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Effects of N-acetylcysteine on human ovarian tissue preservation undergoing cryopreservation procedure

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Summary. The aim of the study was to evaluate the effects of the antioxidant N-acetylcysteine (NAC), added in freezing/thawing solutions, on reactive oxygen species (RRS) levels and on ovarian tissue preservation after cryopreservation. Ovarian samples from 10 subjects suffering from cancer diseases were cryopreserved using the slow freezing/rapid thawing standard protocol without or with NAC supplementation. RRS levels produced during cryopreservation were monitored by electron paramagnetic resonance (EPR) spectroscopy. The preservation of fresh ovarian tissue (t0), thawed tissue (t1 and t1 NAC) and thawed tissue maintained at 4°C for 2 hrs (t2 and t2 NAC) was analysed by light microscopy, transmission electron microscopy, Ki67 immunohistochemical and TUNEL analysis. It was possible to design a maximum peak for RRS production at t1, which slightly decreased at t2. NAC reduced the extent of RRS levels in cryopreserved ovarian tissues if compared with non-supplemented ones, although not restoring RRS production to baseline values. Comparative analysis between the two cryopreservation protocols showed that a better preservation of morphological characteristics, proliferation index and DNA integrity of ovarian tissue was obtained using NAC and no differences between t1NAC and t2NAC were observed. The employment of NAC during cryopreservation procedure could be an useful strategy for preserving the function of endogenous cellular systems. Nevertheless, further studies on the viability of thawed ovarian tissue are needed to support the feasibility of this approach in clinical settings.

Key words: Cryopreservation of human ovarian tissue, Reactive radical species, N-acetylcysteine, Electron paramagnetic resonance spectroscopy, Light microscopy, Transmission electron microscopy, Ki67 immunohistochemical, Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL).

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Introduction

Recent advances in the diagnosis and the introduction of new chemo/radiotherapy protocols have significantly increased the life expectancy of children, adolescent and young women with cancer diseases. However, these treatments are gonadotoxic and can jeopardize or totally destroy ovarian function with consequent premature ovarian failure (Jeong et al., 2012). Premature menopause induces cessation of ovarian sex hormone production with some serious side effects, such as osteoporosis, cardiovascular diseases and psychosexual dysfunctions (Duffy and Allen, 2009). The cryopreservation of ovarian tissue before starting chemo/radiotherapy represents a promising strategy for preserving the reproductive potential of these women and it is the only option for patients suffering from hormone sensitive tumours and for children unable to

undergo alternative treatments (Donnez and Dolmans, 2010).

The cryopreservation procedure causes a reduction in the follicular pool and sub-optimal preservation of stromal cells (Fabbri et al., 2010; Chang et al., 2011). From collection to transplantation, ovarian tissue is subjected to ischemia, hypoxia and toxicity due to cryoprotectants, low temperature and freezing/thawing procedure which cause excessive production of reactive radical species (RRS) (Ahn et al., 2002; Pegg, 2007; Fahy, 2010). This excess can induce DNA fragmentation resulting in apoptosis, lipid peroxidation ending in damaged cell membranes and alterations of structural proteins causing functional cell injury (Rizzo et al., 2012). Alterations of physical and biochemical cellular characteristics developed by oxidative stress involve the antioxidant defence systems (Rahimi et al., 2003). In physiological conditions the cell is protected from excessive RRS levels by endogenous enzymatic (superoxide dismutase, catalase, glutathione peroxidase and reductase) and non-enzymatic (vitamin C, vitamin E, beta-carotene, selenium, zinc, taurine and glutathione) antioxidants (Winyard et al., 2005).

As during the freezing/thawing procedures the endogenous protection system fails, it is reasonable to suppose that the use of exogenous antioxidants during cryopreservation procedure could constitute a helpful strategy to protect the function of endogenous cellular systems.

Indeed, the use of exogenous antioxidant agents seems to improve the preservation and viability of human ovarian tissue after slow freezing (Melo et al., 2011; Sanfilippo et al., 2013; Brito et al., 2014), as well as the viability of frozen-thawed ovarian tissue after avascular transplantation, so preventing the oxidative stress caused by ischemia injury (Demeestere et al., 2009; Friedman et al., 2012).

Among antioxidant agents, N-acetylcysteine (NAC) is widely used in the human field, in-vitro as well as in-vivo studies, for the prevention of RRS damage produced in different diseases such as cancer, cardiovascular diseases, human immunodeficiency virus (HIV) infections, acetaminophen-induced liver toxicity and in neurological disorders (Kelly, 1998; Bavarsad Shahripour et al., 2014).

NAC is a thiol-containing compound that acts as a precursor in the formation of the anti-oxidant gluthatione. It also stimulates the intracellular synthesis of glutathione and glutathione-S-transferase activity. Moreover, the thiol group in NAC has the ability to reduce the free radicals (Saddadi et al., 2014).

Our previous study investigated the effects of NAC on long-term cultures of thawed ovarian tissue, finding that it improved ovarian tissue quality and reduced the activation of the apoptotic pathway after 32 weeks of culture (Fabbri et al., 2007).

On the basis of this evidence, the present study was designed to evaluate the effects of NAC, added to standard cryopreservation solutions, on oxidative stress status and on ovarian tissue preservation.

For the first time, electron paramagnetic resonance (EPR) spectroscopy coupled with an innovative spin-trapping technique (radical probe) was used to estimate RRS levels.

Light microscopy (LM), Transmission electron microscopy (TEM), Ki67 immunohistochemical and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) analysis were employed to assess ovarian tissue preservation.

