

# Vitamin E-analog Trolox prevents endoplasmic reticulum stress in frozen-thawed ovarian tissue of capuchin monkey (*Sapajus apella*)

D. C. Brito · A. B. Brito · S. R. R. A. Scalercio · S. Percário · M. S. Miranda ·  
R. M. Rocha · J. A. P. Diniz · I. C. Oskam · R. Van den Hurk ·  
M. C. J. Paris · S. F. S. Domingues · R. R. Santos

**Abstract** Ovarian fragments were exposed to 0.5 M sucrose and 1 M ethylene glycol (freezing solution; FS) with or without selenium or Trolox. Histological and ultrastructural analyses showed that the percentages of normal follicles in control tissue and in tissue after exposure to FS+50 µM Trolox were similar. Trolox prevented endoplasmic reticulum (ER)-related vacuolization, which is commonly observed in oocytes and stromal tissue after exposure to FS. From the

evaluated stress markers, superoxide dismutase 1 (SOD1) was up-regulated in ovarian tissue exposed to FS+10 ng/ml selenium. Ovarian fragments were subsequently frozen-thawed in the presence of FS with or without 50 µM Trolox, followed by in vitro culture (IVC). Antioxidant capacity in ovarian fragments decreased after freeze-thawing in Trolox-free FS compared with FS+50 µM Trolox. Although freezing itself minimized the percentage of viable follicles in each

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D. C. Brito · A. B. Brito · S. R. R. A. Scalercio ·  
S. F. S. Domingues · R. R. Santos  
Laboratory of Wild Animal Biology and Medicine, Federal  
University of Pará, Belém, Brazil

D. C. Brito · A. B. Brito · S. R. R. A. Scalercio ·  
S. F. S. Domingues · R. R. Santos  
Animal Science Post-graduation Program, Federal University of  
Pará, Belém, Brazil

S. Percário  
Oxidative Stress Research Laboratory, Biological Sciences Institute,  
Federal University of Pará, Belém, Brazil

M. S. Miranda  
Laboratory of In Vitro Fertilization, Biological Sciences Institute,  
Federal University of Pará, Belém, Brazil

R. M. Rocha  
Laboratory of Cell Ultrastructure, Biological Sciences Institute,  
Federal University of Pará, Belém, Brazil

J. A. P. Diniz  
Laboratory of Electron Microscopy, Institute Evandro Chagas,  
Belém, Brazil

I. C. Oskam  
Biokapital, Hamar, Norway

R. Van den Hurk  
Department of Pathobiology, Faculty of Veterinary Medicine,  
Utrecht University, Utrecht, The Netherlands

M. C. J. Paris  
Institute for Breeding Rare and Endangered African Mammals  
(IBREAM), Edinburgh, UK

M. C. J. Paris  
School of Animal Biology, University of Western Australia, Perth,  
Australia

M. C. J. Paris  
Mammal Research Institute, University of Pretoria, Pretoria,  
South Africa

R. R. Santos ( )  
Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104,  
3584 CM Utrecht, The Netherlands  
e-mail: regianers@hotmail.com

solution, Trolox supplementation resulted in higher rates of viable follicles (67 %), even after IVC (61 %). Furthermore, stress markers SOD1 and ERp29 were up-regulated in ovarian tissue frozen-thawed in Trolox-free medium. Relative mRNA expression of growth factors markers was evaluated after freeze-thawing followed by IVC. BMP4, BMP5, CTGF, GDF9 and KL were down-regulated independently of the presence of Trolox in FS but down-regulation was less pronounced in the presence of Trolox. Thus, medium supplementation with 50  $\mu$ M Trolox prevents ER stress and, consequently, protects ovarian tissue from ER-derived cytoplasmic vacuolization. ERp29 but not ERp60, appears to be a key marker linking stress caused by freezing-thawing and cell vacuolization.

**Keywords** Cryopreservation · Pre-antral follicles · Ovarian stroma · Antioxidant · Primates · Capuchin monkey

## Introduction

Cryopreservation of ovarian tissue is an accepted procedure to preserve pre-antral follicles. A crucial step prior to the freezing of ovarian tissue is exposure to cryoprotectants. As the deleterious effect of cryopreservation solutions has been related to oxidative stress (Fahy 2010), supplementation of these mixtures with antioxidant agents probably improves follicular survival and tissue viability. Apart from some initial studies, little is known about the effect of antioxidant agents on the preservation of pre-antral follicle quality after freezing. In earlier studies, ascorbic acid was used as an antioxidant agent but did not improve ovine follicular viability (Melo et al. 2011). Additional studies showed that the supplementation of freezing medium with taurine and L-glutamine (Sanfilippo et al. 2013) and with catalase or Trolox (Luz et al. 2012) preserved the pre-antral follicles morphology of human and caprine ovarian tissue, respectively. From these compounds, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or Trolox appears as a promising candidate to improve freezing because, as a vitamin E analog, it might reduce oxidation in cell membrane lipids and is also a membrane-stabilizing agent (Bradford et al. 2003). Moreover, as is well known, freezing can lead to lipid peroxidation. Selenium is a common component of antioxidant enzymes and has been indicated as one of the compounds responsible for the high post-thaw viability of chimpanzee (*Pan troglodytes*) semen (Younis et al. 1998). Therefore, both Trolox and selenium should be tested to determine whether they can improve ovarian tissue cryopreservation in capuchin monkeys (*Sapajus apella*), a suitable model for New World non-human primates (Santos et al. 2010).

