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From Cell Biology to Tissue Engineering

Epigallocatechin-3-gallate protects the testis from damage generated by experimental cryptorchidism in rabbits

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Summary. Cryptorchidism (CO) is a risk factor for infertility in men. It is associated with an increase in oxidative stress which alters the differentiation of the gonocytes to spermatogonia. Epigallocatechin-3-gallate (EGCG) is an antioxidant that acts as a free radical scavenger and activates the antioxidant enzymes. The aim of this work was to investigate if EGCG plays a role in the protection of the testicle from alterations generated by CO and its possible mechanism. Male rabbits 7 days old were divided into four groups and distributed as follows: 1) control (C) treated with EGCG vehicle (V) (C/V); 2) C with administration of EGCG from 65 to 120 days postpartum (dpp) (C/EGCG); 3) CO induced by administration of 17β-estradiol plus EGCG vehicle (CO/V) and 4) CO plus EGCG administration (CO/EGCG). The animals were euthanized at 120 dpp and their testes were processed to evaluate lipid peroxidation, activities of superoxide dismutase (SOD) and catalase (CAT) enzymes as well as serum testosterone (T) concentrations. In addition, the rates of apoptosis, cell proliferation and histological alterations were determined. The CO/EGCG group showed a significant reduction in lipid peroxidation, a significant increase in the anti-oxidant enzyme activities and concentrations of T. Also, there was a significant decrease in the histological alterations, absence of gonocytes and active spermatogenesis when compared with CO/V group. These results show that EGCG reduces lipid peroxidation and increases the activity of the endogenous anti-oxidant system which protects the testes from alterations produced by oxidative stress generated during experimental CO.

Key words: Cryptorchidism, Epigallocatechin-3-gallate, Testis, Oxidative stress, Antioxidant enzymes

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Introduction

In recent years the worldwide incidence of Cryptorchidism (CO) or undescended testis has increased. It is estimated to occur in 1.8% to 8.4% of live births (Virtanen and Toppari, 2008). The prevalence varies among populations (Virtanen and Toppari, 2008) and specifically in those with different ethnic origin (Thorup et al., 2010). The increase in the incidence of CO may be related with exposure to compounds with estrogenic activity during the gestational stage (Damgaard et al., 2006; Virtanen and Adamsson, 2012; Fernández et al., 2016).

The importance of studying this pathology lies in its long-term effects such as increased risk of developing germ cell neoplasia *in situ* (GCNIS) (Rajpert-De Meyts, 2006; Ferguson and Agoulnik, 2013) and infertility in the adult age. Paternity rates are similar in the general population for men with only one affected testis, but are lower (33-65%) for those with bilateral CO (Barthold, 2008). The histology of CO is normal at birth; however,

after one year of age, it becomes progressively abnormal and at two years 40% of CO begin to lose their germ cells (Hutson, 2006). This event usually occurs when there is a lack of differentiation of the gonocytes (pre-spermatogonia) to spermatogonia in the early postnatal stage (Hadziselimovic et al., 2005; Docampo and Hadziselimovic, 2015), which brings about an interruption in future spermatogenesis.

In CO, the histological alterations have been associated with high oxidative stress, generated by over-production of free radicals and reduction in the activities of antioxidant defense system, such as superoxide dismutase (SOD) and catalase (CAT) enzymes, reported in cryptorchidism (Ahotupa and Huhtaniemi, 1992; Zini and Schlegel, 1997). Therefore, it is of paramount importance to search for agents that would reduce the damage done by oxidative stress in CO.

Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea (*Camellia sinensis*). Among its properties are its ability to act as a free radical scavenger and activator of endogenous antioxidant system (Yang et al., 2001; Büttemeyer et al., 2003; Al-Maghrebi et al., 2012; Dong et al., 2016). Hence, the objective of the present study was to determine if the testicular histological alterations produced as a consequence of experimental CO are reduced with the administration of EGCG by a mechanism that involves a decrease in oxidative stress and the activation of endogenous antioxidant system.

Materials and methods

A total of 20 male newborn rabbits of the chinchilla race (*Oryctolagus cuniculus*) were used. The rabbit is an excellent animal model used in biomedical research including reproduction (Simón et al., 2018). It offers several advantages, among which is that its period of pre-spermatogenesis exceeds that of rat, the species most used in research. This particular characteristic makes the development of rabbit closer to that of human (Vigueras et al., 2013; Manku and Culty, 2015). Moreover, the differentiation of gonocytes to spermatogonia in rabbit occurs after or during testicular descent, which is similar to what happens in humans, but contrary to what is seen in rats, where this process takes place before the end of testicular descent (Vigueras et al., 2013; Manku and Culty, 2015). In addition, non-specific degenerative changes in testes of clinically healthy rabbits are similar to the alterations seen in human spermatogenesis (Holstein, 1986; Sharpe, 2010; Vigueras et al., 2013) which indicate that the rabbit model is closer to human testicular histology, unlike the rat, which presents testicular cytoarchitecture with minimal alterations. This reaffirms the importance of the use of the rabbit as a study model for research in the field of masculine reproductive biology.

The animals were maintained in a room with a 16:08 h light/dark cycle and in stainless steel cage (90x60x40 cm) with their mothers until weaned at 40 days postpartum (dpp). Thereafter, they were separated and individually kept in a stainless steel cage (90x60x40 cm). All animals had free access to food (Purina® rabbit chow, Mexico) and water. Animal handling was carried out in compliance with the Official Mexican Standard Norms NOM-062-ZOO-1999 "Technical specifications for the production, care and use of laboratory animals". The study was approved by the Institution's Animal Care and Use Committee.

