

Effect of Smokeless Tobacco Product, Afzal, on the Reproductive Hormones and Gonadal Pathology of Wistar Rats

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ABSTRACT: Afzal is a common smokeless tobacco product (STP) in Oman, and it is believed to contain toxins that may affect the reproductive hormones and hence reproductive function. This study assessed the effect of Afzal on the gonads of Wistar rats. In order to assess gonad toxicity induced by this STP, an aqueous extract of Afzal was added to drinking water to be administrated orally to Wistar albino rats (n = 72) classified as young (4 weeks old) and adult (20 weeks old) of both genders weighing between 60-80 g and 150-240 g respectively for 8 weeks. The rats were divided into 3 groups; control (received distilled water instead of Afzal extract), low-dose (received 3 mg nicotine/kg body weight/day) and high-dose (received 6 mg nicotine/kg body weight/day). At the termination of the study, the rats were euthanized and their blood samples and ovaries were collected for biochemical and histopathological investigations. Testosterone and estradiol hormones showed a significant decrease ($P<0.05$) in Afzal-treated groups (low and high doses) compared with the control. Histopathological findings revealed the damaging effects manifested as a reduction in the number of the germ cells with deformed organization and in fatty and fibrous degenerations in testes and ovaries. Afzal was found to have adverse effect on the reproductive hormones and gonadal pathology in Wistar rats of both genders, and hence users of Afzal need to consider the risk associated with its frequent use.

Keywords: Smokeless Tobacco Product; Afzal; Wistar Rats; Reproductive Hormones, Testes; Ovaries; Testosterone; Estradiol.

تأثير منتج التبغ الغير مدخن، أفضل، على هرمونات وأمراض الغدد التناسلية لجرذان ويستر

نوال المخيني، طاهر باعمر، الصادق الطيب، عائشة الخياط، نفييلة الريامي، جميلة البلوشي، كوثر العدوي و رومولو سيبرياتو

الملخص: يعد أفضل أحد أنواع التبغ غير المدخن بسلطنة عمان ويعتقد باحتوائه على سموم قد تؤثر على هرمونات التناسل والتي بدورها تؤثر على الوظائف التناسلية. تهدف هذه الدراسة الى تقييم تأثير مادة افضل على أعضاء التناسل لدى فئران التجارب من نوع ويستر. تم إضافة محلول مستخلص منتج أفضل لمياه الشرب ليتم استهلاكه عن طريق الفم بواسطة الفئران من نوع ويستر الأهمق (عدد=72) بتصنيف صغار (بعمر 4 أسابيع) وبالغين (بعمر 20 أسبوع) من كلا الجنسين بأوزان تتراوح من 60-80 جم و 150-240 جم على التوالي لمدة 8 أسابيع. تم تقسيم الفئران إلى 3 مجموعات: التحكم (وتلقت ماء مقطر بدلاً من محلول أفضل)، الجرعة المنخفضة (وتلقت 3 ملجم نيكوتين/كجم من وزن الجسم/يوم) والجرعة المرتفعة (وتلقت 6 ملجم نيكوتين/كجم من وزن الجسم/يوم). بنهاية الدراسة تم قتل الحيوانات بطريقة إنسانية، وأخذ عينات من الدم والخصى والمبايض لعمل تحاليل الكيمياء الحيوية وفحص الأنسجة لتطور المرض. وتم تحليل مستويات هرمون التستوستيرون والإسترايول لجميع مجموعات الفئران. وقد أوضحت تحاليل الهرمونات انخفاض كبير ($P<0.05$) في مستويات التستوستيرون والإسترايول في كلا المجموعتين المعالجتين بأفضل مقارنة بمجموعة التحكم. ولوحظ أن هذا الانخفاض الهرموني يتناسب طردياً مع زيادة تركيز محلول الأفضل وقد تأثرت به جميع مجموعات الفئران (صغار/بالغين، ذكور/إناث) التي تلقت هذا محلول. وكشفت نتائج التحاليل النسيجية المرضية التأثيرات التناسلية المتضررة على شكل نقص عدد وتشوه بالتنظيم في الخلايا الجرثومية التناسلية و تحلل دهني ولفي في كل من الخصى والمبايض. وفي الختام، فقد أظهر منتج التبغ أفضل تأثيره على هرمونات التناسل وسبب أشكال مرضية بكل الجنسين لفئران التجارب من نوع ويستر، ولذلك فإن على مستخدمي أفضل الأخذ بعين الاعتبار المخاطر المرتبطة به مع الاستخدام المتكرر.

الكلمات المفتاحية: منتج التبغ غير المدخن، أفضل، هرمونات التناسل، فئران ويستر، الخصى، المبايض، التوستوستيرون، الإسترايول.



1. Introduction

Afzal is a smokeless tobacco product (STP) which contains toxins and heavy metals. Tobacco consumption has always been associated with stress induction [1]. Physiological and behavioral responses appear as a result of stress exposure [2]. Hormone secretion during stress may directly affect gonadal sex steroids such as corticotrophin releasing hormone (CRH). A stressor can induce corticosteroid secretion and may affect pituitary responsiveness to gonadotrophin releasing hormone (GnRH) [3]. Additionally, STP extract leads to oxidative stress which causes tissue damage and apoptosis [4].

