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Effect of Nordihydroguaiaretic Acid on Spermatogenesis and Fertility in Rats

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Abstract
Nordihydroguaiaretic acid (NDGA) is a naturally occurring lignan with potent antioxidant activity. Currently, it is in clinical trials as anticancer agent. Since there is no earlier report on the effect of NDGA on spermatogenesis and fertility, this study was designed to investigate this aspect. Administration of NDGA to rats for 60 days produced degenerative changes in testis but had no effect on sperm DNA integrity test and androgen receptor expression. Ultrastructural studies revealed loss of integrity of cells in seminiferous tubules, vacuolation and presence of apoptotic bodies. Derangement of the outer dense fibers was noted in some spermatozoa flagella. Acrosome formation appears to be normal. About 13.7% of epididymal spermatozoa had deformations like short tail or rounded head. This may explain the lower fertility index in NDGA-treated group. No external deformations in newborns were noted. In conclusion, NDGA may have adverse effects on spermatogenesis.

Keywords: Nordihydroguaiaretic acid, spermatogenesis, male fertility, androgen binding receptor, transmission electron microscope.

Introduction
Nordihydroguaiaretic acid (NDGA) is a naturally occurring lignan isolated from the desert plant creosote bush (*Larrea divaricata*) (Grice et al., 1968). NDGA is a potent antioxidant and 5-lipoxygenase inhibitor (Blecha et al., 2007). Recently, it was demonstrated that NDGA and its semi-synthetic derivative tetra-O-methylnordihydroguaiaretic acid (terameprocol) are effective in treating several tumors including prostate cancer (Ryan et al., 2008A), breast cancer (Youngren et al., 2005), melanoma (Lambert et al., 2001), lung cancer (Avis et al., 1996) and neuroblastoma (Meyer et al., 2007). Terameprocol is currently in Phase I/II clinical trials in patients with advanced cancers (Kimura and Huang, 2016).

Several in vitro studies have demonstrated that NDGA has multiple effects on sex hormone synthesis and transport. NDGA inhibited testosterone production in purified rat testis Leydig cells after stimulation with LH (Dix et al., 1984). Also, NDGA inhibited aromatase (estrogen synthetase) enzyme (Adlercreutz et al., 1993). Estrogens are produced in male brain by the local aromatization of testosterone in order to exert a negative feedback on the secretion of gonadotropins (Boon et al., 2010; de Ronde and de Jong, 2011). Furthermore, NDGA non-competitively inhibited the binding of testosterone

to human sex hormone binding protein. Therefore, it may disrupt the delivery of testosterone to target tissue (Martin et al., 1996). All these studies raise the question of how NDGA functions in vivo and how it affects the reproductive system.

Another in vitro effect of NDGA is that it has a stabilizing effect on tubulin and it promoted its polymerization (Nakamura et al., 2003). Microtuble-stabilizing agents like taxol are known to disrupt spermatogenesis (Siiderström and Röyttä, 1986). Up to our best knowledge, no previous study has inquired the impact of NDGA on male reproductive system. Therefore, this work was designed to investigate the effect of NDGA on spermatogenesis and male fertility in rats. The ethnopharmacological use of *Larrea,* the plant from which NDGA was isolated, to enhance fertility supports the objective of our study (Heron and Yarnell, 2001).

Materials and Methods

Animals

Thirty two male Wistar rats, 8 weeks old (190-200g), were obtained from the animal house at The University of Jordan, acclimatized for one week and kept under standard animal house conditions Animals were housed in a controlled environment (23 ± 2 ^{o}C, 12:12-h light-dark cycle. Pelleted food (obtained from Hammoudeh Company) and tap water were provided *ad-libitum*. Animal handling was in accordance with the NIH guidelines for the use of laboratory animals.

The animals were randomly divided into four groups of 8 rats each. Group I was given a daily intraperitoneal (i.p) injection of 10mg/kg of NDGA (Cayman Chemical Company) (low-dose group) for 60 days. Group II (negative control group): received 0.1ml/day of the vehicle, dimethyl sulfoxide (DMSO) (obtained from Tedia, USA) for 60 days. The treatment of group I and II lasted for 60 days according to criteria set by the WHO (1983). Group III received 40mg/kg NDGA (high-dose group) for 11 days and Group IV received DMSO for 11 days. Some animals died following high doses (40mg/kg) of NDGA (2 rats). Therefore, we sacrificed the rest on day 11 of the study. We tried low dose (10mg/kg/day) to reach the therapeutic level with minimal side effects. This dose was safe until the end of the experiment which lasted for 60 days (no rats died).

Fertility test

For mating test, virgin, female rats (160-180g) were utilized. After 55th day of the low dose NDGA or vehicle administration, each treated male was housed with two untreated females for 5 days. Then, females were kept in separate cages. Fertility index was calculated by the formula: fertility index= number of impreganted females/total number of mated females x100. At the time of birth, pups were counted, weighed and checked for any external deformations.

Histopathological studies

Immediately after sacrifice, testis and epididymis were weighed and fixed with 10% formalin for histopathological and immunohistochemical staining. Five micrometer-thick paraffin sections were prepared and stained with haematoxylin and eosin. The seminiferous tubules were examined for tubular atrophy, presence of spermatids, vacuoles, intertubular spaces, sloughing of cells, arrest of cell division at any stage,

acrosome formation or any other abnormality. For measuring of seminiferous tubule epithelium height, 100 cross-sections of seminiferous tubules measured in 8-9 tissue sections per rat (6 rats/treatment). The height was measured using MC 170 HD Leica Camera, Switzerland and LAS EZ software at a magnification of 40×. The cauda epididymis was also examined for presence of sperms in their lumens, apoptotic cells, epithelial vacuolation and the integrity of stereocilia.