The primary tested biomarker for measuring oxidative stress caused by cryopreservation is superoxide dismutase

(SOD; Boonkusol et al. 2006; Turathum et al. 2010), which is responsible for the inactivation of superoxide radicals (Matzuk et al. 1998). Although no information is available regarding SOD1 mRNA expression in ovarian tissue exposed to cryopreservation solutions, this enzyme has been shown to protect oocytes against cryoprotectant toxicity (Dinara et al. 2001). Not only oocytes enclosed in the ovarian tissue but also stromal cells are expected to be affected by cryoprotectant-related oxidative stress. Moreover, stromal cells play a role in follicular survival and development after the cryopreservation of ovarian tissue (Gook et al. 2000). Usually, the measurement of antioxidant compounds separately is difficult. Therefore, methods to assess total antioxidant capacity have been developed. These biochemical parameters can be measured by spectrophotometry to calculate the Trolox equivalent antioxidant capacity (TEAC) in the cells, by using Trolox as a standard. Furthermore, the quantification of genes encoding proteins related to stress might offer a suitable tool for assessing follicular quality after exposure to cryopreservation solutions and after cryopreservation. As far as we know, no current information exists about the effect of cryopreservation solutions on gene expression in ovarian tissue.

Changes in the membrane phase caused by cryoprotectants (Spindler et al. 2011) might lead to membrane stress and, subsequently, to the activation of chaperone proteins. Heat shock protein 70 (HSP70) can be used as an immediate biomarker of severe intracellular stress. Expression of HSP70 mRNA has been studied in vitrified murine embryos (Boonkusol et al. 2006) and canine oocytes (Turathum et al. 2010) but not in ovarian tissue, of any species, exposed to freezing solutions (FS).

Cytoplasmic vacuolization is one of the most common signs of damage in cryopreserved oocytes (Nottola et al. 2009; Coticchio et al. 2010) and ovarian tissue (Oskam et al. 2011) and has been adopted as a specific marker for cryodamage (Coticchio et al. 2010). Silva et al. (2000) have suggested that damage to smooth endoplasmic reticulum (ER) leads to vacuolization. As potential biomarkers, we selected ER protein 29 (ERp29) and ER protein 60 (or calreticulin), because (1) both are stress-inducible proteins localized in the ER lumen (Banjerdpongchai et al. 2010) and (2) ERp29 facilitates the process and the transport of proteins (Zhang and Richardson 2011), whereas ERp60 is a  $\text{Ca}^{2+}$ -buffering protein (Lowther et al. 2009). Oocytes exposed to ethylene glycol have been shown to suffer from ER stress related to  $\text{Ca}^{2+}$  release (Larman et al. 2006). Ethylene glycol is widely and successfully used for ovarian follicles vitrification because of its low toxicity (Amorim et al. 2013; Sheikhi et al. 2013; Ting et al. 2013). However, when this cryoprotectant is employed during controlled-rate freezing, an increase in ooplasm vacuolization and multivesicular bodies but a decrease in volume of mitochondria/smooth ER aggregates are observed in human oocytes (Nottola et al. 2008). Therefore, to

minimize cryoprotectant damage, supplementation of the freezing medium with protecting compounds such as antioxidants that might counteract organelle damage will safeguard oocyte quality after freezing-thawing.

As cryopreservation often negatively affects ovarian tissue survival, histological and ultrastructural evaluation of follicles and stromal cells after thawing helps to determine the success of cryopreservation. In addition, short-term *in vitro* culture can be included as a complementary tool to assess ovarian tissue health after freezing and thawing and can be efficiently used to determine cryo-induced damage on the viability of ovarian follicles (Ting et al. 2011). As key markers for pre-antral follicle functionality, growth factors involved in the activation and growth of pre-antral follicles have been used, such as connective tissue growth factor (CTGF), differentiation factor-9 (GDF9), various bone morphogenetic proteins (BMPs), anti-Müllerian hormone (AMH) and stem cell factor or c-Kit ligand (KL; for a review, see Van den Hurk and Zhao 2005).

We describe the morphological effects of ovarian tissue exposure to FS that are either supplemented or not supplemented with antioxidants (selenium or Trolox). Conventional freezing followed by a 24-h *in vitro* culture of ovarian fragments was performed to evaluate the quality status of frozen-thawed ovarian tissue. For both exposure and freezing-thawing procedures, a molecular analysis via quantitative reverse transcription plus the polymerase chain reaction (qRT-PCR) was performed. As a model for our study, we used ovaries from capuchin monkeys.

## Materials and methods

### Source and preparation of ovarian tissue

Our study was approved by the local Ethical Committee in Animal Research (no.029/2009/CEPAN/IEC/SVS/MS). This study comprised of two experimental conditions. For each experiment, ovarian biopsies from healthy sexually mature capuchin monkeys (*Sapajus apella*) were obtained by exploratory laparoscopy (Santana et al. 2013). The removed biopsies were subjected to fragmentation into cortical pieces of 1 mm<sup>3</sup>. These 1 mm<sup>3</sup> pieces were used for all experiments. Selected ovarian fragments were randomly distributed over the experiments. All fragments contained sufficient numbers (more than 30) of pre-antral follicles and no obvious differences in the numbers of pre-antral follicles were observed.