Nicotine, a major component of tobacco, is considered to cause a high incidence of infertility [5]. Acute nicotine administration results in a rise in the plasma glucocorticoid levels [6] via the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland [7]. Nicotine has shown negative effects on both testosterone and estradiol levels in both genders of adult Wistar albino rats [8].

Tobacco use inhibits spermatogenesis and causes decreased steroidogenesis in men and anti-estrogenic effects in women [9-11]. Male users of STPs have exhibited some infertility findings such as reduced semen volume, sperm count, sperm motility and increased frequency of abnormal spermatozoa [12]. Decreased sperm count, sperm viability and sperm motility have also been reported in rats treated chronically with STP extract [13,14].

STP users place it inside the mouth for a period of time and the tobacco-saliva mixture formed most likely ends up in the systemic circulation. Systemic exposure to the harmful ingredients of STPs probably occurs from direct oral absorption or from swallowed saliva or tobacco particulate matter in the digestive tract [15]. Previous chemical analysis of Afzal has shown that it is contaminated with some hazardous substances such as heavy metals [16], high nicotine, nitrate/nitrite, other anions [17] and Tobacco-Specific Nitrosamines (TSNAs) [18]. Among those are some reproductive toxins including nicotine, and several metals, especially Chromium (Cr), Arsenic (Ar), Cadmium (Cd) and Lead (Pb) [19].

The present study was undertaken to evaluate Afzal's reproductive toxicity by assessing its effect on sex hormone (testosterone and estradiol) levels and the morphological changes of testes and ovaries in Wistar rats.

2. Materials and Methods

This study has been approved by Sultan Qaboos University Animal Ethics Committee (SQU/AEC/2013-14/6). Wistar albino rats (n =72) both young (4 weeks old) and adult (20 weeks old) of both genders weighing between 60-80 g and 150-240 g respectively were obtained from the Small Animal House at Sultan Qaboos University. The rats were housed in cages at room temperature (23 ± 2 °C) and a relative humidity of about 60% for 12 hour dark / light cycles and given water and a normal pellet diet *ad libitum*. They were acclimatized for a week prior to the experiment and randomized according to their weight.

A single sample of Afzal weighing 4 kg was purchased personally by the investigators from one source in order to maintain uniformity for chemical and toxicological investigations. This paper presents the results of the toxicological tests. The sample was labelled with the date of purchase and the product was kept refrigerated at 4 °C in plastic bags until analysis.

The rats were treated with aqueous Afzal extract mixed with drinking water for 8 weeks. Afzal extracts were freshly prepared every week according to the modified method of Pramanik [13]. The amount in grams needed for each group was calculated according to their weekly weight measurements, and with reference to the lethal dose of nicotine (LD50)[20]. Extracts were placed in a shaker for 1 hour at 37 °C and then filtered twice using filter paper (Whatman No.1) through a Buchner funnel. The resulting concentrated extracts were diluted with the normal drinking tap water in the ratio of 1:30 ml of the concentrated extract to the tap water.

The rats were divided into 3 groups of 24 each; the control group received distilled water mixed with the drinking water, a low-dose group received 3 mg nicotine/kg body weight/day and a high-dose group received 6 mg nicotine/kg body weight/day.

Blood samples for nicotine/cotinine detection and sex hormone tests were collected in week 2, from the tail vein (1ml), and at sacrifice day (end of week 8) by means of cardiac puncture. At the day of sacrifice, the animals were anaesthetized by intramuscular injection of Ketamil/xylazil-20 and the blood samples and the gonads, testes and ovaries, were collected and dissected out respectively.

Serum was separated by centrifugation at 3000 rpm for 20 min. (CL3 OR-centrifuge, Thermo scientific, UK) and was stored at -80 °C until the analysis. Before hormonal testing, the serum samples were subjected to a verification analysis to prove Afzal consumption by the animals through nicotine/cotinine GC/MS analysis [21].

For nicotine/cotinine analysis, the serum samples from the control and Afzal-treated groups were analyzed by GC/MS using the method of Broadway *et al.*, [21]. The extraction solvent was prepared using 1 ml of dichloromethane and 1 ml of 1:1 petroleum ether (ethyl ether). Another solution used was prepared by making a 2:1 ratio of solvent to serum. The solution samples were left at room temperature in a shaker and then allowed to settle.

The clear upper layer was collected by pipette aspiration. The solution was placed in another test tube for evaporation using a nitrogen stream at room temperature. One ml of methanol was added to the test tube of the nicotine suspension. The contents of the test tube were kept in a vial for general screening run by GC-MS analysis (Perkin

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Elmer Clarus 600 GC System, USA) looking for the nicotine/cotinine peak. This operation was performed under conditions as described in a previous work [17].