Materials and methods

Ovarian tissue collection

Ovarian tissues were obtained from 10 informed and consenting patients, aged 17-25 years (mean age±SD: 26.2 ± 6.5), affected by Hodgkin lymphoma (five patients), breast cancer (four) and Ewing sarcoma (one). All subjects were referred to the Gynecology and Pathophysiology of Human Reproductive Unit, S. Orsola-Malpighi Hospital, Bologna - Italy, for cryopreservation of ovarian tissue before chemotherapy and radiotherapy. The cryopreservation protocol was approved by the Ethics Committee of S. Orsola-Malpighi Hospital (74/2001/O). Ovarian tissue was laparoscopically collected from right and left ovary. For each patient, three pieces (3 mm^2) from each ovarian biopsy were processed for EPR, LM, TEM, Ki67 immunohistochemical and TUNEL analysis (fresh tissue, t0). The remaining tissue was cryopreserved using standard protocol without or with NAC supplementation. After thawing, three pieces for each protocol (t1 and t1 NAC) were processed for the same analysis performed at t0, and other three pieces were maintained at 4°C for 2 hrs in Alpha Minimum Essential Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with antibiotics and 10% of human serum - HS (provided by the Transfusion Centre of S. Orsola-Malpighi Hospital). At the end of this period (t2 and t2 NAC) the samples were processed for the same analysis performed at t0 and t1.

Cryopreservation procedure

All reagents were obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA).

- Standard protocol: samples were cryopreserved using a modified slow freezing/rapid thawing protocol (Fabbri et al., 2010). In brief, ovarian biopsies immersed in Dulbecco's phosphate buffered saline – PBS (Gibco Life Technologies, Paisley, Scotland) added with 10% of HS, were cleaned of medulla and the ovarian cortex was sliced into pieces 1-2 mm thick and 10-20 mm long. Each slice was placed in a cryovial (Intermed Nunc Cryotubes, Denmark) containing the freezing solution (1,26 mol/l 1,2-propanediol – PROH - added with 0,175 mol/l sucrose and 30% HS in PBS), and transferred to a rolling system at 4°C for 60 minutes to allow the

penetration of the cryoprotectant. The cryovials were transferred into a programmable freezer allowing the gradual reduction of temperature from 0 to -9°C at a rate of 2°C/min. At this temperature the formation of the first nucleus of ice (seeding) was manually induced after 10 minutes of stabilization. Subsequently the samples were slowly cooled to -40°C at a rate of -0.3°C/min and then rapidly cooled to -140°C at a rate of 10°C/min. After 10 minutes of temperature stabilization the cryovials were stored in liquid nitrogen for three months. To thaw, the cryovials were air-warmed for 30s and then immersed into 37°C water bath for 2 min. The cryoprotectants were removed at 4°C by stepwise dilution (solution 1: 0,76 mol/l PROH + 0,175 mol/l sucrose with 30% HS in PBS; solution 2: 0,26 mol/l PROH + 0,175 mol/l sucrose with 30% HS in PBS; solution 3: 0,175 mol/l sucrose with 30% HS in PBS). Finally, the tissue pieces were maintained for 20 min in PBS supplemented with 30% HS at 4°C.

- NAC protocol: the samples were cryopreserved using the standard protocol with the addition of 25mmol/l NAC in freezing and thawing solutions.

EPR radical probe measurements

For EPR analysis we used an innovative hydroxylamine 'radical probe' bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate, IAC, capable of efficiently trapping the majority of oxygen-, carbon- and nitrogen-centered radicals, including superoxide and peroxynitrite (Valgimigli et al., 2001). This reaction yields a very persistent nitroxide that can be readily detected and accurately quantified by EPR spectroscopy (Valgimigli et al., 2000, 2001, 2002; Paolini et al., 2003). For each patient, fresh (t0), frozen-thawed (t1 and t1 NAC) and frozen-thawed maintained at 4°C for 2hrs (t2 and t2 NAC) samples were processed for EPR analysis.

Samples were prepared by treating 0.2 g of tissue with 0.5 ml of standard physiological solution containing the hydroxylamine probe (1 mM) and deferoxamine (DFO) (1 mM) as metal chelating agent. After 5 min incubation at 37°C, each sample was snap frozen in liquid nitrogen to stop any reaction and stored at –80°C until EPR measurement was performed. The optimal incubation time and the most appropriate experimental conditions were determined in previous investigations (Valgimigli et al., 2000, 2001, 2002; Paolini et al., 2003). Immediately before measurement, the sample was warmed to room temperature, and about 50 µl of the solution was transferred and sealed in a calibrated capillary glass tube, which was placed inside the cavity of a Bruker ESP 300 EPR spectrometer (Bruker Biospin Srl, Rheinstetten, Germany) equipped with a nuclear magnetic resonance gaussmeter for field calibration, a Bruker ER 033M FF-lock (Bruker Biospin Srl) and a Hewlett-Packard 5350B microwave frequency counter (Hewlett Packard, Houston, TX, USA). The actual amount of solution analyzed was chosen so as to cover the entire sensitive area of the instrument cavity. The spectra of the nitroxide radical, generated by the reaction of the probe with the radicals produced in the tissue, were then recorded using the following instrumental settings: modulation amplitude=1·0 G; conversion time=163·84 ms; time constant=163·84 ms; modulation frequency 100 kHz; microwave power=6.4 mW. The intensity of the first spectral line of the nitroxide (aN=16.60 G; g=2·0056) was used to obtain the absolute amount of nitroxide per ml of sample, after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an ER 4119HS Bruker Marker Accessory as internal standard (Fig. 1). For simplicity, results were expressed as µmol of RRS in g of ovarian tissue.

