

Full Length Research Paper

Testicular damage in Wistar rats caused by methanolic extracts of plants from the North of Mexico

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The aim of this study was to evaluate the effect of methanolic extract of plants from the north of Mexico, on testicular morphology and spermatogenic quality in Wistar rats. Methanolic extracts were orally administered to 4 experimental groups (n=6) of rat for 30 days; a control group not treated with plant extract was included. All animals were sacrificed 24 h after the last dose. Samples of testicles were collected, fixed, processed by histological technique and embedded in paraffin blocks. Histological sections were stained with H&E, Masson's trichrome and histochemical PAS reaction with diastase. Results of the groups treated with methanolic extract of *Tagetes lucida*, *Cynodon dactylon*, *Lippia graveolens* HBK, and *Opuntia ficus-indica* testicle samples demonstrated epithelial detachment and cell fragmentation, an apparent decrease in the diameter of seminiferous tubules, with large empty spaces between them and areas with fragmentation of basal membrane. Sperm quality analysis (concentration, motility and viability) showed a significant decrease of these spermatogenic parameters in all treated groups. In conclusion, this paper demonstrated for the first time that methanolic extracts from plants of the north of Mexico, have toxic effect on testicle and affects spermatogenic parameters of Wistar rats.

Key words: Plants, testicle, spermatogenic quality, methanolic extract, Wistar rat, toxicity.

INTRODUCTION

In traditional medicine, plants have been used for hundreds of years to treat a wide variety of health-related

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disorders. The scientific research of plants used in traditional medicine with a multidisciplinary approach has increased worldwide, with more than 13,000 superior plants having been studied in the past five years, leading to sufficient scientific evidence on the pharmacological properties of these plants (Dahanukar et al., 2000). With this knowledge, the pharmaceutical industry has synthesized from active compounds isolated from plants. About 25% of the most successful drugs in the market are aspirin and tamoxifen (Tripathi, 2005). Numerous plants used frequently in traditional medicine have shown effects like antioxidant and antimutagenic activities (Lee et al., 2002; Martínez-Rocha et al., 2008), antidiabetic activity (Fratini et al., 1990; Singh et al., 2007, 2008; Jarald et al., 2008), neuroprotective and antidepressant effect (Dok-Go et al., 2003; Guadarrama-Cruz et al., 2008), antiulcer activity (Galati et al., 2001), antimicrobial (Salgueiro et al., 2003; Hernández et al., 2006), antifungal and antibacterial (Céspedes et al., 2006) as well as acaricidal effects (Martínez-Velázquez et al., 2011). Also, it has been described that medicinal plants have reproductive regulatory effects like infertility stress-induced (Chidrawar et al., 2011) and sperm DNA fragmentation induction (Meamar et al., 2012); for this reason, it calls for the opportunity to search new alternatives for fertility regulation in human reproduction (Talwar et al., 1997).

These alternatives can associate scientific literature and popular knowledge of plants with spermicidal effect, to suggest a regulator method of fertility from plant origin which retains spermicides advantages without cytotoxicity against epithelial cells (Upadhyay et al., 1993; Kumar et al., 2012). Currently, research has focused on evaluating and developing a regulator of male fertility which is both safe and effective, with reversible production of azoospermia, and suppresses sperm production by hormonal and non-hormonal methods (Lopez et al., 2006). Recently, plant products with low toxicity have been used to regulate fertility (Singh and Singh, 2008). According to Álvarez-Gómez et al. (2007), some plants demonstrating pharmacological fertility control are *Abrus precatorius* (black color pill), *Albizia lebbek* (shadow chickadee or sleepy), *Aloe vera* (sábila), *Ananas comosus* (pineapple), *Anethum graveolens* (dill), *Apium graveolens* (celery), *Azadirachta indica* (neem tree) (Upadhyay et al., 1993), *Bursera fagaroides* (cuijote), *Calendula officinalis* (gold button, crown of king, flamenquilla, dead flower, rose of dead or cempasúchitl), *Carica papaya* (papaya), *Citrus limon* (lemon), *Curcuma longa* (Indian saffron), *Cyclamen persicum* and *Primula vulgaris* (violets from the Andes and springs), *Eupatorium brevipes*, *Gypsophila paniculata* (paniculata or wedding veil), *Momordica charantia* (bitter melon or sibicogén), *Passiflora edulis* (maracuyá or passion fruit), *Pithecellobium saman* (samanor rain tree), etc.

