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Trolox enhances follicular survival after ovarian tissue autograft in squirrel monkey (*Saimiri collinsi*)

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Abstract. The aim of this study was to evaluate ovarian tissue pre-treatment with 50 μM Trolox followed by heterotopic transplantation in squirrel monkeys (*Saimiri collinsi*) and to assess tissue functionality via immunohistochemical analysis of the stroma and ovarian follicles. Five healthy and sexually mature squirrel monkey (*Saimiri collinsi*) females were used. Heterotopic autografting of fresh ovarian tissue with or without previous exposure to the antioxidant Trolox was performed and grafts were recovered for analysis 7 days later. Tissue vascularisation was confirmed by both macroscopic inspection and cluster of differentiation 31 (CD31) staining. Trolox prevented massive follicular activation and kept the percentages of morphologically normal follicles higher than in untreated grafts. Expression of anti-Müllerian hormone in developing follicles was observed only in controls and Trolox-treated grafts. Also, immunostaining for growth differentiation factor-9 was positive only in primordial follicles from controls and from Trolox-treated grafts. Although Trolox improved follicular quality and avoided apoptosis in stromal cells, ovarian tissue fibrosis was increased in Trolox-treated grafts, mainly due to an increase in collagen Type I synthesis.

Additional keywords: apoptosis, fibrosis, grafting, pre-antral follicles, vascularisation.

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Introduction

Different strategies to preserve female fertility and endangered species are in development with the help of animal models (Santos *et al.* 2010; Vanacker *et al.* 2012; von Schönfeldt *et al.* 2012; Luyckx *et al.* 2013). Among the approaches, cryopreservation of ovarian tissue is a promising alternative to storing the large numbers of female gametes enclosed in pre-antral follicles. Indeed, after thawing and grafting, these follicles are able to survive and resume their growth (Santos *et al.* 2009; Amorim *et al.* 2011, 2013; Ting *et al.* 2013).

Despite the successful results obtained with cryopreservation and autotransplantation of ovarian tissue, it is important to highlight the large follicle loss resulting from the latter procedure (Candy *et al.* 1997; Aubard *et al.* 1999; Baird *et al.* 1999). This is probably due to the avascular transplantation of the ovarian tissue, which leads to ischaemic–reperfusion injury in the first days after grafting through the production of reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radical (Karaca *et al.* 2009). These free radicals cause lipid peroxidation, which is followed by cell membrane damage and neutrophil chemotaxis blocking microvasculature by white-cell clogging (Zweier and Talukder 2006). Oxidative stress leads not only to cell damage and death, but also to massive activation of primordial follicles and subsequent burnout of the follicular reserve in the ovarian tissue (Sobinoff *et al.* 2012; Gavish *et al.* 2014).

One of the approaches to prevent ischaemic damage is the systemic or local delivery of free-radical scavengers and vitamins. For this, host or grafts have been treated with vitamin E (Nugent *et al.* 1998; Abir *et al.* 2011; Friedman *et al.* 2012). Nugent *et al.* (1998) reported a higher follicle survival in ovarian xenografts from mice supplemented with vitamin E for 7 days after surgery, but no information on follicular activation was given. These authors showed the importance of tissue analysis 7 days after transplantation and indicated chilling the tissue before transplantation and during tissue treatment to minimise lipid peroxidation. Abir *et al.* (2011) showed that administration of vitamin E (dry form; α-tocopheryl succinate) and gonadotrophins to immunodeficient mice before and after xenografting decreased apoptotic rate in follicles from human ovarian tissue. Noteworthy, granulosa cells from most follicles were positive to proliferating-cell nuclear antigen (PCNA) immunostaining (Abir *et al.* 2011), a reliable marker to determine cell proliferation and, consequently, follicular activation (Wang *et al.* 2013*a*). Friedman *et al.* (2012) showed that graft treatment with vascular endothelial growth factor (VEGF), vitamin E (oil form) and hyaluronic acid-rich biological glue together with host treatment with melatonin improved human ovarian tissue survival grafted in mice for 1 week. However, no effect against accelerated follicular activation was reported.

Although an antioxidant activity is alleged for vitamin E, such a role is questioned (Sylvester 2007; Brigelius-Flohé 2009). The commonly used vitamin E is found as α-tocopherol, its first-recognised and most-abundant isomer in mammalian tissue. However, there are eight chemically different isomers recognised as vitamin E; four are saturated (α-, β-, gamma- and delta-tocopherols) and four are unsaturated (α-, β-, gamma- and delta-tocotrienols) forms (Ahsan *et al.* 2014). All of these isoforms meet the requirements to be classified as antioxidants, but their biological role was observed *in vitro* without evidence of *in vivo* antioxidant activity (Brigelius-Flohé 2009). Furthermore, the metabolites of these multiple agents are sometimes more active than their precursors and present different effects including anti-inflammatory (Jiang *et al.* 2008), as well as ambiguous properties such as pro- or anti-apoptotic (Birringer *et al.* 2010; Abir *et al.* 2011).

Due to the absence of soluble fractions, the vitamin E isomer α-tocopherol can act as an antioxidant only in lipid phases, but not when reactive hydroxyl or alkoxy radicals are taken in account (Brigelius-Flohé 2009). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analogue of vitamin E, is hydro- and liposoluble due to a carboxyl group and is able to inhibit protein damage by hydroxyl radicals (Miura *et al.* 1993). In a complete physicochemical study, Alberto *et al.* (2013) demonstrated that Trolox is a potent scavenger of hydroxyl and alkoxy (a product of hydroperoxide decomposition) radicals regardless the reaction conditions, i.e. aqueous or lipid solution, and of peroxyl radicals more efficiently in aqueous solution. Furthermore, while the aqueous solubility of Trolox allows rapid delivery to grafted tissue (Tan *et al.* 1996), its lipid solubility facilitates membrane permeation (Ross *et al.* 1995; Tan *et al.* 1996). Trolox (50 μM) has been used to preserve the morphology of caprine pre-antral follicles from cryopreserved ovarian tissue (Luz *et al.* 2012) and to prevent endoplasmic reticulum stress in frozen–thawed ovarian tissue of capuchin monkey after *in vitro* culture (Brito *et al.* 2014). Therefore, we hypothesised that Trolox might maintain follicular survival after grafting, as well as avoid exaggerated follicular activation.

