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Green Brazilian propolis effects on sperm count and epididymis morphology and oxidative stress

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ABSTRACT

Basal generation of reactive oxygen species (ROS) is essential for male reproductive function, whereas high ROS levels may be linked to low quality of sperm and male infertility. The number of antioxidants known to inflict damage is growing, and it will be of interest to study natural products, which may have this activity. Since the epididymis is known to play an important role in providing the microenvironment for sperm maturation and storage of sperm, this study was undertaken to evaluate the morphometric-stereological and functional alterations in the epididymis after chronic treatment with low doses of Brazilian green propolis, which is known for its antioxidant properties. For this purpose, forty-eight adult male Wistar rats were treated with 3, 6 and 10 mg/kg/day of aqueous extract of Brazilian green propolis during 56 days and morphological parameters, sperm production and number of sperm in rat epididymis and oxidative stress levels were analyzed. The results showed higher sperm production and greater epithelium height of the epididymis initial segment and no induction of oxidative stress in treated animals. Further studies are needed to fully understand the effects of propolis on the reproductive system but our results showed that it could alter male reproductive function.

1. Introduction

Male infertility is a common and complex problem affecting 1 in 20 men. Despite concentration of research in this field, in many cases, the underlying causes are unknown (Dada et al., 2012). Many natural products are used in folk medicine for treatment of male sexual disorders (Soliman et al., 2012; Mbongue et al., 2011).

Yousef and Salama (2009) described increased activity of 17-ketosteroid reductase and testosterone levels in rats treated with 50 mg propolis/kg BW and suggested increased steroidogenesis, improving sperm proliferation and hence increased fertility. Also, ElMazoudy et al. (2011) and Yousef et al. (2010) showed an increase in testosterone levels for rats and rabbits treated with propolis.

In both situations cited above, propolis has been demonstrated to induce increased testosterone level, and, in another study a significant raise in the level this hormone was induced by *Aframonum melegueta*. The authors suggest the use of plant extracts for male reproductive problems (Mbongue et al., 2011).

It is known that testosterone produced by Leydig cells in the testis is converted into dihydrotestosterone (DHT) in some tissues such as epididymis, probably influencing its metabolism (Garcia et al., 2012). The epididymis has an important role in sperm maturation, protection, transport, concentration and storage because it is actively involved in synthesis and secretion of a wide range of proteins and glycoproteins.

Spermatozoa acquire forward motility and fertilizing capacity during their transit through the epididymis. The maturation process involves modifications of the sperm surface by different proteins that are secreted by a series of specialized regions in the epididymal epithelium (Hermo and Robaire, 2002). Therefore its integrity is essential to balance this physiological process, and abnormalities of epididymal function could lead directly to infertility (Jarvi, 2012).

Furthermore, the fundamental storage and maturation functions of the epididymis are intricately associated with the capacity of mammalian spermatozoa to generate reactive oxygen species (ROS), such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The generation of these highly reactive metabolites is of central importance to the signal transduction pathways that

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control capacitation. It is a major factor in determining how long the spermatozoa can be retained in a functional and viable state as they migrate through, and are stored in the epididymis. ROS are involved in virtually every aspect of sperm function including motility activation, capacitation, the acrosome reaction and hyper activated motility (Aitken, 2002).

On the other hand, damage induced by ROS, particularly H_2O_2 and (O_2^-), has been proposed as a major cause of defective sperm function in cases of male infertility (Sharma and Agarwal, 1996). Elevated levels of ROS in semen are found in up to 40% of infertile man (Goyal et al., 2007). Oxidative stress can induce propagation of a lipid peroxidation chain reaction because spermatozoa contain high concentration of unsaturated fatty acids. These induce sperm plasma membrane to become less fluid and the spermatozoa to loose their function (Aitken, 2002), which plays a key role in the etiology of male infertility (Sharma and Agarwal, 1996). Aitken (2002) suggested that since ROS production is such a potentially dangerous activity for spermatozoa, there must be a powerful biological benefit in developing the specialized biochemical machinery that generates such molecules.

A variety of defense mechanisms encompassing antioxidant enzymes (superoxide dismutase – SOD, catalase – CAT, and glutathione – GSH peroxidase and reductase), vitamins (E, C, and carotenoids), and biomolecules (GSH and ubiquinol) are available. These contribute to the balance of benefits and risks from ROS and antioxidants necessary for the survival and functioning of spermatozoa (Sikka et al., 1995).

Propolis is the generic name for the resinous substance collected by honeybees from various plant sources (Burdock, 1998) that has wide range of biological activities including antibacterial, antiviral, anti-inflammatory, and anti-oxidative properties (Medić-Sarić et al., 2009). In its composition many phenolic compounds were detected that were associated with its antioxidant activity (Sousa et al., 2009).

The aim of this study was to investigate the effect of propolis on morphological parameters of the rat epididymis, on sperm number and production. This information may contribute toward understand the action mechanisms of propolis on this reproductive organ ensuring a beneficial activity, and perhaps contributing to effective therapies with respect to male fertility.

2. Material and methods

2.1. Propolis samples (according Sforcin et al., 2000; Pagliarone et al., 2009)

Propolis was produced from *Baccharis dracunculifolia* D. C. by *Apis mellifera* L. bees in the apiary located in the Araras city (region of Campinas, SP), and collected using plastic nets in February, 2010. This propolis sample was classified as group 12 according Alencar et al. (2005). Propolis was ground and was prepared in sterile conditions (30 g of propolis added to distilled water totaling 100 mL), in the absence of bright light, at room temperature and shaken moderately for 1 week in the Toxicology Laboratory of Centro Universitário Hermínio Ometto (UNIARARAS). After this period, the extract was filtered and the final concentration calculated, obtaining the dry weight of the solution (40 mg/mL).

