

PROTECTIVE ROLE OF JAMBUL (*SYZYGIIUM CUMINI*) FRUIT-PULP EXTRACT AGAINST FLUORIDE-INDUCED TOXICITY IN MICE TESTIS: A HISTOPATHOLOGICAL STUDY

Khawaja R Ahmad,^{a*} Tooba Nauroze,^a Kausar Raees,^b Tahir Abbas,^c Muhammad A Kanwal,^a Shazia Noor,^a Shamsa Jabeen^a
Sargodha, Pakistan

SUMMARY: Ameliorative effects of jambul (*Syzygium cumini*) fruit-pulp extract were studied in mice against fluoride exposure (from NaF) in relation to histopathological and histometric changes of testis and sperm micrometry. The study was conducted on forty 3–4 months old male albino laboratory mice weighing between 25 and 30 g who were fed on a standard laboratory diet and randomly distributed into four groups of 10 mice each as follows: 1) Control, F-free drinking water (15 days); 2) NaF, 50 ppm F ion from NaF in drinking water (10 days) followed by no treatment (5 days); 3) NaF+jambul, 50 ppm F ion from NaF (10 days) followed by 0.25 mL jambul pulp-extract twice a day by gavage (5 days); 4) Jambul, F-free drinking water (10 days) followed by jambul pulp-extract twice a day by gavage (5 days). Results showed that NaF exposure caused a general loss of interstitial tissue, spermatogonia, and spermatogenesis. The average number of spermatogonia per-spermatid cord in the NaF group (0.75) and the NaF+jambul (1.55) groups was significantly ($p<0.05$) lower than the control (2.5) and jambul (2.7) groups. A significant decline ($p<0.05$) in the mean cross-sectional area (CSA) of the seminiferous tubules in the NaF group ($16442.4 \mu^2$) compared to the control ($21017.7 \mu^2$) occurred, whereas the mean CSAs of spermatogonia ($37.29 \mu^2$) and primary spermatocytes ($34.52 \mu^2$) in the NaF group were significantly ($p<0.05$) higher than in the control group ($20.23 \mu^2$ and $24.91 \mu^2$, respectively). Sperm micrometry revealed a significant decline ($p<0.05$) in head length (7.93μ), breadth (3.96μ), tail length (83.25μ), and the length (22.79μ) and diameter (1.06μ) of the middle part in the NaF group compared to the control group (9.79μ , 4.12μ , 101.16μ , 25μ , and 1.198μ , respectively). These latter changes from NaF exposure were significantly reversed on post treatment with jambul fruit-pulp extract. Signs of testicular histopathologies from NaF were effectively reversed with jambul extract treatment.

Keywords: Fluoride exposure; Mice testicular pathology; Sperm micrometry; *Syzygium cumini* (jambul).

INTRODUCTION

1. Background:

The main sources of fluoride (F) intake include drinking water, foodstuffs, industrial dust and smoke, pesticides, and F-containing dental products.¹⁻⁵ F is also a potent industrial toxicant.^{6,7} The production of reactive oxygen species and radicals (ROS),^{3,8-11} disturbance in the activity of Na^+/K^+ ATPase,¹²⁻¹⁴ and damage to proteins and DNA are major outcomes of F intoxication.^{5,9,15,16} F also promotes lipid peroxidation that alters membrane fluidity and can thus potentially disrupt intracellular compartmentalization of spermiogenesis¹⁷ leading to a general loss of spermatogenesis.¹⁸

Jambul (*Syzygium cumini*) is a common medicinal food plant of the family *Myrtaceae*.^{19,20} Its fruit pulp has excellent antioxidant properties²¹⁻²³ because it contains fairly large amounts of hydrolysable tannins, flavonols, and

^{a*}For correspondence: Khawaja Raees Ahmad, Assistant Professor of Zoology, Department of Biological Sciences, University of Sargodha, Sargodha Pakistan; E-mail: kraees@uos.edu.pk; ^bPrincipal Government College (for women) Farooq Colony Sargodha, Pakistan; ^cDepartment of Biology, Government College 75SB, District Sargodha, Pakistan.