Tissue analysis was performed 7 days after transplantation, a crucial time-point for tissue re-vascularisation in large mammals (Santos *et al.* 2009; Dath *et al.* 2010; Friedman *et al.* 2012; Wang *et al.* 2013*a*), encompassing the critical hypoxic period post-transplant (Nugent *et al.* 1998; Dath *et al.* 2010). Also, as suggested by Nugent *et al.* (1998), tissue was kept at low temperature (4°C) to minimise oxidative stress before grafting. A time exposure of 30 min was chosen to simulate the time necessary for ovarian tissue transport and preparation before grafting.

Hence, our aim in the present study was to evaluate the effect of Trolox on follicle survival after heterotopic autotransplantation, as well as the ability of this antioxidant to inhibit or minimise massive primordial follicle activation. Follicular survival was evaluated considering morphology, activation, immunolocalisation of growth markers and apoptosis detection, while ovarian stroma quality was used to assess tissue vascularisation, proliferation, apoptosis and fibrosis.

Materials and methods

Chemicals

Unless mentioned otherwise, the chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Our study was approved by the local Ethical Committee on Animal Research from the Evandro Chagas Institute (IEC/CEU no. 0009/2011) and by the Brazilian Institute for Wildlife and Environment (SISBIO/IBAMA no. 32632–1/2012) and followed the guidelines of the National Council of Control Animal Experimentation of Brazil. Five healthy and sexually mature squirrel monkey females (2.5–5 years old, weight range: 0.4–0.7 kg) born in captivity at the National Primate Center, Pará, Brazil, were selected for our study. Females were selected from the reproductive group, kept indoors under a natural photoperiod and housed individually in cages (80 × 90 × 80 cm), for proper management and to avoid fights among them during recovery from surgery. Their daily diet consisted of fresh fruits, vegetables and commercial monkey pellets (MEGAZOO P18; Betim, Minas Gerais, Brazil). Tap water was available *ad libitum*. At the end of experiments, females were returned to their reproductive groups.

Ovarian tissue collection and exposure to Trolox

All procedures were performed under general anaesthesia. Each animal was anaesthetised with ketamine hydrochloride (Cetamine, 10 mg kg^{-1}, intramuscular (i.m.); Köning SA, Avellaneda, Argentina) and xylazine hydrochloride (Anasedan, 1 mg kg^{-1}, i.m.; Köning SA). Anaesthesia was maintained with 2% halothane (Tanohalo; Cristália, São Paulo, Brazil). The females were placed in dorsal recumbence and one ovary was

collected by exploratory laparotomy. For this, a ventral midline skin incision was made to reach the ovaries, as described by Domingues *et al.* (2007). The right ovary from each female was completely removed, cut into halves and the medulla was removed. The remaining cortical tissue was then cut into strips (13 pieces of ~4 × 4 × 1 mm) in a Petri dish containing minimal essential medium (MEM) modified with 25 mM HEPES (GIBCO BRL, Carlsbad, CA, USA). One fragment was immediately fixed in 4% paraformaldehyde for histological and immunohistochemical analyses (fresh control), while the other 12 fragments were kept at 4°C for 30 min in MEM modified with 25 mM HEPES supplemented or not supplemented with Trolox (six fragments each treatment). A concentration of 50 µM Trolox was used based on a previous study (Brito *et al.* 2014). After exposure, cortical fragments were submitted to autografting (details below). After surgery, the animals received antibiotics for 1 week (Chemitril; Chemitec, São Paulo, Brazil), and ibuprofen (Ketofen; Merial Saúde Animal Ltda, São Paulo, Brazil) was used to treat pain in the 2 days following surgery.

Ovarian tissue autografting

Under anaesthesia, grafting of ovarian tissue was performed subcutaneously in the dorsal region. Before re-implantation, ovarian strips were stitched using non-absorbable sutures (6/0 Prolene; Ethicon, Diegem, Belgium). Each animal received six non-treated (right side) and six Trolox-treated (left side) grafts. After 1 week, the animals were anesthetised, as described above, grafts were recovered and fixed in 4% paraformaldehyde for histological and immunohistochemical analyses.

Analyses

Histological analysis

After overnight fixation in 4% paraformaldehyde (pH 7.2) at 4°C, ovarian fragments were dehydrated in ethanol, clarified with xylene and embedded in paraffin wax. Serial sections (5 µm) of ovarian tissue were cut and every tenth section was mounted on glass slides and stained with haematoxylin–eosin (Merck, Darmstadt, Germany). Follicle quality was evaluated based on the morphological integrity of the oocyte, granulosa cells and basement membrane as previously described (Santos *et al.* 2006). To avoid counting a follicle more than once, pre-antral follicles were counted in the sections where their oocyte nucleus was observed. Extra sections were mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany) and prepared for immunohistochemical analysis. All sections were examined using a light microscope (Olympus, Tokyo, Japan) at a magnification of 400×.

Pre-antral follicular density

Follicular density in the ovarian cortex was calculated as the total number of follicles divided by the total tissue area and expressed as the number of follicles per mm^2 of ovarian tissue. For this, sections were scanned using a Mirax Scan (Zeiss, Jena, Germany) and pictures were taken at a magnification of 100×. With the help of the image processing and analysis program Image J (National Institutes of Health, Bethesda, MD, USA), primordial, primary and secondary follicles were counted and density calculated per mm^2.