The animals were randomly distributed into 4 groups and treated as follows: 1) control (C) plus EGCG vehicle (V) ip from 65 to 120 dpp (C/V); 2) C plus administration of EGCG at 5 mg/kg ip from 65 to 120 dpp (C/EGCG); 3) CO induced plus EGCG vehicle ip from 65 to 120 dpp (CO/V) and 4) CO plus administration of EGCG at 5 mg/kg ip from 65 to 120 dpp (CO/EGCG).

CO was induced by subcutaneous administration of 0.12 mg of 17 β-estradiol (Sigma-Aldrich, St Louis, MO, USA) dissolved in a solution of 50 µl of propylene glycol vehicle and administered every three days from 7 to 60 dpp (Veeramachaneni, 2006; Vigueras et al., 2016). The testicular descent is approximately at 50 dpp in the chinchilla rabbit. In this model, the second phase of the testicular descent is inhibited. The administration of 17β-estradiol vehicle in a control group was not considered, since propylene glycol used as vehicle does not generate any alteration in the testes (Vigueras et al., 2016). EGCG (Cayman Chemical, Ann Arbor, Michigan, USA) (5 mg/kg ip) was dissolved in physiological saline solution (0.9% NaCl) and was administered (100 μl) from 65 to 120 dpp. In this last age (120 dpp), the body weight of the animals were measured and registered, and immediately sedated with xylazine (10 mg/kg, im, PiSA, Atitalaquia Hidalgo, Mexico) followed by an overdose of sodium pentobarbital (90 mg/kg, ip; Pfizer, Toluca, Estado de Mexico, Mexico) diluted in physiological saline solution (1:1) to induce euthanasia. Blood was immediately obtained through cardiac puncture and used to determine serum testosterone. The testes of the animals were extracted, weighed and dissected to determine gonadosomatic index (GI) defined as the ratio of testicular weight/body weight. The determination of lipid peroxidation (indirect measure of oxidative stress), the activities of SOD and CAT enzymes, apoptosis and cellular proliferation, as well as morphological analysis were performed from samples obtained from the animal testicular tissue.

Evaluation of lipid peroxidation as an indirect measure of oxidative stress

The production of thiobarbituric acid-reactive substances (TBARS) was measured using the modified technique described by Rios and Santamaria (1991). 1 ml aliquot containing the homogenized testis was added to 2 ml of thiobarbituric acid (TBA) reagent (TBA 0.375 g, trichloroacetic acid 15 g and HCl 2.5 ml in 100 ml of

distilled water), and the final solution (total volume=3 ml) was heated in a boiling water bath for 30 min. Samples were cooled in ice bath and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatants was spectrophotometrically measured at 532 nm. Concentrations of TBARS were calculated by the interpolation of malondialdehyde periodic oxidation standard curve. The final result was expressed as nmol of TBARS per mg of protein. For the protein concentration, the Lowry method was used with a Protein Kit (Micro Lowry, Modification) (Sigma-Aldrich) following the manufacturer's instructions. The results of lipid peroxidation were normalized to the protein content in each sample.

Determination of superoxide dismutase (SOD) enzyme activity

The activity of SOD was determined using superoxide dismutase colorimetric detection kit (Enzo LifeSciences, Inc., Farmingdale, New York, USA). This test determines the capacity of SOD to reduce the concentration of superoxide ions generated from the conversion of xanthine and oxygen into uric acid and hydrogen peroxide by the action of xanthine oxidase enzyme. The activity of SOD was determined from the percentage of inhibition of the formation rate of WST-1-formazan, a product that absorbs light at 450 nm. The absorbance was read every minute for 10 min at a room temperature (Pan et al., 2015). The amount of SOD in the samples was calculated by correlating the inhibition percentage of WST-1-formazan formation with the logarithm of SOD units in a standard calibration curve. The results of SOD activity were expressed in U/mg of protein.

Determination of catalase (CAT) activity

The activity of CAT was determined using Catalase Fluorometric Detection Kit (Enzo LifeSciences). This was performed through H_2O_2 degradation reaction catalyzed by CAT at room temperature and was calculated in terms of μmol of H_2O_2 consumed per minute (U) per mg of protein. A non-fluorescent detection reagent was used to measure the remaining substrate (H_2O_2) of the reaction. The results of CAT activity were expressed as U/mg of protein (Sun et al., 2016).

Determination of testosterone

Testosterone (T) levels were determined by automated chemiluminescent microparticle immunoassay, the Architect® 2nd generation testosterone (Abbott Diagnostics, Abbott Park, IL, USA), as describe by the manufacturer. The functional sensitivity was calculated to be 0.14 ng/mL (95% confidence interval of 0.11 to 0.17 ng/mL).

Processing testicular tissue for morphological analysis

Testicular tissue samples were fixed with Karnovsky solution, postfixed with 1% OsO_4 and processed for inclusion in EPON 812 (Ted Pella, Inc., Redding, CA, USA). Semi-thin 1 µm-thick sections of the processed testes were obtained with ultramicrotome Leica UCT (Leica, Vienna, Austria) and stained with 5% toluidine blue and mounted on slides. The analysis was performed by a single observer with an Olympus BX 51 optical microscope (Tokyo, Japan). For each individual, 30 cross sections of tubes and/or seminiferous cords were analyzed.