proanthocyanidins.²⁴ In silica-intoxicated rats, jambul extracts effectively diminished a general increase in the serological parameters of aspartate amino transferase, alanine amino transferase, alkaline phosphatase, glucose, protein, and cholesterol.²⁵ In mice, hexane extracts (15 mg/kg) of the flower buds of *Syzygium aromaticum* (a related species) enhanced testicular functions, produced an increase in steroid dehydrogenase, alcohol oxidoreductases, and serum levels of testosterone in mice.²⁶ With jambul, apoptosis in germ cells in mouse testes induced by hyperthermia²⁷ is reported to improve semen quality because of its anti-hyperthermic and anti-oxidative properties.²⁸

In the present study, effects of jambul fruit-pulp extract for protection against testicular histopathologies and structural dynamics of spermatozoa from NaF exposure in mice are reported.

MATERIALS AND METHODS

2.1 Chemicals:

Standard laboratory-grade sources of sodium fluoride (NaF), ethanol, xylene, picric acid, glacial acetic acid, formaldehyde, embedding wax, Canada balsam, hematoxylin, eosin, etc., were used for various experimental preparations in this study.

2.2 Animal care and maintenance:

The study was conducted on forty 3–4 months old male albino laboratory mice weighing between 25 and 30 g, born and nurtured in the Animal House, Department of Biological Sciences, University of Sargodha, in Sargodha, Pakistan. The prevailing housing conditions were at $23\pm 3^{\circ}\text{C}$, 45% relative humidity, and a 12-hr dark-light cycle. The animals were provided free access to a standard laboratory mouse diet and low-F water, strictly following the animal care and experimental guidelines of the University of Sargodha.

2.3 Preparation of jambul (*Syzygium cumini*) fruit-pulp extract:

Ripe fruit of *Syzygium cumini* was purchased from the local market from which fully ripe berries were carefully selected. These were washed thoroughly in cooled boiled drinking water for 5 min, air dried, and finally the pulp was softened and separated from the seeds by means of vigorous shaking in a tightly closed sterilized wide mouth glass jar. The seeds were discarded and 100 g of the pulp was blended with an electric juicer in 100 mL of cooled boiled drinking water for 5 min. The resulting juicy material was centrifuged at 500 rpm for 10 minutes to separate the deep purplish supernatants from the bottom-settled fibrous pulpy mass. The supernatants were immediately placed at -30°C in sterilized 5-mL ice-cube dishes. The frozen cubes (one each) were then placed in sterilized (airtight) plastic bags and stored at -30°C . For each treatment, extract from a freshly thawed (at room temperature) cube was used.

2.4 Preparation of NaF solutions:

Analytical grade sodium fluoride was used to prepare the required aqueous NaF solutions. A 1000-ppm F stock solution was prepared by dissolving 2.21 g of NaF in 1 L of water. Feeding dilution (50 ppm) was prepared by adding 95 mL of water to 5 mL stock solution as per requirement.

2.5 Animal groups and dose administration:

Mice were randomly divided into four groups of 10 mice each. These groups were:

2.5.1 Control (untreated) group: Animals were maintained without any treatment throughout the study period (15 days).

2.5.2 NaF group: Animals were provided 50 ppm F in drinking water for 10 days followed by simple water (non-fluoridated) for the next 5 days.

2.5.3 Jambul group: Animals were maintained on non-fluoridated drinking water throughout the study period (15 days). For the last 5 days of the study they were given 0.25 mL jambul pulp extract at 12-hr intervals by gavage.

2.5.4 NaF+jambul group: Animals were given F in drinking water as in NaF group (2.5.2) and pulp extract as in the jambul group (2.5.3).

