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Histopathological and flow cytometry of nitrate-induced testicular toxicity in adult mice and the protective effects of garlic oil

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ABSTRACT

Nitrate is a common contaminant in groundwater aquifers. Current study aimed at evaluating the potential testicular toxicity of sodium nitrate in adult mice and treated with garlic oil. Sodium nitrate was given orally to mice at doses of 900 mg/kg/day for 35 consecutive days. Sperm count and motility, viability and testis weight were significantly decreased. Testicular activities of hormones (Testosterone, LH and FSH) were significantly inhibited in $NaNO_3$ treated group. The proportions of diploid and tetraploid cells decreased in $NaNO_3$ treatment, but showed an increase after treatment with garlic oil. The decrease in sperm count and motility and other parameters were confirmed by histopathological studies which indicated congested dilated interstitial blood vessels, degenerations in the seminiferous tubules and intercellular dissociations of germ cells, and necrosis in spermatocytes. In conclusion, exposure of mice to sodium nitrate results in testicular toxicity as evidenced by decreased sperm count and motility, and testis weight, inhibited activity of enzyme markers of spermatogenesis and induction of histopathological changes. These effects are attributed, at least partly, to testicular oxidative stress. Also, garlic treatments appear able to cure the sodium nitrate-induced testicular toxicity. The protective effects should be associated with its antioxidative activities.

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KEYWORDS

Sodium nitrate;
Testis;
Flow cytometry;
Spermatogenesis.

INTRODUCTION

Nitrate is the most common chemical contaminant in the world's groundwater aquifers[29]. Groundwater is the source for >50% of drinking water supplies, 96% of private water supplies, and an estimated 39% of public water supplies. An estimated 42% of the U.S. population uses groundwater as their drinking water supply. However, the World Health Organization drinking water guideline value for nitrate has been set at 45 mg/L[35]. In the European Union, the maximum admissible nitrate level in drinking water has been set at 50 mg/L[1].

Ingested nitrate is reduced to nitrite, which binds to hemoglobin to form methemoglobin. Infants are particularly susceptible to developing methemoglobinemia[20]. Nitrate is a precursor in the formation of N-nitroso compounds (NOC), a class of genotoxic com-

Regular Paper

pounds, most of which are animal carcinogens[6]. Animal studies suggest that nitrate at high doses can induce hypertrophic changes in the thyroid[5]. In a human, consumption of water with nitrate levels was associated with thyroid hypertrophy[33], increased blood pressure[27], and acute respiratory tract infections[14]. Adverse reproductive outcomes of nitrates in drinking water have been reviewed[20]. Nitrate contamination of drinking water may increase cancer risk, because nitrate is endogenously reduced to nitrite and subsequent nitrosation reactions give rise to N-nitroso compounds; these compounds are highly carcinogenic and can act systemically[34].

High nitrate concentrations in drinking water caused a decline in sperm count and motility in mice. This was accompanied with alteration in testicular 17-β hydroxysteroid dehydrogenase and gamma glutamyl transpeptidase[25].

Garlic (*Allium sativum*) belongs to family *Alliaceae* was considered one of the most important plants that has been used for decades by the Egyptian population; its characteristic odor arises from allicin (allyl 2-propene thiosulfinate or diallyl thiosulfinate) and other oil-soluble sulphur components[2]. Garlic extracts and its abundant organosulphur molecules (S-allyl cysteine sulfoxide) have been shown to exert antioxidant effects through its ability to scavenge reactive oxygen species ROS[7]. The wealth of scientific literature supports the proposal that garlic and its preparations help in preventing or reducing the risk of cardiovascular complications, stroke, and cancer[28].

Garlic oil (GO) exerts its effects by modulating lipid peroxidation and anti-oxidants. It has got differing effects in the target organ and host tissues that reflect its modulatory role in cell proliferation. Garlic oil has been reported to scavenge free radical species and modulate the levels of lipid peroxidation and antioxidants[16]. To date, it has been reported that heated garlic juice was effective in recovery of testicular function after experimental testicular hypogonadism but other laboratories have reported that powder[11] or crude[15] garlic preparations impaired testicular and male reproductive tract functions. Moreover, garlic metabolites such as diallyl trisulfide have been reported to have spermicidal effects[8]. The mechanisms of garlic action on male reproduction function, and particularly on spermatogenesis, however, remain unknown.

Therefore, the present study was undertaken to investigate the antioxidant activity of garlic oil in evoking positive responses in the toxic model produced by $NaNO_2$ in the testes of male albino mice.

MATERIALS AND METHODS

Sodium nitrate (98.5% pure) was procured from Merck Chemicals, Germany. A test solution in drinking water was prepared daily 24 hours prior to feeding at a concentration of 900 ppm[25]. Sexually mature (7 weeks) white Swiss male mice weighing 20±2 g bred at Fayoum faculty of science animal house colony were used. They were fed *ad libitum* on a pellet diet and maintained under standard laboratory conditions. Mice were divided into four groups. Control group received tape water. Garlic oil group received orally 200 mg/kg bw garlic oil (Egyptian garlic oil was obtained from El-Captain Company, Egypt). Sodium nitrate group received 900 mg/ kg bw of $NaNO_3$ in drinking water freshly daily prepared, treated group received 900 mg/kg bw of $NaNO_3$ in drinking water and treated with garlic oil with dose 200 mg/kg bw[37] for 36 days. The animals were sacrificed by cervical dislocation on the end of the experiment.

