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Ameliorative effects of nano *Moringa* on fluoride-induced testicular damage *via* down regulation of the StAR gene and altered steroid hormones

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ABSTRACT

Fluoride is a common environmental contaminant that has harmful effects on human health when it is present in high concentrations. Fluoride enters the bloodstream after being absorbed by the gastrointestinal system when fluoride-contaminated groundwater is consumed by people. The aim of the present study was to determine whether polyphenol-rich nano *Moringa oleifera* (NMO) could protect rat testicles from sodium fluoride (NaF) damage by evaluating sperm quality, sex hormones, testicular oxidative status, histopathology, and StAR gene expression. Twenty-eight adult Wistar rats were divided equally and randomly into four groups: group one received distilled water; group two received NMO at a dosage of 250 mg/kg/body weight; group three received NaF at a dosage of 10 mg/kg/body weight; and group four received NaF and NMO. The rats were orally administrated daily for a duration of eight weeks. The study's findings demonstrated that, in comparison to rats exposed to NaF alone, co-administration of NMO and NaF enhanced sperm motility and viability, decreased sperm morphological changes, restored the balance between oxidant and antioxidant status, improved testosterone and dehydroepiandrosterone, improved testicular histology, raised the Johnson score, and upregulated the StAR gene in testicular tissue. These findings show that NMO is promise as a prophylactic medication against sodium fluoride-induced testicular damage because administration of NMO had no adverse effects and enhanced reproductive health.

1. Introduction

All naturally occurring water contains fluoride in varying amounts, and groundwater in many nations has been found to have much higher concentrations of fluoride than the 1.0–1.5 mg/L range [1,2]. Some of which can reach values of 30–60 mg/L [1]. Additionally, using water with high fluoride content for irrigation purposes can cause the bioaccumulation of fluoride in crops, and as a result, the daily intake of fluoride in the local population in such an area far exceeds recommended safe levels [3–7]. The widespread use of fluoride-containing products (such as cosmetics and dental implants), inhalation of fluoride-contaminated air from production facilities, and the intake of fluoride-containing foods have increased [7].

Previous studies have shown that approximately 50 % of male infertility cases are caused by testicular toxicity [8,9]. According to Messer et al. [10], mice's fertility was improved by a high fluoride diet, whereas their infertility was exacerbated by a low-fluoride diet. However, research has also shown that fluoride is mutagenic, genotoxic, and can result in chromosomal abnormalities [3,11,12]. According to Chinoy & Sequeira [13], NaF therapy in mice alters the shape of sperm and the histology of reproductive organs in addition to inducing biochemical changes [14].

In the twenty-first century, the proverb "Let your food be your medicine and your medicine be your food" has proven to be valid [15]. *Moringa oleifera* (MO) *Lam.* (Moringaceae), along with its 12 related species, is a highly regarded and often-used ethnomedicinal plant species [16–18]. MO leaves contain abundant levels of minerals such as calcium, potassium, zinc, magnesium, iron, and copper [19]. MO also contains vitamin A (beta-carotene), vitamin B's pyridoxine, nicotinic acid, and folic acid, and as vitamins C, D, and E [20,21]. Additionally, MO contains a variety of potent phytochemicals such as tannins, sterols, terpenoids, flavonoids, saponins, anthraquinones, and alkaloids which produce antioxidant activity and the capacity to prevent oxidative damage [22,23]. MO's ability to scavenge free radicals at concentrations of 250 and 500 mg/kg for 60 days helped diabetic rats subjected to cold stress improve sperm quality, and as a result they produced more

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offspring [24]. Previous studies show that the most successful treatment for restoring the diameter and epithelial thickness of the seminiferous tubules was administration of MO at 500 mg/kg [25].

Steroidogenic acute regulatory (StAR) protein is crucial for the intracellular transport of cholesterol to the mitochondrion in endocrine organs such as the adrenal gland, ovaries, and testes, and it is required to produce all steroid hormones [26]. StAR is generated in large quantities by Leydig cells, and it interacts with a protein complex containing the translocator protein at the outer mitochondrial membrane (TSPO) [27].

Nano M. oleifera (NMO) was used in this investigation because it possesses unique benefits for bioavailability and stability, as afforded by its nano-configuration, and it is anticipated that NMO will be used as a highly effective antioxidant. This study sought to verify whether NMO could maintain the reproductive health of male rats exposed to NaF.

2. Materials and methods

2.1. Moringa oleifera (MO) harvest and extract preparation

Young, fresh MO leaves was provided by the Egyptian Scientific Society of the Moringa plants, National Research Centre, Dokki, Giza, Egypt. The leaves were air dried before being pulsed through an electric blender to create a powder. To make the extract, 250 g of this powder was soaked in 2.5 L of methanol over the course of six hours at 70 °C. The extract was then mixed with methanol with a 10 % (W/V) concentration.

2.1.1. Nano Moringa oleifera (NMO) preparation

Top-down methods for the synthesis of green materials involve the reduction of large mineral particles into small ones. The multi-step ball milling technique was utilized to create green material particles. 10 g of material particles were put into 40-cm long stainless-steel vials for ball milling. The ball mills used in this research were made of stainless steel and silicon carbide balls set on a vibrating plate. First, 50 g of 0.1-cm diameter stainless-steel balls were added to the ball mill chamber with green materials that are around 50 m in size. Wet milling was performed for 15 h, and then for an additional 15 h the 0.05-cm diameter silicon carbide balls crashed repeatedly with the plate and the barite powder inside the vial [28].

2.1.2. Characterization of Moringa oleifera (TEM)

The morphology of the NMO was assessed (TEM, Jeol JEM-1400) using Transmission electron microscope (TEM). A drop of the diluted sample was applied to a copper grid and stained with phosphor tungstic acid at a 2 % (w/v) concentration for analysis. At 120 kV, a 40,000x magnification was used to view TEM pictures.