Sex hormone levels of testosterone and estradiol were estimated using the serum samples. Estradiol (REF 33540) and testosterone (REF 33560) were assayed using immunoassay by an automated UniCel™Dxi 600 (Beckman Coulter, USA) analyzer used in the Biochemistry Laboratory, Sultan Qaboos University Hospital. This analytical method used antigen-antibody reactions (immune complexes) to detect or measure the specific hormones in the serum samples of the rats [22, 23].

Quality control standards for estradiol and testosterone were maintained in the analyzer, and the calibration data updated automatically daily. For estradiol, they were as follows: r-value 0.99, accuracy or recovery coefficient 110%, with the analytical range of the lower limit of detection and the highest calibrator value being 73-17,621 pmol/L. Those for testosterone were: r-value 0.98, the accuracy or recovery coefficient 105%, with the lower limit of detection and the highest calibrator value being 0.35-55.5 nmol/L. The system uses a six-point calibration curve, controls, and 10 replicates of the zero calibrator in multiple assays. The six calibration levels were 0.0, 0.5, 1.5, 4.0, 8.0 and 16.0 ng/mL. The calibrators were prepared gravimetrically from testosterone/estradiol and a buffered bovine serum albumin (BSA) matrix. An assay calibration curve was prepared and measured daily, and from it, the amount of the analyte in the sample was determined.

Testes and ovaries were fixed in Karnovsky fixative, dehydrated in a series of acetone and embedded in resin. Semi-thin sections (0.5 μ m), stained with toluidine blue, and ultra-thin sections (70 nm) were cut using ultramicrotome (Reichert-Jung, Canada). The ultra-thin sections were collected on grids, and stained with uranyl acetate and lead citrate according to the method of Boozola and Russell [24]. They were then screened under a transmission electron microscope (JEOL JEM-1230, Japan).

Data were expressed in standard error of the mean (SEM). The significance of differences in the mean between control and treated animals was determined using one way ANOVA (Analysis of Variance) and followed by multiple comparison tests for observed means, Least Significant Difference (LSD) and Duncan post-test, using IBM-SPSS statistics-version 21 software. P values <0.05 were considered significant.

3. Results

The serum of Afzal-treated Wistar rats showed several peaks for nicotine and/or its secondary metabolites (Figure 1). The nicotine peak was obvious in the serum samples collected in both week 2 of treatment and at the termination of study. This is a clear verification of the ingestion of Afzal by the tested rats.

Levels of testosterone and estradiol hormones in serum samples of 8 weeks Afzal-treated rats showed significant differences ($P < 0.05$) compared with those of the control groups (Figure 2). Testosterone of the control group ($34.58 \text{ nmol/L} \pm 22.32$) was higher than both the low dose group ($14.36 \text{ nmol/L} \pm 11.51$) and the high dose group ($3.69 \text{ nmol/L} \pm 1.24$). The control level of testosterone was approximately 9 fold higher than that of high dose and 2.4 fold higher than that of low dose groups. Similarly, the estradiol of the control group ($0.288 \text{ nmol/L} \pm 0.07$) was higher than both the low dose group ($0.133 \text{ nmol/L} \pm 0.03$) and the high dose group ($0.045 \text{ nmol/L} \pm 0.02$).

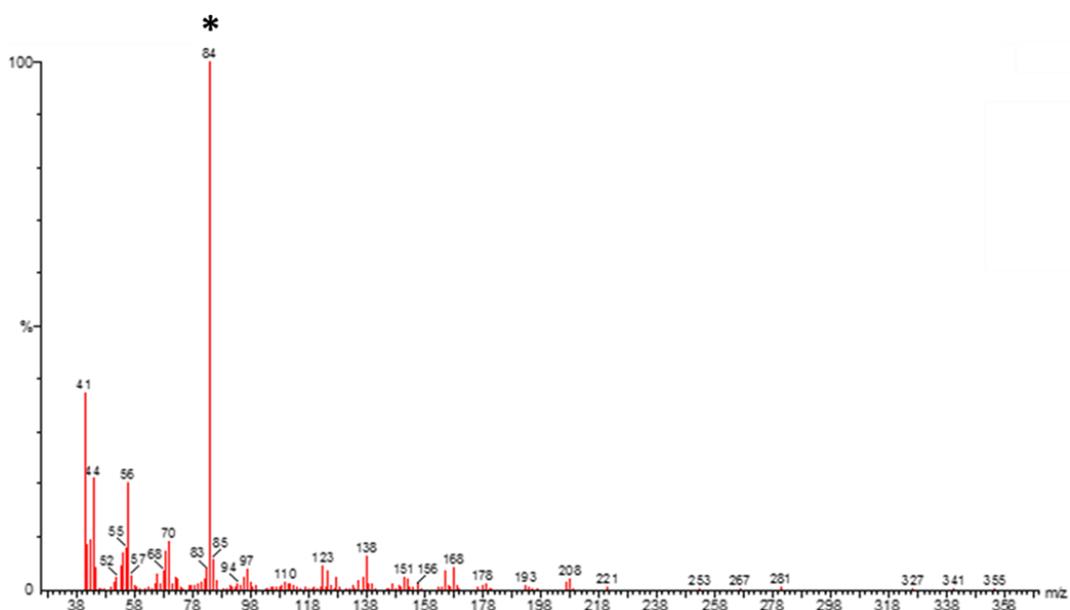


Figure 1: GC/MS chromatography showing the peak of nicotine (with asterisk and the spectrum mass of 84 m/z) seen in the serum of Afzal-treated rats only, while the control serum samples did not show peaks.