Studying sperm morphology and chromatin integrity

The cauda epididymis was opened by a blade in 1 ml Hank's balanced salts solution, Sigma to exude epididymal contents. After 5 minutes, smears were made and sperm morphology was examined immediately under light microscope. In order to evaluate chromatin integrity of sperms, DNA integrity test was performed as follows: Epididymal sperm smears were air-dried and fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) for 2 hours. Then, samples were stained by freshly prepared acridine orange, Gurr (0.19 mg/ml in Mcllvain phosphate-citrate buffer (pH=4) for 10 min (Talebi et al., 2014). Smears were examined within 2 hours using Nikon fluorescent microscope 460-nm filter. One hundred sperms were counted on each slide. Sperms having normal DNA are the green ones and those with damaged DNA are the yellow to red ones. The percentage of sperms with damaged DNA was calculated as red, yellow-orange/total×100%. In order to study sperm abnormalities, at least 100 sperms were counted/ rat.

Immunohistochemical staining of androgen receptor

Paraffin-embedded sections of the testis of NDGA-treated (N=8) and control rats (N=8) were mounted onto HIER slides (Biocare medical, USA). Antigen retrieval was performed according to the method of Shi et al. (1993). Sections were incubated with 10µg/ml anti-androgen receptor monoclonal mouse IgG antibody catalog number MAB58761 (R&D System, USA) specific for human, rat and mouse at 4◦C overnight. Streptavidin–biotin-peroxidase immunostaining was performed using Cell and Tissue Staining Kit HRP-DAB System (R&D System, USA) as in Tahtamouni et al. (2011).

Preparation of ultrathin sections for Transmission Electron Microscopy

Tiny pieces of tissue were fixed in 5% glutaraldehyde, kept at 4◦C and prepared as in (Abbas et al., 2013). Tissue specimens were post-fixed with 1% osmium tetroxide (Riedel-de Haën) and embedded in Spurr's medium. Ultrathin sections were stained later with aqueous uranyl acetate/lead citrate and studied using FEI transmission electron microscope at 30kV at the Faculty of Medicine/The University of Jordan. 6-10 seminiferous tubules and epididymal sections were observed with EM per rat (6 Rats per treatment).

Statistical analysis

Statistical analysis was performed using SPSS, 22nd version. Unless otherwise stated, all statistical tests were performed with two-sample two-tailed t-tests with 14 (=8+8-2) degrees of freedom. Data was presented as mean ± standard error of the mean (SEM). Differences were considered significant when $p<0.05$.

Results

No animals died in control and low-dose NDGA groups while 2 animals died in high-dose NDGA treated group. After 60 days of treatment, no statistically significant difference in relative paired testis weight was found at $P \leq 0.05$ (0.75%±0.06 for the control group and 0.81%±0.11 for the low dose NDGA-treated group) and for relative cauda epididymis weight (0.15%±0.007 for the control group and 0.11%±0.04 for the low dose NDGA-treated group). Similarly, no statistically significant difference in the percentage of orange red/total sperms was found between control and the low dose NDGA dose group.

Light microscopic examination of epididymal sections showed that the pseudostatified columnar epithelium has stereocilia in both control and all NDGA-treated rats given the drug for 60 days. Furthermore, some spermatozoa had rounded heads or short tails (Figure 2). Abnormality in epididymal spermatozoa in NDGA low dose group was 13.7% ± 3.3 compared to 4.6± 2.4 % in the control while it was 9.5± 6.3 % in NDGA high dose group.

In the group treated with 10mg/kg NDGA for 60days, the testes showed degenerative changes (Figure1D, F). The percentage of damaged seminiferous tubules was 11.48% while no damage was observed in the control group. If the height of damaged tubule was not included in measurements, no statistically significant difference in the height of epithelium of intact seminiferous tubules was found between control (89.4± 23.3µm) and NDGA treated-group (93.15±21.1µm) at $p<0.05$. However, no such changes were observed in high-dose NDGA-treated group treated for only 11 days. Using immunohistochemical method, it was demonstrated that NDGA does not affect androgen receptor expression in all testis sections of NDGA-treated rats (Figure 3).

Ultrastructural studies

Electron micrographs of testes of control rats showed intact lamina basalis of seminiferous tubules, normal myoid cells, interstitial cells, Sertoli cells, spermatogonia, spermatocytes and spermatids. In NDGA-treated group, degenerative changes were massive in 60-day study but much less dramatic in 11-day study (Figure 4). Spermeogenesis and acrosome formation appears to be normal in NDGA-treated groups. However, some spermatozoa had derangement of the outer dense fibers in their flagellum (Figure 4F). No apparent changes were observed in Leydig cells in NDGA-treated groups.

Effect of NDGA on fertility of male rats

Fertility index (number of pregnant females/total number of females x 100) was lower in NDGA-group treated for 60 days (56%) compared to control group (77%). Number of newborns was not statistically different ($P \leq 0.05$) for females impregnated with NDGA-treated males for 60 days compared with their control group (Table 1). Similarly, weight

of newborns was not statistically different for females impregnated with NDGA-treated males compared with their control group (Table 1). No gross abnormalities in newborns were detected in any group.