Studies in cattle reported that incorporation of *Cynodon dactylon* (Bermuda grass) in the diet decreased the re-

production rate (Banta et al., 2008). Furthermore, ethnobotanical studies in Mexico and Mediterranean area of Europe, reported the traditional use of *Ceterach officinarum* (Doradilla) as a method of contraception, but this has not been fully tested (Márquez et al., 1999; Guarrera et al., 2008). Same information was reported in studies with an extract of *Lippia graveolens* (oregano), which is known to be toxic in high doses and is traditionally believed to affect reproduction when ingested as a tea (Longe, 2002). There is empirical evidence that communities in the State of Durango in Mexico use *Tagetes lucida* (yerbanis) and *Opuntia ficus-indica* (prickly pear) as reproductive control methods; but to date, there is no scientific evidence of anti-reproductive properties of these plants (González et al., 2004). Little studies have been done on the flora from the North of Mexico, and the literature provides few references on the study of their pharmacological activities and possible contribution to the ethnopharmacology. The objective of this study was to evaluate by morphological methods, the effect of methanolic extracts of *T. lucida*, *O. ficus-indica*, *C. dactylon*, and *L. graveolens* HBK, from the North of Mexico on testicle of male Wistar rats.

MATERIALS AND METHODS

Collection of plants

About 5 kg of leaves or stalks of *T. lucida*, *C. dactylon*, *L. graveolens* HBK and *O. ficus-indica* were collected in the rural area of Torreón Coahuila in the north of Mexico, on an altitude of 1,140 m above the sea. To collect plants tested, Guidelines for Good Practice Plant Collection proposed by the World Health Organization (WHO, 2003). Identification of the specimens were performed by a botanic specialist from the Universidad Autónoma Agraria Antonio Narro, Campus Torreón.

Preparation of methanolic extracts

Plant material was dried in a solar drier with aeration for 5 days. When dried, leaves and stalk were ground and passed through a sieve with 20 mesh (inch 0030, MM 08600) to obtain a fine powder as proposed by Navarro et al. (2006). Then, 30 g of dry ground plant material was weighed and added to 300 ml of methanol (analytical grade) (JT Baker, USA) in an Erlenmeyer flask (Payrex, USA). Flask was capped with parafilm, stirred at low speed and maintained at 37°C for 24 h. Mash was filtered on Kitazato flask with vacuum pump, process was repeated three times with same amount of fresh solvent each time. The filtrate obtained was distilled in a rotavapor (Rotavapor Buchii-215®, USA) to evaporate the solvent; crude extract was completely dried in a hot air oven at temperature lower than 50°C for 7 days (Benchmark Scientific Mini Incubator, USA). Dried extracts were stored at 4°C, in amber vials until use (Nostro et al., 2000; Navarro et al., 2006).

Animals and study groups

In this study, 30 Wistar male rats were used with 280 to 300 g weight, and were 60 days old. Age of animals was selected

according to events of maturation in reproductive cycle of the rat. Rats per group were placed in plastic boxes. The animals were kept in a controlled environment with room temperature between 25 and 28°C (temperature control: 2H/JT-03, Lennox^{MR}, Mexico), relative humidity between 30 and 70% (Minisplit, Lennox^{MR}, Mexico), light/darkness cycles of 12 h (Photoperiod was regulated by an electric timer and light intensity was at least 300 lx, positioned laterally to the eyes of the animals; Electric R/F, China), with free access to food (NUTRI-CUBES, Agribands Purina®, Mexico) and water *ad libitum*. Rats were subjected to a period of acclimatization for 7 days before the first administration of extracts. Extracts were suspended in distilled water and were orally administered daily with gastric probe for 36 days; a pilot assay was conducted to determine the effective dose that resulted in 50 mg/kg for all extracts; this dose was administered to the following groups.

Animals were organized in 5 groups as follows: Control group (n:6), group without treatment; Group 1 (n:6), treated with 50 mg/kg extract of *T. lucida*; Group 2 (n:6), treated with 50 mg/kg extract of *C. dactylon*; Group 3 (n:6), treated with 50 mg/kg extract of *L. graveolens HBK*; Group 4 (n:6), treated with 50 mg/kg extract of *O. ficus-indica*.

Experiments were carried out in accordance with the International Guidelines on the Appropriate Use of Experimental Animals, and according to Mexican Norm NOM-062-ZOO-1999 on the Technical Specifications for Production, Care and Use of Laboratory Animals (SAGARPA, 2010) and cared for by expert veterinarian (professional license: 4807528). The protocol was approved by the Bioethical Committee of the Faculty of Medicine of the Autonomous University of Coahuila, Torreon Campus, Coahuila in Mexico (Number of approval by the Secretaría de Salud and Comisión Nacional de Bioética in Mexico: CONBIOETICA07CEI00320131015).