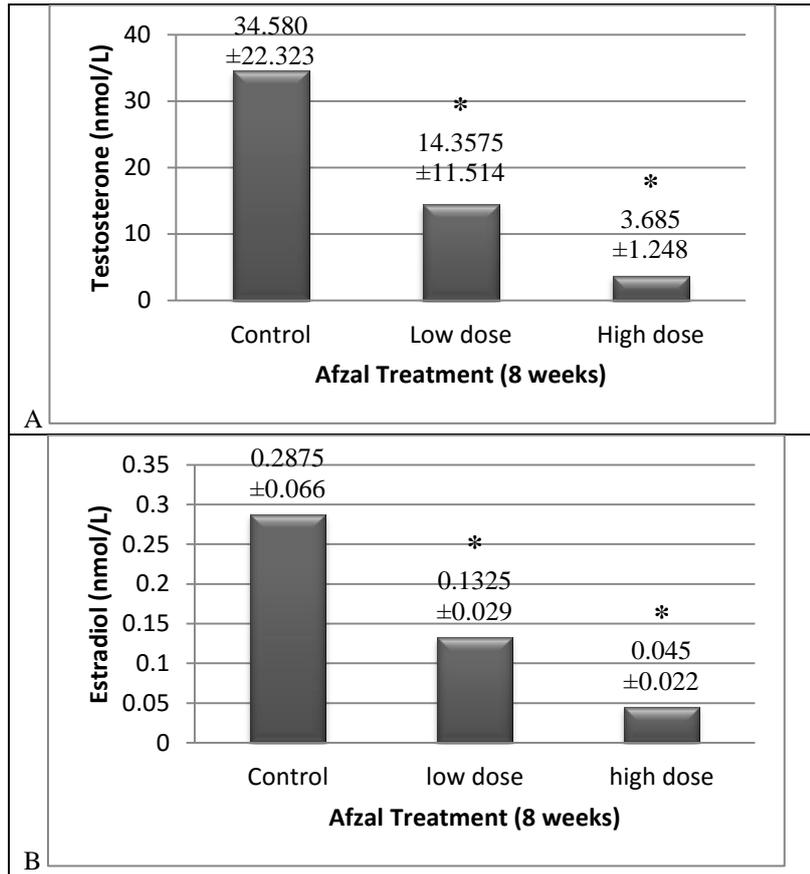


Figure 2. Sex hormone levels in 8 weeks Afzal treated rats' serum samples and their control counterpart groups. A. testosterone hormone, B. estradiol hormone. All treated groups were significantly different from the control, $P < 0.05$, in the two hormone levels (* asterisks: refer to significant differences compared with the control). Values are expressed on the top of each bar as the mean \pm SD, and animals were 8/group.