Discussion and conclusion

NDGA is a natural potent antioxidant (Grice et al., 1968). It is well known that antioxidants have protective effect on testis (Abbas, 2017). Many plants such as black maca (*Lepidium meyenii*) have beneficial effects on sperm production due to their antioxidant components (Yucra et al., 2008). Similarly, wenshen shengjing decoction composed of Chinese plant mixture including *Panax ginseng* and *Radix astragali* protected testis from oxidative stress caused by cyclosporine A (Pan et al., 2017). Despite its antioxidant activity, NDGA produced harmful effects on rat testis. The mechanism of action by which NDGA exerted its action in vivo needs further clarification.

Nowadays, infertility is regarded as one of the important public health issues because of its prevalence and that leads to social problems (Khaki and Ainehchi, 2017). According to Ouladsahebmadarek et al. (2016), the main cause of male infertility is impairment of sperm production due to different factors including the use certain medications. The present study highlights, for the first time, that NDGA may have negative effects on male rat fertility. Light and electron microscopic studies revealed the presence of degenerative changes in testis of NDGA-treated rats. Some seminiferous tubules were affected more than others (Figure1D). It is well known that testicular toxicants are often cell and stage specific (Gopinath and Mowat, 2014). Apoptotic bodies were observed in testis. NDGA was reported to induce anoikis-like apoptosis due to disruption of the actin cytoskeleton and activation of stress activated protein kinases (Seufferlein et al., 2002). Loss of cell–matrix interactions causes cell cycle arrest and a specific form of caspase-mediated apoptosis (Guadamillas et al., 2011).

NDGA possesses several pharmacological activities including 5-lipoxygenase (Meyer et al., 2007, insulin-like growth factor-1 receptor (IGF-1) inhibitory activities (Ryan et al., 2008B). It has been recently reported that IGFs are main regulators of Sertoli cell number, testis size and FSH action (Pitetti et al., 2013). Also, they mediate steroidogenic function of adult Leydig cells (Griffeth et al., 2014). Furthermore, NDGA has microtuble stabilizing activity in vitro (Arasaki et al., 2007). Previous studies have shown that NDGA arrest mitotic cells at the metaphase-anaphase stage (Arasaki et al., 2007). Therefore, at least theoretically, NDGA may interfere with germ cell formation and therefore male fertility.

The studied drug; NDGA, is insoluble in water. It is soluble in DMSO and organic solvents such as ethanol (Lundberg et al., 1944). Since ethanol is toxic to the testis (Cobb et al., 1978), DMSO was used instead. A water-soluble semi-synthetic derivative of NDGA; tetra-O-methylnordihydroguaiaretic acid (terameprocol), was developed recently and can be taken orally by humans (Kimura and Huang, 2016). Despite differences in the pharmacokinetics of oral and i.p routes, this study sheds light on the possible harmful effect of NDGA and its derivatives on testis. Future studies are needed to investigate the effect of NDGA oral derivatives on human male reproductive system.

Despite concerns over DMSO toxicity, no report on its toxicity on male reproductive system of the rat was reported. In our study, DMSO was given in a minimal dose of 100µl/rat/day which equals 0.5ml/kg. According to Gad et al. (2006), DMSO is well tolerated in rats when given i.p for 4 weeks by a dose of 5ml/kg. DMSO was used as a vehicle in several in vivo studies. According to Sahinturk et al. (2007) "The vehicle (DMSO)-treated control rats showed the typical morphological organization of the adult rat testis". Similarly, Yucra et al. (2008) found no difference between rats treated with DMSO or distilled water. Pan et al. (2017) administered D**MSO** for 30 days and found that spermatogenic cells in control and DMSO have normal histological architecture and abundant sperms inside seminiferous tubule lumen. Also, the number of apoptotic spermatogenic cells in DMSO group was not significantly different from that in control group. In fact, DMSO is not toxic for spermatozoa, it was found to be the best preservative for cryopreservation for testicular tissue banking (Baert et al., 2013).

In this study, no difference was detected in androgen receptor immunostaining between NDGA-treated and control animals (Figure 3). Absence of staining in some seminiferous tubules in the control was noted (Figure3A,B). It is well established that nuclear immunostaining of Sertoli cells increased progressively in intensity from stages II through VII of the spermatogenic cycle, and then declined during stage VIII to become poorly detectable in stages IX-XIII (Bremner et al., 1994).

Acrosome formation appears to be normal in NDGA-treated groups despite the fact that the acrosome is a structure derived from the Golgi apparatus. NDGA is a potent agent blocking protein transport in the secretory pathway and it disrupts the structure and function of the Golgi complex (Fujiwara et al., 1998). It has been found that NDGA interferes with membrane traffic by disturbing the microtubule motor dynein-dynactin complex and its auxiliary proteins (Arasaki et al., 2007).

NDGA is unique in that it is the first case of a compound that can modulate dynein-dynactin–related processes (Arasaki et al., 2007). It has been reported that dynactin 1 plays an important role in mouse spermiogenesis, and mainly affects the formation of the tail of spermatozoa. According to Zheng et al. (2011), sperm tail abnormalities were higher in dynactin 1 siRNA group compared to the control group. This agrees with the results of the present study in which short tailed sperms were present in cauda epididymis smears of NDGA-treated group. Also, derangement of the outer dense fibers was observed in cross sections of some spermatozoa. Nine outer dense fibers surround the axoneme in the middle and principal pieces of the spermatozoan tail. These fibers are essential for the elastic structure and recoil of the sperm tail. Furthermore, they protect the tail from shear forces. According to (Petersen et al. 1999) defects in the outer dense fibers results in abnormal morphology of sperm and may lead to infertility. The presence of tail and sperms head deformations in rats treated with NDGA may explain the lower fertilization capability of NDGA-treated rats and therefore, a lower pregnancy rate. Even though, no external deformations were observed in newborns.