In order to evaluate the extent of oxidation of the hydroxylamine by atmospheric oxygen under our experimental conditions, reference samples containing only the hydroxylamine and DFO in physiological solution were prepared for each sample and treated in the same way. The amount of nitroxide detected in the reference solution was subtracted from the amount recorded in the actual samples to eliminate results from the spontaneous autoxidation of the probe.

The hydroxylamine probe was bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate dihydrochloride (CAS no. 314726-62-0), which was prepared as previously described (Valgimigli et al, 2000, 2001). All other compounds were commercially available (Sigma-Aldrich Co., Milan, Italy).

Light and transmission electron microscopy analysis

For each patient, fresh (t0), frozen-thawed (t1 and t1 NAC) and frozen-thawed maintained at 4°C for 2hrs (t2 and t2 NAC) samples were fixed in a 4% paraformaldehyde solution in 0.1 mol/l sodium cacodylate buffer (pH 7.4) overnight at 4°C. After 1% osmium tetroxide post-fixation and dehydration through a graded series of alcohol, the samples were embedded in Araldite epoxy resin (Fluka, Buchs, Switzerland) and then sectioned with an ultramicrotome (Ultracut; Reichert, Vienna, Austria). For each sample, one 0.5 µm thick section (semithin section) out of every 50 was collected and stained with toluidine blue for LM examination.

According to Gougeon criteria (1996), follicles were classified as resting (primordial, intermediary and small primary) and growing (primary, secondary, preantral and antral). Follicular density (total follicle number per mm^2 of the overall section area), follicle preservation and stromal integrity degree was evaluated (Fabbri et al., 2010). Follicle and stromal interstitial injuries were analysed on at least 10 random microscopic high power fields taken at x400 magnification and scored using the method previously described in Fabbri et al. (2010). Semithin sections (0.5 µm thick) were observed in a blind fashion by two different operators in a Leitz Diaplan light microscope equipped with CCD JVC video camera and digitalized images were analysed with Image ProPlus software (MediaCybernetics).

Sixty-nm thick sections were collected on 200 mesh

grids, stained with uranyl acetate followed by lead citrate, and viewed using a Philips 410 T transmission electron microscope at 80 kV, in order to evaluate the ultrastructural features of oocytes, granulosa and stromal cells before and after freezing/thawing, according to Fabbri et al., (2010) criteria.

Ki67 Immunohistochemical and TUNEL analysis

For each patient fresh (t0), frozen-thawed (t1 and t1 NAC) and frozen-thawed maintained at 4°C for 2 hrs (t2 and t2 NAC) samples were processed using routine histologic techniques. Four-µm thick sections were stained with hematoxylin and eosin (Merck, Darmstadt, Germany), immunostained with Ki67 monoclonal antibody for proliferation assessment (Ventana, Roche, USA), and processed with TUNEL assay for apoptosis evaluation (POD, Roche, Germany).

Ki67 Immunohistochemical analysis was performed according to Vasuri et al. (2012) and evaluated according to Fabbri et al. (2003). TUNEL assay was performed according to the manufacturer's instructions and evaluated according to Fabbri et al. (2014). ki67 and TUNEL stained sections were observed and analyzed in a double blind fashion using a Leitz Diaplan light microscope equipped with a CCD JVC video camera. Digitized images were analyzed with Image ProPlus software (MediaCybernetics).

Statistical analysis

For each of the two variables (follicle damage and stromal interstitial oedema) at least 10 fields were scored on an integer scale of 0-3. Differences in the mean score among the slow freezing/rapid thawing protocols were analyzed statistically using Kruskal Wallis test followed by Dunn multiple comparison test (GraphPad Prism version 4). The Student's t-test was used to analyze the percentage of Ki67 and TUNEL stained cells observed in fresh, frozen-thawed and frozen-thawed maintained at 4°C samples.

Since the distribution of RRS could not be approximated by a Gaussian curve, non-parametrical tests were used for statistical analysis. For comparison of

Table 1. Follicle distribution and follicular density.

Follicular stage	Number of follicles (%)				
	t0 (%)	t1 (%)	t1 NAC (%)	t2 (%)	t2 NAC (%)
Primordial / Small primary	292 (98.32)	237 (98.75)	274 (98.56)	253 (98.83)	245 (98.39)
Primary	4 (1.34)	0 (0)	2 (0.72)	2 (0.78)	4 (1.61)
Secondary	1 (0.34)	2 (0.83)	1 (0.36)	1 (0.39)	0 (0)
Antral	0	1 (0.42)	1 (0.36)	0 (0)	0 (0)
Total follicles	297	240	278	256	249
Follicular density (mean±SD)	3.8±1.7	3.4±1.9	3.1±1.2	4.3±2.1	4.1±2.2

t0: fresh; t1: frozen-thawed w/o NAC; t1 NAC: frozen-thawed w NAC; t2: frozen-thawed w/o NAC maintained at 4°C for 2hrs; t2 NAC: frozen-thawed w NAC maintained at 4°C for 2hrs. Follicular density: total follicle number/mm^2

Table 2. Patient characteristics and morphometric analysis of ovarian tissue damage.