### Experimental design

#### *Experiment I: exposure of ovarian tissue to FS*

After the collection of biopsies from four paired ovaries, fragments of 1 mm<sup>3</sup> were submitted to Experiment I. From

each ovarian pair, 18 fragments were harvested and divided as follows. Two fragments were randomly selected as the control; one piece was immediately fixed for microscopic and ultrastructural examination of the pre-antral follicles and of the ovarian stroma, whereas the other piece was snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The remaining 16 fragments were kept for 20 min (control FS group) in TCM-199, which was supplemented with 1 M ethylene glycol (EG)+0.5 M sucrose (FS; Santos et al. 2006a). The FS was either supplemented or not supplemented with selenium (2.5, 5, or 10 ng/ml) or Trolox (25 µM, 50 µM, or 100 µM). Two fragments were exposed to each treatment and after cryoprotectant removal (CR), one fragment was submitted to morphological and ultrastructural analysis and the other to qRT-PCR (see experimental design in Supplementary Table 1). For CR, solutions were prepared prior to use. CR-1, CR-2 and CR-3 solutions, respectively, consisted of TCM-199 with 0.250 M sucrose, TCM-199 with 0.125 M sucrose and TCM-199 only. Each fragment was immersed in CR-1 at 37 °C in a water bath for 3 min followed by CR-2 (5 min) and CR-3 (7 min). Tissue exposed to TCM-199 was subjected to washes in TCM-199 only at 37 °C. After being rinsed, ovarian fragments from each group were subjected to morphological and ultrastructural analysis and qRT-PCR.

#### *Experiment II: freezing of ovarian tissue*

Ovarian fragments were individually collected from five animals. From each ovarian pair, 16 fragments of 1 mm<sup>3</sup> were frozen-thawed in FS only or in FS supplemented with 50 µM Trolox as shown in Supplementary Table 2. A previously described protocol was applied for the freezing of ovarian tissue (Santos et al. 2006a). Prior to the freezing procedure, fragments were placed in straws (0.5 ml) and equilibrated in a controlled-rate programmable freezer (Freezer Programs by CryoLogic, Victoria, Australia) for 20 min at 20 °C in 0.5 ml TCM-199 supplemented with 0.5 M sucrose and 1.0 M EG alone or in combination with 50 µM Trolox. Straws were sealed and cooled at 2 °C/min from 20 °C to -7 °C; ice-induction (seeding) was manually performed by touching the straws with forceps pre-cooled in liquid nitrogen. After seeding, the straws were held at this temperature (-7 °C) for 10 min and then cooled at 0.3 °C/min to -30 °C, after which the straws were plunged immediately into liquid nitrogen (-196 °C) and stored for at least 1 month. When required, the straws were thawed in air for 1 min at room temperature (~25 °C) and then immersed in a water bath at 37 °C until the cryopreservation medium had completely melted. The cryoprotectant was then removed as described in Experiment I. After CR, ovarian frozen-thawed fragments were submitted to *in vitro* culture, viability analysis, Trolox equivalent antioxidant capacity (TEAC) measurement and qRT-PCR.

Assessment methods used in Experiments I and II

*Morphology of ovarian tissue exposed to FS (Experiment I)*

Control and treated ovarian tissue biopsies were fixed in Karnovsky's solution (2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for 3 h. Thereafter, tissue was post-fixed in 1 % osmium tetroxide solution buffered with sodium cacodylate (0.1 M, pH 7.2) for 2 h at room temperature. The fragments were then dehydrated in a grade acetone series and infiltrated and embedded in EPON. Semi-thin sections of 1  $\mu\text{m}$  thickness were stained with toluidine blue. Pre-antral follicles were defined as follicles without an antrum. Follicular quality was evaluated based on the morphological integrity of the oocyte, the granulosa cells and the basement membrane as described previously (Santos et al. 2006b). To avoid counting a follicle more than once, pre-antral follicles were counted in the sections in which their oocyte nucleus was observed. All sections were examined by using a light microscope (Olympus, Tokyo, Japan) at a magnification of  $\times 400$ .

*Ultrastructure of ovarian tissue exposed to FS (Experiment I)*

Based on morphological analysis, fragments of control ovaries and ovaries that were exposed to FS either with or without 50  $\mu\text{M}$  Trolox were submitted to ultrastructural analysis. For this, ultra-thin sections (60–70 nm) were contrasted with uranyl acetate and lead citrate and examined under a Jeol JEM 100C (Carl Zeiss Microscopy, Jena, Germany) transmission electron microscope.

*Assessment of follicular viability (Experiment II)*

Capuchin pre-antral follicles were mechanically isolated from fresh and freeze-thawed ovarian fragments according to the procedure described by Domingues et al. (2003). Pre-antral follicles smaller than 100  $\mu\text{m}$  were collected under a dissecting stereomicroscope (SZ-ST5, Olympus, Tokyo, Japan) and transferred to TCM-199. Isolated follicles were incubated in this medium for 10 mins at 37 °C in a mixture of 2  $\mu\text{M}$  propidium iodide (Molecular Probes Europe, Leiden, The Netherlands) and 10  $\mu\text{M}$  Hoechst 33342 (Sigma) to detect membrane integrity and to enable the counting of the nuclei, respectively. After being labeled, stained follicles were washed three times in TCM-199, mounted on a glass microscope slide and examined by epifluorescent microscopy (BH2-RFCA microscope, Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix900, Nikon Instruments Europe, Badhoevedorp, The Netherlands). The emitted fluorescent signals of propidium iodide and Hoechst were collected at 490/635 nm and 472 nm, respectively. Oocytes and granulosa cells were classified as viable if the ooplasm was

unlabeled with propidium iodide. Follicles with a viable oocyte surrounded by  $\geq 90$  % viable granulosa cells were considered to be viable (Santos et al. 2006a).