The choice of doses used in this work was based on previous report on the intraperitoneal LD_{50} of NDGA in mice which was found to be 550mg/kg (Lehman et al., 1951) and a more recent study using 100 mg/kg/day of NDGA given orally, for 8 weeks in mice (Lee

et al., 2010). In our study, some animals died following high doses (40mg/kg) of NDGA(2 rats). Therefore we sacrificed the rest on day 11 of the study. Low dose (10mg/kg/day) was used to reach the therapeutic level with minimal side effects. Since the duration of spermatogenesis -the time taken by one type A spermatogonium to form mature spermatozoa- in rats takes 48–58 days (Cheng and Mruk, 2012), treatment of group I and II in the present work lasted for 60 days according to the criteria set by the WHO (1983). The low dose (10mg/kg/day) was safe until the end of the experiment which lasted for 60 days.

In a recent study, the dose of NDGA used to treat patients with non-metastatic hormone-sensitive prostate cancer was 2000 mg/day given for 28 days (Friedlander et al., 2012). According to Ryan et al. (2008A), 2500 mg/day of NDGA has been shown to be safe and well tolerated in patients. Assuming that the average weight of patients was 70kg, therefore the safe dose of NDGA in humans equals 35.7 mg/kg/day. In this study, the 40mg/kg/day i.p dose resulted in fatality of some rats. In order to compare our results and that of Ryan et al. (2008A), the oral dose of NDGA should be multiplied by a conversion factor that takes into consideration the different surface areas of the two species. In general, the i.p dose is much lower than the oral dose when applied to similar species. More pharmacokinetic studies of NDGA and its derivatives are needed since research in this field is scarce and inconclusive (Friedlander et al., 2012).

In vitro, NDGA had a stabilizing effect on tubulin and it promoted its polymerization (Nakamura et al., 2003). Microtuble-stabilizing agents disrupt spermatogenesis by different mechanism including spermatid retention, inhibition of spermatid apical movement, residual body retention, seminiferous epithelium vacuolization and seminiferous epithelium atrophy (Johnson, 2014). The results of the present study show the presence of vacuoles (Figure 1D). According to Chapin et al. (1983) vacuoles may be present preferentially associated with stages XII, XIII, IX, and I of the spermatogenie cycle. This may explain the presence of vacuoles in some but not all seminiferous tubules in this study.

Future studies are needed to determine (1) the specific apoptotic pathway involved in testis (2) the specific role of dynactin 1 in spermeogenesis of rat and human sperms and how NDGA affects it in vivo (3) the effect of NDGA administration and its other pharmacological forms on sperm count, motility, morphology, and DNA integrity in humans.