Patient	Pathology	Age	Follicle injury					Stromal interstitial oedema				
			t0	t1	t1NAC	t2	t2NAC	t0	t1	t1NAC	t2	t2NAC
1	HL	27	0	1	0	2	1	1	2	0	2	1
2	HL	19	1	2	1	3	1	0	3	1	3	1
3	HL	20	0	2	1	3	1	1	2	0	3	0
4	HL	26	0	2	1	3	2	0	2	1	2	1
5	HL	22	1	2	1	2	2	1	2	1	3	1
6	BC	34	1	2	1	3	1	1	3	1	3	1
7	BC	35	1	3	1	3	1	0	3	1	3	1
8	BC	33	0	1	1	3	1	1	2	1	3	1
9	BC	29	0	1	1	2	1	0	2	1	2	1
10	ES	17	0	2	1	2	1	1	3	0	3	0

Score for semi-quantitative light microscopy analysis according to Fabbri et al., 2010: 0= 0-25%; 1= 26-50%; 2= 51-75%; 3≥75%. Score 0= best; score 3= worst. t0: fresh; t1: frozen-thawed w/o NAC; t1 NAC: frozen-thawed w NAC; t2: frozen-thawed w/o NAC maintained at 4°C for 2hrs; t2 NAC: frozen-thawed w NAC maintained at 4°C for 2hrs. HL: Hodgkin Lymphoma; BC: Breast Cancer; ES: Ewing Sarcoma. Follicle injury: t1 vs. t0 P<0.001; t1 NAC vs. t0 P=NS; t1 vs. t1 NAC P<0.01; t2 vs. t0 P<0.001; t2 vs. t1 P=NS; t2 NAC vs. t0 P=NS; t2 NAC vs. t1 NAC P=NS; t2 vs. t2 NAC P<0.01. Stromal Interstitial oedema: t1 vs. t0 P<0.001; t1 NAC vs. t0 P=NS; t1 vs. t1 NAC P<0.01; t2 vs. t0 P<0.001; t2 vs. t1 P=NS; t2 NAC vs. t0 P=NS; t2 NAC vs. t1 NAC P=NS; t2 vs. t2 NAC P<0.01.

two groups of individuals, the Wilcoxon Signed Rank test was employed. All results are reported as mean ± SD for the different groups of patients. Statistical significance was set at $P<0.05$.

Results

EPR radical probe measurements

RRS level in ovarian tissue at t0 was 9.72±2.10 μmol/g (Fig. 1A). At t1, RRS measurement of samples cryopreserved using standard protocol resulted 2.5-fold significantly higher with respect to t0 (t1 24.30±7.92 vs. t0 9.72±2.10 μmol/g, $P<0.003$, Fig. 1B). When NAC was added, the values seen in the standard samples were reduced, resulting only 1.5-fold greater than t0 (t1 NAC 14.76±4.40 μmol/g vs. t0 9.72±2.10, $P<0.003$, Fig. 1C).

RRS measurement after 2 hrs at 4°C (t2) was 2-fold higher than t0 (t2 20.46±5.33 vs. t0 9.72±2.10 μmol/g, $P<0.003$, Fig. 1D), and further decreased following NAC