2.1.3. LD_{50} of nano Moringa oleifera determination

OECD Guideline for testing acute oral toxicity of chemicals–fixed dose procedure was used to determine LD_{50} cut-off value of nano-moringa. The numbers of animals, study strategy, and management of animals were revised, and supported by the Cairo University ethics committee (CU/IF 13/20).

2.2. Experimental animals

The current trial research involved 28 male albino rats (*Rattus norvegicus*). The National organization for drug control and research, in Giza, Egypt, provided sexually mature Wistar rats (8 weeks old) weighing 170–180 g. These rats received a typical pellet diet and water while being kept in a microbe-free environment with typical temperatures (20–25 °C), relative humidity (50–70 %), and a 12-hour light/dark cycle.

2.3. Ethical consideration

This The Institutional Animal Care and Use Committee of Cairo University's Faculty of Science gave its approval for this study (CU/IF 45/20).

2.4. Study groups

There were four groups of rats (7 animals per group). According to [29], the trial's 56-day duration coincides with the end of spermatogenesis. The groups were comprised as follows:

Control group: rats given 1 ml oral distilled water.

NMO group: rats given MO leaf extract nanoparticles at a dose of 2500 mg/kg body weight (1/2 LD_{50}).

NaF group: rats given oral doses at 10 mg/kg body weight [30].

NaF + NMO group: rats given oral doses of NMO and NaF.

Following the final dosage, intraperitoneal injections of pentobarbital (10 mg/kg body weight) were used to anesthetize all rats. Through the heart puncture, blood was drawn from each sedated rat and put into a test tube. The testicles, epididymis, and seminal vesicles of the animals were exposed after being dissected. The tissues were weighed, dried, and rinsed with regular saline.

2.5. Preparation of serum

After allowing a blood sample to clot at room temperature for an hour, serum was obtained by centrifuging the sample for ten minutes at a speed of 3000 rpm in an electrical centrifuge.

2.5.1. Hormone analysis

The levels of testosterone (TS), dehydroepiandrosterone (DHEA), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in sera were measured by the competitive enzyme immunoassay technique (ELISA kits) corresponding to the command procedure of Sun Long Biotech Co., LTD, China (Mainland).

2.6. Count of epididymal sperm

The cauda portion of each epididymis was taken out and put in a Petri dish with 1 ml of physiological saline solution. With a pair of razor-sharp scissors, each portion was immediately macerated, then allowed to release its spermatozoa into the saline solution. A microscope and an upgraded Neubauer chamber were used to count the sperm [31].

2.6.1. Evaluation of sperm motility

A drop of semen was inserted on a tidy, previously warmed slide. A cover slip was placed over the slide, and the sperm motility was viewed under a microscope with a 40 X objective [32].

2.6.2. Determining the morphology and viability of sperm

Semen in the amount of 10 μl was put on a slide, spread with another slide, and then allowed to dry. The dried samples were stained using Eosin and Nigrosin stains. When spermatozoa are dead, the membrane is usually ruptured, which allows the dye to enter the sperm's cytoplasm [33,34]. The frequency of overall anomalies was reported, including amorphous head or tail shape or conformation.

2.7. An assessment of the oxidative state

To prepare the tissue homogenate, the left testis of each rat was excised and homogenized in PBS (1:10 ml). The solution was centrifuged at 4000 rpm for 20 min, and the supernatant residue was used to calculate catalase (CAT) [35], malondialdehyde (MDA) [36], glutathione (GSH) [37], and super oxide dismutase (SOD) [38].

2.8. Testicular histopathology

The testes were separated from the surrounding tissue and then excised. For fixation, the tissue was stored in a 10 % neutral formalin solution. Following the 24-hour period, the tissue was washed for 24 h under running water, dehydrated using descending alcohol concentrations, cleaned in xylene, and then embedded and blocked-in paraffin. Hematoxylin and eosin were used to stain sections of 4–5-m thickness before they were inspected under a microscope [39]. An AmScope light microscope was used to examine the slides, and a magnification device connected to a digital camera was used to take pictures.

2.8.1. Johnsen's score

A previously described technique [40] was applied by [41] to determine the mean Johnsen's testicular biopsy score (MJTBS). (Table. 1).

2.9. Analysis of StAR gene expression

2.9.1. cDNA synthesis and RNA extraction

Each frozen testicular tissue sample was first processed to extract the entire cellular RNA using the QIAamp RNA microkit (Qiagen, Germany) in accordance with the manufacturer's RNA concentrations were measured using a NanoDrop ND 1000 UV Spectrophotometer. Using a random primer and the RevertAidTM First Strand cDNA Synthesis Kit, 1 g of RNA was reverse transcribed into cDNA (Fermentas).

2.9.2. Polymerase chain reaction in quantitative real-time (q RTPCR)

A typical real-time PCR was used to determine the StAR gene's mRNA transcript expression and to quantify reverse transcribed cDNAs. SYBR green-based real-time PCR was used to amplify the StAR and actin genes, and the results were detected using the 7500 Fast Real-Time PCR equipment (Applied Biosystem 7500, Clinilab, Egypt) and the primers listed in Table 2 [43,44].

After activating at 95 for 10 min, there were 40 cycles of 95 for 10 s, 60 s, and 72 s, followed by melt curve analysis. 15 microliters of reaction volume, 0.75 µl of cDNA, 1x Maxima SYBR Green qPCR master mix, and 7.5 pmol of each primer were used.

A mixture of 12.5 ÂµL of 2x Quanti Tect SYBR Green PCR Master Mix (Qiagen Inc, Valencia, USA), 2.5 ÂµL of freshly synthesized cDNA, 1 µl of primer mixer, and 8 ÂµL of PCR grade water were used in each PCR reaction. Following a preliminary step of heat activation at 95 °C for 15 min, a 40-cycle denaturation at 95 °C for 15 s, annealing, and elongation at 60 °C for StAR and actin PCR thermal protocol was carried out. The target gene was relative quantified using the comparative delta-delta CT method ($2^{-\Delta\Delta CT}$) was used for relative quantification of the target gene as follows: ΔΔCT = (CT of the target gene − CT of β-actin) for the treated sample − (CT of the target gene − CT of βactin) for the calibrator (normal control). Following the method's validation, the results were reported for each sample as fold changes in the target gene copies relative to the copy number of the target gene in the calibrator using the following equation: 2 CT is equal to the target quantity.