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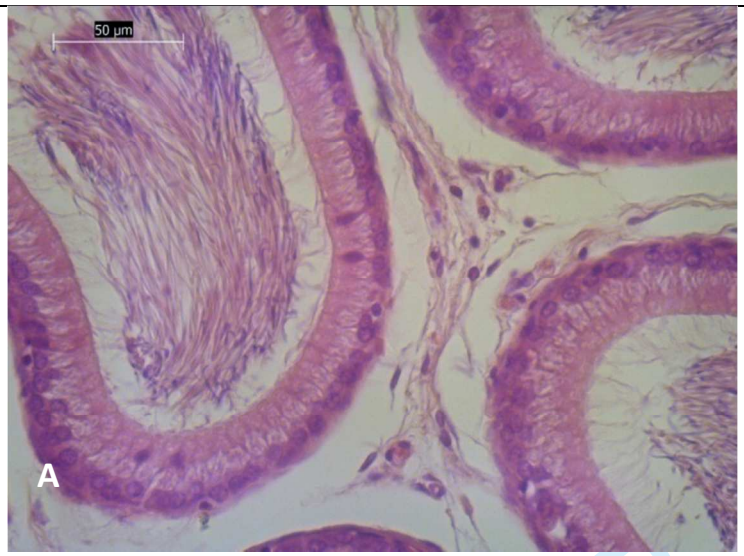
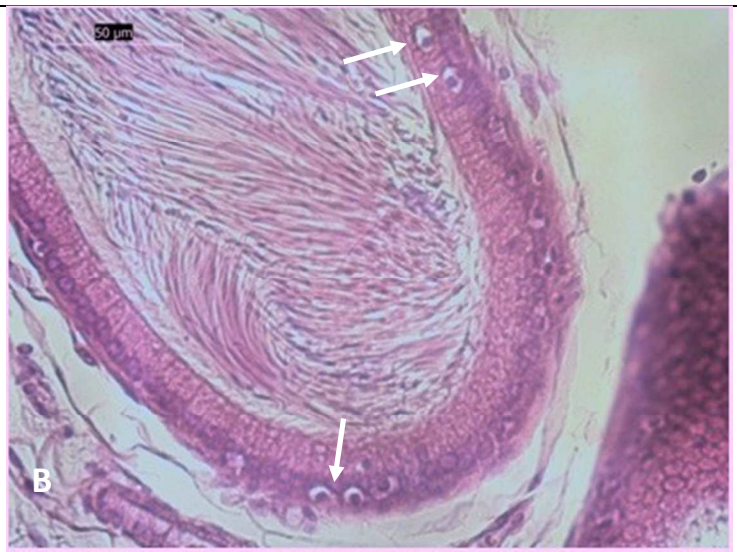
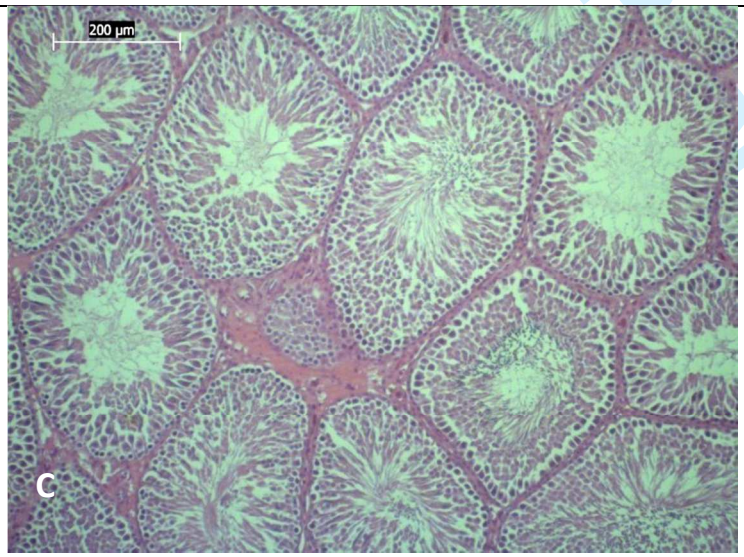
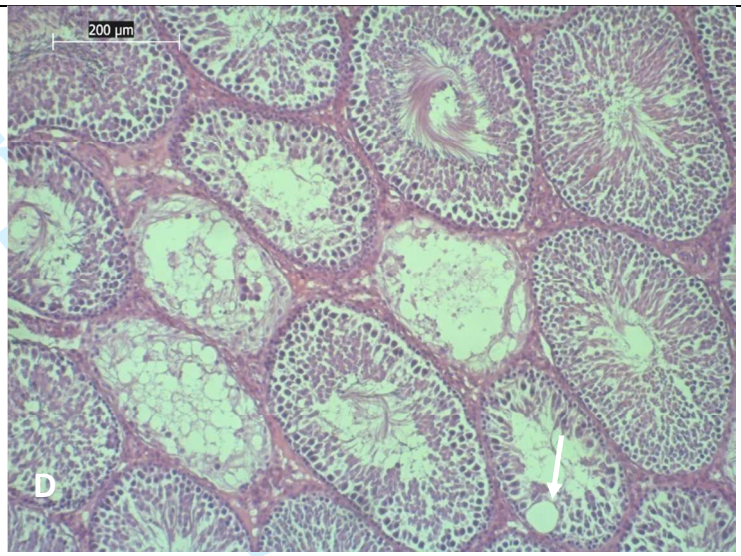
Negative control group	NDGA treated group (10mg/kg, 60 days)
	
	