Tissue samples collection

24 h after the last dose had been administered, all animals were sacrificed by cervical dislocation. Testicle samples were placed in 10% formalin with phosphate buffered saline (PBS) pH 7.2 to 7.4, processed by conventional histological technique and embedded in paraffin blocks.

Morphological evaluation

5-micron histological sections were obtained, mounted, and stained with hematoxylin and eosin (H&E) and Masson's trichrome; pretreatment with diastase and periodic acid Schiff (PAS) was used for histochemical analysis. Samples were analyzed by light microscopy to evaluate the morphology of different stages of development of spermatogonia, Sertoli cells and Leydig cells (Gilbert et al., 1986).

Collection of sperm from the epididymis

Sperm cells were collected from the epididymis in test tubes and immediately washed with 3 ml of physiologic solution (NaCl 0.9%), then washed again with HamF-10 culture medium (Sigma-Aldrich, San Luis MO, USA) and incubated at 37°C for 30 min. Concentration of cells was carried using bright field microscopy according to Zhou et al. (2006). To determine the percentage of sperm motility, a drop of sperm cell suspension was placed in a Neubauer chamber; sperm were counted in 10 different fields and the number of sperm with lineal mobility was determined with total mobility. A total of 200 sperm per sample were evaluated as described by Zhou et al. (2006).

Data analysis

Analysis of variance, and means comparison Pearson test with $P \leq 0.05$ were performed. The statistical software SPSS version 20.0 for Windows was used to analyze the variables.

RESULTS

Morphological alterations

In testicle samples of control group stained with H&E, a normal histology was observed: seminiferous tubules with epithelium without peeling or artifacts (Figure 1A). In group treated with the methanolic extract of *T. lucida*, destruction of some seminiferous tubules, characterized by epithelial detachment with cell fragmentation was observed (Figure 1B). In group 2 that received extract of *C. dactylon*, same alterations described earlier were observed alternating with areas without damage in the same seminiferous tubule (Figure 1C and D). In groups 3 and 4 treated with *L. graveolens HBK* and *O. ficus-indica*, respectively, same alterations described were observed, with an apparent decrease in the diameter of the seminiferous tubules, accompanied with large empty spaces between them (Figure 1E). With Masson trichrome method, no differences were observed among the control group (Figure 1F) and treated groups (Figure 1G and J). In samples analyzed with histochemical reaction of PAS with diastase from the control group, basal membrane was observed as a continuous line in magenta color (Figure 1K). In treated groups, areas with fragmentation of the basal membrane related to the areas of epithelial disruption were observed (Figure 1L and O).

Sperm quality analysis

Results of sperm quality analysis showed a significant decrease in sperm concentration in the group treated with *O. ficus-indica* (Figure 2A). Significant decrease in sperm motility was observed in groups treated with *T. lucida*, *L. graveolens HBK* and *O. ficus-indica* (Figure 2B). Groups treated with *C. dactylon*, *L. graveolens HBK* and *O. ficus-indica* showed a marked decrease in sperm viability, this was more evident in group treated with *O. ficus-indica* (Figure 2C).

DISCUSSION

This study, reports for the first time that methanolic extracts of *T. lucida*, *C. dactylon*, *L. graveolens HBK* and *O. ficus-indica* from the north of Mexico, have toxic effects on testicle and affect spermatogenic parameters of Wistar rats. These results correlate with other studies that demonstrated these same injuries after administration of extracts of *Lagenaria breviflora* (Saba et al., 2009), *Carica papaya* (Lohiya et al., 2002), *A. precatorius* (Sinha and Mathu, 1990), *Martynia annua* (Mali et al., 2002),