The area of seminiferous epithelium was determined using Image-Pro® Plus (version 5.1. Media Cybernetics, INC. MD, USA) program and the presence of gonocytes and spermatogonia was quantified and expressed as the number of cells in 10,000 μm^2 of tissue. The maturation index of the seminiferous epithelium was determined using Johnsen Index. A score from one (no cells) to ten (complete spermatogenesis with many late spermatids) was assigned to each seminiferous tubule depending on the main cell-type present (Johnsen, 1970). Moreover, the histopathological index was determined. This was done by assigning values from 1 to 6 to the damage observed and then the index was calculated by the sum of the scores (Vigueras et al., 2009).

Determination of apoptosis and cell proliferation

Testicular tissue samples were fixed in paraformaldehyde at 4% for 24 h and then processed for inclusion in paraffin. Tissue sections of 5 µm thick were obtained with a microtome (Leica RM 2155; Microsystems, Nussloch Gmbh, Germany) and mounted on slides covered with poly-l-lysine.

To determine apoptosis, slides with tissue sections were deparaffinized with xylene, hydrated in a graded ethanol series and placed in a blocking solution of 0.3% H_2O_2 in methanol. Thereafter, the slides were removed from the blocking solution and rinsed with phosphate-buffered saline solution (PBS; 0.1M, pH 7.4) and incubated with permeation solution of Triton X-100 at 0.1% (Sigma-Aldrich). Subsequently, the sections were washed with PBS and incubated in TUNEL solution (*In situ* Cell Death Detection Kit, Roche Diagnostic Corporation, IN, USA). Thereafter, they were rinsed with PBS and mounted with fluorescent medium with DAPI (DAKO, USA, Fluorescent Mounting Medium, Carpinteria, CA, USA).

To evaluate cell proliferation, paraffin sections of 5 μm thickness of each animal were deparaffinized and hydrated. Tissue sections were immersed in boiling antigen retrieved solution (sodium citrate buffer, 10 mM, pH 6.0). The sections were then washed in distilled water and PBS. Sections were incubated in a humid chamber with proteinase K (Boehringer Mannheim, GMBH, Germany) and washed with PBS. Nonspecific

binding sites were blocked with donkey serum albumin at 5% (Jackson Immunoresearch, USA) for 2 h. Subsequently, the tissue sections were incubated with mouse-monoclonal antibodies against Ki67 (DAKO) at a dilution of 1:100 for 24 h at 4°C. Then, the sections were incubated with biotinylated goat anti-mosue IgG (Invitrogen®, USA) at a dilution of 1:200 for 2 h at room temperature. The sections were incubated in avidin-biotin-horseradish peroxidase solution (ABC kit, Vectastain Vector Labs, Burlington, ON, Canada) at room temperature for 1h. The antigen-antibody-peroxidase complex was visualized with diamino-benzidine (Biocare Medical, Concord, CA, USA) in accordance with the manufacturer's instruction. The sections were counterstained with haematoxylin, dehydrated and coverslipped with Entellan (Merck, KGaA, Darmstadt, Germany). Apoptosis and proliferation index were determined in at least 30 tubes or seminiferous cords per animal by quantifying the number of positive cells expressed per 10,000 μm^2 of tissue.

The data were expressed as the mean ± SEM. The parametric data were analyzed using one-way ANOVA and Tukey *post hoc* comparison. The non-parametric data were compared using Kruskall-Wallis test. Values of $p<0.05$ were considered significant.

Results

The testes of the control animal groups (C/V and C/EGCG) were found to be located in the scrotum following treatment, unlike the animals in the CO group where the testes had a bilateral inguinal position.

With regard to sexual maturity, there was a significant reduction ($p<0.05$) in the gonadosomatic index (GI) of CO/V group when compared with the rest of the groups ($p<0.05$) (Table 1). In CO/EGCG group, the administration of EGCG significantly increased by twice the values of GI when compared with CO/V group ($p<0.05$); nevertheless, this increase did not reach the values of the control groups. It is necessary to consider that the CO/V and CO/EGCG groups continued with cryptorchidism. A similar effect was observed in C/EGCG group, where the IG increased significantly ($p<0.05$) by 16.6% when compared with C/V group (Table 1).

Lipid peroxidation values in C/V group was observed to be basal (Table 1) but decreased in C/EGCG group. In CO/V group, there was a significant increase ($p<0.05$) in the lipid peroxidation value when compared with what was observed in the control groups. The reverse of this occurred in the animal group CO/EGCG where lipid peroxidation value was found to decrease significantly ($p<0.05$) by 28% with respect to CO/V group, although without reaching the values observed in the C/V group.

The activities of CAT and SOD enzymes showed a significant increase ($p<0.05$) in the C/EGCG group when compared with the C/V group. These activities witnessed a significant reduction in CO/V group in the order of 83% for SOD and 6% for CAT with respect to what was seen in the C/V group. In the group of CO/EGCG, the activities of these enzymes increased significantly ($p<0.05$) by 318% for SOD and 6% for CAT when weighed against what was observed in CO/V group. The activity of CAT was similar between the groups CO/EGCG and C/V (Table 1).

With regard to testosterone, the concentration was found to decrease significantly ($p<0.05$) in CO/V group with respect to the rest of the groups. However in C/EGCG group, there was a significant increase ($p<0.05$) in the concentration of this hormone when compared with the rest of the groups (Table 1).