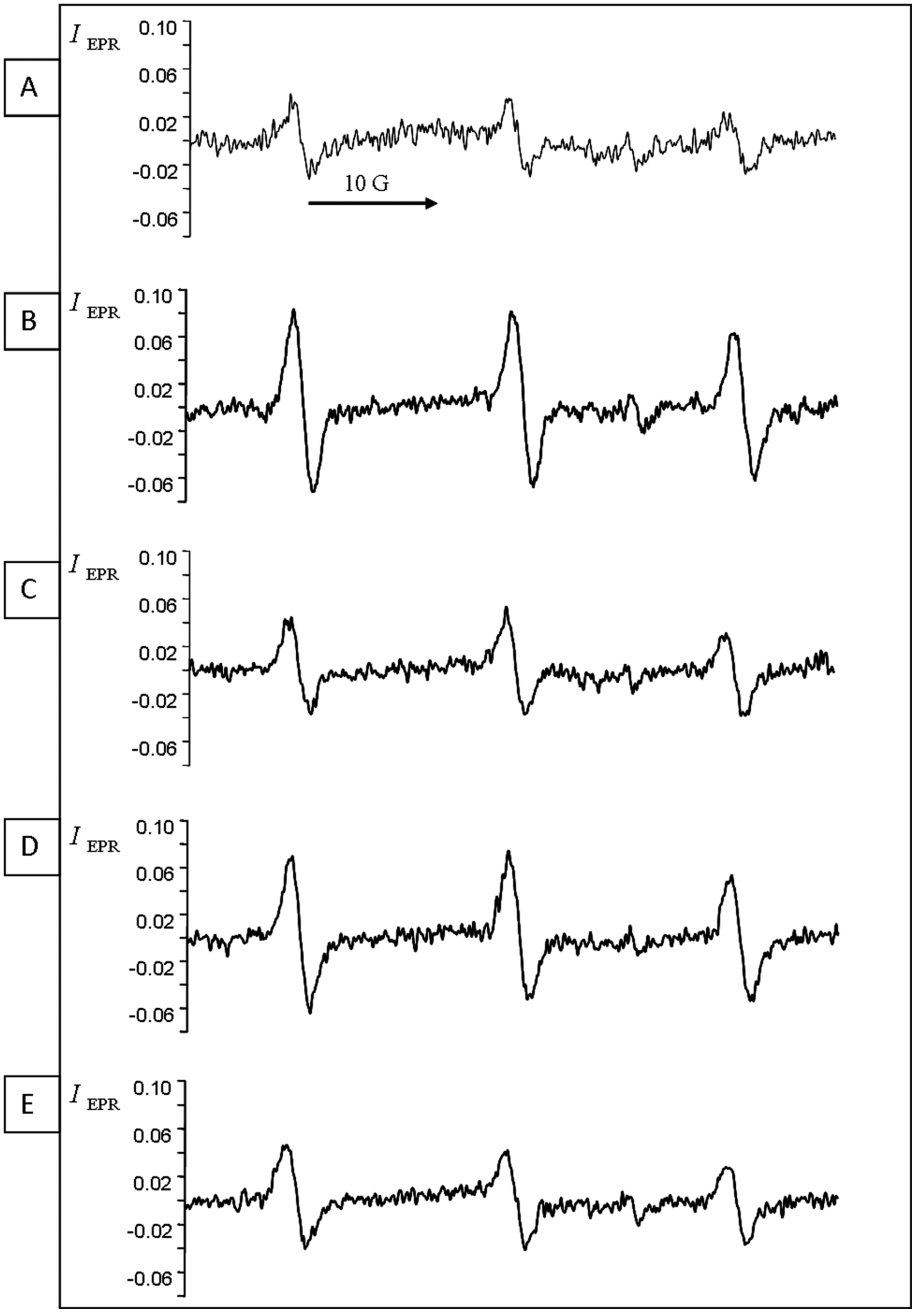

Fig. 1. EPR spectra of nitroxide radicals observed at t0 **(A)**, t1 **(B)**, t1 NAC **(C)**, t2 **(D)** and t2 NAC **(E)**. The intensity of the first spectral line of the nitroxide (aN=16.60 G; g=2.0056) was used to obtain the absolute amount of notroxide per ml of sample, after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an ER4119HS Bruker Marker Accessory as internal standard. For simplicity, results were expressed as μmol of RRS in g of ovarian tissue. t0: fresh; t1: frozen-thawed w/o NAC; t1 NAC: frozen-thawed w NAC; t2: frozen-thawed w/o NAC maintained at 4°C for 2hrs; t2 NAC: frozen-thawed w NAC maintained at 4°C for 2hrs.

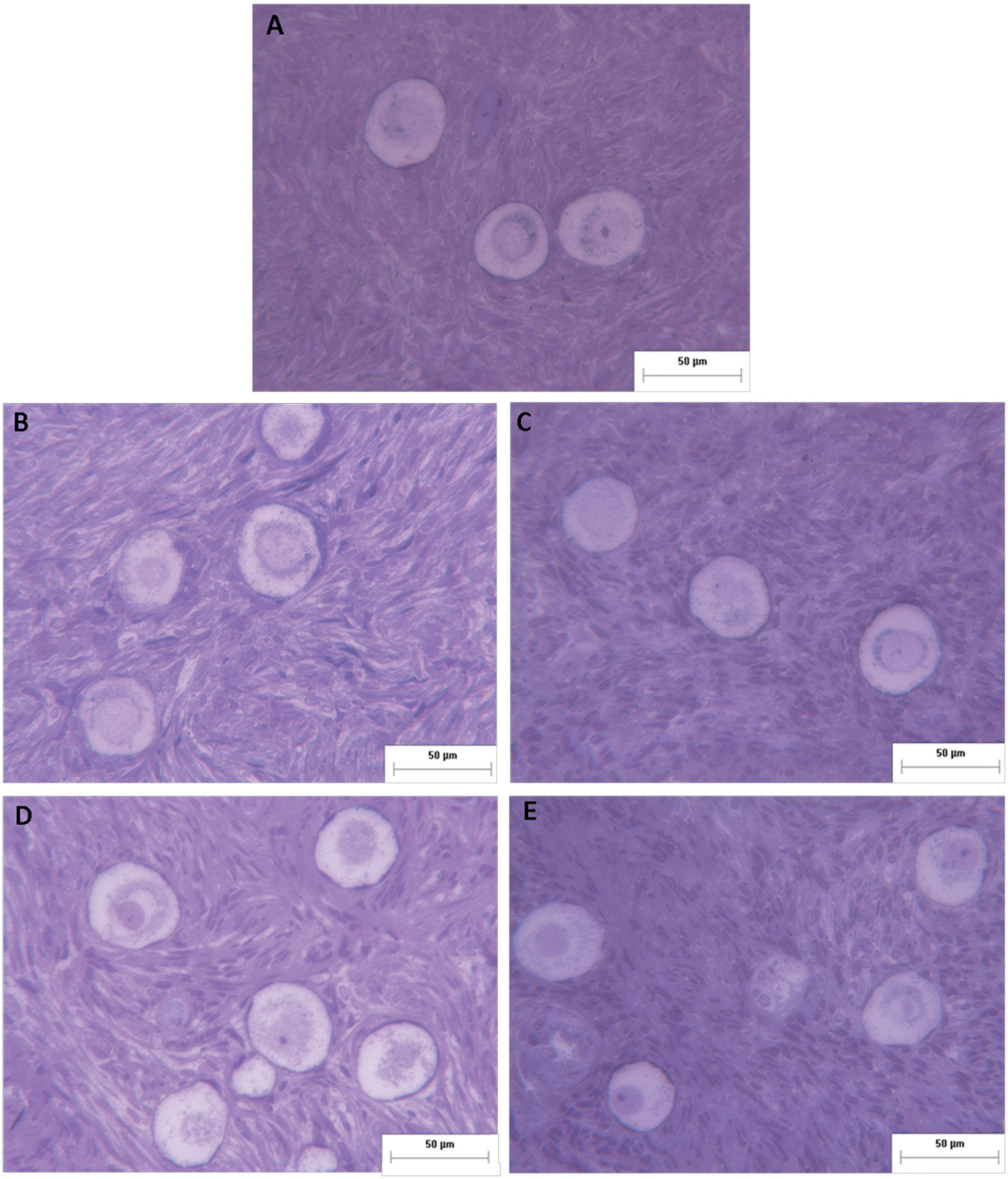

Fig. 2. Light microscopy of ovarian cortex at t0 **(A)**, t1 **(B)**, t1 NAC **(C),** t2 **(D)** and t2 NAC **(E)**. t0: fresh; t1: frozen-thawed w/o NAC; t1 NAC: frozen-thawed w NAC; t2: frozen-thawed w/o NAC maintained at 4°C for 2hrs; t2 NAC: frozen-thawed w NAC maintained at 4°C for 2hrs. Bar: 50 μm.