Immunohistochemistry

The following markers were selected to assess cell proliferation, follicle growth and function, stromal cell apoptosis and graft vascularisation: Ki67, anti-Müllerian hormone (AMH), growth differentiation factor-9 (GDF-9), c-kit and cluster of differentiation 31 (CD31). Paraffin sections were deparaffinised with Histosafe (Yvsolab SA, Beerse, Belgium) and rehydrated in alcohol series. After blocking endogenous peroxidase activity with 3% H_2O_2 diluted in methanol, a demasking step was performed for 75 min at 98°C with citrate buffer and Triton X100 before the sections were subjected to an antigen retrieval step. Antigen retrieval steps, antibody dilutions and incubation conditions were performed as previously described (Scalercio *et al.* 2015). Diaminobenzidine was used as a chromogen (Vector Laboratories, Peterborough, UK). The slides were then counterstained with haematoxylin and mounted with DPX (Distrene, Plasticiser, Xylene) neutral mounting medium (Prosan, Merelbeke, Belgium). Negative controls consisted of the dilution solution without any primary antibody and the following positive controls were used: hepatic carcinoma for GDF-9, human antral follicles for AMH, testicular germ-cell tumour tissue for c-kit, human uterus for CD31 and proliferative endometrium for Ki67. All positive controls were processed in the same way as the tested tissues. For quantitative analysis of GDF-9, AMH, c-kit and Ki67 protein expression, primordial, primary and secondary follicles were evaluated in all animals. Follicles were considered to be GDF-9-positive when the oocyte cytoplasm was immunostained; AMH-positive follicles presented at least one granulosa cell immunostained; Ki67 staining was used to attest the growing status of follicles and to ensure if follicles classified as primordial were indeed at resting stage or being activated. Follicles containing at least one Ki67-positive granulosa cell were considered as belonging to the growing pool. Ki67 was also used to detect stromal cells proliferation by counting positive cells in random areas of 100 mm^2 in every tissue section applying Image J software. For CD31 immunostaining, depending on the size of the graft, between three and five slides were scanned by Mirax Scan (Zeiss) and visualised using the Mirax Viewer software (Carl Zeiss MicroImaging GmbH, Jena, Germany). The area around each ovarian tissue graft was defined and all vessels present in the grafts were counted.

Fibrosis

Relative areas of fibrosis were evaluated using trichrome and picrosirius red stain. Regarding Masson's trichrome analysis, fibrotic areas were characterised by poor cellularity, as evidenced by a low number of cell nuclei and collagen deposits as previously described (Dath *et al.* 2010). Masson's trichrome staining turns the tissue green, showing that it has been replaced with collagenous connective tissue, making fibrotic areas easily recognisable. Sections were scanned by Mirax Scan and fibrotic areas and total section areas were delimited with the freehand tool and then measured using the Mirax Viewer program.

Fibrosis was determined by calculating the percentage of fibrotic areas per analysed area.

To evaluate collagen density, picrosirius red staining plus polarisation microscopy were applied. Fibrotic areas were characterised by rich collagen deposits. Picrosirius is a strong anionic dye that attaches to collagen fibers turns the tissue green showing that it has been replaced with collagenous connective tissue, becoming fibrotic areas easily recognisable. When evaluated by light microscopy, collagen is stained red, while cytoplasm is stained in yellow. Under polarised light microscopy, collagen type I is stained yellow orange birefringence, while collagen type III is stained green birefringence. Collagen area was determined as previously described (Rich and Whittaker 2005). In brief, types I and III collagen were calculated as a percentage of the area of each image (expressed in pixels). To avoid counting non-collagen fibers, a colour separation on the original (circularly polarised) images, resolving each into its cyan, yellow, magenta and black components. Collagen area calculation was performed automatically with the help of the image processing and analysis program Image J.

Analysis of DNA strand breaks for the detection of apoptotic cells

Apoptosis was analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) to detect DNA fragmentation. Paraffin sections were deparaffinised with Histosafe, rehydrated in alcohol series and washed in running de-ionised water. The slides were subsequently pre-treated with 20 $\mu g\,mL^{-1}$ proteinase K working solution (Roche Applied Science, Penzberg, Germany) in 10 mM TRIS-HCl (pH 7.5) for 30 min at 37°C in a humidified chamber. DNA fragmentation was detected by means of the *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and analysis was performed as described previously (Martinez-Madrid *et al.* 2007). Human tonsil tissue was used as a positive control and negative control sections were incubated with label solution without enzyme solution. Follicles with ≥49% of their granulosa cells staining positive for TUNEL were considered to be atretic, as were those follicles with TUNEL-positive oocytes. The population of stromal cells was analysed in a 100 mm^2 area randomly assigned in the sections.

Statistical analyses

All data are presented as mean ± standard error (s.e.m.) and the follicle, vessels or the stromal cells were the unit of analysis. The results were analysed using GraphPad Prism 6.04 software (La Jolla, CA, USA) and compared with two-way ANOVA. Values of at least $P < 0.05$ were considered to be statistically significant.

Results

Graft harvesting

One week after transplantation all grafted fragments were recovered from the five squirrel monkeys. They had the same size and shape as the tissue before transplantation and were connected to the surrounding tissue through numerous vessels. No antral follicle nor corpus luteum was seen. All females were cycling after the study.