Table 1
Johnsen's mean testicular biopsy score (MJTBS) [42].

Description	Score
Tubular sclerosis, no seminiferous epithelial cells	1
Only Sertoli cells, no germ cells	2
Only spermatogonia	3
No spermatids, arrest of spermatogenesis at the primary spermatocyte stage	4
Many spermatocytes, but no spermatids	5
No late spermatids, arrest of spermatogenesis at the spermatid stage	6
Many early spermatids, but no late spermatids	7
Few late spermatids	8
Disorganized tubular epithelium with several late spermatids	9
Full spermatogenesis	10

Table 2
lists the StAR and actin Wistar rat cDNA primer sequences.

Gene	Sense 5' - 3'	Antisense 5' - 3'	Product size (bp)
β-actin	ATGGTGGGTATGGGTCAG	CAATGCCGTGTTCAATGG	97
StAR	CCTGAGCAAAGCGG TGTCAT	GCAAGTGGCTGG CGAACTCTA	187

2.10. Statistical analysis

The statistical results were expressed as mean SE, and one way ANOVA with the post hoc Tukey test was used to compare the values of the various parameters. The level of significance was set at 5 % ($P < 0.05$).

3. Results

3.1. LD_{50} of NMOs

NMO had an LD_{50} of more than 5000 mg/kg (Table 3).

3.2. TEM result

TEM Images of the NMO particles demonstrate their spherical nature. The NMO displayed a nearly sphere-shaped with an average diameter of 37-nm Fig. 1(A&B).

3.3. Effect of NMO on body weight change and the weight of reproductive organs

Compared with the control and NMO groups, the change in body weight was significantly less in the NaF group (32.80 ± 3.48) when compared to the control (65.8 ± 2.87), NMO (72.8 ± 8.74) and NaF + NMO (108.00 ± 6.18) groups. The absolute (0.61 ± 0.06) and relative weight (0.23 ± 0.03) of the seminal vesicles significantly decreased in the NaF group when compared to the control (Absolute; 1.03 ± 0.05) (Relative; 0.39 ± 0.02) and NMO (Absolute; 0.93 ± 0.01) (Relative; 0.35 ± 0.01) groups. The relative weight of the left (0.59 ± 0.01) and right (0.57 ± 0.01) testes in the NaF-treated group showed no significant increase compared to the control group (Left; 0.56 ± 0.02) (Right; 0.55 ± 0.01). The absolute weight of the left (1.56 ± 0.02) and right (1.53 ± 0.02) testes increased significantly in the NaF-treated group in comparison to the NMO group (Left; 1.34 ± 0.05) (Right; 1.33 ± 0.04). The absolute weight of the right cauda epididymis of the NaF group did not significantly differ from the other groups, and the relative weights of the left and right cauda epididymis of the NaF group did not significantly differ from the other three groups. The absolute weight of the left cauda epididymis of the NaF group (0.25 ± 0.01) increased significantly when compared to the control group (0.20 ± 0.01). The body weight change of the treated group in the NaF + NMO group was significantly higher

Table 3
Showing evaluation of LD_{50} of NMOs.

Dose Responses to dose	5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg	5000 mg/kg
No. of animals	5	5	5	5	5
No. of live animals	5	5	5	5	5
No. of dead animals	0	0	0	0	0
Sings of toxicity	no	no	no	no	no
Body weight of animals at the day of dosing	192.8	192	190.5	190.4	196
Body weight of animals after the first week	208.9	203.9	200.5	199.5	204.4
Body weight of animals after the second week	225	215.8	210.7	208.6	212.8
Body weight change	32.2	23.8	20.3	18.2	16.8

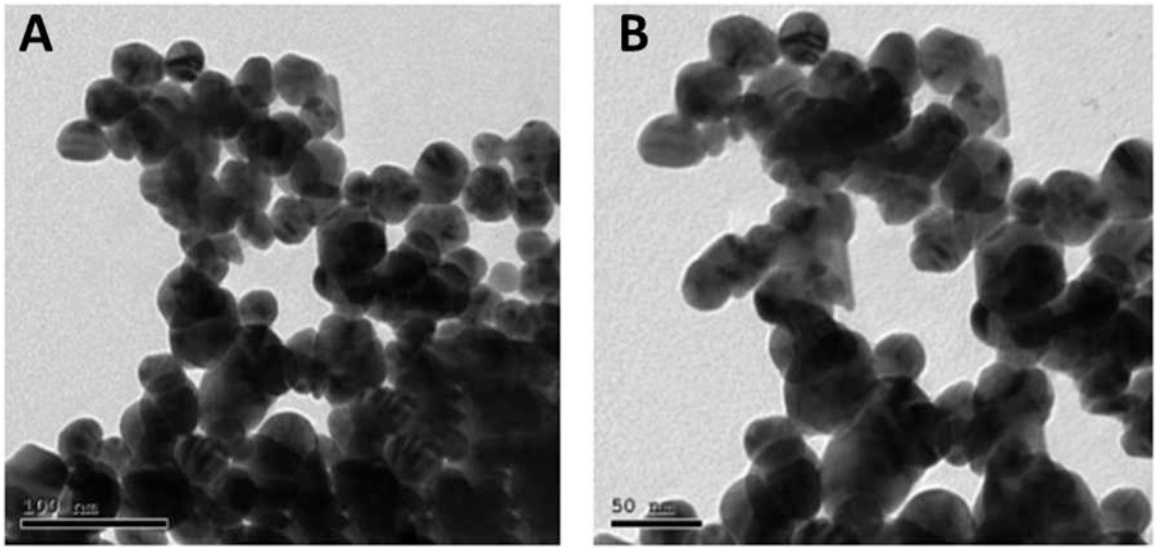

Fig. 1. HR-TEM image of NMO shows spherical shape particles with average diameter around 37 nm.

than that in the other groups, and the absolute weight of the seminal vesicle improved. In the NaF + NMO group (1.50 ± 0.05), the absolute weight of the left testis (1.47 ± 0.01) insignificantly decreased. In comparison to the control group (0.55 ± 0.01), the relative weight of the right testis in the NaF+ NMO group (0.45 ± 0.02) significantly decreased (Tables 4 and 5).