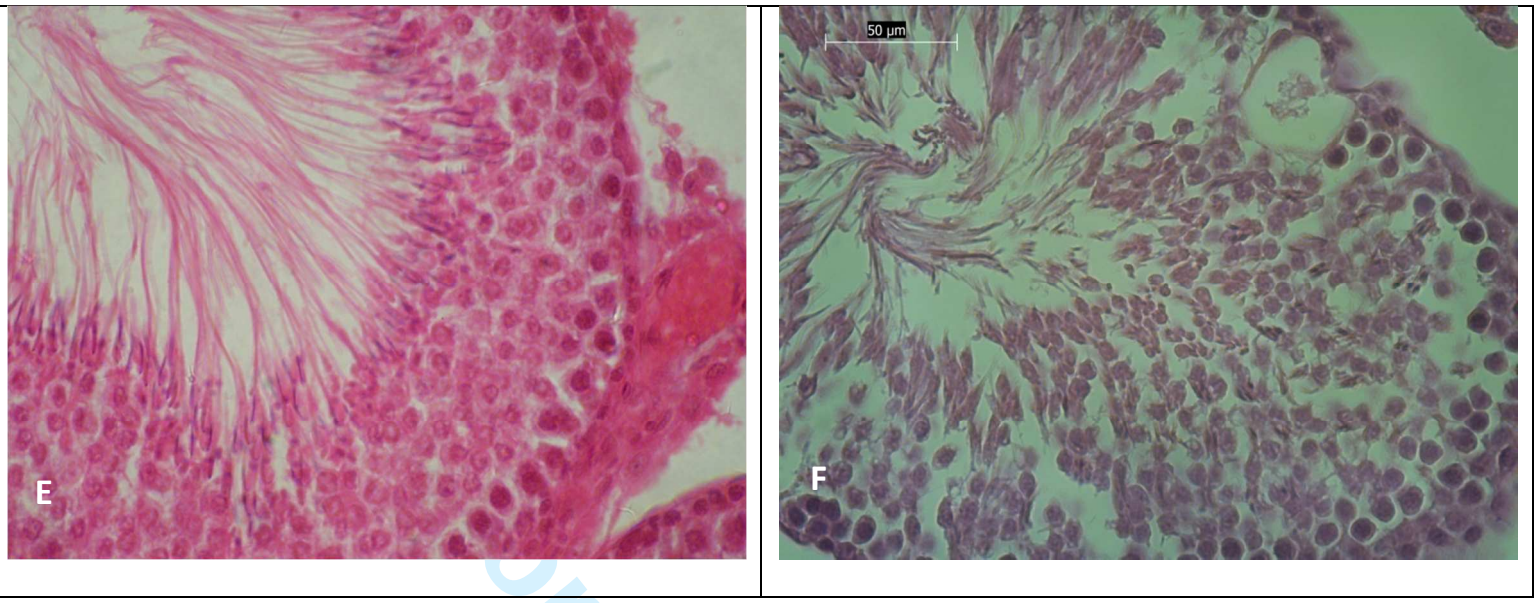


Figure 1 Light microscopic sections of control and NDGA-treated groups. Epididymis (A) control group (B) NDGA-treated group (10mg/kg for 60 days). Note that the epididymis is lined by pseudostatified columnar epithelium with stereocilia in A and B. However, some apoptotic cells are seen in B (arrows). (C,E) Testis of control group. (D,F) Testis of NDGA-treated group (10mg/kg for 60 days). Note the presence of vacuoles in D (arrow). Note degenerative changes in testis in D and F (haematoxylin & eosin stain).

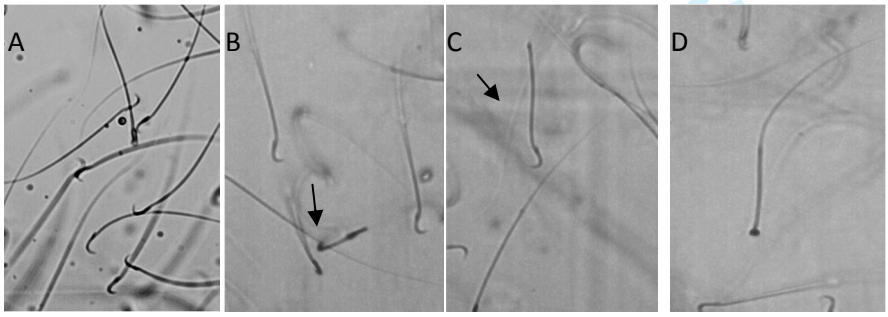


Figure 2 Epididymal spermatozoa deformations in NDGA treated rats. A) Control group: spermatozoa heads are hooked in rat. B, C) Short tail-spermatozoa were seen in NDGA-treated groups (arrow). D) Rounded head in NDGA-treated groups.

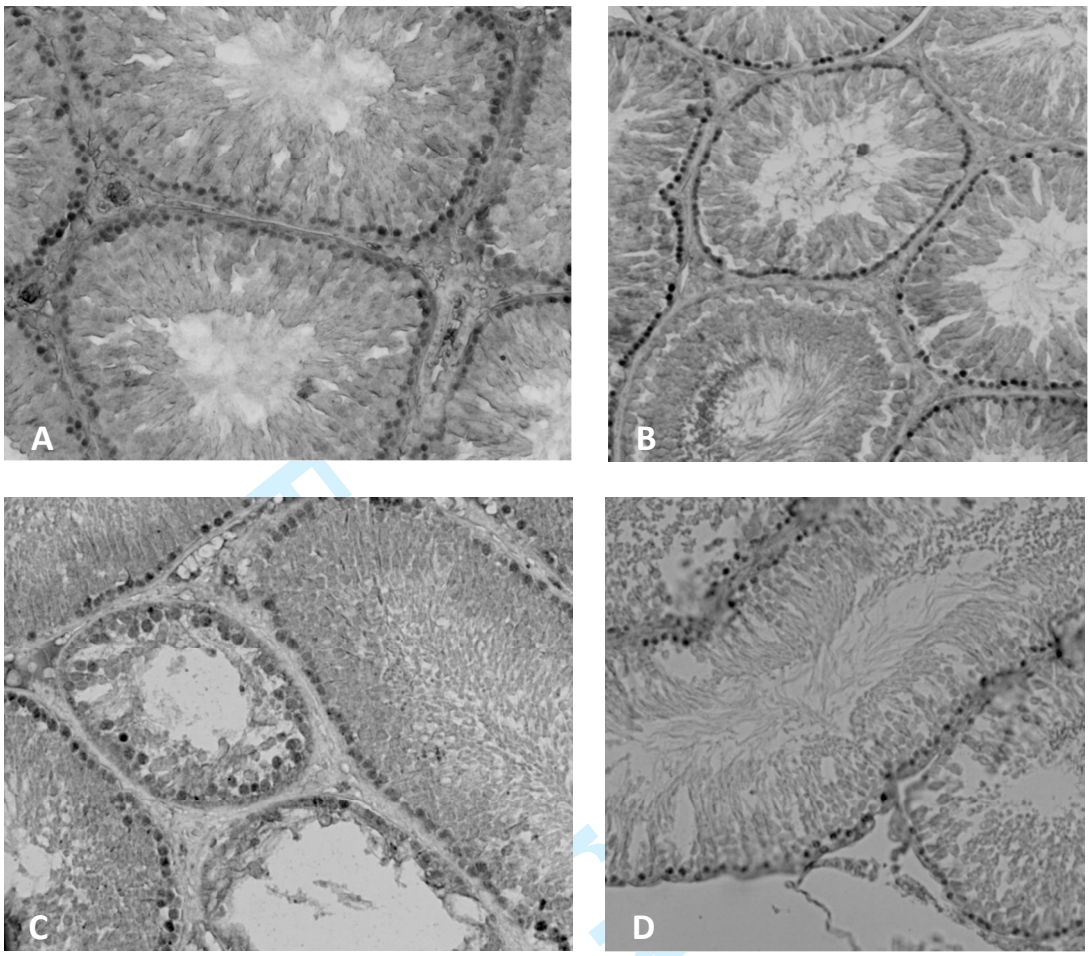
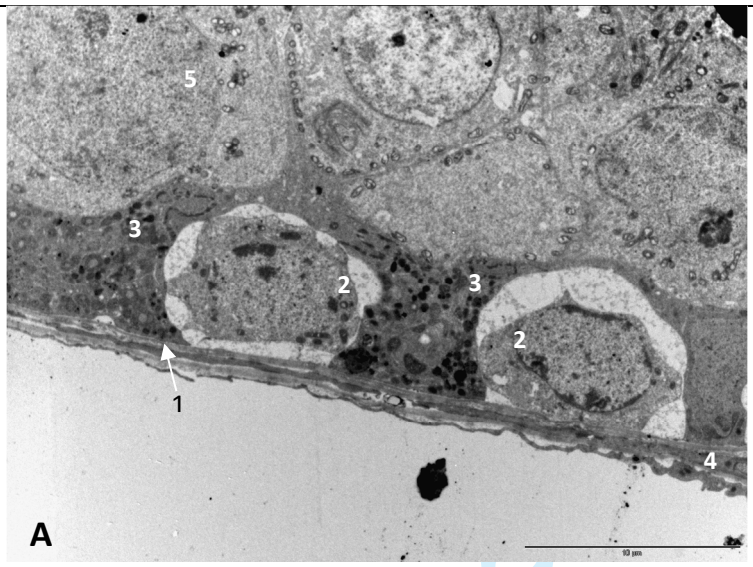
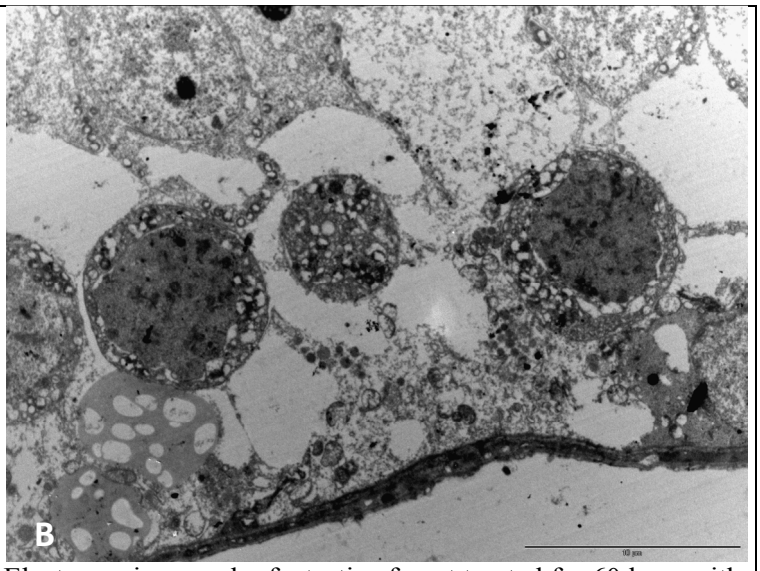