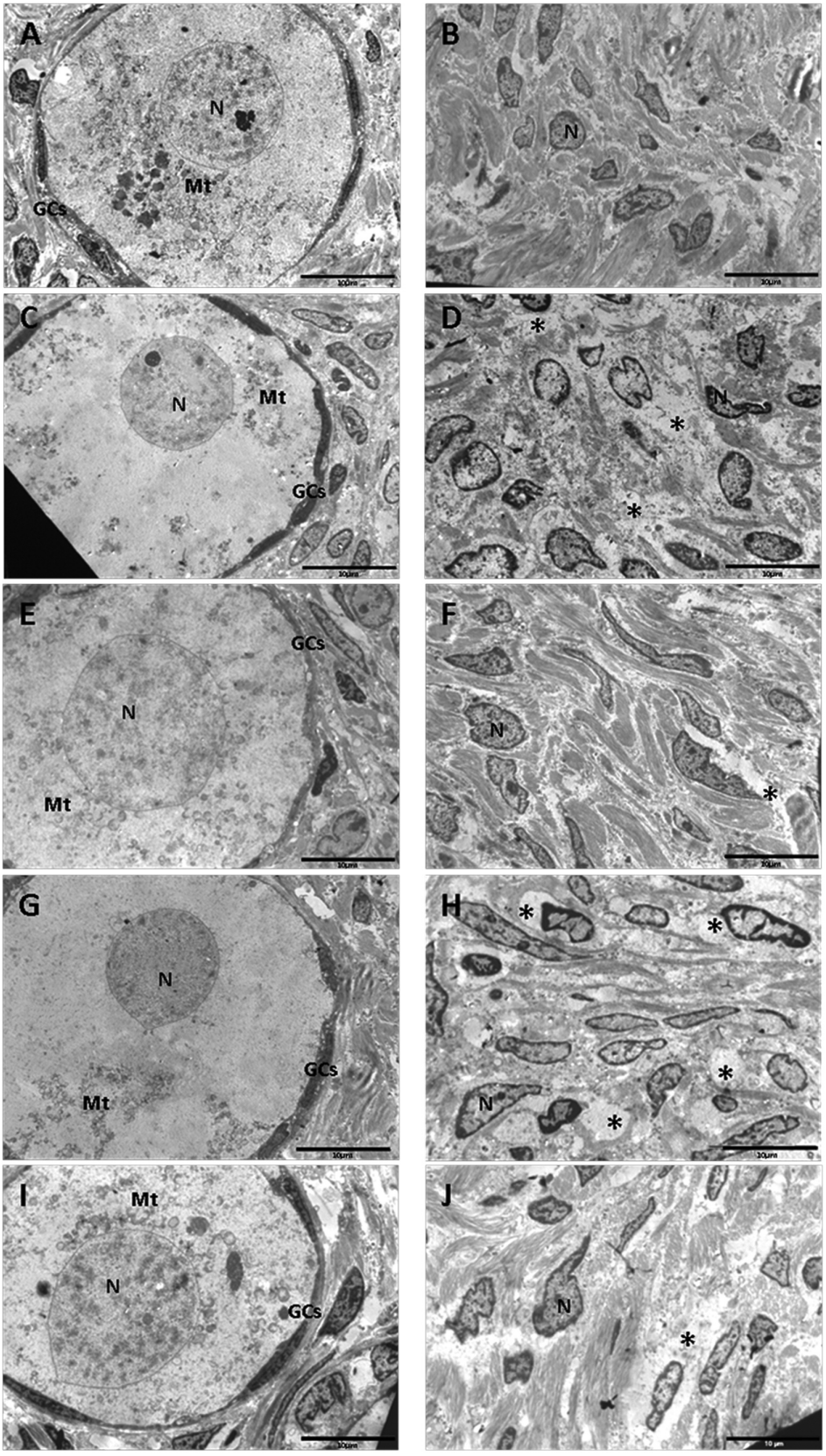

Fig. 3. Transmission electron microscopy of follicles and stroma at t0 **(A, B)**, t1 **(C, D)**, t1 NAC **(E, F)**, t2 **(G, H)** and t2 NAC **(I, J)**. t0: fresh; t1: frozen-thawed w/o NAC; t1 NAC: frozen-thawed w NAC; t2: frozen-thawed w/o NAC maintained at 4°C for 2hrs; t2 NAC: frozen-thawed w NAC maintained at 4°C for 2hrs. **A.** t0 follicle showing a well-preserved oocyte and granulosa cells (GCs). The oocyte has a regularly shaped nucleus (N) and with the membrane intact and mitochondria (Mt) located around the nucleus. **B.** t0 stroma showing stromal cells with dispersed nuclear chromatin without interstitial oedema or vacuolizations. **C.** t1 follicle showing empty oocyte cytoplasm with scattered mitochondria (Mt) and granulosa cells (GCs) with chromatin clumping. **D.** t1 stroma showed interstitial oedema (*) and cells with frequently euchromatin condansation. **E.** t1 NAC oocyte shows nucleus (N) with dispersed chromatin and perinuclear aggregates of mitochondria (Mt). **F.** t1 NAC stroma presents a slight interstitial oedema (*) and cells with less chromatin clumping than samples cryopreserved without NAC. The same ultrastructural characteristics of t1 and t1NAC samples were observed in t2 **(G, H)** and t2NAC **(I, J)** samples, respectively. Bar: 10 μm.