3.4. Semen profile outcome

NaF consumption significantly decreased sperm count, viability, and motility when compared with the other groups (Table 6). The NaF group experienced significant increases in general abnormalities, including an aberrant tail and hookless sperm (Table 7 & Fig. 2). Treatment with NMO significantly decreased numerous sperm defects as compared with the NaF group, and it had an impact on overall abnormalities in a statistically meaningful way. Additionally, it brought back the average levels of sperm count, motility, and vitality.

3.5. Reproductive hormones

NaF administration created considerable disparities in reproductive hormone levels. The NaF-treated rats had significantly decreased TS, DHEA and FSH levels while having significantly greater LH concentrations when compared with the control group and NMO groups. When compared to the control group, the NaF + NMO group showed a non-significant increase in TS and DHEA. level, and when compared to the NaF group, the FSH and LH levels were restored to normal levels (Table 8).

Table 4
displaying body weight change and absolute weights of reproductive organs.

Groups Parameters	Control	NMO	NaF	NaF + NMO
Body Weight Change/g	65.8 ± 2.87	72.8 ± 8.74^{c}	32.80 ± 3.48^{a,b,c}	**108.00 ± 6.18^{a,b}**
Seminal Vesicles/g	1.03 ± 0.05	0.93 ± 0.01	0.61 ± 0.06^{a,b}	**0.76 ± 0.06^{a}**
Left Testis/g	1.47 ± 0.01	1.34 ± 0.05^{c}	1.56 ± 0.02^{b}	**1.50 ± 0.05**
Right Testis/g	1.44 ± 0.02	1.33 ± 0.04	1.53 ± 0.02^{b}	**1.45 ± 0.05**
Left Cauda Epididymis/g	0.20 ± 0.01	0.23 ± 0.02	0.25 ± 0.01^{a}	**0.24 ± 0.01**
Right Cauda Epididymis/g	0.20 ± 0.003	0.19 ± 0.005	0.20 ± 0.004	**0.23 ± 0.01**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

Table 5
displaying relative weights of reproductive organs.

Groups Parameters	Control	NMO	NaF	NaF + NMO
Seminal Vesicles %	0.39 ± 0.02	0.35 ± 0.01^{c}	0.23 ± 0.03^{a,b}	**0.22 ± 0.02^{a}**
Left Testis %	0.56 ± 0.02	0.51 ± 0.04	0.59 ± 0.01^{c}	**0.47 ± 0.02**
Right Testis %	0.55 ± 0.01	0.51 ± 0.03	0.57 ± 0.01^{c}	**0.45 ± 0.02^{a}**
Left Cauda Epididymis %	0.07 ± 0.004	0.08 ± 0.008	0.09 ± 0.004	**0.07 ± 0.003**
Right Cauda Epididymis %	0.07 ± 0.003	0.07 ± 0.002	0.07 ± 0.002	**0.07 ± 0.005**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

Table 6
showing sperm quality outcome.

Groups Parameters	Control	NMO	NaF	NaF + NMO
Viability (%)	59 ± 4.30	55 ± 5.24	22 ± 4.37^{a,b,c}	**45 ± 1.61**
Motility (%)	54 ± 09	55 ± 4.47	26 ± 5.09^{a,b,c}	**47 ± 3.39**
Sperm Count (No. of sperm×10^{6}/mm)	125.20 ± 11.93	110.60 ± 9.48	55.00 ± 3.36^{a,b,c}	**93.20 ± 6.05**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

3.6. Oxidative state in the testicles

According to the data for oxidative stress parameters, Malondialdehyde (MDA) generation, an indicator of lipid peroxidation, increased dramatically following NaF administration compared with the control

Table 7
Presenting sperm morphological abnormalities.

Groups Parameters	Control	NMO	NaF	NaF + NMO
Total abnormality sperm %	7.80 ± 0.86	8.20 ± 0.58	13.60 ± 0.92[a,b,c]	**8.20 ± 0.58**
Hook less %	1.80 ± 0.37	2.20 ± 0.58	6.60 ± 0.50[a, b,c]	**3.60 ± 0.92**
Abnormal tail %	0.60 ± 0.24	1.00 ± 0.31	2.60 ± 0.24[a, b]	**1.40 ± 0.50**
Small head %	0.60 ± 0.40	1.00 ± 0.54	2.60 ± 0.92	**1.00 ± 0.31**
Large head %	0.20 ± 0.20	0.40 ± 0.24	0.60 ± 0.24	**0.40 ± 0.24**
Banana head	1.00 ± 0.31	1.60 ± 0.24	1.60 ± 0.24	**1.00 ± 0.31**
Amorphus head %	1.20 ± 0.37	2.00 ± 0.31	2.40 ± 0.40	**1.60 ± 0.24**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

group. Compared with the control group, catalase (CAT), glutathione (GSH) levels, and SOD significantly decreased after NaF intubation. Administration of NMO reduced the reactive oxygen species (ROS) that was brought on by consuming NaF. In comparison to the NaF group, NMO was able to dramatically boost CAT, GSH, and SOD activity and decrease the MDA level (Table 9).