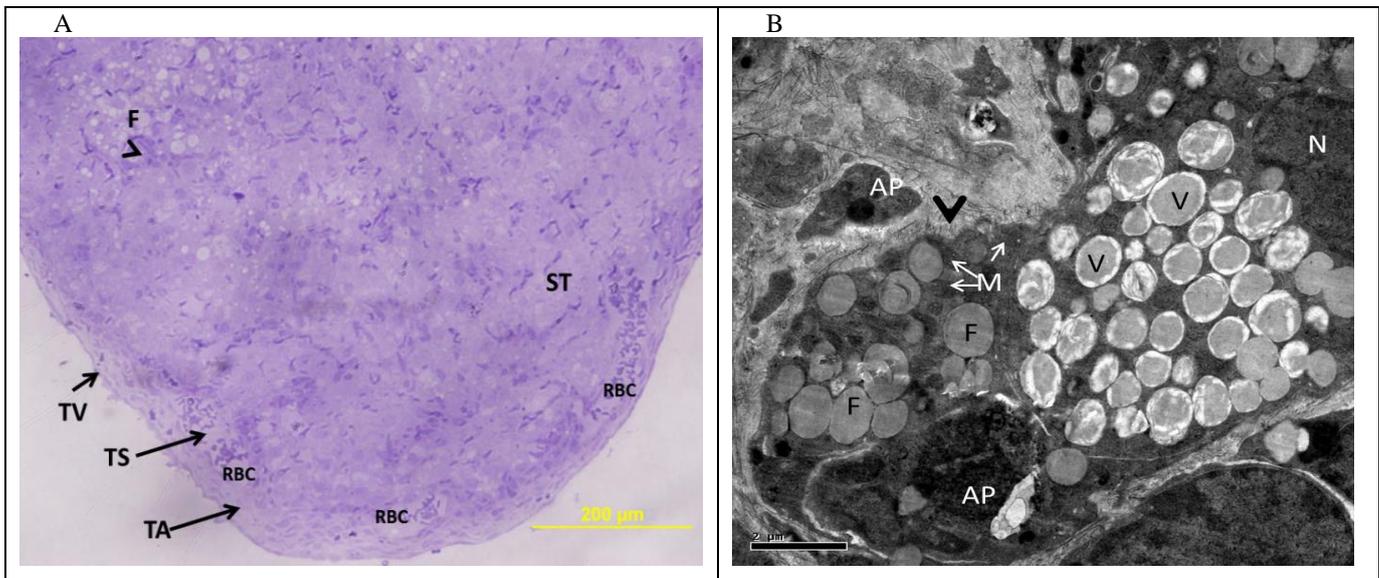


Figure 3. Micrographs of 8 weeks treated adult male rat testes of the control (A) and Afzal-treated (B) groups. The control group shows compact seminiferous tubules within the testicular capsule which is composed of tunica vaginalis (TV), tunica albuginea (TA) and tunica vasculosa (TS) with the interstitial cells of Leydig (arrow head) (scale bar =200 μ m). The treated low dose group shows a Sertoli cell engulfing the apoptotic cells (AP) and forming multiple vacuoles, and their microtubules (arrowhead) exhibiting their intercellular communications (scale bar =2 μ m). Red blood cells (RBC), seminiferous tubules (ST), lipid droplets (F), nucleus (N), myoid cells (M), vacuoles (v).

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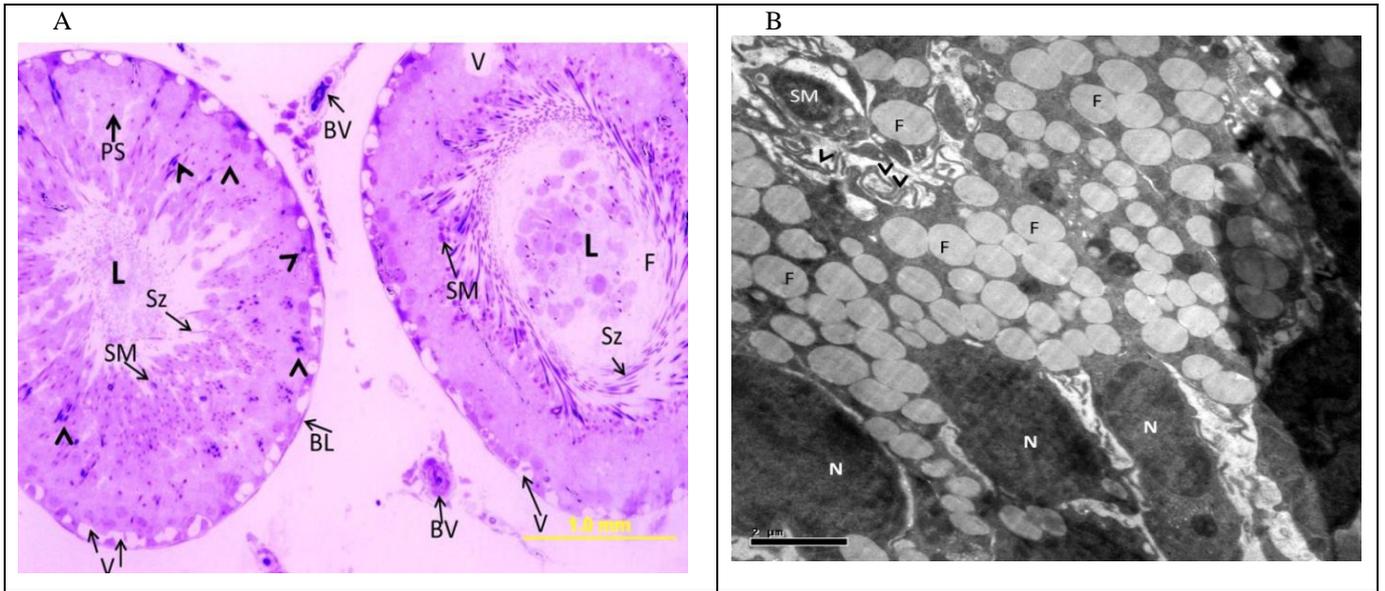


Figure 4. Micrographs of 8 weeks Afzal-treated adult male rat testis. (A) shows seminiferous tubules of a rat treated with the low dose showing degenerating germ epithelia and nuclear pyknosis (arrowheads). The lumen of the right tubule shows exfoliated germ cell and fat, while the lumen of left tubule shows a lower number of spermatids than in that on the right (scale bar =1.0mm). (B) shows a high-dose treated rat showing Sertoli cells with prominent nuclei engulfing the deformed spermatid cell. Sertoli cells show a large amount of fat droplets in the cytoplasm and many cellular extensions (arrowheads) (scale bar =2μm). Lipid droplets (F), nucleus (N), primary spermatocytes (PS), spermatids (SM) and spermatozoa (Sz), vacuoles (v), seminiferous tubule basement membrane layer (BL), and lumen of seminiferous tubule (L).