Analysis of the testicular histology showed a normal cytoarchitecture characterized by complete spermatogenesis with germ cells in different stages of development, from spermatogonia to elongated spermatids, in the C/V group. Nonspecific histological alterations, characteristics of the species (Fig. 1); the presence of cell proliferation (Fig. 2) and apoptosis (Fig. 3) were also observed in this group. The testes of the animals in C/EGCG group showed a significant increase ($p<0.05$) in the area of seminiferous epithelium and in the Johnsen and proliferation index when compared with C/V group. Moreover, a significant reduction ($p<0.05$) was observed in the histopathological index (Table 2) of

Table 1. Effects of the administration of epigallocatechin-3-gallate (EGCG) in an experimental cryptorchidism model on gonadosomatic index, lipoperoxidation, antioxidant enzymes activity and serum testosterone concentrations.

Group	GI %	Lipid peroxidation (nmoles of TBARS/mg prot)	CAT Activity (U/mg prot)	SOD Activity (U/mg prot)	Testosterone (ng/ml)
C/V	0.119±0.003	26.00±2.51	0.33±0.001	5.23±0.04	3.20±0.29
C/EGCG	0.143±0.013[ab]	19.75±0.62[ab]	0.34±0.000[ab]	6.00±0.03[ab]	5.38±0.45[ab]
CO/V	0.026±0.010[a]	34.60±1.29[a]	0.31±0.001[a]	0.83±0.05[a]	0.13±0.01[a]
CO/EGCG	0.045±0.002[ab]	24.80±0.97[b]	0.33±0.000[b]	3.47±0.14[ab]	1.62±0.38[ab]

The data are expressed as mean ± SEM of 5 animals/group. [a]: Statistically significant difference compared with C/V group $p<0.05$. [b]: Statistically significant difference compared with CO/V group $p<0.05$. C/V: control group + EGCG vehicle; C/EGCG: control group + EGCG; CO/V: Induced cryptorchidism + EGCG vehicle; CO/EGCG: cryptorchidism induced + EGCG. GI: Gonadosomatic Index; TBARS: Thiobarbituric Reactive Substances; CAT: Catalase; SOD: Superoxide Dismutase.

Table 2. Effects of the administration of epigallocatechin-3-gallate (EGCG) on various testicular histological parameters in an experimental cryptorchidism model.

Group	Epithelial Area (μm^2)	Gonocytes (in 10,000 μm^2)	Spermatogonia (in 10,000 μm^2)	Johnsen Index	Histopathological Index	Apoptosis Index	Proliferation Index
C/V	18277.7±457.04	0.00±0.0	3.02±0.10	8.00±0.00	4.66±0.13	0.25±0.02	29.62±1.760
C/EGCG	31283.8±748.3^{ab}	0.00±0.0b	5.10±0.09^{ab}	8.45±0.09^{b}	3.28±0.11^{ab}	0.25±0.01	43.93±1.22^{ab}
CO/V	6852.2±103.1^{a}	3.82±0.15^{a}	1.33±0.10^{a}	2.26±0.03^{a}	12.19±0.17^{a}	4.26±0.16^{a}	13.85±0.77^{a}
CO/EGCG	12653.1±277.6^{ab}	0.32±0.09^{b}	2.60±0.08^{ab}	3.24±0.04^{ab}	8.61±0.21^{ab}	0.89±0.07^{ab}	18.66±0.87^{ab}

The data are expressed as mean ± SEM of 5 animals/group. [a]: Statistically significant difference compared with C/V group $p<0.05$. [b]: Statistically significant difference when compared with CO/V group $p<0.05$. C/V: control group + EGCG vehicle; C/EGCG: control group + EGCG; CO/V: induced cryptorchidism + EGCG vehicle; CO/EGCG: group with induced cryptorchidism + EGCG.