2.6 Recovery of testes:

For removal of testes, the animals were euthanized by cervical dislocation on day 16. Both testes in each case were retracted in the visceral chamber and removed intact through a medial abdominal incision. One randomly chosen testis in each case was used for smear preparation while the other was placed in alcoholic Bouin's fixative for 48 hr and then processed for wax embedding and microtomy.

2.7 Testicular smear preparations:

To study sperm morphology and micrometry, one testis from each animal was cut into two equal halves from the middle across the long axis and gently crushed on a clean glass slide with a blunt glass rod. Normal saline (2–3 drops) was added to this material and mixed thoroughly. The curdy material thus obtained was used to make thin smears. The smears were air-dried and stained with hematoxylin and eosin.

2.8 Sperm micrometry:

Photographs of 100 randomly chosen, morphologically normal and intact spermatozoa from testicular smears in each group were obtained at 1000× (optical magnification) with a 7.2 MP (mega pixel) digital camera (Sony) mechanically affixed on a trinocular research microscope (Labomid CXR2). Dimensions of the sperm cells in terms of length and breadth of the head, tail length, and the length and thickness of the middle part were measured from these digital images in CorelDRAW11 graphics software. For calibration, digital images of stage micrometer, obtained on the optical and digital specifications fixed for photography of spermatozoa, were used.

2.9 Testicular histology:

Testes fixed in alcoholic Bouin's fixative were processed for dehydration, wax embedding and serial sectioning in a routine way. Histological sections (6 μ) were obtained on a rotary microtome. These were affixed on albuminized glass slides for haematoxylin and eosin staining. Digital photographs of these testicular sections (100, 400 and 1000×) were obtained to highlight histopathological outcomes and to obtain histometric data. (See sections 2.10 and 2.11 below.)

2.10 Spermatic cords:

Successive generations of spermatogonia from 10 spermatic cords were counted in each of the 10 randomly selected seminiferous tubules from every individual animal (100 spermatic cords/ animal). Mean values for each animal were used to obtain the group means \pm SEM.

2.11 Histometry:

To obtain the cross-sectional area (CSA) of the seminiferous tubules, spermatogonia, and primary spermatocytes, calibrated measurements of diameter were obtained from two right-angle positions in each case. The following formula was employed to calculate the cross-sectional area in each case.

$$\text{CSA} = \frac{(\text{length} \times \text{width})}{4} \pi$$

Ten randomly selected tubules and 100 cells from each animal were used for these measurements to obtain mean values.

2.12 Statistical analyses:

From histometric and sperm micrometric data, group means \pm SEM were obtained. The data were further subjected to the analysis of variance and post-hoc comparative analysis of group means (Duncan's multiple range tests).

RESULTS

3.1 Testicular histology:

The histological sections in the control group showed seminiferous tubules surrounded by dense interstitial tissue. All along the basement membranes, in each seminiferous tubule, spermatogonia were arranged in concentric layers (typically representing the zone of mitosis), followed by similar concentric layers of spermatocytes immediately inward to the spermatogonial layers (representing the zone of meiosis), while the core area contained differentiating spermatozoa (identified as the zone of spermiogenesis). The most central luminal part of the seminiferous tubules was occupied by mature spermatozoa (Figure 1A). Similar histological layout was obvious in the NaF+jambul group (Figure 1D).

In the jambul-only treated group all histological dispositions (of the control group) were clearly visible with an impression of increase in the thickness of the zone of mitosis and denser luminal space filled with spermatozoa (Figure 1C). By contrast, the NaF group showed signs of various pathological alterations including necrosis and obliterations of the interstitial tissue and a drastic decline in the number of spermatogonial cells resulting in the absence of all internal layers except the only one present along the basement membrane, and even in this layer the spermatogonia were scattered leaving empty spaces in-between. Inner to the spermatogonia irregularly arranged spermatocytes were seen. Wide luminal spaces with a few scattered clusters of spermatozoa were observed in the centre of each seminiferous tubule (Figure 1B). Signs of necrosis and unusual lateral mitosis in spermatogonial cells, leakage of the nuclear contents, and resultant necrosis of the spermatocytes and involutions of spermatozoa were the obvious histopathological observations of NaF exposure (Figure 2).

