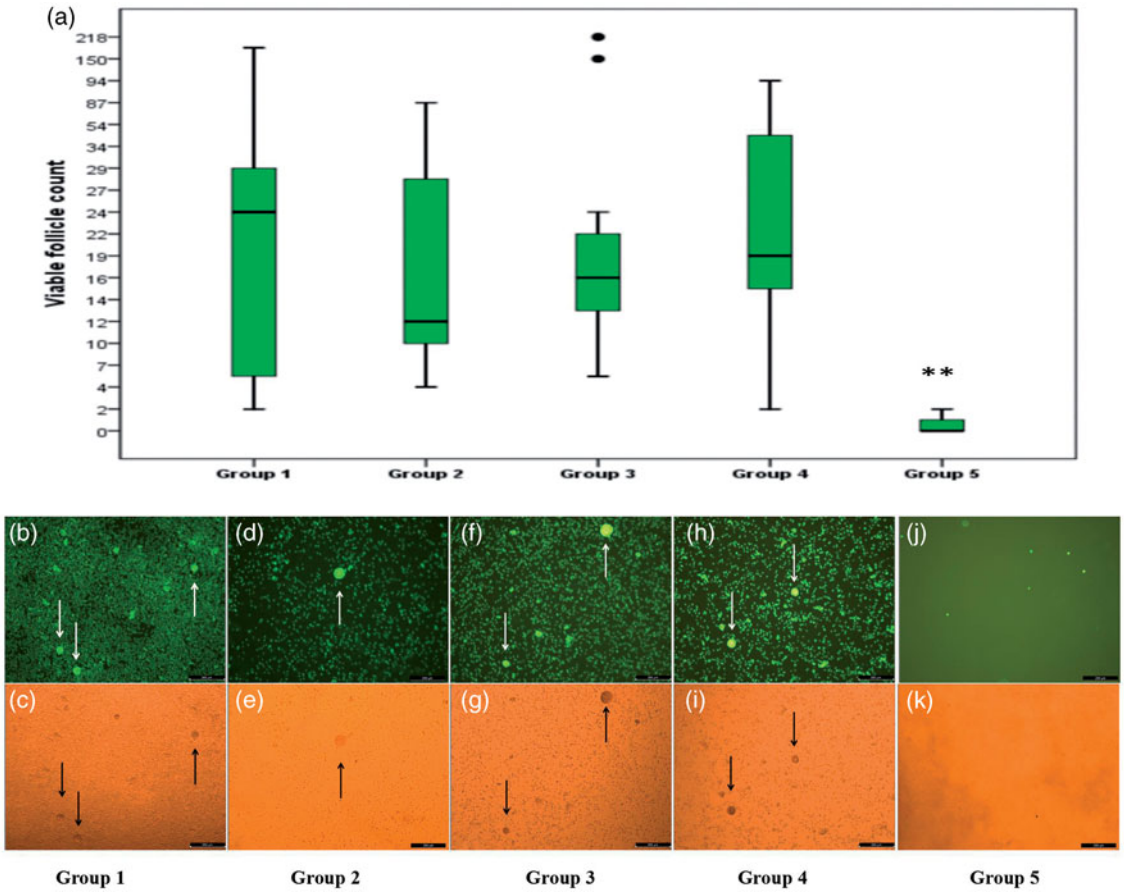


Figure 1. Viable follicle count and representative images of live follicles with calcein-AM staining in each group. (a) Box-plots showing the viable follicle count in each group by median and interquartile range (spot = outlier). **Viable follicle count in Group 5 significantly lower compared to the other four groups ($p < 0.05$). No statistical difference between Groups 1, 2, 3, and 4 ($p > 0.05$). (b–k) Pictures in the same column are representative images of the same sample but under different microscopy. Upper pictures show follicles and stroma cells from Group 1 to Group 5 with Calcein-AM staining under fluorescence microscopy (b, d, f, h, j). White arrows show viable follicles. Lower pictures show follicles and stroma cells under light microscopy (c, e, g, i, k). Black arrows show viable follicles. Scale bar = 200 μm. Magnification 100×.

calcein-AM staining under fluorescence microscopy (Figure 1). Only live cells have various esterases, which could cleave the non-fluorescent calcein-AM to calcein, emitting fluorescent green[31]. We suggest that the addition of 25 mM NAC to our cryomedium did not maintain tissue viability despite preservation of morphology.

### *Reactive oxygen species and total antioxidant capacity*

ROS levels decreased with increase of NAC up to a concentration of 5 mM. Although the 5 nM and 25 mM groups had significantly less ROS production than the control group, 25 mM was not as effective as 5 mM for inhibiting ROS. Group 4 with 5 mM achieved the highest level of TAC among the five groups, suggesting that our modified protocol showed better protective effect in reducing ROS production and antioxidative capability.