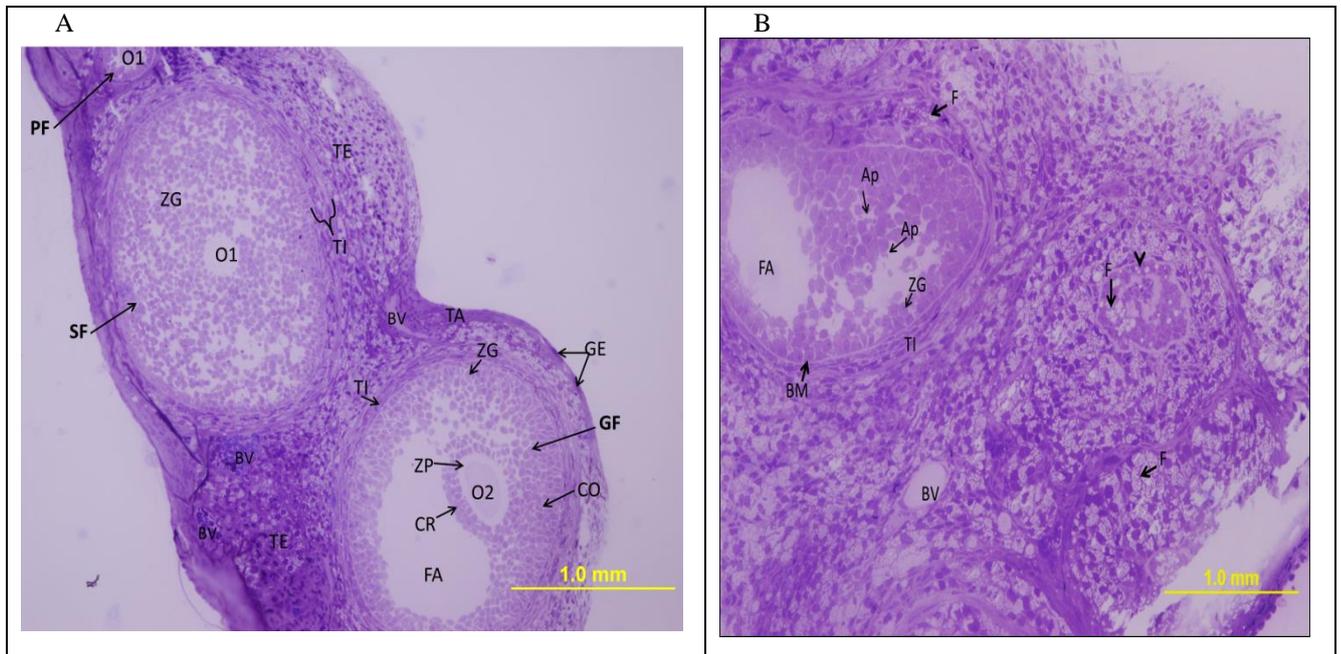


Figure 5. Micrographs of 8 weeks Afzal-treated adult female rat ovaries. (A) shows the control with primary follicle (PF), secondary follicle (SF) and Graafian follicle (GF) (scale bar = 1.0mm). (B) shows a low dose treated rat with impaired development of ovarian follicles with plenty of fat droplets accumulating in the follicular cell of zona granulosa cytoplasm along with multiple intracellular vacuolations (arrow head) and some surrounding deformed follicles (star) (scale bar = 1.0mm). Primary oocyte (O1), Zona Granulosa (ZG), Theca Interna (TI), Theca Externa (TE), blood vessels (BV), Follicular Antrum (FA), Secondary Oocyte (O2), Cumulus Oophorus (CO), Corona Radiata (CR), Zona Pellucida (ZP), germinal epithelial layer (GE), Basement Membrane (BM), apoptotic cells (AP), vacuoles (v).