3.7. Histopathology of the testis

The testicular tissue made up of tightly packed seminiferous tubules and presenting with the typical characteristics of a normal structure was visible in the control (Fig. 3A) and NMO (Fig. 3B) groups. The seminiferous tubules included spermatogenic cells that were typically organized, such as spermatogonia that were laying on the basement membrane, and spermatocytes, spermatids, and spermatozoa inside the lumen. Groups of Leydig interstitial cells were seen in the interstitium. The testis of the NaF group showed seminiferous tubule disruption, a significant reduction in the spermatogenic cell sequence, and a significant number of seminiferous tubules devoid of sperm, spermatids, and secondary spermatocytes. Within the lumen of some seminiferous tubules, injured cells were seen to be exfoliating. The inter-tubular connective tissue had vacuolation, a rise in the size of interstitial regions, and a decrease in interstitial cells together with edema. Pyknotic nuclei

Table 8
showing reproductive hormones level.

Groups Parameters	Control	NMO	NaF	NaF + NMO
Testosterone (ng/ml)	4.45 ± 0.53	3.28 ± 0.20	1.83 ± 0.07[a, b]	**3.02 ± 0.03[a]**
DHEA (ng/dl)	23.66 ± 0.33	23.16 ± 0.60	16.66 ± 0.33[a,b]	**21.66 ± 2.72**
FSH (IU/L)	3.3 ± 0.16	3.2 ± 0.16	0.80 ± 0.15[a, b,c]	**3.9 ± 0.51**
LH (IU/L)	0.23 ± 0.05	0.24 ± 0.00	4.9 ± 0.27[a,b, c]	**0.24 ± 0.03**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

Table 9
showing level of testicular oxidative stress markers.

Groups Parameters	Control	NMO	NaF	NaF + NMO
CAT (U/mg protein)	97.00 ± 5.81	102.50 ± 4.64	39.75 ± 1.75[a, b,c]	**118.00 ± 6.49**
GSH (U/g tissue)	3.98 ± 0.12	5.23 ± 0.44	2.70 ± 0.10[b,c]	**4.38 ± 0.60**
SOD (U/gm tissue)	1058.50 ± 41.61	981.75 ± 18.60	632.00 ± 35.45[a,b,c]	**942.75 ± 42.98**
MDA (nmol/g tissue)	95.75 ± 3.81	99.00 ± 6.17	511.50 ± 51.59[a,b,c]	**129.50 ± 8.06**

Each value represented as means ± SEM. Significance level (P < 0.05). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

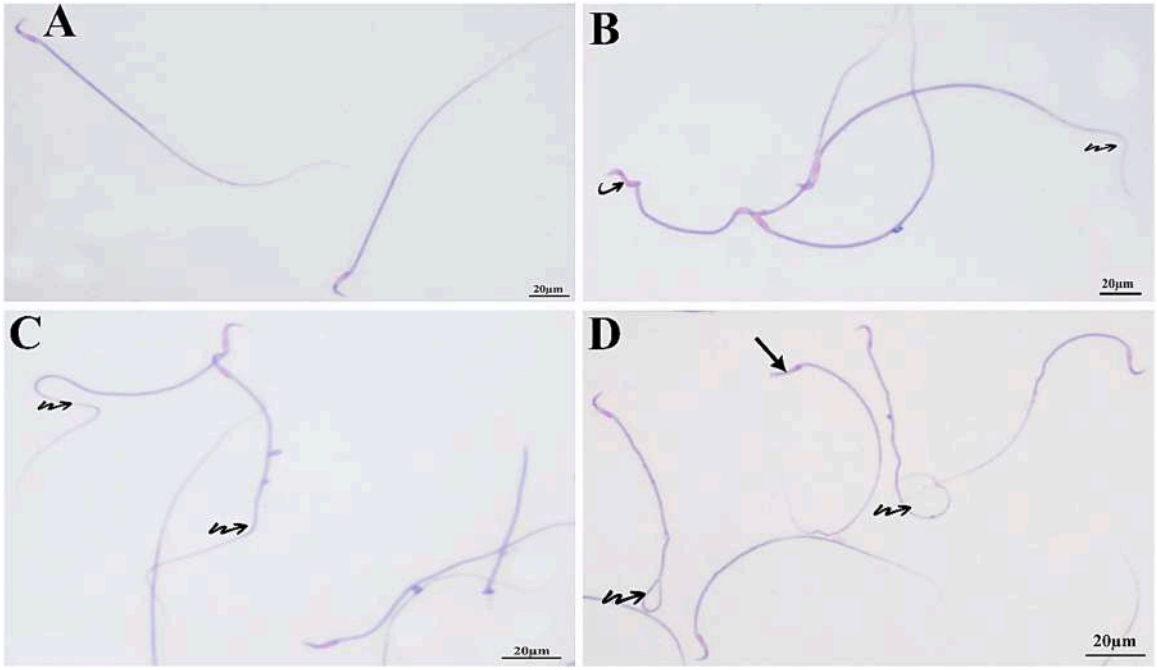

Fig. 2. showing sperm morphology of rat. Normal sperm with intact shape (A), sperms with morphological abnormalities; head without acrosome (arrow), bent tail (zigzag arrow) and amorphous head (curved arrow).