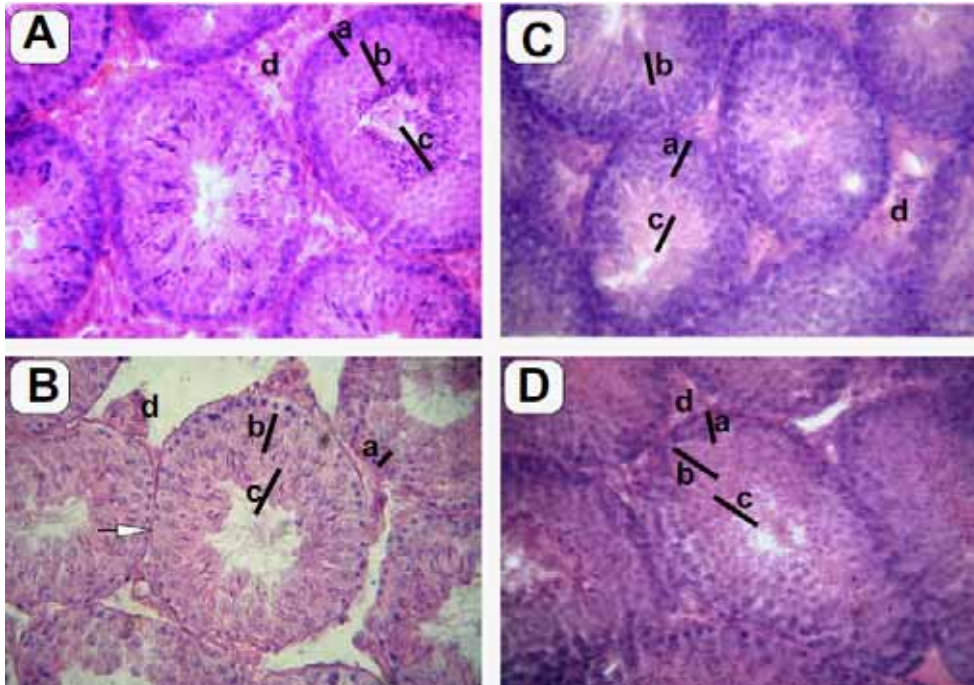


Figure 1. Histological sections of testis (400 \times). A: control; B: NaF; C: jambul; D: NaF+jambul treated groups. a: spermatogonia (zone of mitosis), b: spermatocytes (zone of meiosis), c: spermiogenesis (zone of transformation), d: interstitial tissue. White arrow: basement membrane with no spermatogonia.