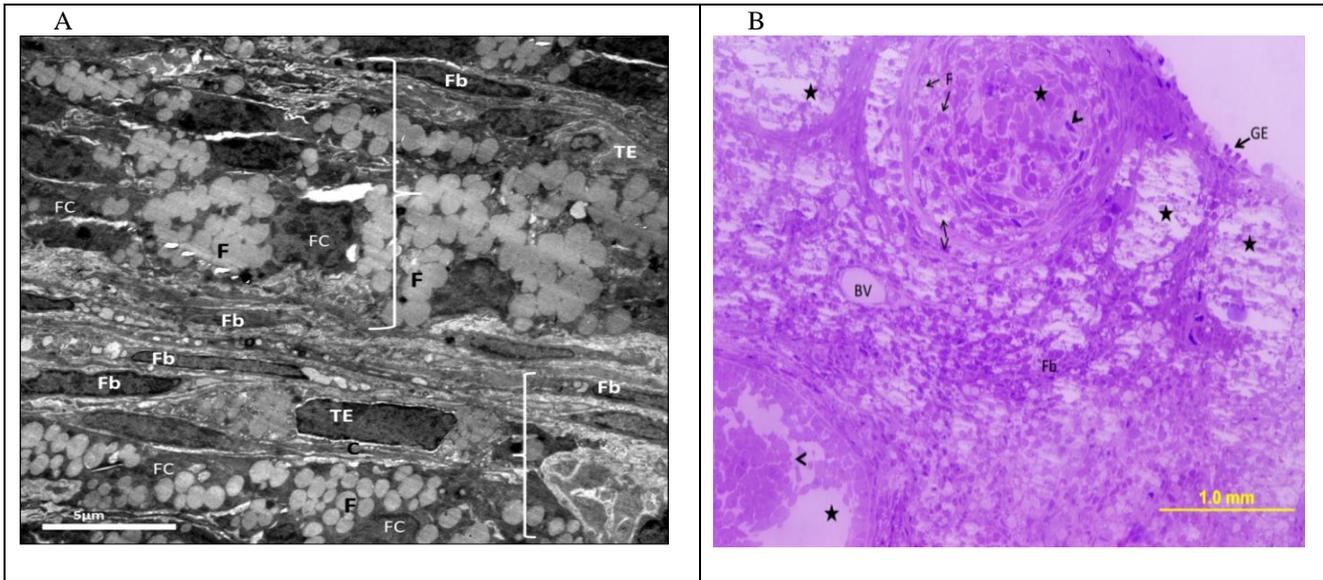


Figure 6. Micrographs of 8 weeks Afzal-treated adult female rat ovaries. (A) shows a low dose treated rat with degenerated atretic follicle (brackets) with high lipid droplets within the follicular granulosa cells (scale bar = 5µm). (B) shows a high dose treated rat with atretic follicles (star) with many vacuolations and lipid droplets. It also shows loss of granulosa cells, some with apoptotic and pyknotic nuclei (arrow head). The stroma shows the large increase of fibroblast activity (scale bar = 1.0mm). Theca Externa (TE), blood vessels (BV), Follicular Antrum (FA), germinal epithelial layer (GE), Follicular Granulosa Cells (FC), Fat Tissue or droplet (F), Vacuolations (V), Fibroblast cells (Fb) and Collagen fibers (C).

The control group showed normal features of testicular tissue with their seminiferous tubules containing germinal epithelial layers (Figure 3A). The testicular capsule is composed of different layers which are; tunica vaginalis (inner layer) (Figure 3A), tunica albuginea (middle layer) and tunica vasculosa (inner layer). Fat tissues can be seen within the interstitial spaces.

Testes of the adult Wistar rats treated with Afzal for 8 weeks in both low and high doses groups showed degeneration and damage (Figure 3B and Figure 4A and B).

The low dose Afzal-treated rats showed fatty degeneration occupying the majority of the interstitial spaces (Figure 3B and Figure 4A). Moreover, some seminiferous tubules showed small and deformed lumen and fat tissue between the germ cells, which may affect spermatogenesis. Some seminiferous tubules showed deformations in their lumen and their walls (Figure 4A). These deformations include indentations of nuclei, loss of cell-to-cell contact and multiple vacuoles in the cytoplasm and some apoptotic cells (Figure 3B).

The high dose group showed similar degeneration of spermatogenesis stages and focal disorganization of seminiferous tubules with marked depletion of the spermatogenic cell populations (Figure 4B). Moreover, the degenerated seminiferous tubules showed the exfoliation of damaged germ cells in the lumen with fat tissue. Some seminiferous tubules lacked spermatids and secondary spermatocytes.

The control group showed normal covering of the ovaries by a thick connective tissue capsule (Figure 5A). The ovarian cortex showed the presence of the developing follicles, and a vascular interstitial space. The cortex exhibited various developing oocytes.

The low dose showed impaired development of ovarian follicles (Figure 5B and Figure 6A). The follicles contained lipid droplets and vacuolations. Some ovarian follicles showed apoptotic characteristics (Figure 5B). Other degenerated atretic follicles were also observed (Figure 6A).

In the high dose group, tissues of adult female rats showed different forms of atretic follicles (Figure 6B). Some of those follicles appeared as cystic-like structures. Secondary follicles showed loss of normal granulosa cells arrangement and some displayed a deformed antral cavity, and apoptotic and dark small (pyknotic) nuclei. Other degenerating ovarian follicles and cells exhibited various vacuolations and lipid droplets in their cytoplasm (Figure 6B). Generally, the high dosage group showed fatty degeneration and loss of the usual organization, and large spaces between the cells reflecting the loss of cell junctions.

Gametes are vulnerable organs which showed STP associated tissue damage [12, 14]. Tobacco use leads to oxidative stress which has been established as one of the causes of male infertility [25]. Oxidative stress in testes was found to be increased in STP treated Wistar rats when compared with tobacco smoke or nicotine [26].

Stress induces impairment in the pituitary function causing the release of GnRH, which may affect gonadal secretion of sex steroids, such as corticotrophin releasing hormone (CRH) and corticosteroids [3].