*In vitro culture of cryopreserved ovarian tissue (Experiment II)*

In vitro culture of ovarian tissue was performed as previously described by Brito et al. (2013a). In brief, ovarian fragments were placed in 500  $\mu\text{l}$  culture medium in 24-well plates for 24 h. The basic culture medium consisted of TCM-199 (pH 7.2 – 7.4) supplemented with 10 % fetal calf serum, 100 ng/ml epidermal growth factor, 10  $\mu\text{M}$  beta-mercaptoethanol, 100 ng/ml BMP4 and 25 IU pregnant mare serum gonadotropin. Incubation was carried out at 37 °C in 5 %  $\text{CO}_2$  in air for 24 h. After in vitro culture, all fragments were divided into two equal parts and submitted to viability analysis or qRT-PCR.

*Trolox equivalent antioxidant capacity (Experiment II)*

In order to access the TEAC, ovarian tissue was homogenized in phosphate-buffered saline by using a mixer. The final volume (15 – 20  $\mu\text{l}$ ) was submitted to TEAC determination. The antioxidant potential was determined according to its equivalence to a potent antioxidant known as Trolox (Aldrich Chemical; 23881-3), a vitamin E hydro-soluble synthetic analog. The method modified by Re et al. (1999) was followed. This method involves a colorimetric technique based on a reaction between 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium (ABTS) with  $\text{K}_2\text{S}_2\text{O}_8$  directly producing radical cation  $\text{ABTS}^+$ , a green/blue chromophore with a maximum absorbance at 734 nm. The addition of antioxidants to this pre-formed radical cation reduces it, once again, to ABTS, the extension and time-scale being dependent on the antioxidant capacity, concentration of the antioxidants and duration of the reaction. This can be measured by spectrophotometry by observing the change in the absorbance read at 734 nm (800 XI, FEMTO, Brazil) over 5 min. Thus, the extension of discoloration as an index of the inhibition of the radical cation  $\text{ABTS}^+$  is determined as the total antioxidant activity of the sample, after which its relationship with Trolox reactivity is calculated as a standard under the same conditions. TEAC values lower than 2 mM were considered as being decreased based on control standard measurements.

*RNA extraction and cDNA synthesis (Experiments I and II)*

Total RNA was isolated from ovarian tissue homogenized in Trizol reagent (Invitrogen, Carlsbad, Calif., USA) as described by Nilsson and Skinner (2003). The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer

(NanoDrop ND-1000 and ND-8000 8-Sample, NanoDrop, Wilmington, Del., USA). For each sample, the RNA concentrations were adjusted to 45 ng/ml and used to synthesize cDNA. Reverse transcription was performed in a total volume of 20  $\mu$ l composed of 10  $\mu$ l sample RNA, 3.2  $\mu$ l de-ionized water, 2  $\mu$ l reverse transcriptase buffer (High Capacity Reverse Transcription kit, Applied Biosystems, Foster City, Calif., USA), 0.8  $\mu$ l dNTP Mix (10 mM), 2  $\mu$ l random primers, 1  $\mu$ l MultiScribe reverse transcriptase and 1  $\mu$ l RNase inhibitor (High Capacity Reverse Transcription kit, Applied Biosystems). The mixture was incubated at 42 °C for 1 h and subsequently at 80 °C for 5 min and was finally stored at -20 °C. The negative control was prepared under the same conditions but without addition of the nucleic acid.

#### qRT-PCR (Experiments I and II)

qRT-PCR was performed in a thermocycler (ABI PRISM 7500 Real Time PCR system, Applied Biosystems). The primers, chosen to carry out the amplification of the various reference and target genes are shown in Table 1. Amplification efficiency was determined per plate by using LinRegPCR (Ruijter et al. 2009). Data were analyzed by the efficiency-corrected Delta-Delta-Ct method (Pfaffl 2001). The fold-

change values of the genes encoding AMH, CTGF, BMP4, BMP15, ERp29, ERp60, GDF9, HSP70, KL and SOD1 were normalized by means of the geometric average of the fold-change values of two reference genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1) and the *TATA box* binding protein (TBP).

#### Statistical analyses

Data were analyzed with Prism 4 software (GraphPad). For morphological data, *P*-values were calculated by using one-way analysis of variance (ANOVA) and the unpaired *t*-test. The viability of oocytes and granulosa cells was analyzed by the chi-square test and the numbers of total nuclei per follicle were compared by one-way ANOVA. Percentages of primordial and developing follicles and molecular data were calculated by using one-way ANOVA and the unpaired *t*-test. For statistical analysis of differential gene expression, an empirical Bayes method was used to moderate the standard errors of the log-fold changes. A Benjamini-Hochberg correction was applied to the *P*-values of the log-fold changes to correct for multiple testing (Benjamini and Hochberg 1995). All error bars refer to the SEM and differences were considered significant when *P* < 0.05.