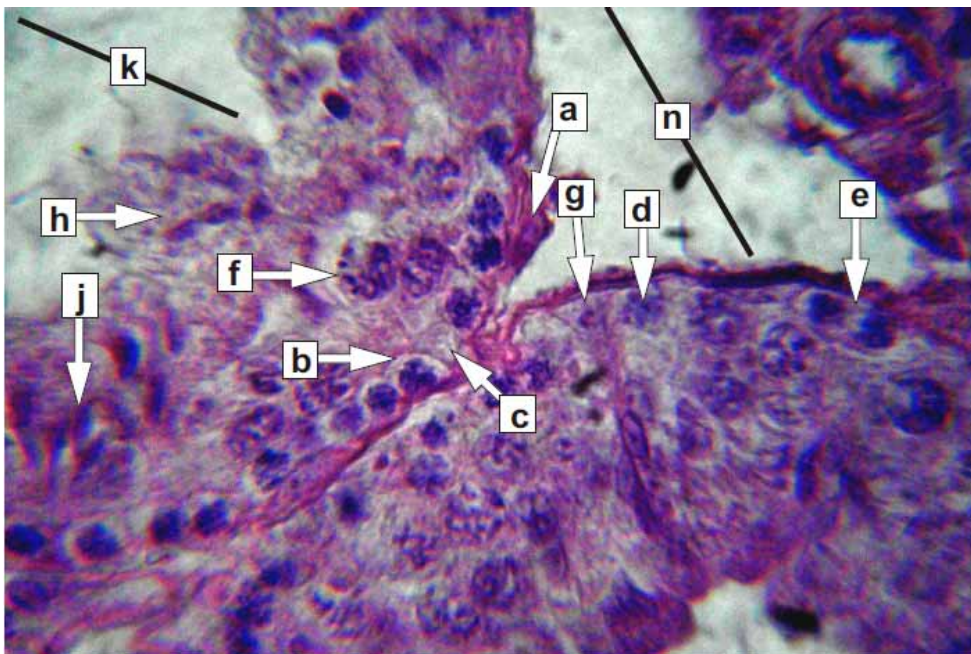


Figure 2. Selected area of the histological section of NaF treated testis showing various histological abnormalities (1000 \times). a: basement membrane cell, b: a typical primary spermatogonium, c: apoptosis of a primary spermatogonium, d: spermatogonium under necrosis, e: lateral mitosis in primary spermatogonium, f: necrotizing spermatocyte, g: a nurse or Sertoli cell, h: spermatozoon involution, j: halted meiosis/spermiogenesis, k: wide luminal space of the seminiferous tubule, n: hollow interstitial space produced by necrotic death of the Leydig cells.

3.2 Testicular histometry:

Data pertaining to the CSAs (cross-sectional areas) of the seminiferous tubules showed a trend towards approaching significant variation among the groups ($p < 0.13$), whereas post-hoc analysis of the data (Duncan's Multiple Range Test ($p < 0.05$)) indicated significant variations of the NaF group compared to the data for the control group and the jambul-only group (Table 1). Although no significant variation in the data for number of spermatocytes was noted, the spermatogonial count along the spermatoc cords showed highly significant variation among the groups ($p < 0.0001$). Post-hoc analysis revealed a significant difference between the NaF and NaF+jambul groups, and at the same time these two groups differed significantly from the control and jambul-only groups (Table 1).

Table 1: Histometry of the tubular size and the number spermatogonia and primary spermatocytes per spermatoc cord

Histometric parameters	Groups: Means±SEM			
	Control	NaF	Jambul	NaF+Jambul
Cross-sectional area of the seminiferous tubules	21017.7 μ^2 ±1078.01 [†]	16442.4 μ^2 ±807.9 [*]	21403.34 μ^2 ±895.58 [†]	18690.14 μ^2 ±1712.78 [†]
No. of spermatogonia per spermatoc cord	2.5±0.115	0.75±0.123 [*]	2.7±0.128	1.55±0.135 [†]
No. of spermatocytes per spermatoc cord	4.05±0.235	4±0.271	3.8±0.172	3.95±0.17

[†]Any two groups not sharing a common symbol differ significantly ($p < 0.05$) from each other.

3.3 Germ cells micrometry:

Statistical analysis of the data on spermatogonial size indicated an overall highly significant variation ($p < 0.0001$) among the groups. Post-hoc analysis of the means revealed a significant difference ($p = 0.05$) between the NaF and NaF+jambul groups, while, at the same time, these two groups also differed significantly from the control and jambul-only groups. Also seen was an overall significant difference ($p < 0.01$) among the groups for CSA of primary spermatocytes. Post-hoc comparative analysis also indicated a significantly ($p = 0.05$) higher mean cell size in the NaF group compared to that of the other three groups (Table 2).

In spite of an overall significant variation ($p < 0.05$) among the groups for sperm head breadth data, the post-hoc analysis of the means indicated no significant variation between any two groups. On the other hand, sperm head length data revealed no significant variation among the groups. However, the post-hoc analysis showed a significant ($p = 0.05$) decline in mean head length of the NaF-treated group compared to that of the other three groups. For the parameters of length and diameter of the middle part of spermatozoa, no significant variation was noted among the groups, whereas post-hoc analysis indicated a significant difference ($p = 0.05$) between the NaF and the jambul-only groups, while at the same time these groups also differed significantly from the control and NaF+jambul groups. Similarly no significant variation among the groups was seen

for tail length of spermatozoa, although post-hoc analysis of the means showed a significant decline ($p=0.05$) for the mean tail length in the NaF group compared to that of the control and jambul groups (Table 2).