### *Role of antioxidants in fertility preservation*

Physiologically a balance exists between prooxidants (free radical species) and scavenging systems (antioxidants). The major forms of ROS include superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (•OH), and hydrogen peroxide ($H_2O_2$). If antioxidants fail to counteract, ROS can damage various substances including lipids, enzymes, and proteins, leading to dysfunction of physiological activities[15,32].

However, ROS, in adequate concentrations, can also serve as key mediators in physiological processes including hormone signaling, follicular development, oocyte maturation, ovulation, corpus luteum function, luteolysis, and follicular atresia[33]. This might explain why, although the concentration of NAC in Group 5 was five-fold compared to Group 4, the antioxidant capability and reduction of ROS was better in Group 4.

Many studies have demonstrated the protective efficacy of antioxidants used in human fertility preservation, including sperm[34], oocyte[35], and embryo[36] cryopreservation, as well as OT vitrification[37] or controlled-rate cryopreservation[17]. The most commonly used antioxidants are based on catalase, l-ascorbic (vitamin C), L-carnitine, alpha-lipoic acid, and tocopherol[38].

For controlled-rate cryopreservation it was shown that the ovarian integrity and functionality were well preserved with the supplementation of L-glutamine and taurine in the freezing medium[17]. Another study showed that OT frozen–thawed with vitamin E-analog Trolox could result in higher rates of viable follicles and upregulation in stress markers[39].