**Table 1** Pairs of primers used in quantitative reverse transcription plus the polymerase chain reaction for quantification of reference and target genes in ovarian tissue from capuchin monkeys

Gene (abbreviation)	Accession number	Sequence	Fragment size (bp)	Primer position	Reference
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	S43335.1	5'- TTATGGACAGGACTGAACGTCTTGCT 3'- TCCAGCAGGTACAGCAAAGAATTATA GC	107	59	Brito et al. 2013b
<i>TATA box</i> binding protein (TBP)	AB178959.1	5'- TGCACAGGAGCCAAGAGTGAA 3'- CACATCACAGCTCCCCACCA	132	889	Brito et al. 2013b
Anti-Müllerian hormone (AMH)	XM_002761553.1	5'- CTGCTGCGTGCCACCGCCTA 3'- CAGCCACACTCGGTGGCCAC	106	1575	Brito et al. 2013a
Bone morphogenetic protein (BMP4)	XM_002805069.1	5'- CGAGAGAGACGCAGACGCAGA 3'- AATGGCTCCATGTTCCCGAG	82	56	Brito et al. 2013a
Bone morphogenetic protein (BMP15)	XM_001083980.2	5'- GCCACTGTGGTTTACCGCCA 3'-GGGAAGGTTTTGAGGAATCTCCTTC	130	446	Brito et al. 2013a
Connective tissue growth factor (CTGF)	NM_001901.2	5'- TGCATCTTCGGTGGCACG GT 3'- ATGCTGCACAGGGGCATGCA	110	513	Brito et al. 2013a
Endoplasmic reticulum protein 29 (ERp29)	NM_001195735.1	5'- GCCCTTCCCCTGGATACGGTCA 3'- CATCTGCTTCTCACCGTAGGGGT	103	55	This paper
Endoplasmic reticulum protein 60 (ERp60)	NM_004343.3	5'- GCCCGCCATGCTGCTATCCG 3'- AGTCCACCCGTCTCCGTCCA	108	74	This paper
Differentiation factor-9 (GDF9)	NM_001168763.1	5'- GCCTTCCCAGGGTCTGACCA 3'- TGACCTCTGCGGTGACGGTGA	105	861	Brito et al. 2013a
Heat shock protein 70 (HSP70)	NM_001193708.1	5'- TGACCCTCCTGTTGGCTGGCT 3'- CCAACGGAACAATAGGTGGTGCC	100	52	This paper
c-Kit ligand (KL)	NM_003994.5	5'- GCTCGGGCTACCCAATGCGTG 3'- CGCTGCGATCCAGCACAAACAG	112	72	Brito et al. 2013a
Superoxide dismutase 1 (SOD1)	AB220492.1	5'- GCCGCACATTGGTGGTCCATGAA 3'- AAGCCAGACGACCTCCAGCGT	96	425	This paper

## Results

### Experiment I: exposure of ovarian tissue to FS

In total, 2143 pre-antral follicles (at least 100 per treatment) were histologically examined in the control tissues and in the tissues exposed to FS either with or without selenium (2.5, 5, or 10 ng/ml) or Trolox (25  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M). The only similar ( $P > 0.05$ ) percentage of morphologically normal follicles was observed between control ovarian fragments (83 %) and treated ovarian fragments with FS+50  $\mu$ M Trolox (79.2 %) and FS+100  $\mu$ M Trolox (61.3 %). However, the other treatments led to a significant decrease in the percentages of morphologically normal pre-antral follicles, with the lowest ( $P < 0.05$ ) percentages being observed after ovarian tissue exposure to FS+10 ng/ml selenium (19.5 % of normal pre-antral follicles; Fig. 1). The relative expression of genes encoding HSP70, SOD1, ERp29 and ERp60 in the ovarian tissue of capuchin monkeys after exposure to TCM-199 and freezing procedures is also presented in Fig. 1. SOD1 was up-regulated when ovarian tissue was exposed to FS+10 ng/ml selenium. The other genes and treatments were unaltered.

All degenerating pre-antral follicles exposed to FS with or without selenium exhibited vacuolated oocytes and stroma, whereas those from the control group or exposed to FS plus Trolox did not (Fig. 2). To characterize follicular degeneration caused by FS with or without 50  $\mu$ M Trolox properly, ultra-structural analysis was performed. Normal pre-antral follicles presented oocytes with a large, well-delimited and pleiomorphic nucleus, large mitochondria with irregular cristae and continuous membranes and elongated mitochondria

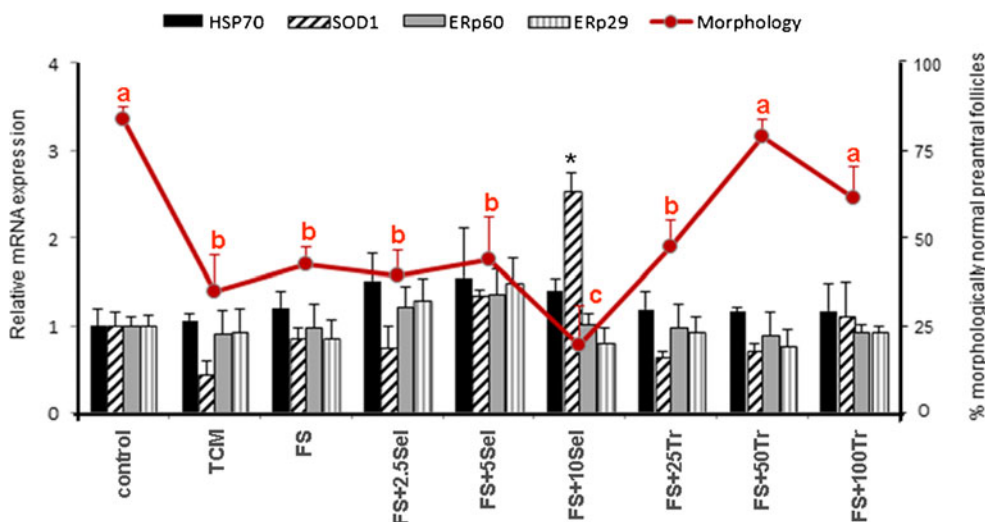
with parallel cristae. Granulosa cells around these oocytes had irregularly shaped nuclei. When ovarian tissue was exposed to FS only, cytoplasmic vacuolization was observed in areas in which normal rough and smooth ER would have been expected. The addition of 50  $\mu$ M Trolox to FS, however, reduced ooplasmic vacuolization but the detachment of oocytes from granulosa cells was not prevented (Fig. 3).