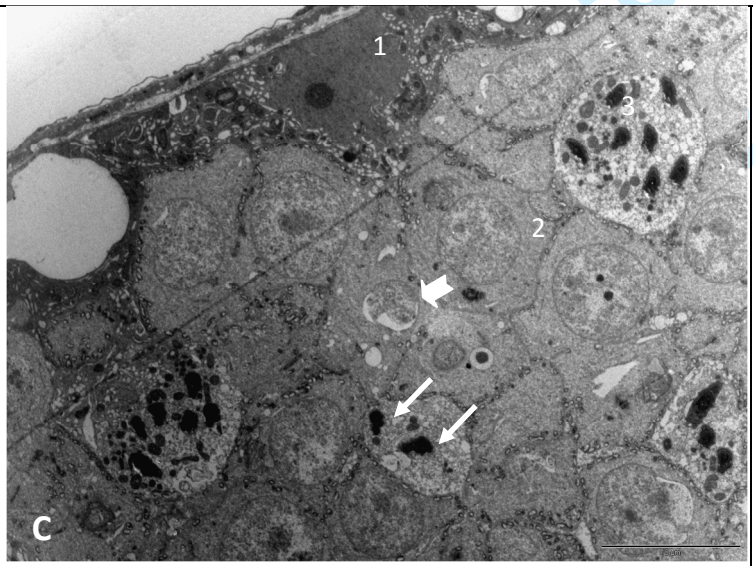
Figure 3 Immunohistochemical staining of the androgen binding receptor. Cells having androgen receptors are stained with dark color. A, B) control C) NDGA low dose, 60 days D) NDGA high dose, 11 days .



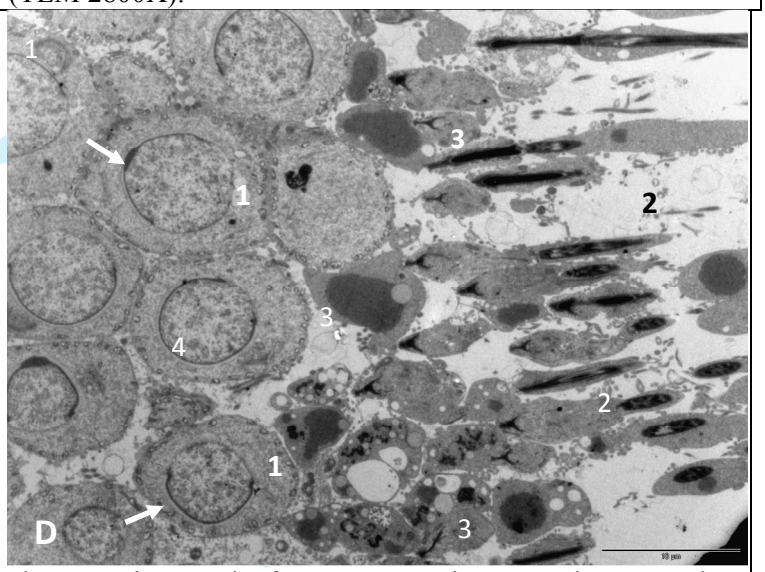
Electron micrograph of a testis of a control rat showing Lamina basalis (1); spermatogonia (2); Sertoli cell (3); myoid cell (4) and primary spermatocyte (5) (TEM 2800X).