Ovarian follicle analyses

Morphology and density of pre-antral follicles

A total of 1066 pre-antral follicles was evaluated: 689 from control tissue, 162 from tissue grafted without Trolox exposure and 215 from Trolox-treated graft. The percentages of morphologically normal primordial, primary and secondary follicles in control ovarian tissue were 72.6 ± 9.1%, 21.4 ± 7.2% and 6.0 ± 2.0%, respectively. Similar proportions were observed in Trolox-treated grafts, i.e. 72.7 ± 8.9%, 24.8 ± 7.3% and 2.5 ± 2.0% of primordial, primary and secondary follicles, respectively. When compared with control, grafts without Trolox pre-treatment presented a significant decrease in the proportion of morphologically normal primordial follicles (49.9 ± 5.1%), with a concomitant significant increase in the proportion of primary ones (47.4 ± 4.9%); rates of secondary follicles were similar to control (2.7 ± 2.0%) (Fig. 1). Independently of the Trolox treatment, grafting resulted in a significant decrease in the density of primordial follicles when compared with control (Fig. 1.).

Follicle survival, growth and function

The proportion of apoptotic primary and secondary follicles was significantly higher in grafted tissue without Trolox pre-treatment than in all the other groups (Fig. 2.). Follicle growth after grafting was confirmed by Ki67 immunostaining. The population of recruited primordial follicles (i.e. containing Ki67-positive granulosa cells in untreated grafts) was significantly higher compared with control, where none of the primordial follicles were positive for Ki67. No differences were observed when the percentages of proliferating granulosa cells in primordial follicles from Trolox-treated grafts were compared with control ones or with untreated grafts. The percentage of primary and secondary follicles with Ki67-positive granulosa cells was higher in the group that was not supplemented with Trolox, but this difference was not significant (Fig. 2).

AMH staining was absent in primordial follicles, independent of the treatment. Primary and secondary follicles from control samples expressed AMH, whereas those from untreated grafts did not. Trolox-treated grafts, however, presented secondary follicles positively stained for AMH. GDF-9 staining ranged from faint (primordial and primary follicles) to strong (secondary follicles) and it was observed in the oocyte cytoplasm and granulosa cells. All primordial, primary and secondary follicles from control samples were positively stained. Similarly, follicles from grafts, either treated or not treated with Trolox, were positive for GDF-9, except for the primordial follicles, which were not stained. Immunostaining was performed to identify c-kit protein in the oolemma of pre-antral follicles. In all treatments, c-kit immunostaining was found to be positive in primordial, primary and secondary follicles. Images of immunostaining are depicted in Fig. 3.

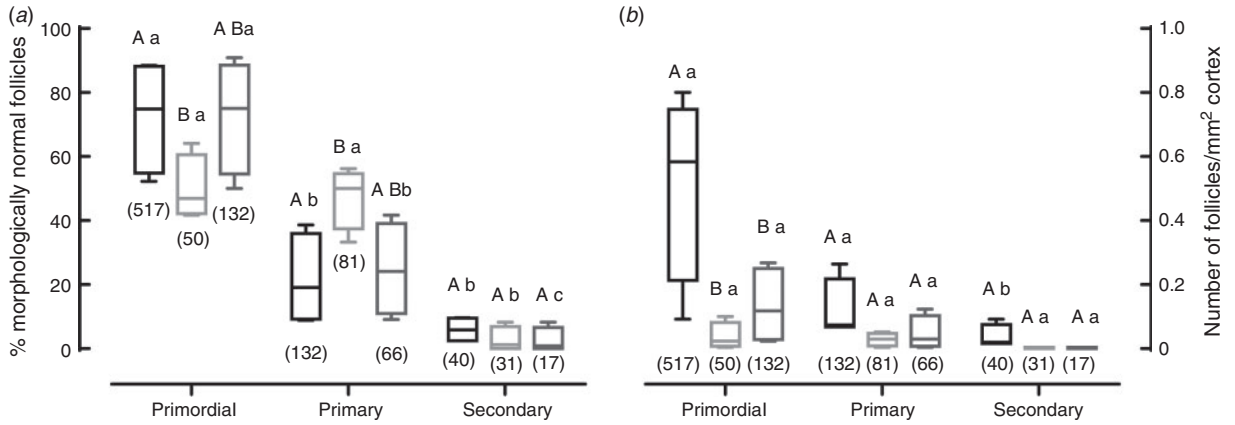

Fig. 1. (*a*) Percentages of morphologically normal follicles (primordial, primary and secondary) in control tissue (black box) and after grafting without (light grey box) or with (dark grey box) Trolox pre-treatment. (*b*) Density of follicles (primordial, primary and secondary; number per mm^2) in control tissue (black box) and after grafting without (light grey box) or with (dark grey box) Trolox pre-treatment (right panel). The number of evaluated follicles per class is given within the brackets under each box plot. Different upper-case letters (A, B) indicate significant differences between groups within the same follicular class. Different lower-case letters (a–c) indicate significant differences between follicular classes within the same treatment group.

Ovarian tissue analyses

Tissue vascularisation after grafting

Tissue vascularisation was confirmed by counting the number of vessels per mm^2 in the histological sections. The mean number of vessels per mm^2 in the sections identified in the ovarian tissue grafted without (0.43 ± 0.16) and with Trolox (3.35 ± 2.54) did not differ significantly from the number of vessels found in control (0.41 ± 0.22; Fig. 4*a*). Representative immunostaining images for CD31 in control and after grafting of tissue pre-treated with Trolox are shown in Fig. 4*b*, *c*.

Stromal cell survival and proliferation

Grafting did not result in increased apoptosis, but exposure to Trolox significantly decreased the percentage of apoptotic stromal cells. While control tissue and grafts not pre-treated with Trolox presented mean percentages of $14.13 \pm 2.96\%$ and $12.50 \pm 2.84\%$ apoptotic cells, respectively, exposure of tissue to Trolox resulted in $1.15 \pm 0.39\%$ apoptotic cells (Fig. 4*d–f*).