### Experiment II: freezing of ovarian tissue

Based on the data from Experiment 1, ovarian tissue was frozen-thawed in the presence or absence of 50  $\mu$ M Trolox and subsequently cultured in vitro. Ovarian fragments were submitted to TEAC analysis, follicular viability evaluation and qRT-PCR.

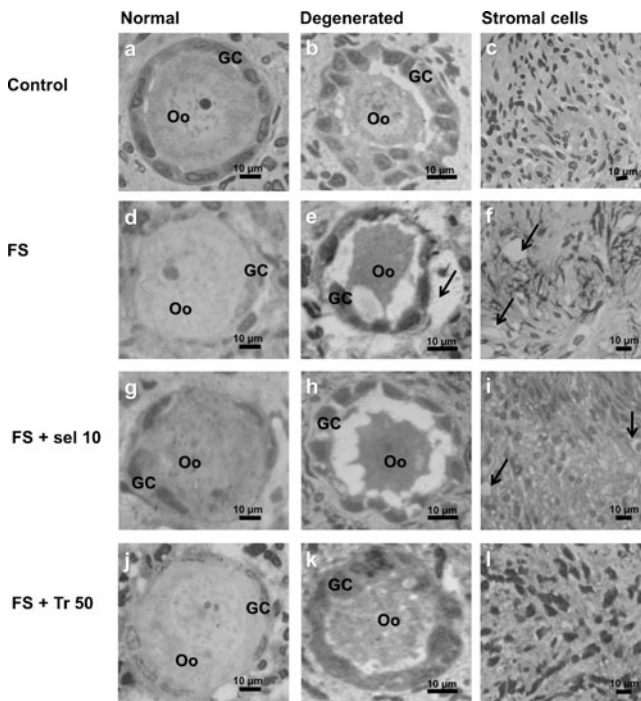
TEAC values were within the normal range only when ovarian tissue was cryopreserved in the presence of 50  $\mu$ M Trolox and evaluated after thawing ( $2.9 \pm 0.06$  mM) or after in vitro culture ( $2.6 \pm 0.03$  mM). Samples cryopreserved without Trolox and evaluated immediately after thawing or in vitro culture presented TEAC values of  $0.1 \pm 0.03$  mM and  $0.09 \pm 0.03$  mM, respectively.

Follicles enclosed in frozen-thawed ovarian tissue were immediately evaluated for their viability after being thawed or after in vitro culture for 24 h. In total, 293 pre-antral follicles (at least 30 per treatment) were examined. The percentage of viable follicles was significantly diminished after freezing when compared with control ovarian fragments (89 %). However, FS supplementation with Trolox resulted in higher rates of viable follicles (67 %) when compared with FS only (39 %). A similar effect was also observed after



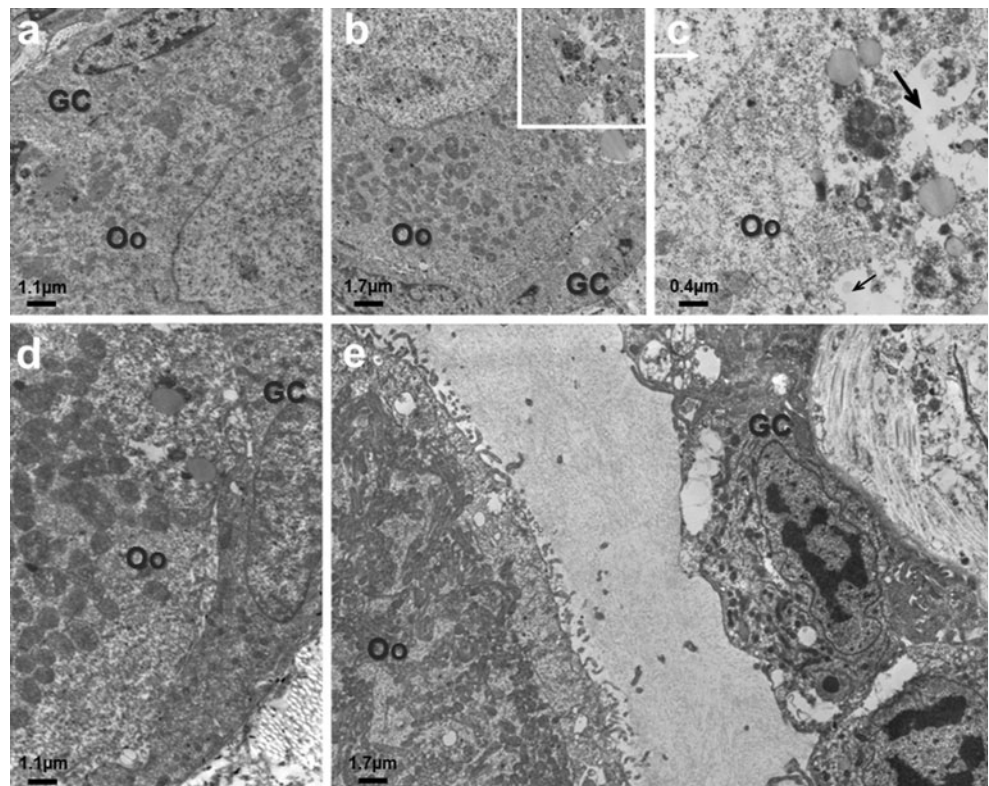
**Fig. 1** Comparison of the average relative expression (bars) of heat shock protein 70 (HSP70), superoxide dismutase 1 (SOD1), endoplasmic reticulum protein 60 (ERp60) and endoplasmic reticulum protein 29 (ERp29) and percentages of morphologically normal pre-antral follicles (red line) in ovarian tissue from the control group, exposed for 20 min to medium (TCM) only or exposed to freezing solution (FS) with or without