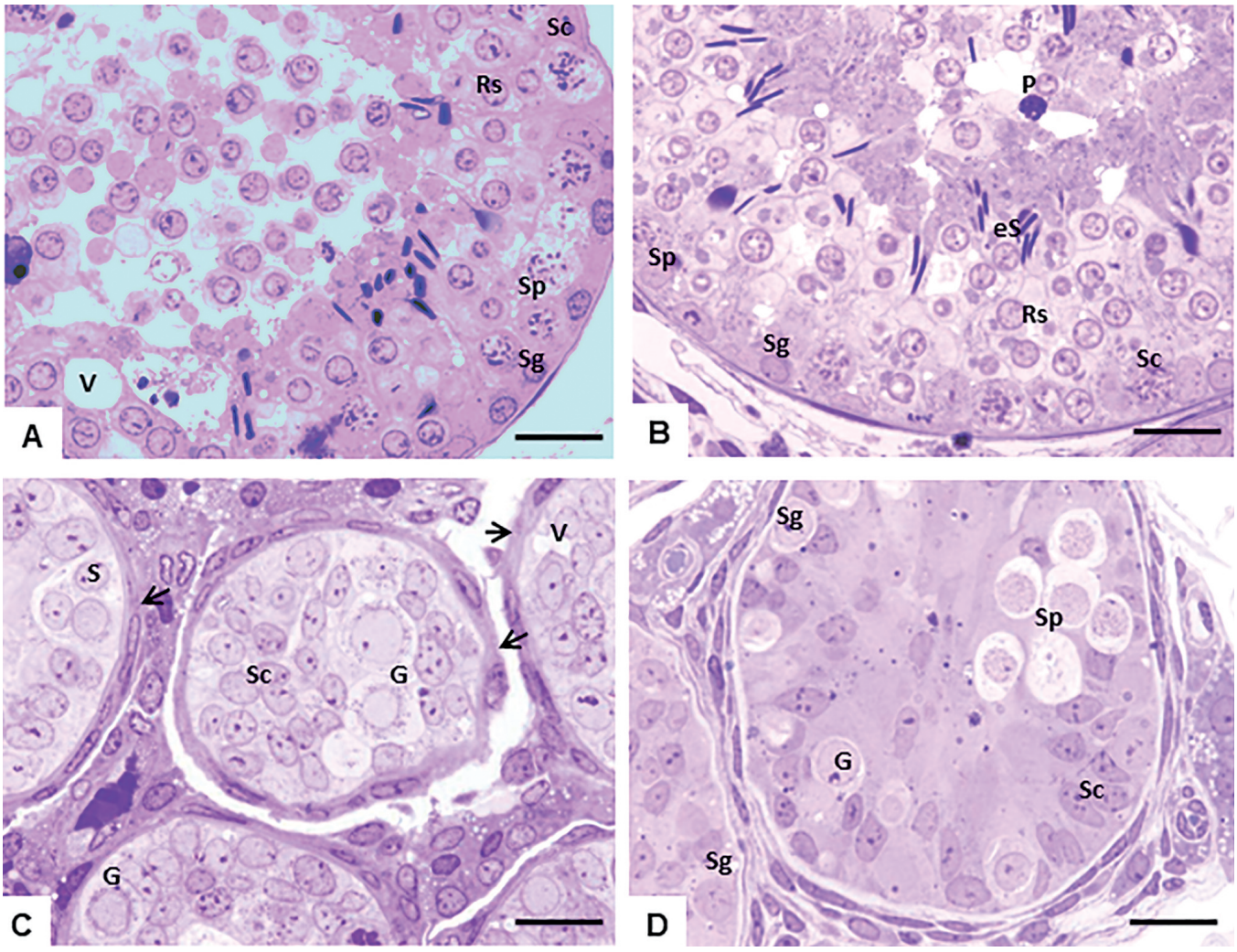

Fig. 1. Seminiferous tubes of the animal groups studied. **A.** C/V group where seminiferous tubes with a normal cytoarchitecture according to age can be observed. Also the presence of Sertoli cells and germ cells in different stages of development as well as histological alterations characteristics of the species can be seen. **B.** C/EGCG group where the cell types of the C/V group with reduced non-specific alterations can be observed. **C.** CO/V group depicting small seminiferous tubes with thickening of the basal membrane layer and peritubular cells (arrow), nuclei of Sertoli cells and the presence of gonocytes with their characteristic mitochondria surrounding the nucleus. **D.** CO/EGCG group with the presence of Sertoli cell nuclei, spermatogonia and spermatocytes. Sertoli cell (Sc), gonocytes (G), spermatogonia (Sg), spermatocytes (Sp), round spermatids (Rs), elongated spermatids (eS), syncytial cells (S), vacuolization (V) and picnosis (P). Toluidine blue. Scale bars: 20 μm.

this group. In the CO/V group, there were histological alterations consisting of a significant reduction ($p<0.05$) in the area of seminiferous epithelium associated with cellular desquamation (Table 2); basal membrane/peritubular cell layer folding; and spermatogenesis arrest depicted by the presence of only Sertoli cells and gonocytes, some of which were in degeneration (Fig. 1). In addition, the number of gonocytes, apoptosis and histopathological index parameters were significantly higher ($p<0.05$) while the area of seminiferous epithelium, Johnsen and proliferation index were significantly lower ($p<0.05$) in comparison to what were observed in the C/V group (Table 2). In the group with CO that received EGCG (CO/EGCG), the presence of Sertoli cells, spermatogonia and spermatocytes with few gonocytes were observed (Fig. 1). Moreover, this group presented a significant increase ($p<0.05$) in the parameters of the number of spermatogonia, area of seminiferous epithelium, Johnsen and proliferation index and witnessed a significant reduction ($p<0.05$) in the parameters of apoptosis and histopathological index when compared with CO/V group; however, these values did not reach the values observed in the C groups (Table 2, Figs. 1-3).

Discussion

Testis with CO is subjected to higher temperature than the scrotal testis. This condition leads to the production of free radicals such as superoxide anion, hydroxyl radical, nitric oxide and hydrogen peroxide in CO testis (Zini and Schlegel, 1997; Kumagai et al., 2002; Ishii et al., 2005; Paul et al., 2009) and to a reduction in the endogenous antioxidant system