2.2. Animals

Forty-eight adult male Wistar rats (90 days) were maintained throughout the experiment at the Center of Animal Experimentation, Centro Universitário Hermínio Ometto (UNIARARAS), on a 12-h light/12-h dark cycle at a temperature of 25 °C and air humidity of 60%. The animals received water and Purina standard chow, *ad libitum*. The animals were divided into four groups with 12 animals in each group, distributed in two different cages (A and B) with six rats in each. The control group (Co) ($n = 12$) received only filtered water and the three experimental groups T1, T2 and T3 ($n = 12$ for each group) were treated with 3, 6 and 10 mg/kg/day, respectively, of the aqueous propolis extract. The study was approved by the Ethics Committee of Centro Universitário Hermínio Ometto, UNIARARAS (protocol 860/2009) and was conducted in accordance with the ethical guidelines of the Brazilian College of Animal Experimentation (COBEA).

2.3. Doses and administration route

The animals were weighed, and received by gavage, a solution of propolis for 56 days, which is the period necessary to complete a spermatogenic cycle (Russell et al., 1990). Subacute doses were prepared daily using the solution of 40 mg/mL to obtain a final concentrations of 3, 6 and 10 mg/kg/day, as described in the literature (Mani et al., 2006).

2.4. Tissue preparation

The experimental animals were subjected to Ketamine (70 mg/BW) and Xylazine (10 mg/BW) anesthesia and the animals from cages A were fixed by perfusion. After a brief saline wash to clear the blood vessels, they were perfused with glutaraldehyde 4% and paraformaldehyde 4% in 0.1 M sodium caccodylate buffer (pH 7.2) for 25–30 min, according Sprando (1990). Epididymis were dissected out and fixed overnight in the same solution, then prepared for embedding in hydroxyethyl methacrylate (Historesin, Leica), using routine technique. For morphological and morphometrical evaluation, the epididymis were sectioned at 3 μm thickness and stained with toluidine blue in 1% sodium borate (TB). The cage B animals, that were not perfused, after anesthesia, had their epididymis dissected out, weighed and the left epididymis was frozen at −20 °C for sperm count while the right epididymis was frozen at −80 °C for biochemical analysis.

2.5. Daily sperm production (DSP) and DSP per gram of testis

The left testis of unfixed rats was weighed and homogenized for 3 min, using a Ultra-Turrax T18 (IKA) homogenizer, in ice-cold saline-triton (0.9% NaCl and 0.05% Triton X 100 v/v), at 24,000 rpm, after removal of the albuginea (Robb et al., 1978). The homogenate was used to count the number of homogenization-resistant spermatids/sperm in each sample, analyzed in duplicate using a Neubauer hemocytometer (Kempinas et al., 1998). DSP was calculated by dividing the total number of spermatids per testis by 6.1 days, which is the duration of step 19 spermatids in the seminiferous tubules (Robb et al., 1978).

2.6. Epididymal sperm number

The frozen, unfixed left epididymis was used for this purpose. Sperm of caput/corpus and cauda portions of the epididymis were counted in a Neubauer chamber (4 fields per animal) after homogenization for 3 min, at 24,000 rpm (Ultra-Turrax homogenizer – Janke & Kunnkel IkaWerk), in a mixture of 0.9% NaCl, 0.05% Triton X 100 (v/v), according to the method described previously (Robb et al., 1978). One milliliter of this solution per 100 mg of cauda and 1 ml for every 200 g of caput/corpus weight were used. The spermatozoa were counted according to the methodology described in Kempinas et al. (1998).

2.7. Biometry, morphometry and stereology

The weight of the testicular parenchyma was obtained subtracting the mass occupied by the albuginea from the total right testis weight, thus providing the net weight of the organ's functional portion. The gonadosomatic index (GSI) was expressed as a percentage of the total body weight in relation to the testis weight, GSI = (testes weight/total body weight) × 100. The initial segment and cauda epididymis epithelium height was measured in ten tubules per animal at 200× magnification. The stereological analysis of the epididymis was made at 12 random epididymal cross sections per animal. This analysis was performed with a 120 point grid to determine the proportion of the epididymal components (epithelium, lumen and interstitium) in the experimental groups.

2.8. Transmission electron microscopy (TEM)

After whole body perfusion fixation, the caput and cauda epididymis pieces were post fixed in the same fixative for 24 h. Then the specimens were rinsed three times with 0.1 M sodium phosphate buffer, pH 7.2, post fixed in 1% osmium tetroxide, rinsed and dehydrated in an ascending acetone series and embedded in epoxy resin. Ultrathin sections were cut and contrasted with 2% uranyl acetate and 2% lead citrate prior to observation with a transmission electron microscope (Zeiss, Leo906).

2.9. Evaluation of epididymis oxidative stress

Oxidative stress of the right epididymis collected from unfixed animals was evaluated by the determination of catalase activity (CAT), reduced sulphydryl group levels (–SH) and lipid peroxidation by TBARS formation. Protein concentration was measured according to biuret method (Gornall et al., 1949). CAT activity was assayed according to the method of Aebi (1974). The levels of reduced groups of total protein (–SH) were measured using 1.0 mg protein liver homogenate in 25 mM Tris–HCl buffer, pH 8.2, and 20 mM EDTA, and 20 mM EDTA, as described by Ellman (1959). Absorbance was measured immediately and after 15 min at 412 nm (Allameh et al., 1997). The lipid peroxidation assay was used as described by Buege and Aust (1978).