selenium (2.5Sel, 5Sel, 10Sel 2.5, 5, or 10 ng/ml, respectively) or Trolox (25Tr, 50Tr, 100Tr 25  $\mu$ M, 50  $\mu$ M or 100  $\mu$ M, respectively). \*Differs from control in relative mRNA expression ( $P < 0.05$ ). <sup>a,b</sup> Different red lower-case letters differ significantly between treatments in the morphologically analysis ( $P < 0.05$ )



**Fig. 2** Semi-thin sections representing normal and degenerated pre-antral follicles and ovarian stroma (*Oo* oocyte, *GC* granulosa cell, *arrows* vacuolization) in control tissue (**a–c**, tissue exposed to freezing solution (*FS*; **d–f**), tissue exposed to FS supplemented with 10 ng/ml selenium (*FS+sel10*; **g–i**) and tissue exposed to FS supplemented with 50 nM Trolox (*FS+Tr50*; **j–l**). Original magnification  $\times 400x$

**Fig. 3** Electron microscopy of ovarian tissue exposed to freezing solutions. **a** Control (*Oo* oocytes, *GC* granulosa cells). **b** Pre-antral follicle after exposure to freezing solution, indicating vacuolization of the oocyte as empty areas in which ER should be located (*inset*). *Inset* in **b** is shown at higher magnification in **c** (*arrows* vacuolization). **d** Pre-antral follicle after exposure to freezing solution plus 50  $\mu M$  Trolox with normal distribution of mitochondria and normal endoplasmic reticulum. **e** Pre-antral follicle after exposure to freezing solution plus 50  $\mu M$  Trolox presenting displacement of granulosa cells



in vitro culture following which 61 % of the follicles were viable after freezing-thawing in the presence of Trolox and 36 % in FS only (Fig. 4).

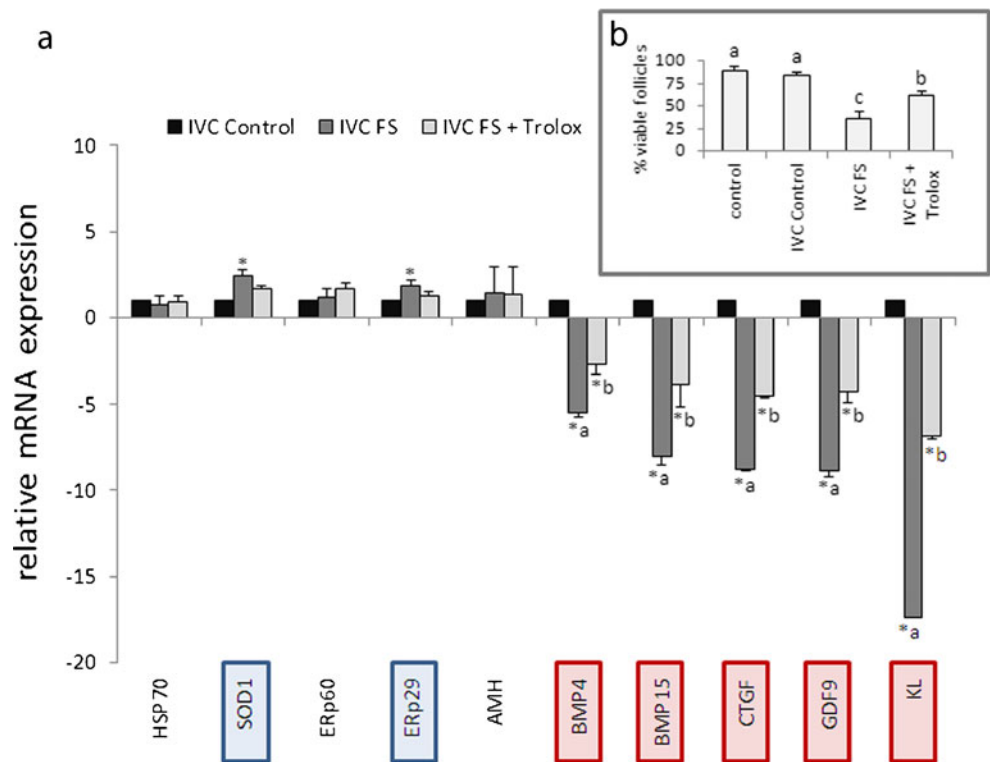
As in vitro culture did not affect follicular viability when compared with control and as cell changes attributable to cryopreservation could be seen even after short-term in vitro culture, the relative expression of genes encoding markers for stress (HSP70, SOD1, ERp29 and ERp60) and follicular and ovarian tissue quality (AMH, BMP4, BMP5, CTGF, GDF9 and KL) was evaluated (Fig. 4). SOD1 and ERp29 were up-regulated in ovarian tissue that had been frozen-thawed in the absence of Trolox. Except for AMH, freezing-thawing led to the down-regulation of BMP4, BMP5, CTGF, GDF9 and KL independently of the presence of Trolox in the freezing medium. However, KL down-regulation was less pronounced when ovarian tissue was frozen in the presence of Trolox.