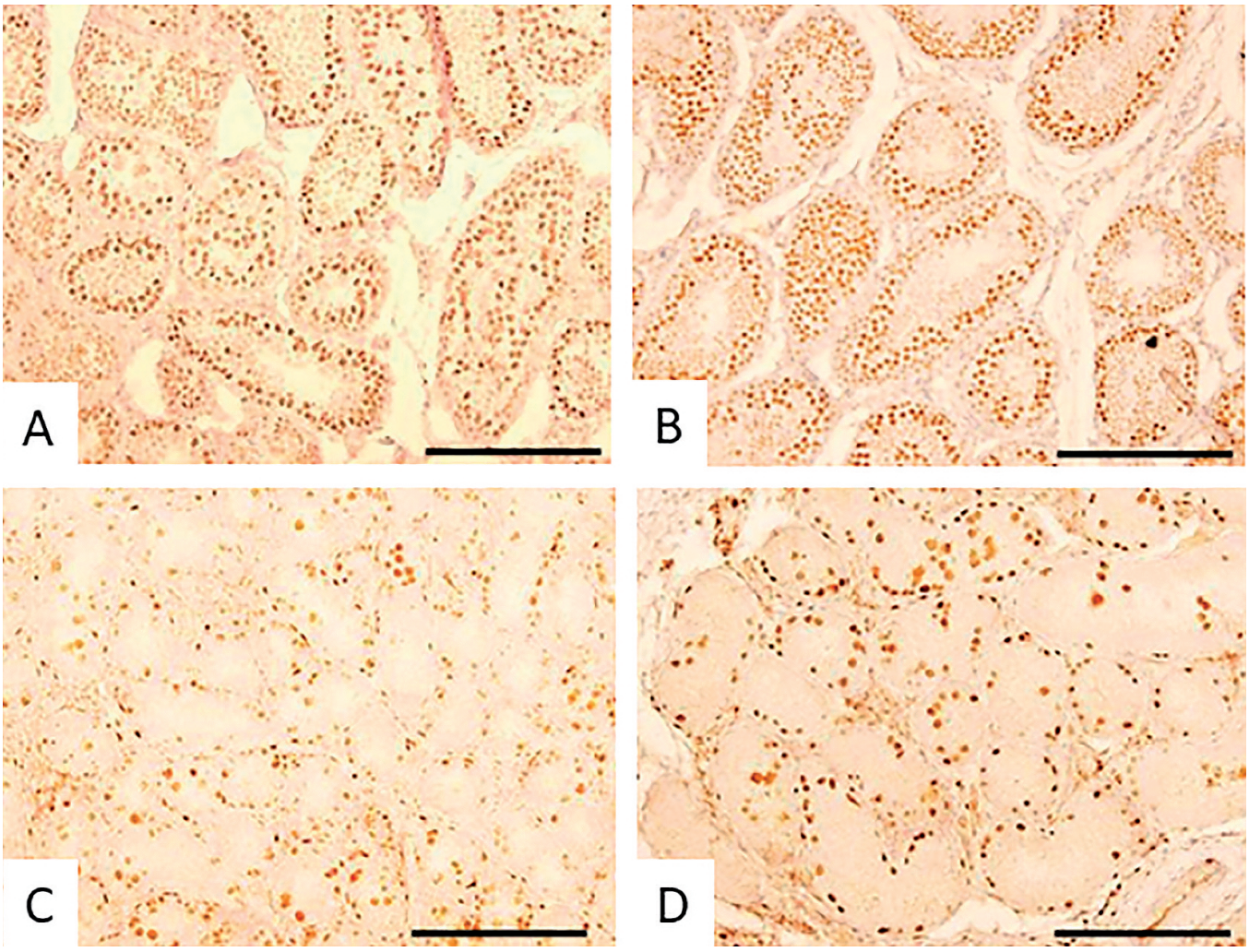

Fig. 2. Seminiferous tubes showing protein immunoreactivity Ki67, indicating greater proliferation in the C/V **(A)** and C/EGCG **(B)** groups. The CO/V group **(C)** showed little proliferation and CO/EGCG group **(D)** presented increase in the proliferation when compared with CO/V group. Scale bars: 250 μm.