After grafting, cell proliferation was significantly increased compared with control ($0.47 \pm 0.20\%$ Ki67-positive stromal cells). In untreated grafts, the percentage of Ki67-positive stromal cells was $1.99 \pm 0.50\%$ and in Trolox treated grafts $1.37 \pm 0.25\%$ of stromal cells were Ki67-positive (Fig. 4*g–i*). However, cell density was not significantly increased. While in control tissue there were 55.6 ± 8.3 stromal cells per mm^2, grafts without and with Trolox pre-treatment presented 81.8 ± 18.5 and 74.5 ± 6.3 stromal cells per mm^2, respectively.

Quantification of ovarian tissue fibrosis

Fibrosis was expressed as fibrotic area visible after Masson's trichrome or Picrosirius staining. Total evaluated areas measured 3282 ± 1573 mm^2, 3161 ± 495 mm^2 and 2371 ± 496 mm^2 in control, untreated graft and Trolox-treated graft, respectively. Based on Masson's trichrome staining, fibrotic areas increased significantly in Trolox-treated grafts ($31.1 \pm 1.5\%$) when compared with control ($3.3 \pm 0.7\%$) or untreated grafts ($15.5 \pm 4.7\%$; Fig. 4*j–l*).

Picrosirius staining revealed that the area with fibres increased in grafted tissue without ($30.8 \pm 2.1\%$) and with ($26.5 \pm 1.7\%$) Trolox pre-treatment when compared with control ($18.5 \pm 2.8\%$). When distribution of collagen Types I and III was evaluated, grafts pre-treated with Trolox presented significant higher levels of collagen I ($7.5 \pm 1.2\%$) than untreated grafts ($1.4 \pm 0.4\%$) or control ($0.6 \pm 0.1\%$). No differences were observed when levels of collagen Type III were evaluated (Fig. 4*m–o*).

Discussion

In the present study we have shown the importance of treating ovarian tissue with Trolox before grafting. As the initial moment of grafting survival is decisive to follicular survival, we evaluated both follicular and stromal quality 7 days after transplantation. It is imperative to note that, in a pilot study (data not shown), ovarian fragments ($\sim 4 \times 4 \times 1$ mm) were collected for the evaluation of follicular quality immediately after tissue collection and exposure to medium with and without Trolox, i.e. before grafting. As no differences in the percentages of morphologically normal follicles were observed from control, complete data analyses herein presented are focussed on the effect of tissue pre-incubation with Trolox followed by grafting.

No particular changes were observed in the grafts at the moment of harvesting, i.e. no tissue losses or shape changes, nor absence of vascularisation. Not surprisingly, no antral follicles were found in the transplants. Loss of follicles in more advantage stages is common because of their greater susceptibility to ischaemia than primordial follicles (Demeestere *et al.* 2009).

Although primordial, primary and secondary follicles were found in the recovered transplants, their density was decreased in the grafts as previously reported in fresh transplants of ovaries from mice (Liu *et al.* 2008), sheep (Salle *et al.* 1999), goats