## Discussion

This study was designed to investigate the influence of ovarian tissue exposure to FS with or without the addition of selenium or Trolox. Moreover, the quality of pre-antral follicles and oxidative stress was assessed in the in-vitro-cultured ovarian tissue after freezing-thawing in the presence or absence of Trolox.

Ovarian tissue exposed to FS showed an increase in follicular and stromal degeneration, whereas medium supplementation

**Fig. 4** Comparison of the average relative expression of HSP70, SOD1, ERp60, ERp29, AMH, BMP4, BMP15, CTGF, GDF9 and KL (a) and viability of ovarian follicles (b). For an explanation of gene abbreviations, see Table 1 (IVC in vitro culture, FS freezing solution, blue boxes significant up-regulation, red boxes significant down-regulation). \*Differs from control. <sup>a,b</sup>Different lower-case letters differ significantly between treatments ( $P < 0.05$ )



with 50  $\mu$ M Trolox appeared to decrease vacuolization in oocyte and stroma cells. Similarly, previous studies have shown the value of antioxidants in protecting ovarian tissue from ischemia following transplantation (Kim et al. 2004) or their usefulness in freezing medium to improve follicular preservation (Melo et al. 2011; Sanfilippo et al. 2013). Vacuolization has been reported after cryopreservation of ovarian tissue from sheep (Santos et al. 2006b; Oskam et al. 2010), bovine (Celestino et al. 2008) and human (Marsella et al. 2008), with indications of damage in the ER (Silva et al. 2000). In agreement with this evidence, Oskam et al. (2010) found oocyte vacuolization together with an extreme decrease in or even total absence of smooth and rough ER in cryopreserved sheep ovarian tissue.

Ovarian tissue exposure to freezing medium supplemented with 50  $\mu$ M Trolox did not affect the percentages of morphologically normal pre-antral follicles or gene expression. However, degenerated follicles after exposure to cryoprotectant solutions with or without Trolox showed oocytes detached from their granulosa cells. Dolmans et al. (2006) presented similar micrographs after the exposure of follicles to collagenase and considered this degeneration to be the result of the uncoupling between follicular cell prolongations and oocyte microvilli.

When freezing medium was supplemented with 10 ng/ml selenium, ovarian tissue exposed for 20 min exhibits an up-regulation of SOD1, together with oocyte and stroma vacuolization. Up-regulation of the antioxidant enzyme

SOD1 indicates that cells might be suffering from oxidative stress. This finding supports that of Uğuz et al. (2009), who have previously shown that cell incubation with selenium at concentrations as high as 1 mM leads to severe ER stress.

mRNA expression of ER-related chaperones (ERp29 and ERp60) appeared to be unaltered in exposed ovarian tissue, even when presenting vacuolization. Probably as a result the 20-min exposure, stress was not sufficient to change the regulation of these proteins. However, after freezing-thawing in Trolox-free medium followed by in vitro culture, SOD1 was up-regulated together with ERp29 as a response to ER-derived cytoplasmic vacuolization. mRNA expression of ERp60 remained unchanged. Furthermore, ERp60 has been demonstrated to be activated when cells are under osmotic stress (Bibi et al. 2011), suggesting that the present freezing medium did not compromise  $Ca^{2+}$  release caused by osmotic stress. Moreover, mRNA expression of HSP70 was not affected by ovarian tissue exposure to cryoprotectants or freezing-thawing, probably because this chaperone protein is not regulated by dehydration or rehydration of the ovarian tissue (Lopez-Martinez et al. 2009).

ERp29 has a role not only in the ER stress response but also in protein processing (Hubbard et al. 2000). This might explain the induction of ERp29 and its up-regulation under ER-stress, which is accompanied by the down-regulation of the mRNA expression of BMP4, BMP15, CTGF, GDF9 and KL. AMH is unchanged because of its low mRNA expression in all treatments. One of the functions of the ER is protein



synthesis. Therefore, the findings that (1) mRNA expression is more negatively regulated after freezing-thawing in a Trolox-free medium than in medium with Trolox, (2) ovarian tissue frozen-thawed in the presence of Trolox presents the highest TEAC values and (3) Trolox-free frozen-thawed cultured ovarian tissue is more susceptible to degeneration suggest that this compound protects ER from cryo-related stress.

We therefore conclude that freezing medium supplementation with 50  $\mu$ M Trolox protects ovarian tissue from ER-derived cytoplasmic vacuolization. However, the rates of follicular viability are still too low to allow this Trolox-containing medium to be considered as suitable for maintaining the viability of follicles after freezing and their subsequent in vitro culture. Thus, both the cryopreservation solution for ovarian tissue and the medium in which it is cultured once it is thawed have to be improved to guarantee normal follicular development and to optimize the freezing procedure for ovarian tissue.

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