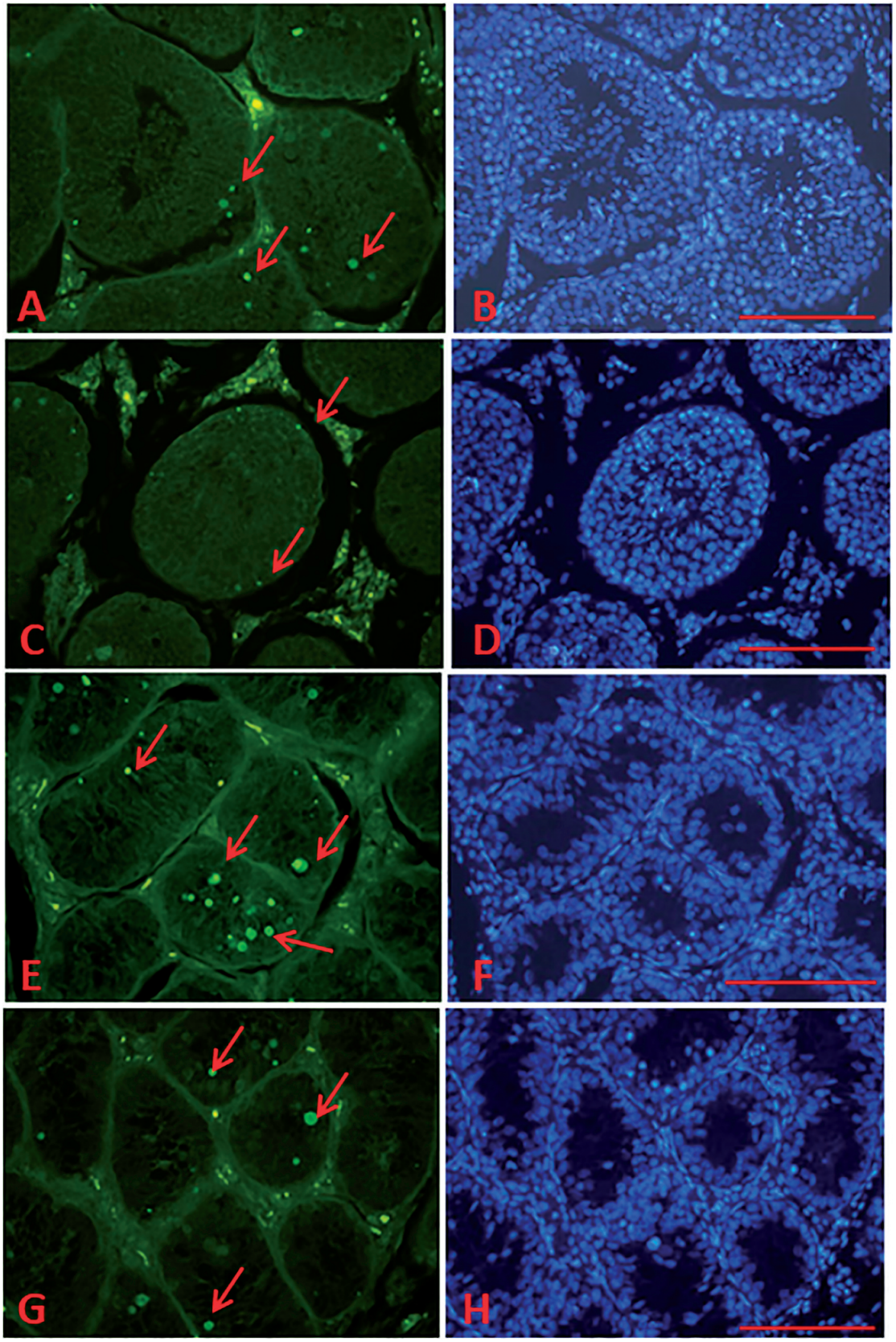

Fig. 3. Seminiferous tubes showing cells in apoptosis (arrow) in the different groups studied. In groups C/V **(A, B)** and C/EGCG **(C, D)**, apoptosis is reduced compared with group CO/V **(E, F)**. In group CO/EGCG **(G, H)**, apoptosis is reduced when compared with CO/V group. Tunnel technique **(A, C, E and G)**; DAPI contrast **(B, D, F and H)**. Scale bars: 150 μm.

(Ahotupa and Huhtaniemi, 1992; Zini and Schlegel, 1997). These events induce an increase in lipid peroxidation (Janero, 1990; Ahotupa and Huhtaniemi, 1992; Vigueras et al., 2011) and apoptosis of the germ cells (Aitken and Roman 2008), thus generating the histological alterations characteristic of cryptorchidism (La Vignera et al., 2009; AbouZeid et al., 2011; Koni et al., 2014). Testicular oxidative stress plays an important role in future fertility (Vigueras et al., 2011; Jung et al., 2015; Tsounapi et al., 2016) by probably affecting the survival of gonocytes and their differentiation to spermatogonia. When the gonocytes remain undifferentiated, they are programmed to die by apoptosis, thereby, leaving the testis depopulated of germ cells (Cobellis et al., 2014). In some cases, the gonocytes that survive develop germ cell neoplasia *in situ* (GCNIS) (Papparella et al., 2010; Hutson et al., 2013). Antioxidant treatments could increase endogenous antioxidant defense system of the cells by scavenging the free radicals and hence produce a reduction in the alterations (Thornalley and Vasak, 1985; Palamanda and Kehrer, 1992). It has been shown that polyphenols, specifically EGCG of green tea, reduce the alterations generated by oxidative stress in various neurodegenerative diseases by exerting free radical inhibiting and scavenging roles and serving as activator of the endogenous antioxidant system (Levites et al., 2001; Rezai-Zadeh et al., 2008). Nevertheless, its role in the survival, differentiation and proliferation of germ cells in a model of cryptorchidism in rabbits has not been studied.

The results of the present study confirm the role of the increase in lipid peroxidation and the reduction in the activity of the antioxidant system on the histological alterations in testis with CO. The administration of EGCG in the animal group with CO (CO/EGCG) increased the activity of CAT and SOD and reduced the lipid peroxidation and the histological alterations. The role of EGCG or green tea has also been demonstrated in animal models in which oxidative stress has been induced but in a different route from CO (Shi et al., 2000; Na and Surh, 2008; Khan et al., 2009; Messarah et al., 2013). Studies of electron paramagnetic resonance spectroscopy showed that EGCG reacts with the superoxide anion (O_2^-) oxidizing the D-ring (Severino et al., 2009). The analysis of electron paramagnetic resonance spectroscopy also showed that this molecule can scavenge hydroxyl radical (HO•) and superoxide anion (O_2^-) (Shi et al., 2000). This action is carried out by polyphenol mainly through hydrogen atom transfer or single electron transfer reactions, or both by the participation of hydroxyl group. These groups are constituents of the B- and D-rings of EGCG. As chain-breaking antioxidants, tea catechins are thought to interrupt deleterious oxidation reactions by hydrogen atom transfer mechanisms, the most important being lipid peroxidation (Shi et al., 2000; Severino et al., 2009).

The increase in SOD and CAT activities observed by the administration of EGCG in the CO/EGCG group, in comparison with CO/V group, has also been demonstrated in mice and rats to which oxidative stress was induced by $CdCl_2$ administration. In these models, the catechins of the green tea increased the activities of SOD (Sharma and Goyal, 2015; Abdelrazek et al., 2016) and CAT (Sharma and Goyal, 2015), and in this way reduced the testicular damage. The increase in SOD activity allows a greater capacity for O_2^- to be reduced to hydrogen peroxide (H_2O_2) thus preventing the conversion of O_2^- to HO• which is highly harmful (Aitken and Roman, 2008). The elimination of H_2O_2 is either effected by CAT or glutathione peroxidase, with the latter predominating in the case of the testes (Peltola et al., 1992; Zini and Schlegel, 1996). Therefore the role of CAT in the testicle is less important compared with that of SOD. The importance of testicular SOD and its behavior in this work are based on the fact that this enzyme is produced by both Sertoli and germ cells in its different conventional, cytosolic, mitochondrial and extracellular forms (Aitken and Roman, 2008). The protective role of SOD in heat stress in germ cells has been demonstrated in SOD1-knockout mice to which cryptorchidisms were induced and there was greater death of germ cells compared with wild-type mice (Ishii et al., 2005).