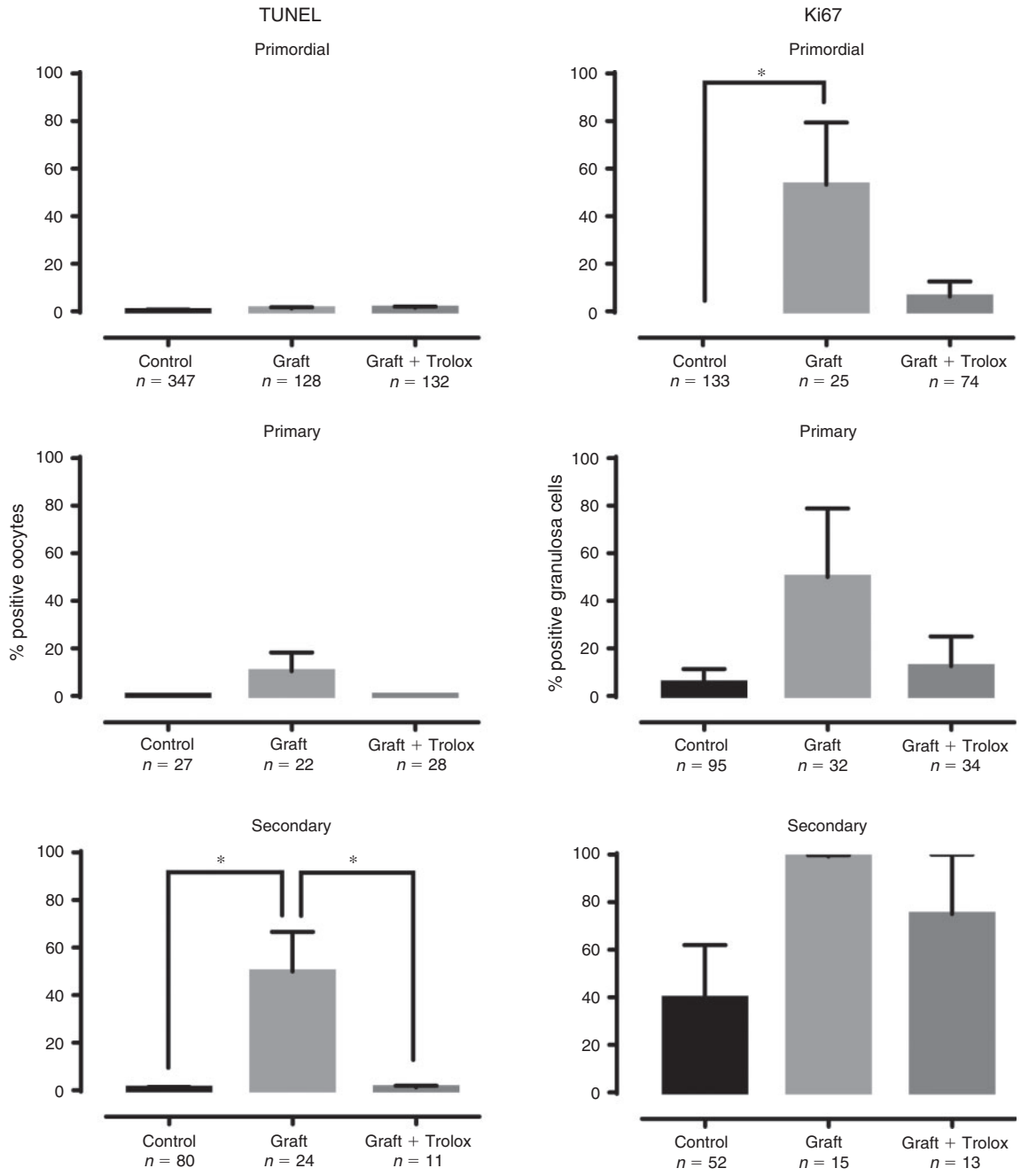

Fig. 2. (*a*) Proportion of follicles with apoptotic oocytes after TUNEL immunostaining. (*b*) Proportion of follicles with Ki67-positive granulosa cells. * indicates significant differences ($P < 0.05$) between treatment groups within the same follicular class.

(Santos *et al.* 2009), cows (Gavish *et al.* 2014) and women (Oktay *et al.* 1998). All these authors agree that follicular loss is a result of ischaemia before revascularisation of the graft. However, this is not the single source of follicular depletion; it is also due to the massive recruitment of primordial follicles (Gavish *et al.* 2014). Comparably to Gavish *et al.* (2014), we also observed that grafting resulted in a decrease in the population of primordial follicles with an associated increase in the number of primary follicles. In the present study, recruitment of a large number of primordial follicles in the untreated grafts was confirmed by the Ki67 immunostaining of their granulosa cells. Loss of follicles after transplantation can be partially due to lipid peroxidation, which leads to ischaemia–reperfusion injury (Liu *et al.* 2002). When tissue was treated with Trolox before grafting, no extreme follicular activation was observed. As Trolox has a protective effect on cells through inhibition of

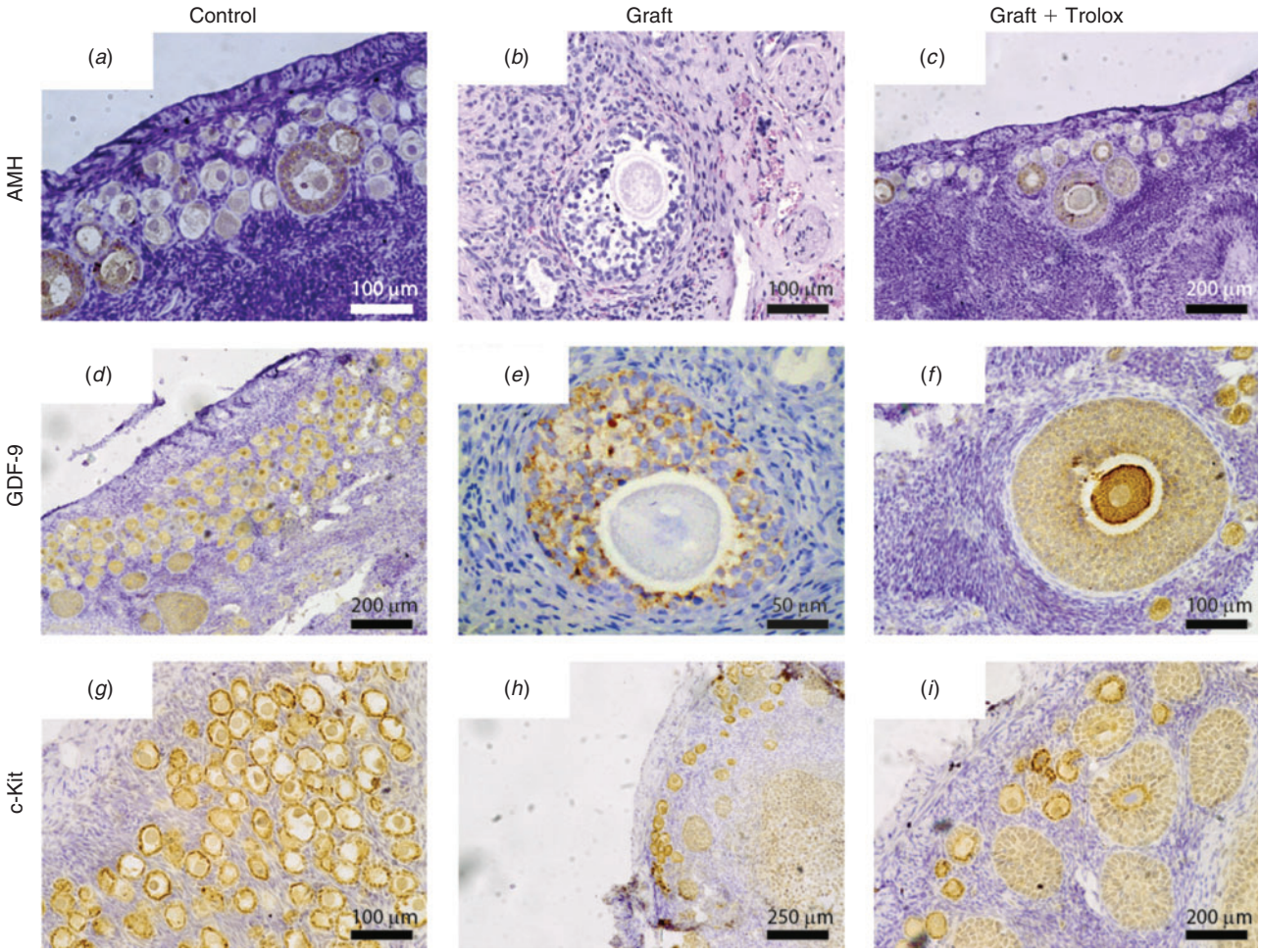

Fig. 3. Immunohistochemical staining for (*a*–*c*) AMH, (*d*–*f*) GDF-9 and (*g*–*i*) c-kit in granulosa cells of secondary follicles in (*a*, *d*, *g*) control, grafted tissue (*b*, *e*, *h*) without and (*c*, *f*, *i*) with previous exposure to Trolox.