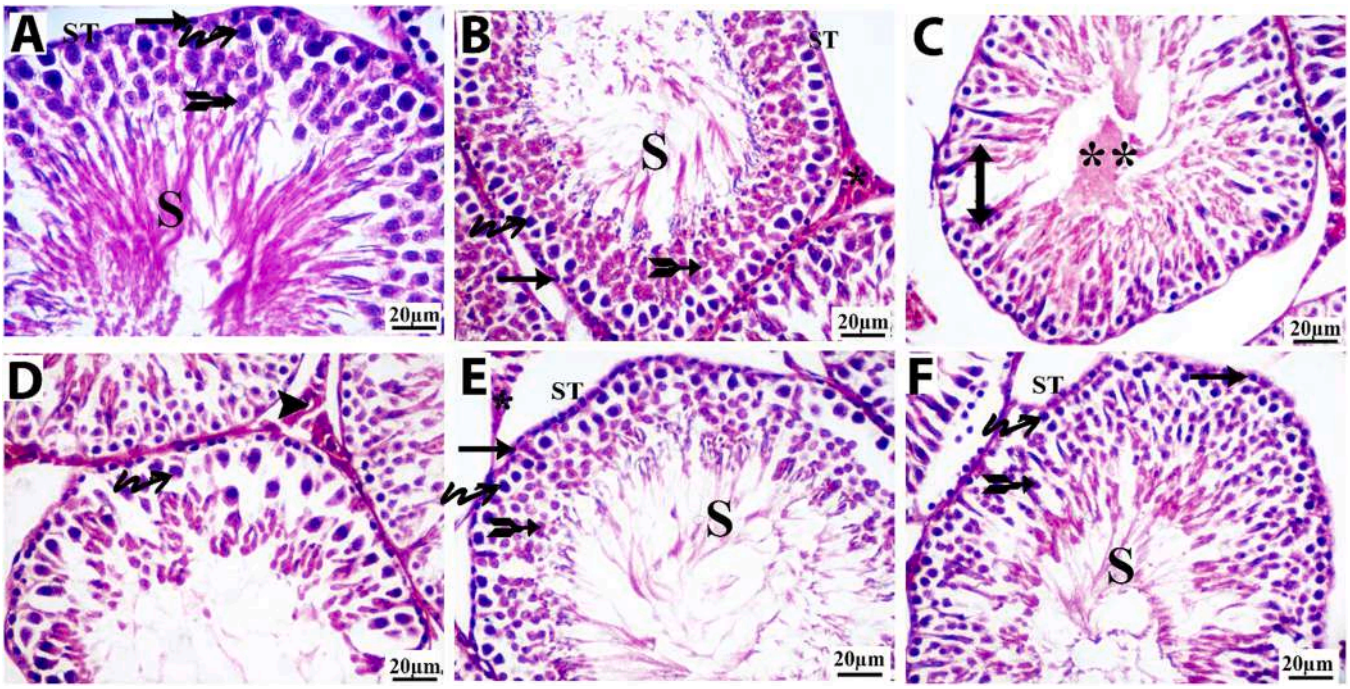

Fig. 3. Showing testicular histopathology of rat. Testes specimens from the control (A) and NMO groups (B) had a natural appearance of testicular tissue, consisting of firmly coordinated seminiferous tubules (ST) and representing the basic characteristics of normal structure. The spermatogenic cells in the seminiferous tubules were well-organized, with spermatogonia (thin arrow) on the basement membrane, spermatocytes (zigzag arrow) and spermatids (bifid arrow) in the lumen, and spermatozoa (S) in the lumen. In the interstitial space, Leydig interstitial cells (asterisk) in groups were discovered. The NaF group (C&D) had disoriented seminiferous tubules and a significant loss in spermatogenic cell (double head arrow) sequence in their testis's tissues. There was no sperm in most of the seminiferous tubules. The number of interstitial cells in the inter-tubular connective tissue decreased with vacuolation (arrowhead). Some degenerated seminiferous tubules with obliteration lumen filled with damaged cells and casts materials (double asterisk). The combined group's testes (E&F) showed a significant improvement in histological assessment, with multiple rows of various stages of spermatogenic cells in certain seminiferous tubules. The area of the spaces between seminiferous tubules was diminished. Seminiferous tubules (ST) with spermatogonia (thin arrow), spermatocytes (zigzag arrow), spermatids (bifid arrow) and spermatozoa (S) in the lumen. Leydig interstitial cells (asterisk) were discovered.

were present in the majority of spermatogenic cells, and some seminiferous tubules had inappropriate basement membranes (Fig. 3C&D). Histological analysis of the combined group's testes revealed a remarkable improvement, including numerous rows of spermatogenic cells in different stages in certain seminiferous tubules and seminiferous tubule spaces with a smaller surface area. Additionally, spermatogenic cells in certain seminiferous tubules had retreated (Fig. 3E&F). Johnson Score outcomes supported the findings. When compared to the control, the Johnson score for the NaF group significantly decreased, whereas the Johnson score for the NMO group significantly increased (Fig. 4).

3.8. StAR gene expression levels

When compared to the control group, the NaF group's StAR gene expression levels were considerably ($P < 0.05$) lower than that of the control group. This suggests that NaF significantly lowers the mRNA levels of the StAR gene. StAR gene levels were restored following NMO ingestion compared with the NaF group (Fig. 5).

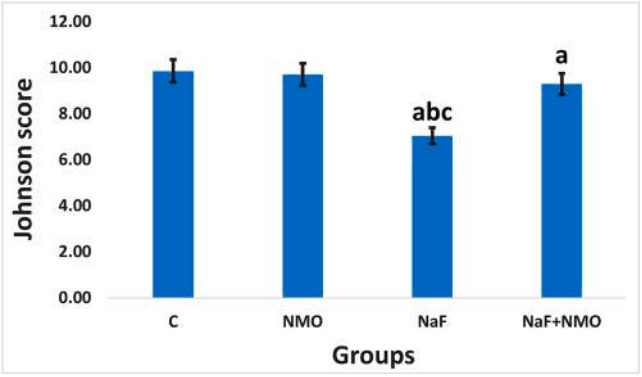

Fig. 4. showing Johnson score among different experimental groups. Each value represented as means ± SEM. Significance level ($P < 0.05$). a = compared with control group. b = compared with NMO group. c = compared with NaF + NMO group.

4. Discussion

The goal of the current study was to evaluate the ability of NMO leaf extract in vivo to ameliorate the toxic effects of NaF on testicular tissue in male albino rats.

In low levels, fluoride encourages healthy bone formation and is crucial for dental hygiene as it guards against tooth decay and dental caries [45]. However, in excessive amounts, fluoride exposure is harmful to human health and can cause fluorosis, a disorder [46] that causes damage to the liver, lungs, kidneys, blood, nerves, brain, and gastrointestinal system in addition to causing tooth mottling and debilitating skeletal deformities [47,48]. National Research Council (NRC) listed several exemplary cases in its 2006 report, including [49,50]: (1) cessation of spermatogenesis and changes in the epididymis and vas deferens were seen in rabbits given NaF at a dose of 10 mg/kg body weight per day for 29 months [51]; (2) effects on Leydig cells and decreased serum TS were seen in rats exposed to NaF at a dose of 10 mg/kg body weight per day for 50 days [52].