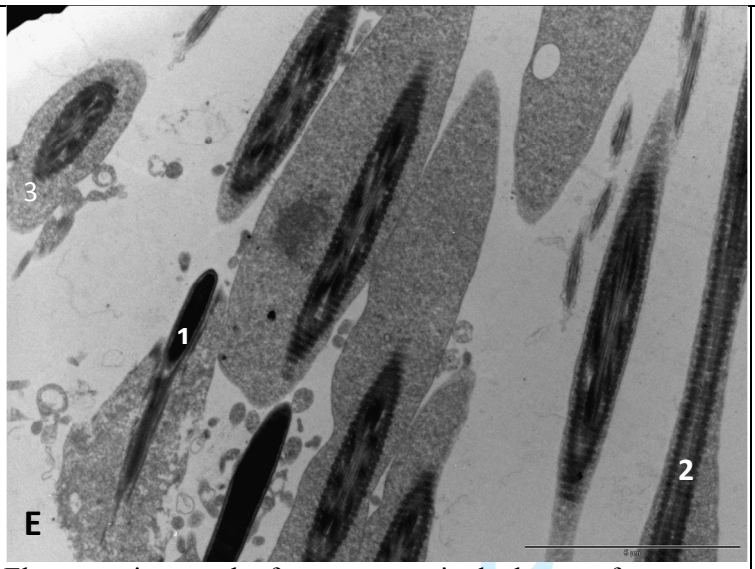
Electron micrograph of a testis of a rat treated for 60days, with NDGA (10mg/kg) showing intact basal lamina, disintegration of cells, vacuolation of cytoplasm and degenerative changes (TEM 2800X).



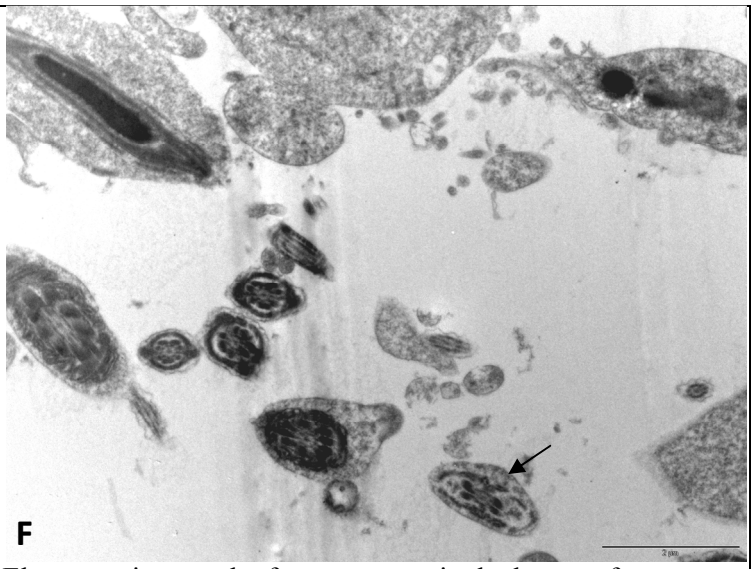
Electron micrograph of a testis of NDGA-treated rat (40mg/kg, 11 day) showing intact basal lamina, Sertoli cell (1); round spermatids (2); elongated spermatids (3); vacuolation of cytoplasm (arrow head); apoptotic bodies (arrow), disappearance of certain stages and degenerative changes (TEM 1800X).



Electron micrograph of spermeogenesis process in a control rat testis showing round spermatids (1) with their large rounded nuclei. Their position is close to the lumen (2). The acrosome spread over part of nucleus. The nuclear membrane is highly electron dense under the acrosomal cap (arrow). Also, elongated spermatids (3) are seen (TEM 1800X).



Electron micrograph of spermatozoa in the lumen of a seminiferous tubule in testis of a control rat showing head of a spermatozoan containing nucleus (1); longitudinal section (2) and cross section (3) of spermatozoa (TEM 5600X).



Electron micrograph of spermatozoa in the lumen of seminiferous tubule in testis of a NDGA-treated rat (40mg/kg, 11 day). Derangement of the outer dense fibers is seen in some cross sections of spermatozoa (arrow) (TEM 8900X).

Figure 4 Electron micrographs of testes and epididymides of control and NDGA-treated rats.

Table 1: Results of fertility study

	Control (vehicle)	NDGA (10mg/kg,60days)
Fertility index[1]	77%	56%
Number of newborns[2]	7.8±2.9	7.5±3.9
weight of newborns (gm)[2]	5.65 ±0.62	5.56 ±0.52

[1] Fertility index (number of pregnant females/total number of females x 100)
[2] No statistically significant difference in number and weight of newborns at $p \leq 0.05$.