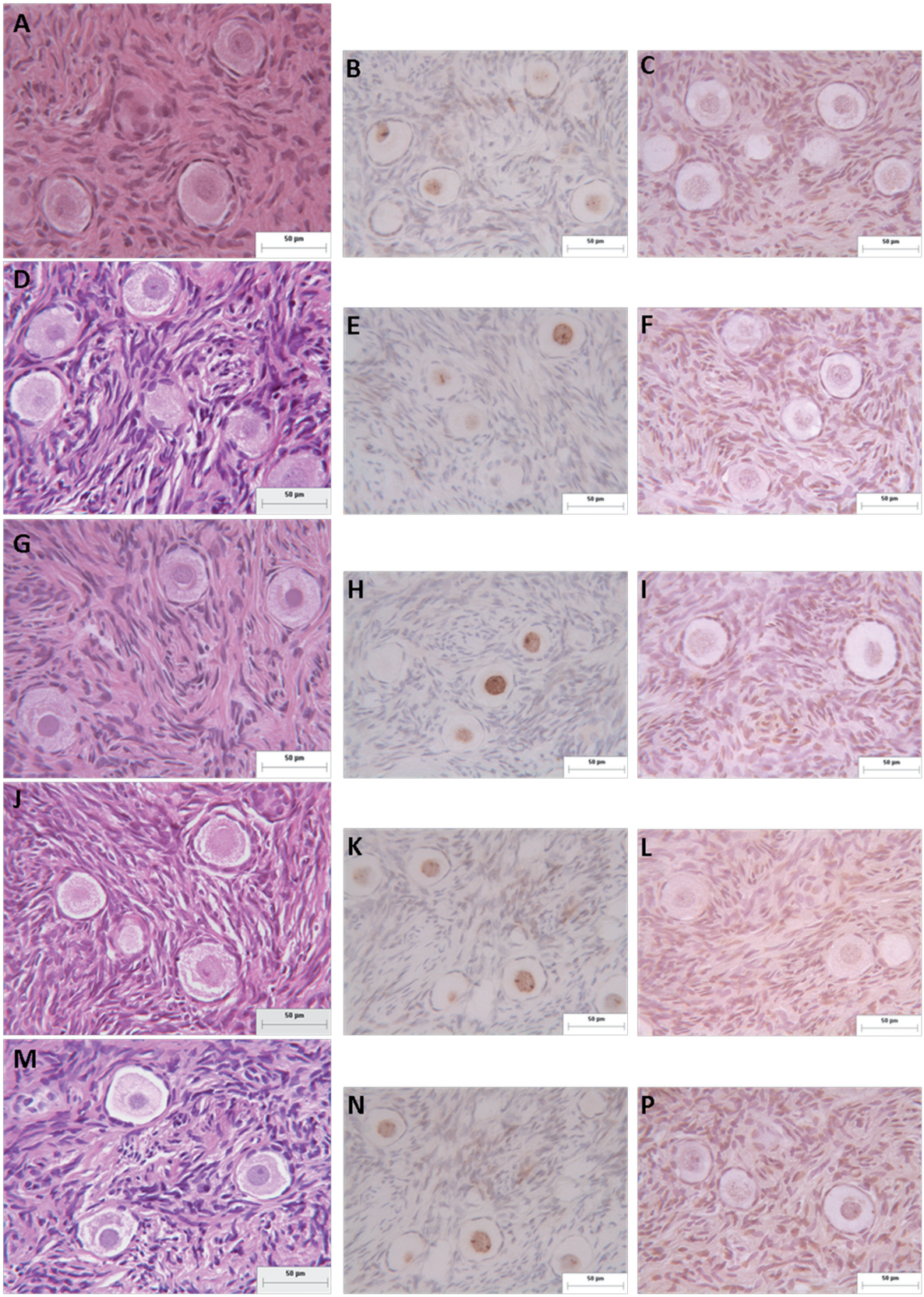

Fig. 4. Histological, Ki67 Immunohistochemical and TUNEL analysis of ovarian tissue at t0 **(A, B, C),** t1 **(D, E, F),** t1 NAC **(G, H, I),** t2 **(J, K, L)** and t2 NAC **(M, N, P)**. Bars: 50 μm.

addiction (t2 NAC, 14.82±2.83 μmol/g, vs. t0 9.72±2.10, P<0.003, Fig. 1E).

These comparisons showed that even though NAC was unable to restore RRS production to baseline values (t0), at both time points (t1 NAC and t2 NAC), it reduced the intensity of oxidative stress in the ovarian tissue, when compared to standard protocol samples (t1 and t2).

Light and Transmission Electron Microscopy Analysis

Out of 1320 follicles examined, the majority was resting both in fresh and frozen-thawed tissues (98.3% vs. 98.7%) and the remaining was growing (1.7% vs. 1.3%); follicular density did not vary among the experimental groups (P=NS; Table 1).

For each patient, the semi-quantitative light microscopy analysis of follicle and stromal interstitial injuries was performed and reported in Table 2.

When compared to fresh samples (t0, Fig. 2A), thawed samples (t1, Fig. 2B) showed oocytes with empty cytoplasm and granulosa cells with an increased chromatin density. The stroma presented interstitial oedema and chromatin clumping was evident in the stromal cells. On the contrary, samples cryopreserved with NAC (t1 NAC, Fig. 2C) showed well preserved oocytes with round euchromatic nuclei and perinuclear mitochondria aggregates. The granulosa cells had a normal morphological appearance. The stromal cells showed nuclei without chromatin clumping; slight interstitial oedema was seen.