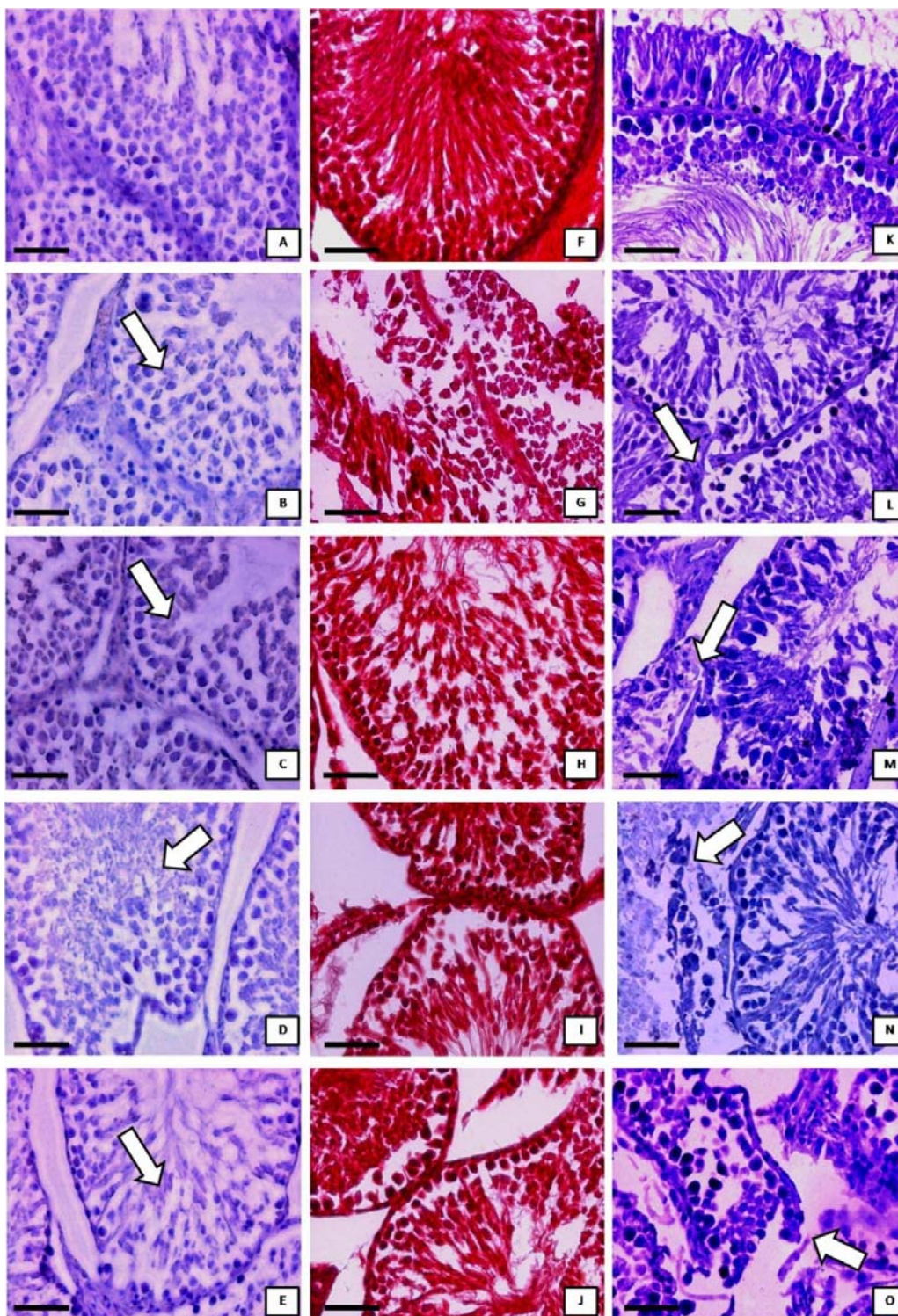


Figure 1. Methanolic extracts induced histopathological alterations in testicle: (A) Control group shows a normal histology. Groups treated with methanolic extracts: (B) *Tagetes lucida*, (C) *Cynodon dactylon*, (D) *Lippia graveolens* HBK, and (E) *Opuntia ficus-indica*. Varying degrees of detachment and epithelial cells destruction are observed (white arrows). H&E. Methanolic extract did not cause fibrosis: (F) Control group shows few fibers of collagen, no differences were observed between control and treated groups (G-J). Masson's trichrome. Fragmentation of basal membrane: (K) Intact basement membrane in control group is observed. In treated groups (L-O), areas of fragmentation of basal membrane (white arrows) are observed in relation to areas of epithelial damage. PAS with diastase. Paraffin embedded, light microscopy. Bar: 50 μ m.