peroxidation (Du *et al.* 2014), it probably had a positive effect avoiding the exaggerated activation of primordial follicles caused by ischaemia–reperfusion and oxidative stress (Baird *et al.* 1999; Kim *et al.* 2004; Soleimani *et al.* 2011; Friedman *et al.* 2012). Oxidative stress not only leads to uncontrolled activation of primordial follicles in grafts, but is also related to follicular apoptosis (Tilly and Tilly 1995). This is in line with our study, in which we also observed increased apoptotic rates in primary and secondary follicles from grafts not treated with the antioxidant Trolox, which works on the apoptosis pathway (Forrest *et al.* 1994; Salgo and Pryor 1996; Quintanilla *et al.* 2005).

We investigated follicle function using AMH, GDF-9 and c-kit, as they are expressed by granulosa cells, oocyte cytoplasm and oolemma, respectively, in follicles from squirrel monkeys, and play an indispensable role in the folliculogenesis process (Scalercio *et al.* 2015). AMH is a member of the transforming growth factor β (TGFβ) superfamily, known to have an inhibitory effect on primordial follicle activation (Visser *et al.* 2006). Absence of AMH expression in primordial follicles from squirrel monkeys was expected, as it was recently reported (Scalercio *et al.* 2015). A low AMH level, normally produced by the pool of developing follicles in intact ovaries and after ovarian tissue transplantation, promotes massive follicular recruitment (Visser and Themmen 2005). In the present study, growing follicles from untreated grafts were negative for AMH immunostaining. Based on this, we can suggest that the lower population of primordial follicles found in the untreated group was probably due to the absence in AMH expression by growing follicles. GDF-9, another member of the TGFβ superfamily, has a fundamental function in early folliculogenesis (Dong *et al.* 1996), participating in recruitment of primordial follicles and proliferation of granulosa cells (Paulini and Melo 2011). Except primordial follicles from untreated and Trolox-treated grafts, most primary and secondary follicles from both groups were positive for GDF-9 staining. In a previous study, Wang *et al.* (2013*a*) demonstrated the absence of GDF-9 expression in primordial follicles from transplanted mouse ovaries, which was probably an indicator of low-quality follicles with possible subsequent retarded development. c-Kit immunostaining was