Table 2: Micrometry (CSA) of the spermatogonia, primary spermatocytes and various dimensions of the spermatozoa

Micrometric parameters	Groups: Means± SEM			
	Control	NaF	Jambul	NaF+Jambul
Spermatogonial size	20.23 μ^2 ±0.919	37.29 μ^2 ±2.011*	21.89 μ^2 ±1.019	33.84 μ^2 ±1.94 [†]
Spermatocytic size	24.91 μ^2 ±1.943	34.52 μ^2 ±2.898*	26.85 μ^2 ±1.065	28.69 μ^2 ±1.85
Sperm head length	9.79 μ ±0.5208	7.93 μ ±0.465*	9.67 μ ±0.548	9.88 μ ±0.61
Head breadth	4.12 μ ±0.052	3.96 μ ±0.058	4.11 μ ±0.059	4.16 μ ±0.04
Middle piece length	25 μ ±1.57	22.79 μ ±2.49*	29.5 μ ±1.77 [†]	25.5 μ ±1.46
Middle piece diameter	1.198 μ ±0.052	1.06 μ ±0.031*	1.42 μ ±0.087 [†]	1.23 μ ±0.06
Tail length	101.16 μ ±12.52 [†]	83.25 μ ±11.43*	98.82 μ ±8.43 [†]	91.13 μ ±17.72 [†]

*†Any two groups not sharing a common symbol differ significantly ($p<0.05$) from each other.

DISCUSSION

Elevated intake of F has been shown to interfere with the anatomical structure and physiological activity of testis, epididymis, and associated duct system.²⁹ Experimental data have shown NaF exposure-related degenerative changes mainly affecting shape, motility, viability, and capacitation of spermatozoa in laboratory animals.^{30,31} In the present study, we observed a general decline in the number of spermatogonia resulting from the exposure of male mice to NaF in their drinking water with interference with and diminished mitosis. Moreover, the NaF exposure showed severe loss of interstitial tissue as a plausible outcome of the oxidative stress it inflicted on steroidogenesis and apoptosis in the Leydig cells.³²

In detail, spermiogenesis is a delicate stage in spermatogenesis involving subtle biosynthetic activities and sub-cellular alterations including (1) the biosynthesis of enzymes and formation of compact acrosomes, (2) production of tubulin and dyanin proteins and their peculiar structural arrangement to form biomechanical elements of the sperm tail, and (3) removal of excessive cytoplasts and the typical arrangement of mitochondria to produce the middle pieces.³³ Our findings indicate histological signs of involutions of the spermatids at various stages of spermiogenesis. The micrometric analysis revealed NaF exposure caused a decline in various dimensions of sperm cells. Taken together, these findings can be seen as partial outcomes of NaF exposure related to increased membranous lipid peroxidation and elevated oxidative stress in these cells at the verge of the height

of metabolic activity with a simultaneous decline in the biosynthesis and availability of male sex steroids.^{32,34,35}

Treatment with jambul fruit pulp extract has shown clear histologic, histometric, and micrometric signs of recovery from NaF exposure-related deteriorations in germ line cells in mice indicating its ameliorative potentials in male sex related toxicology.

CONCLUSIONS

In this study we report reclamation of spermatogonia and the interstitial tissue after jambul extract treatment for 5 days in mice following losses from NaF exposure. Our findings clearly indicate revival of germ plasm and a gradual rehabilitation of micrometric dimensions of spermatozoa with jambul fruit-pulp extract. Based on these findings, we propose jambul consumption revitalizes steroidogenesis and spermatogenesis in males following exposure to environmental contaminants like F that disrupt these vital functions.

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