After 2 hrs at 4°C, the morphology of samples (t2, Fig. 2D) was comparable to that of t1 samples. Similarly the morphology of samples cryopreserved with NAC (t2 NAC, Fig. 2E) was similar to that of t1 NAC samples. Overall, follicle and stroma features of t1 NAC and t2 NAC samples were comparable to t0 samples.

TEM confirmed the improved preservation of follicles and stroma in samples cryopreserved with NAC (Fig. 3A-J). In particular, follicles at t1 NAC (Fig. 3E) and t2 NAC (Fig. 3I) showed oocytes with perinuclear clustering of mitochondria and a smaller amount of cytoplasm vacuolization; granulosa cells had less chromatin clumping than samples without NAC. A reduced amount of interstitial oedema was also appreciable (Fig. 3F,J).

Immunohistochemical and TUNEL analysis

Histological analysis showed further the presence of stromal oedema in t1 e t2 samples (Fig. 4D,J) that appeared less pronounced in samples with NAC (Fig. 4G,M).

Regarding cellular proliferation, no significant differences were observable in follicles and stroma of all experimental conditions (Fig. 4B,E,H,K,N).

Compared to fresh tissue (Fig. 4C), apoptosis appeared slightly increased after cryopreservation in t1 (Fig. 4F) and t2 (Fig. 4L) experimental conditions, whereas it was equivalent in t1 NAC (Fig. 4I) and t2 NAC (Fig. 4P). In particular, apoptosis was observable in granulosa and stromal cells and the respective percentages resulted 11% and 10% in t0, 15% and 19% in t1, 12% and 15% in t1 NAC, 15% and 18% in t2, 13% and 15% in t2 NAC.

Discussion

It is conceivable that some morpho/functional deficits found in ovarian cryopreserved tissue are a consequence of hypoxia or production of free radicals during sampling and freezing/thawing procedures (Hatami et al., 2014).

The scientific literature is completely devoid of studies on the measurement of RRS in ovarian tissue before and after cryopreservation.

Due to the RRS high reactivity and, consequently, limited life-time, the detection of free radicals in-vivo is still troublesome and techniques able to directly assess RRS in human tissues are still scarcely available.

A method for the measurement of RRS in human tissue was developed by Paolini et al., (1996). This approach is based on the EPR detection of the stable nitroxide radical produced by the reaction of the highly lypophilic hydroxylamine spin-probe bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate, IAC, with RRS generated in human ovarian tissue. The hydroxylamine appears to have the correct lypophylicity to cross the cell membrane and distribute on both intracellular and extracellular compartments, thus scavenging RRS wherever they are generated. This technique has been successfully applied in microsomes, cell cultures, human tissues and animals (Canistro et al., 2010; Mancarella et al., 2008). In the present study, for the first time, the EPR technique has allowed to monitor the presence and to quantify the free radicals to which the ovarian tissue is exposed during the different phases of the standard cryopreservation procedure, filling an important gap, which is essential to optimize the cryopreservation process.

Studies from the literature, in human and animal models, report the beneficial effects of the addition of antioxidant agents in freezing solutions on preservation and viability of cryopreserved ovarian tissue: L-Glutamine and Taurine lead to good preservation of human ovarian integrity and functionality after slow freezing (Sanfilippo et al., 2013); the vitamin E prevents endoplasmic reticulum stress in monkey ovarian tissue after freezing and, consequently, protects tissue from endoplasmic reticulum-derived cytoplasmic vacuolization (Brito et al., 2014); the ascorbic acid increases the percentage of sheep primordial follicles with normal morphology and high viability after vitrification followed by short-term culture (Melo et al., 2011).

On the basis of these results, this study aimed to evaluate the effect of NAC, supplemented to the cryopreservation solutions, on RRS levels, by the EPR

technique, and on morphology, proliferation and apoptotic features of cryopreserved ovarian tissue, by LM, TEM, ki67 immunohistochemical and TUNEL analysis.

Using standard cryopreservation procedure, RRS detection showed that oxidative stress in the human ovarian tissue significantly increased after freezing-thawing as compared to basal level, with consequent morphological and ultrastructural damage of follicles and stroma.

The maintaining of thawed ovarian tissue at 4°C for 2 hrs allowed only a slight decrease of RRS levels and the morphological and ultrastructural features remained similar to those of just thawed tissue.

NAC, supplemented to the cryopreservation solutions, had a protective effect against oxidative damage on follicles and stroma, preserving DNA integrity, cytoplasmic architecture and organelle distribution in the oocytes, as well as oedema formation in the stromal compartment.

In the presence of NAC, the levels of apoptosis were comparable to that of fresh tissue. In particular, apoptosis was not observable in the oocytes but only in granulosa and stromal cells. The immunohistochemical analysis for the proliferation index also showed a positivity in the oocytes of all experimental conditions, indicating that the oocyte nucleus is not affected by freezing and thawing procedures and, after cryopreservation, it might resume the mitotic cycle.

Based on these data, it is reasonable to believe the NAC might have an important role during the cryopreservation of ovarian tissue. However, as highlighted by the EPR technique, the non-achievement of oxidative baseline level (9.72±2.10 μmol/g) suggests that the NAC concentration used in this study was not sufficient to scavenge the free radicals generated during the cryopreservation procedure.

Further research is necessary to identify the best concentration of NAC or a more efficient exogenous radical scavenger able to reduce RRS to basal level, preventing cryodamage.

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