In the current study, rats exposed to NaF had significantly lower body weights and absolute and relative seminal vesicle weights but had significantly higher absolute left cauda epididymis weights and the left

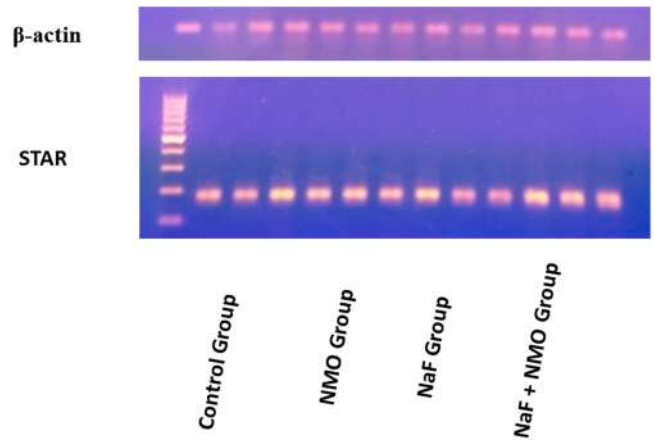

Fig. 5. Semiqualitative real-time PCR assay of StAR mRNA expression in the rat testicular tissue of control, NMO, NaF, and combined (NaF+ NMO) groups.

and right testes only slightly increased. The general metabolic abnormalities seen in NaF-treated rats may be due to the rapid effects of NaF on the gastrointestinal tract and inadequate nutrient absorption brought on by decreased feeding efficiency [53]. The inhibition of various enzymes by sodium fluoride [54,55] subsequently disrupted various metabolic processes such as glycolysis, protein synthesis, and antioxidative pathways. Body weight then decreased due to these modifications and the reduction in food intake [56,57]. Possible contributors to reduced seminal vesicle weight include atrophic changes in the glandular epithelium and decreased secretion, lowered serum TS levels, suppression of 5-alpha reductase or vesicles, and/or prostate-impairing androgen-specific receptors [58]. Excess interstitial fluid can influence hemodynamics, harm vascular endothelial cells, impede lymphatic drainage, and/or increase ductular fluid, which then inhibits fluid absorption by the rete, efferent ducts, and caput epithelium and this contributes to higher epididymal weight [58]. However, the mean testicular weights of rats administered fluoride at 100 and 300 ppm for 12 weeks did not significantly differ from the control group, and the lower level of fluoride could be the cause [14,59]. According to Ghosh et al., fluoride intake at 20 mg/kg for 29 days increased the relative testicular weight of rats when compared to a control group, and this may have been the result of a compensatory alteration or fluid buildup in the testis [14,60]. Their results showed that only the high NaF dosage group and long NaF intake duration group showed a significant decrease in testicular weight and an epididymal weight decrease when compared with the control group. Similarly, other research found that fluoride-fed rabbits had significantly lower epididymal weight [14,61].

According to the results of the present research, sperm count, motility, and viability in the epididymis all significantly decreased, while sperm abnormalities increased. This was consistent with the findings of **Kim et al.** [62] **and Sun et al.** [63], who observed that treatment with NaF dramatically decreased sperm motility and viability and increased morphological abnormalities. Additionally, research by **Al-Sabaawy & Al-Kaisie** [64] **and Tetsatsi et al.,** [65] revealed a significant decrease in sperm concentration, count, and motility following treatment with sub-lethal sodium fluoride concentrations. One reason for these findings may be a reduction in fructose levels in the seminal vesicles and vas deferens, as fructose fuels motility and is affected by changes in carbohydrate levels. Another factor in decreased sperm motility is reduced levels of androgen carrier proteins, which are involved in sperm motility [14]. Therefore, it is suggested that NaF has an impact on the systemic spermatozoa energy production mechanisms that are needed to sustain good sperm motility. Fluoride-induced oxidative stress results from excessive ROS/RNS production, decreased antioxidant enzymes, and fluoride poisoning. This stress causes extensive peroxidation of membrane lipids and cellular proteins as well as causing frequent observable micromorphological abnormalities [7,66,67].

The results of the current study revealed a barely detectable drop in TS and DHEA levels, a striking decrease in serum FSH, and a noticeable rise in LH levels. TS and FSH levels were reported to have been significantly decreased [64]. These results demonstrate that sodium fluoride can impair TS, DHEA, LH, and FSH secretion, which in turn can hinder sperm maturation and production and cause the malfunctions in the reproductive system's physiology. Additionally, fluoride may inhibit androgen receptor mRNA expression in Sertoli cells, which results in a decrease in androgen receptors, through which TS acts [68]. Or, fluoride may cause a significant decrease in epidermal growth factor and its receptor (EGFR) in Leydig's cells during spermatogenesis, or it may lower TS levels by lowering testicular zinc levels, which impair angiotensin-converting enzyme [69].

Some drugs, chemotherapy, hypothalamus-pituitary axis illnesses, primary hypogonadism, cryptorchidism, and orchitis, as well as hereditary illnesses like as Klinefelter and Kallmann syndrome, can reduce testosterone production [70]. Increased production, underproduction, receptor insensitivity, or poor testosterone metabolism include testosterone-related pathology. To have a deeper comprehension of certain of these illnesses, it is essential to recognize the distinctions between testosterone and dehydroepiandrosterone (DHEA). DHEA is an androgen generated by the adrenal glands and ovaries/testes that is fairly weak. DHEA is a progenitor to hormones such as testosterone and oestrogen [70]. Androstenedione, also known as 4-Androstene-3–17-dione (4 A), is a naturally present steroidal hormone generated by the gonads and adrenal glands of both sexes [71] and serves as an intermediate in the generation of testosterone [72,73]. 17-hydroxysteroid dehydrogenase [74] catalysis the conversion of androstenedione to testosterone from dehydroepiandrosterone. The adrenal cortex converts DHEA to androstenedione, which can then be de-hydrogenated in the liver to produce testosterone [73,75]. DHEA has an essential physiological role in sustaining steroidogenesis by serving as the accessible progenitor that is transformed to testosterone and estrogens in numerous peripheral tissues [75]. Approximately 95 % of androgen production and release in males happens in the interstitial Leydig cells of the testis. Previous reports have demonstrated that functional alterations in Leydig cells, as opposed to cell death, are responsible for the detected decrease in serum testosterone levels [75,76]. Rat Leydig cells contain 3-HSD, 17-HSD, and aromatase, which catalyze the production of testosterone and estradiol [75,77].