In this work, the lipid peroxidation generated by oxidant stress in the cryptorchidism group was reduced with the administration of EGCG. This has also been demonstrated in animal models with induction of testicular oxidant stress using cyclophosphamide and administration of green tea (Zanchi et al., 2015). By reducing the lipid peroxidation, the cell membrane is conserved (Paul et al., 2017). This is important for cellular adhesion and paracrine communication (Wong and Cheng, 2009; Neto et al., 2016). In this sense, the ability to regulate the essential components of tight, adhesion and gap junctions is also attributed to EGCG and this favors the differentiation of germ cells after oxidative stress by irradiation in mice (Ding et al., 2015).

The data from our studies suggest that EGCG plays an important role in the survival and differentiation of germ cells, reflected by the increase in the parameters of GI and area of seminiferous epithelium. The differentiation of gonocytes to spermatogonia in CO/EGCG group may be related to the direct action of polyphenol on the gonocytes. It has been demonstrated that EGCG promotes the differentiation of keratinocytes *in vitro* (Balasubramanian et al., 2005), odontoblasts (Kwon et al., 2017) and neural stem cells (Zhang et al., 2016). According to Zhang et al., (2016), the mechanism by which EGCG promoted the differentiation of neural stem cells is through the activation of phosphoinositol 3-kinase-mediated pathways (PI3K/Akt).

In our study, the alterations observed in the CO group such as the reduction in GI, area of seminiferous epithelium, Johnsen Index and persistent gonocytes coincide with what were reported in humans and animal

models with this condition (La Vignera et al., 2009; Vigueras et al., 2009, 2011; AbouZeid et al, 2011; Koni et al., 2014). These alterations are possibly due to the fact that spermatocytes and spermatids are the germ cells most vulnerable to damage caused by free radicals (Bauché et al., 1994; Ikeda et al., 1999; Ishii et al., 2005).

The CO/EGCG group in this study showed reduction in the histological alterations and onset of spermatogenesis, corroborated by the presence of spermatocytes. These observations were similar to what were reported in animal models, where experimental oxidative stress was induced by ionizing radiation plus doxorubicin and subsequently treated with EGCG or green tea, which gave rise to the recovery of spermatogenesis and reduction of the testicular alterations in the animals (Sato et al., 2010; Ding et al., 2015). On the other hand, negative effects on the testicular function such as inhibition of spermatogenesis, by elevated consumption of green tea of up to 5.0 g, equivalent to 20 cups of tea/day (Chandra et al., 2011) and 5.0% green tea leaf extract, 1 ml/100 g of body weight, (Das and Karmakar, 2015) have been reported.

Cryptorchidism is associated with an increase in the number of testicular cells in apoptosis (Hikim et al., 2003, Somwaru et al., 2004), as we observed in this work. The role of EGCG as protector of apoptosis in our CO model may involve the following mechanisms: a) EGCG may have activated the survival protein 140. This protein inactivates the NFkB pathway, since this pathway favors the expression of p53 and Bax (proapoptotic proteins) and induces an inhibition of Bcl-2 (antiapoptotic proteins) (Chu et al., 2011; Jeyasuria et al., 2011) which leads to the survival of germ cells (Al-Ajmi et al., 2013). A reduced expression of mRNA and survivin protein of the variants 140 and 40 in bilateral experimental cryptorchidism in murine which leads to apoptosis in testicular cells and impaired spermatogenesis has been demonstrated (Absalan et al., 2010). The protective effect of EGCG through this protein has also been observed in animal models with induction of oxidative stress by testicular torsion (Al-Ajmi et al., 2013). b) By inhibition of caspase-3 through mitogen-activated protein kinase/BCL2 family/caspase-3 pathway as mentioned by Ding et al. (2015) and Abdelrazek et al. (2016), who on administering EGCG and green tea respectively, reduced testicular apoptosis experimentally induced by oxidative stress. However, due to our experimental design, our results do not have the scope to reach these mechanisms.

In vivo and *in vitro* treatments with catechins, including EGCG, have been reported to increase the concentration of testosterone in rats by the activation of AMPc (Yu et al., 2010). On the other hand, there are reports where it was documented that CAT can be an important enzyme in the metabolism of lipids, especially cholesterol, and can work with other peroxisomal enzymes in the steroidogenesis (Ihrig et al., 1974). This is reinforced with the data found in the present work, since the concentrations of testosterone coincided with CAT increase in C/EGCG and CO/EGCG groups.

It should be noted that C/EGCG group presented increases in GI, area of seminiferous epithelium, Johnsen Index, proliferation index and in testosterone concentrations compared with the rest of the groups. Hijazi et al., (2015) mentioned that the administration of green tea to rats increased testicular weight. Martins et al. (2014) found that the administration of white tea which contains EGCG as the main constituent, improves the production of lactate and therefore spermatogenesis. It is possible that this is as a result of the increase in the production of lactate, an important cellular component, as the metabolic substrate of the Sertoli cells for the development of germ cells (Courtens and Plöen, 1999).

Conclusion

EGCG reduces the alterations produced by oxidative stress generated in cryptorchidism, possibly by increasing the endogenous antioxidant system (SOD and CAT) and by reducing lipid peroxidation.

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