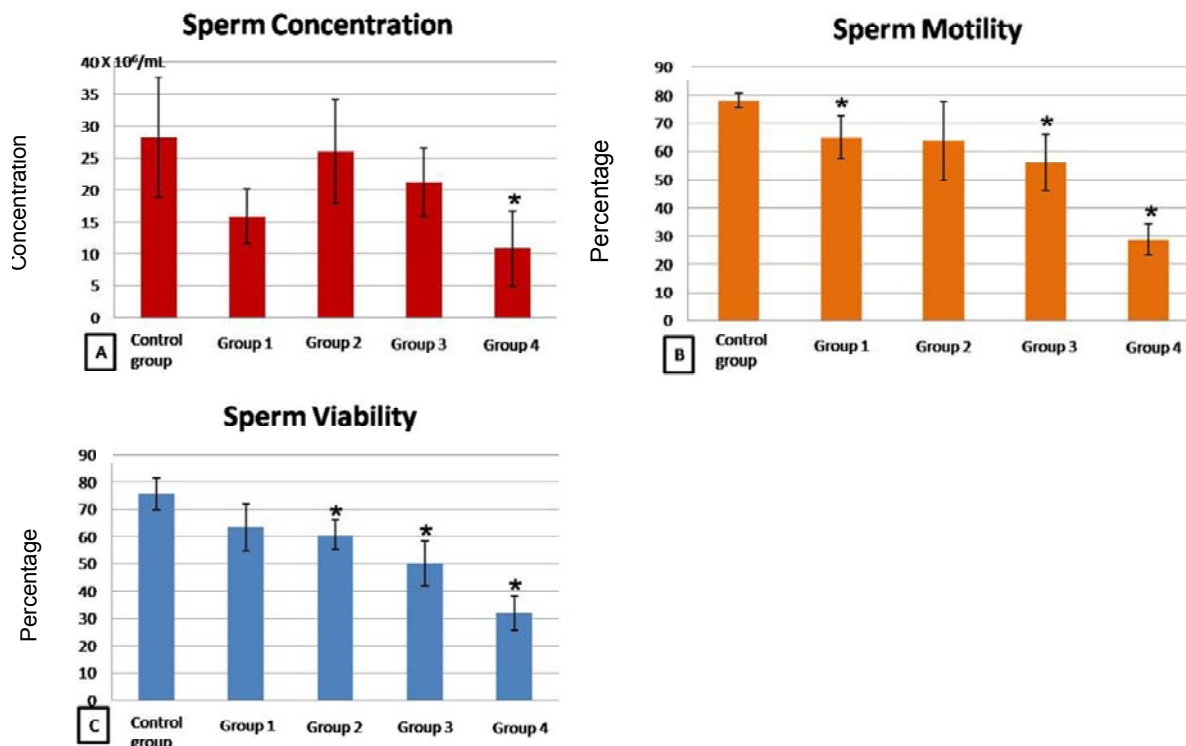


Figure 2. Graphs show (A) sperm concentration, (B) sperm motility, and (C) sperm viability. Control group and groups treated with *Tagetes lucida* (Group 1), *Cynodon dactylon* (Group 2), *Lippia graveolens* HBK (Group 3), and *Opuntia ficus-indica* (Group 4), respectively. Statistically difference is observed in some groups (* $P < 0.05$).

among others (Kamal et al., 2003; D'Cruz et al., 2010). The mechanism of damage to the testicular epithelium has not been fully established, but several studies tested other plant extracts and described selective damage to spermatogonia (Sinha and Mathu, 1990), Sertolice cells (Lohiya et al., 2002) and Leydig cells (Oluyemi et al., 2007), as well as a decrease in serum levels of testosterone (Ashok and Meenakshi, 2004; Takizawa et al., 2004; Yakubu et al., 2007; Shkukani et al., 2007) and spermatogenic toxicity (Tellez-López et al., 2014). In our study, the decrease in sperm quality was more pronounced in groups treated with *L. graveolens* HBK and *O. ficus-indica*, this correlates with the degree of damage to the testicular epithelium observed in histopathological sections.

The damage observed in testicular epithelium, as well as the decrease in sperm quality were not in the same intensity in the different study groups; they were more marked in groups treated with *L. graveolens* HBK and *O. ficus-indica*, and less evident in group treated with *C. dactylon*. The degree of damage to the testicular epithelium has been described as dose dependent (Gupta et al., 2004; Tellez-López et al., 2013); our findings suggest that an increased dose of *C. dactylon*, could cause similar damage to that observed with extracts of *L. graveolens* HBK and *O. ficus-indica*. Finally, the accentuated damage in testicular epithelium, and the

adverse effect on sperm quality parameters caused by extracts from *L. graveolens* HBK and *O. ficus-indica*, makes them good candidates for male fertility control; however, other physiological studies are required to support their use in humans. Several studies describe that extracts of *Azadirachta indica* (Shaikh et al., 1999), *Dendrophthoe falcata* (Gupta and Kachhawa, 2007) and *Taraxacum officinale* (Tahtamouni et al., 2011), affect the structure and sperm function that results in azoospermia (Purandare et al., 1999). To date, current studies are carried out to elucidate the mechanism of damage on testicular epithelium, and how the methanolic extract evaluated in this study decreased the sperm quality. Also we are investigating if morphological alterations in testicle of treated rats, are reversible or not after discontinuation of treatment.

Conflict of interest

The authors declare that they have no conflicts of interest.

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