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Afzal is contaminated with some hazardous substances such as heavy metals, [16] high nicotine, [17] and some carcinogens (TSNAs) that might cause reproductive toxicity [18]. Several metals found in Afzal, especially Cr, Ar, Cd and Pb, are considered to be reproductive toxins [19].

It has been suggested that nictines act as a central nervous system stimulant and also interferes with endocrine secretion of gonadotropins (FSH and LH) and prolactin from the pituitary and causes a feedback decrease in testosterone level [27, 28]. Nicotine has been reported to have a dose-dependent deleterious effect on the sperm characteristics. Aprioku and Ugwu found that smoking or nicotine treatment results in testicular degeneration, deficiency of male sex hormone and reduction in sperm count [26]. Thus the low sperm count would probably be a result of the decrease in, or absence of, androgens and FSH, which steer the process of spermatogenesis.

Additionally, it has been claimed that nicotine can cause a high incidence of female reproductive disorders [29]. A similar conclusion was that nicotine exerts a reproductive dysfunction affecting the female sex hormones [1]. It has also been observed that nicotine administration can cause an elevation in plasma glucocorticoid levels by releasing (ACTH). [6,7] Our study is in agreement with the above findings.

Chromium has been detected in Afzal samples [16] and it has been found that exposure to this metal affects and alters both male and female reproductive organs and their function in Wistar rats. [30-31] In addition, a significant decrease in estradiol levels was found to occur due to Cr treatment of female Wistar rats for 6 months [31].

In this study the testosterone level was found to be significantly lower in the Afzal-treated group ($P < 0.05$). Some studies attributed the relationship of tobacco use and nicotine in inhibiting the spermatogenesis and causing decreased steroidogenesis [9]. The findings of this study are in agreement with Yamamoto *et al.*, [32] and Oyeyipo *et al.*, [33].

It has been established that nicotine administration decreases the level of testicular androgenic enzymes along with plasma testosterone and sperm counts [32-33]. Furthermore, the fertilization ability of sperm has been found to be impaired due to tobacco use [32]. Additionally, Galam *et al.*, reported that nicotine alone affects male fertility and causes significant reduction of sex hormones [14]. Those results are in agreement with the high nicotine levels found in Afzal and its effects in impairing the reproductive organs in male rats.

Testes of Wistar rats displayed the damaging effects of exposure to Cr, which caused membrane damage and lipid peroxidation in testes [30]. The membrane damage was attributed to the alteration of the proportions of cholesterol and phospholipid in the membrane structure.

The results of this study confirm the finding that sex hormones are negatively affected by the presence of tobacco or its products. Moreover, our results clearly show that the administration of the aqueous extract of Afzal to adult male rats induced variable pathological changes in the testes. The present investigation has revealed that some of the seminiferous tubules appeared devoid of sperm, most probably due to alteration or inhibition of the process of spermatogenesis, and such changes may be dependent on the stage in the process at which the tobacco extract exerts its effect [34].

Apoptosis, disorganization, marked loss of spermatogenic cells and maturation arrest in the seminiferous epithelia of rats treated with Afzal were all clearly manifested. Afzal also caused degeneration of spermatocytes at various stages of maturation. These features and other degenerative changes in seminiferous tubules and spermatocytes are in agreement with reports on the testes of vitamin A-deficient rats and testes of mice exposed to halogenated diamines [35].

In females, the levels of the sex hormone estradiol were found to be significantly decreased in the Afzal-treated group. Tanko and Christiansen reported similar tobacco anti-estrogenic effects in women [11]. Some studies attribute this effect to the main tobacco alkaloid, nicotine, and some to Cr toxicity, both of which were abundant in Afzal. [8, 31]. Nicotine has been found to exert a negative effect on both testosterone and estradiol levels in adult Wistar albino rats [8]. Nicotine has adverse effects on female sex hormones by mediating the reproductive dysfunction [1]. However, it was also found that a significant decrease in estradiol levels in female Wistar rats occurred due to Cr treatment for 6 months [31].

Extracts from STPs have been reported to induce oxidative tissue damage and apoptosis [4]. Our findings in assessing Afzal's toxicity in ovarian tissues are in line with those of Kilinc *et al.* [4] and Iranloye and Bolarinwa [29].

Stress and chronic tobacco use are associated with the disturbance of body lipid patterns [1]. An effect of lipid disturbance has also been observed as fat accumulation within the surrounding follicular cells in ovarian tissues of Afzal treated groups.

Conclusion

The present study has shed light on the adverse health consequences associated with Afzal in Wistar rats, including impaired functional and structural outcomes for the main reproductive organs (testes and ovaries). Functionally, the sex hormone (testosterone and estradiol) levels showed significant decrease due to Afzal treatment compared to the control groups. Structurally, the degenerated features of testicular and ovarian tissue were evident in this study. The main destructive features are reduction in the germ cells population with fatty and fibrous degeneration in testes and ovaries. It can be concluded that the frequent administration of Afzal causes significant reduction in the testosterone and estradiol levels and may lead to a compromised reproductive activity in Afzal users.

Conflict of interest

The authors declare no conflict of interest.

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