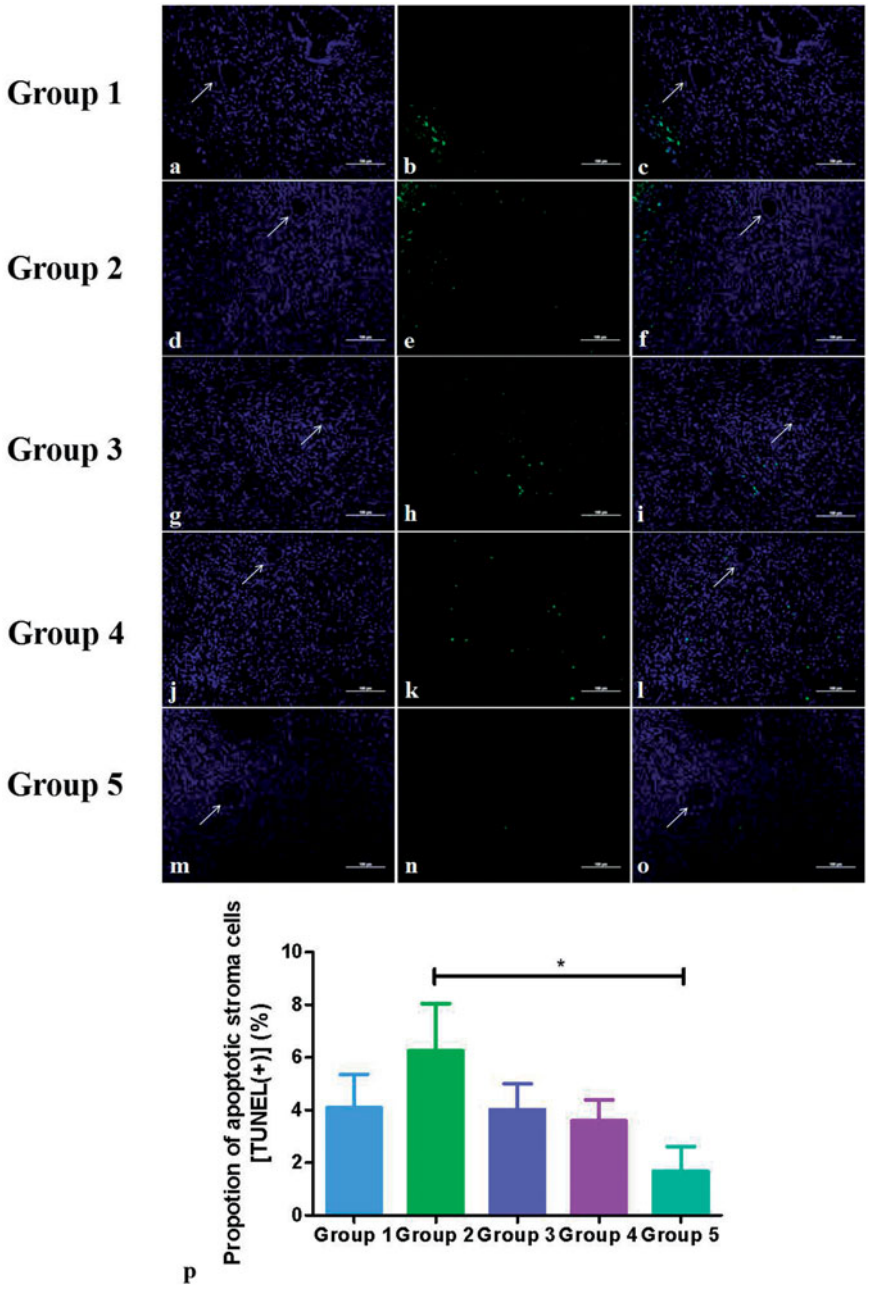


Figure 2. Representative images of stroma cells and follicles with DAPI and TUNEL staining. (a–o) Pictures in the same row are of the same samples under the same fields of microscope, but stained with different methods. Pictures on the left side show that all cell nuclei stained positive with DAPI (blue stain); apoptotic cell nuclei presenting green fluorescence with TUNEL are displayed in the middle panel. For each row, pictures on the right side are merged from the left picture and middle picture. The follicles were not visible with TUNEL stain, which means follicles were not apoptotic in all groups. White arrows show follicles. Scale bar = 100 μm. Magnification 200×. (p) Group 2 has a significantly higher proportion of apoptotic stroma cells than Group 5 ($p < 0.05$). There was no significant difference between the other groups. DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling.

### *Controversial results with NAC as antioxidant*

In the presence of NAC, glutathione synthesis is improved, resulting in increased fertilization rates of mouse oocytes[20,40]. NAC administration resulted in higher estradiol levels and increase of antral follicles[41].

The Italian study by Fabbri et al.[19] used a protocol based on PROH and 25 mM NAC. They found better preservation of morphological characteristics, a better proliferation index, a slight increase of apoptosis, and a slight decrease of ROS levels, the latter two not significant. To optimize our freezing–thawing protocol, we used the same concentration of NAC. We obtained similar results in terms of morphological characteristics and apoptosis, but a stronger and significant decrease of ROS. Surprisingly, we found that all follicles and stroma cells were non-viable after calcein-AM staining. Therefore, we decided to seek an appropriate concentration of NAC, getting the controversial result that 5 mM is the optimal concentration. In contrast to the Italian study[19], we also assessed the follicle viability and stroma cells, showing that 5 mM NAC maintains viable follicles and stroma cells.

The explanation for these controversial results may be the different cryoprotectant – PROH in the Italian study[19] versus DMSO in our study. Another explanation may be the

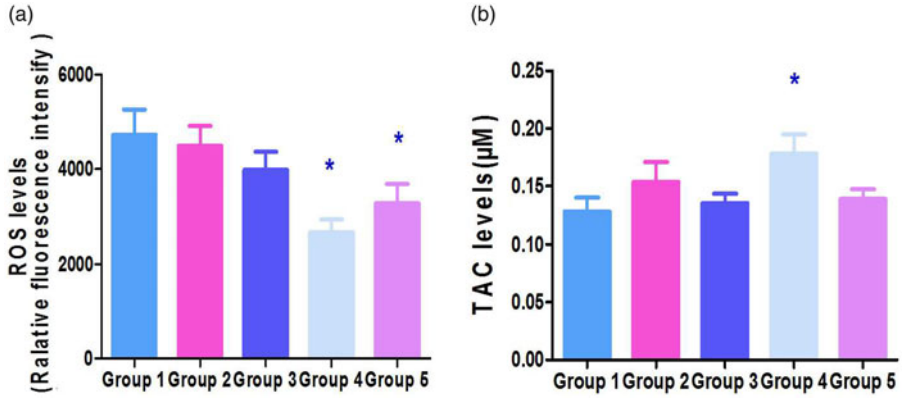


Figure 3. ROS levels and TAC levels in each group. (a) Group 4 had lower ROS levels than Groups 1, 2, and 3 ($p < 0.05$). ROS levels in Group 5 were lower than in Groups 1 and 2 ($p < 0.05$). (b) Group 4 obtained higher TAC levels than Groups 1, 3, and 5. No significant difference was observed between Groups 2 and 4 ($p = 0.191$). *$p < 0.05$ vs. Group 1 (control group). ROS, reactive oxygen species; TAC, total antioxidant capacity.

difference in the control group – they used fresh tissue, we used frozen–thawed tissue without NAC as the control group.

### *Limitations*

The limitation of our study is that no concentration of NAC between 5 and 25 mM was investigated. Thus, we do not know whether the optimal concentration would be within this range. Due to ethical constraints, only less than 10% of the OT could be used for research. Hence, we could not obtain enough tissue to increase the number of study groups including more concentrations. Further studies are needed to assess whether other concentrations of NAC or any other antioxidant may achieve stronger protective efficacy. Finally, only the clinical practice, namely successful transplantations, could conclude which is the best protocol for ovarian cryopreservation.

## Summary and conclusion

Fertility protection using OT preservation has been established recently in the Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing – the first official center in China. After this technique has been assessed internationally just recently as an effective routine method for fertility protection, other centers will be established in the near future, and it seems important to describe in detail the decisive steps, which are the controlled-rate cryopreservation and thawing of OT. The aim of this study was to test the potential benefit of adding NAC at various concentrations to the currently used cryopreservation and thawing solutions. With respect to the four main endpoints of this study – follicle viability, apoptosis, ROS, and TAC – we consider 5 mM NAC to be the best concentration in our cryomedium. Our results need to be verified with a higher sample size and using other antioxidants.

*Conflict of interest* The authors disclose no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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