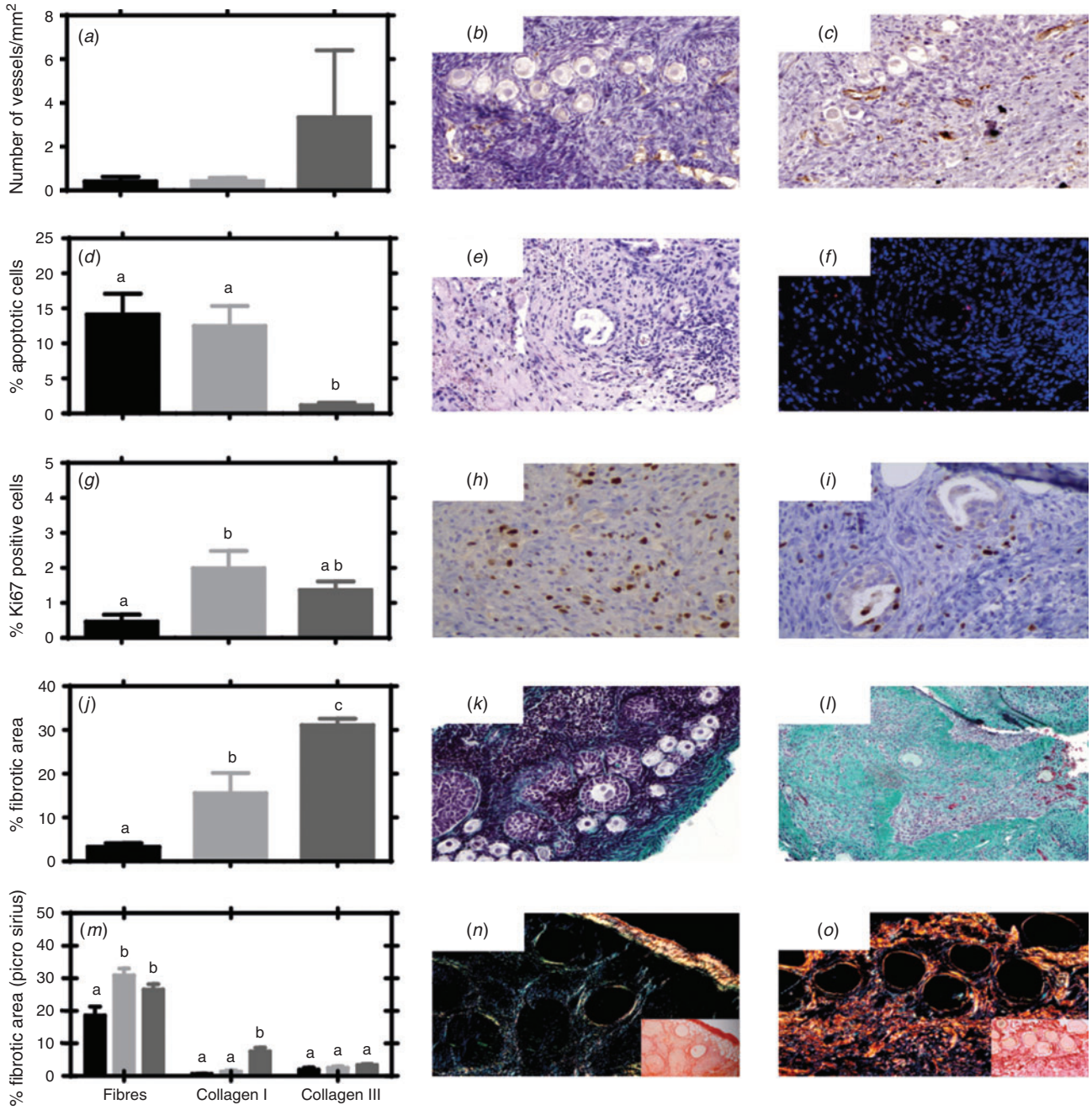

Fig. 4. (*a*) Mean (± s.e.m.) number of vessels per mm^2 and percentages of (*d*) apoptotic cells, (*g*) proliferating cells, (*j*) fibrotic area in control tissues as well as after grafting with and without pre-treatment with Trolox. (*m*) Detailed fibre staining with picrosirius to detect fibres and collagen Types I and III. Representative immunostaining images for (*b*) CD31 in control and (*c*) after grafting of tissue pre-treated with Trolox; (*e*) stromal cells stained with haematoxylin–eosin; (*f*) TUNEL staining to detect apoptotic cells (4′,6-diamidino-2-phenylindole (DAPI) counterstaining (cells in blue) and TUNEL staining (cells in red) are merged); representative images of Ki67 positively stained (brown) stromal cells in (*h*) control and (*i*) Trolox-treated graft; fibrotic tissue evidenced by Masson's trichrome staining in (*k*) control and (*l*) Trolox-treated graft (green staining is a marker for fibres); picrosirius staining of (*n*) control and (*o*) Trolox-treated graft (images were obtained after polarisation and small boxes indicate staining without polarisation. Different lower-case letters (a–c) indicate significant differences between treatment groups.

observed at all stages of follicle development after grafting in both groups, with a similar pattern of expression of this factor when compared with control ovarian tissue. As with our results, David *et al.* (2011) demonstrated c-kit expression in the oolemma of pre-antral follicles; the pattern of expression of c-kit was similar after cryopreservation and transplantation to that of fresh ovarian tissue. However, is important to bear in mind that expression of c-kit was investigated only in morphologically

normal follicles, so we cannot assert if any alteration in c-kit expression would have played a role in the death of follicles after grafting (Jin *et al.* 2005).

No difference was found in the number of vessels between control and grafts, treated or untreated. Although Trolox did not exert any effect on tissue revascularisation, its combination with angiogenic factors appears suitable as a strategy for the next procedures of ovarian tissue grafting. Schnorr *et al.* (2002) suggested that treatment of cryopreserved grafts with VEGF improved tissue vascularisation but not tissue viability. In a 6-week study, human fresh grafts were xenotransplanted to rabbits. Both tissue and hosts were treated with a combination of VEGF and basic fibroblast growth factor (bFGF), resulting in increased tissue vascularisation and survival, but follicular loss was inevitable (Wang *et al.* 2013*b*). These authors also observed that graft treatment decreased apoptotic rates, even when compared with control tissue. Similarly, in the present study, Trolox was also responsible for decreasing apoptosis not only in ovarian follicles but also in stromal cells, due to its anti-apoptotic effect (Forrest *et al.* 1994; Salgo and Pryor 1996; Quintanilla *et al.* 2005). Independently of the pre-treatment with Trolox, grafts presented an increased rate of stromal cell proliferation when compared with control, probably because Trolox had no effect on vascularisation. Studying xenografts, Soleimani *et al.* (2011) showed a positive correlation between ovarian stromal cell proliferation and tissue vascularisation. Although we have observed increased proliferation in the graft stromal cells, cell density was decreased. Improving vascularisation, therefore, may also improve stromal cell survival, as shown by Dath *et al.* (2011).

An intriguing finding in the present study was the significant increase in fibrotic tissue after exposure to Trolox followed by grafting. Oxidative stress plays an important role in the development of fibrotic responses. Although the antioxidant Trolox is claimed to be an anti-fibrotic agent (Galicia-Moreno *et al.* 2008, 2013), it is well known that antioxidant vitamins, mainly E and C, increase collagen turnover (Archile-Contreras *et al.* 2011) and protect collagen against damage (Plessinger *et al.* 2000). This was confirmed in the present study after picrosirius staining, where although the levels of collagen Type III were unchanged in the grafts, grafts exposed to Trolox presented a significant increase in the synthesis of collagen Type I. Fibrosis may also indicate damage in the ovarian structure and, consequently, loss of ovarian follicles, including primordial ones (Meirow *et al.* 2007). However, in the present study, follicular survival was increased in the presence of Trolox and massive activation was avoided. Likewise, Nisolle *et al.* (2000) showed that ovarian tissue fibrosis does not affect the population of healthy primordial and primary follicles. However, we assume that concerns should be taken into account when considering further development of these follicles.

In conclusion, this study shows that graft pre-incubation with Trolox improves survival of pre-antral follicles, protects primordial follicles from massive activation, decreases apoptotic rates in ovarian follicles and stromal cells, but also increases fibrotic areas in the tissue, which might affect follicle density in the tissue. As Trolox did not improve tissue revascularisation, its combined use with angiogenic factors might improve cell density after grafting. Moreover, the beneficial influence of graft tissue pre-treatment with Trolox needs to be confirmed after long-term grafting.

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