In the present study, NaF raised the MDA level and significantly decreased antioxidants like CAT, GSH, and SOD. Damage to mitochondrial DNA (mtDNA) can result from elevated ROS levels [78], affecting physiological function of sperm [79,80]. Excessive MDA generation from LPO can react with amino substances such as protein, nucleic acid, and cerebral phospholipid to cross-link them, and this can change the accumulation, structure, and dynamics of lipid membranes [80].

No mature spermatozoa were visible in the lumens, and complete cessation of spermatogenesis was noted after NaF administration. Moreover, the number of sperms decreased and the spermatogenic cells were disorganized in the testis, which supports earlier studies showing that NaF significantly caused testicular histopathology lesions in varying degrees [81]. These results are also in line with those of Sun et al. [82] who found that fluoride exposure caused a noticeable alteration in the structure of the testis, including the rearrangement of spermatogenic cells and a decrease in luminal sperm. Some of the ways that fluoride impacts the cell include the generation of superoxide anions, mitochondrial toxicity [83], the discharge of cytochrome c from mitochondria, and the triggering of apoptosis leading to inhibition of sperms from migrating following exposure to fluoride [50,84].

The current study showed that the NaF treatment decreased gene expression of the StAR protein. Reduced StAR gene expression was discovered to be connected to testicular injury, as reflected in prior research [85–87]. The damage to the Leydig cells that is linked to the

structural abnormalities in the testes [34] may be the mechanism behind the reduction in StAR protein expression [88]. By altering mRNA expression levels of the transcription factors, NaF decreased the expression of steroidogenic genes, and proteins [89]. In the present study, the reduction of StAR expression led to a significant decrease in TS level as StAR expression is predominantly linked to steroidogenic tissues in vertebrates, and hence the synthesis of steroids. As a result, the StAR protein is essential for the regulation of steroid hormones required for preserving the reproductive ability of gonadal and adrenal steroids [90].

Nano-formulation varies from 1 to 1000 nm, and a decrease in material size results in a larger surface area to volume ratio. This then increases conductivity and dictates bio-distribution across tissues and organs, as well as the elimination rate [91]. Therefore, the NMO used in this study may have greater bioactivity because of its small particle size (37 nm).

In this study, there was a recovery in sperm count, motility, abnormalities, and percentage of alive sperm. Also, the TS level remained similar to the control level, even though Moringa reduces the weight of reproductive organs to the control level. For 60 days, MOLE extract supplements significantly increased sperm viability and density [24].

Previous studies demonstrated that oral administration of MO extracts at doses of 200 and 400 mg/kg/body weight into the high-fat diet of rats for 12 weeks significantly inhibits the levels of lipid peroxidation and balances the antioxidant enzyme activities which enhances reproductive features [92]. Antioxidants found in MO leaves include quercetin, polyphenols, and flavonoids [93,94]. Flavonoids help protect the male reproductive system from damage [95]. Additionally, MO flavonoids and triterpenoids have been shown to diminish testicular diseases brought on by exposure to ROS, prevent protein oxidation, and scavenge free radicals [96–98].

The present research demonstrated that NMO was effective at restoring levels of TS, DHEA, FSH, and LH after NaF toxicity in rats, and these results were consistent with earlier findings by Dafaalla et al. [99].

These results indicate that NMO ameliorated testicular histopathological changes induced by NaF exposure, elevated the Johnson score, and improved spermatogenesis and sperm production. In Moringa leaf extract, vitamin C plays an antioxidant or free radical scavenger role [100,101].

The current study also revealed that NMO increased the level of StAR gene expression as compared with the NaF group. In Leydig cells, Creb1 is a key stimulator of the production of steroidogenic genes, including StAR [102–104]. A previous study demonstrated that luteolin can boost StAR expression in Leydig cells, which in turn increases steroidogenesis [105–107]. The ability of luteolin to improve cholesterol import into the mitochondria by elevating StAR protein levels in MA-10 Leydig cells has been similarly documented by others [108]. Additionally, as has been shown for other flavonoids [108], luteolin increases StAR transcription by suppressing the expression of Dax-1 [107]. Quercetin also enhances Creb1 transcriptional activity, which supports its favorable impact on steroidogenesis [105,107]. Quercetin was found to boost StAR mRNA levels, StAR promoter activity, and steroid hormone synthesis from MA-10 Leydig cells, according to other reports [108]. By lowering the amounts of Dax1 protein in Leydig cells, quercetin may promote StAR gene expression in response to cAMP stimulation [107,108]. Because MO contains phenolic and flavonoid compounds, which act as free radical scavengers and protect against oxidative changes of many toxic substances, this may be the cause of these improvements.

5. Conclusion

This study demonstrates the antioxidant potency of NMO. Following exposure to NaF, NMO improved sperm profile characteristics, decreased testicular histopathology, restored reproductive hormone concentrations, increased antioxidant activity, and controlled the StAR gene. These findings demonstrate that NMO is an effective antioxidant capable of shielding sperm.

Competing interests

The authors say they have no competing interests.

Data Availability

On reasonable request, the corresponding author will provide the information supporting the study's conclusions.

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We sincerely hope that everyone will help the Zoology Department at the Universities of Al-Azhar and Cairo to complete this job.

Contributions of authors

Every author made a similar contribution.

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