SPERM COUNT AND VIABILITY

To measure the sperm reserves, cauda epididymis were minced, and homogenized for 1 min in 5.0 ml of physiological saline solution or phosphate buffer saline solution[23, 36]. The homogenate was filtered and then 0.1 ml of filtrate was diluted with 2.0 ml of saline solution containing 4% trypan blue. From this solution, 20 µl aliquots were placed on the Neubauer hemacytometer for counting the number of sperms/mg of cauda epididymis tissue.

The ratio of live to dead spermatozoa or sperm viability was determined using 1% trypan blue as described in the method of[32]. Briefly, undiluted sperm samples (0.2 ml) used for counting were incubated with 0.2 ml of 1% trypan blue stain for 15 min at 37°C. A drop of the suspension was placed in a Neubauer chamber under a cover slip, allowed to settle for 1 min, and observed under a Nikon binocular microscope. The

numbers of stained and unstained spermatozoa were scored in 10 to 20 separate fields.

SPERM MOTILITY ANALYSIS

The sperms were collected as quickly as possible after a mouse was sacrificed. The cauda epididymis was placed in 1 ml of 37^0C phosphate buffer saline solution and cut by surgical blades into approximately 1 mm^3 pieces. The solution was pipetted several times to homogenize the sperm suspension. One drop of the suspension was placed on a slide, covered by a 24 x 24 mm coverslip, and evaluated under a microscope at 200x magnification. The sperms were categorized based on their motility as ''motile'' or ''immotile''. The results were recorded as the percentage of sperm motility.

EPIDIDYMAL SPERM MORPHOLOGY

After evaluating epididymal sperm motility, the sperm suspension was used for the analysis of sperm morphology. Thus, one drop of the suspension was smeared onto a glass slide and stained with 1% eosin was analysed microscopically for morphological abnormalities. A total of 200 sperms from each mouse were examined[25]. In total, 2000 sperms on each slide were evaluated and the results were recorded as the percentage of abnormal sperm on each slide. Abnormal heads and tails were evaluated by using the criteria of[21, 22, 24].

HORMONE ASSAYS

The blood samples were obtained from the animals by heart puncture. The serum level of testosterone, luteinizing hormone, and FSH were determined[12, 26] using radioimmunoassay kits produced from Immunotech (Marseille, France).

HISTOPATHOLOGY

For histopathological examination, the testicular tissues were dissected, and the tissue samples were fixed in 10% neutral buffered formalin solution for 24 h, then, processed by using a graded ethanol series, and embedded in paraffin. The paraffin sections were cut into 5 μm thick slices and stained with hematoxylin and eosin for light microscopic examination. The sections were viewed and photographed by using a light microscope.

FLOW CYTOMETRY ANALYSIS

Flow cytometry analysis was carried out as described previously[17]. Testes were mechanically dissociated and suspended in the citrate buffer (4°C) and cell number was estimated with a Coulter Z1 counter (Coulter Electronics Limited, Luton, Beds, England). Samples were adjusted to a cell count of 2 x 10^7 cells/mL and centrifuged at 800 *g* for 10 min. The supernatant was removed and 0.5% trypsin in the spermine tetrahydrochloride buffer was added and gently rocked for 10 min at room temperature. A solution containing trypsin inhibitor and ribonuclease A (12 mg/mL) was added to stop trypsin action and remove double-stranded ribonucleic acid and the specimen was again rocked for 10 min at room temperature. Finally, propidium iodide (50 mg/mL) in spermine-tetrahydrochloride buffer solution were added and rocked for 10 min in a dark room. A minimum of 10,000 cells were analyzed by a FACSort (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA). The excitation wave length was 488 nm at 150 mW, 10,000 nuclei/specimen. Histogram analysis of the red fluorescence emitted by the propidium iodide was accomplished manually by setting markers around the haploid (n), diploid (2n), and tetraploid (4n) peaks and calculating the percentage of each ploidy compartment. The data from each time point as presented as mean and standard deviation.

STATISTICAL ANALYSIS

The data were analyzed by using SPSS 11.0 for Windows. The significance of differences was calculated by using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

THE RESULTS

The body weights of the $NaNO_3$ treated mice were significantly decreased when compared to the other groups of animals. The significant differences were found

Regular Paper

in weights of the testis and cauda epididymis after $NaNO_3$ treatment for 35 days. However, body, testis, and cauda epididymal weights were significantly altered by GO treatment. With GO administration, these weights were as similar as to those in control mice (TABLE 1).

TABLE 1 : Body weight (g) and organ weights (mg) of control and experimental animals groups for 35 days.

Parameters	Control	Garlic oil (GO)	$NaNO_3$	$NaNO_3$+GO
Body weight (g)	23±0.60	23±0.77	19±0.34*	22±0.81
Testis (mg)	120±3.90	122±4.22	87±5.21*	110±4.23
Cauda epididymis (mg)	40±2.23	41±3.11	28±2.14*	38±3.93

Mean±SE of ten mice in each group. *p<0.05.

Sperm count and motility from the cauda epididymis were markedly and significantly reduced by $NaNO_3$, but was comparable to normal levels when GO was administered with $NaNO_3$. The frequency of non-viable and no-motile sperm increased markedly and significantly with $NaNO_3$ treatment, and when compared with control, but with no significant effect associated with $NaNO_3$ administered and garlic oil treatment (TABLE 2).

TABLE 2 : Effect of nitrate exposure on sperm motility, viability and total epididymal sperm count of mice treated for 35 days.

Parameters	Control	Garlic oil (GO)	$NaNO_3$	$NaNO_3$ +GO
Sperm motility	75.8±2.25	75.23±3.11	50.39±4.31*	69.41±3.91
Viability (%)	65.22±3.09	64.29±2.99	45.45±3.21*	60.98±3.87
Sperm count X 10^6/ml	42.45±1.65	45.21±2.78	29.67±3.13*	39.99±4.22

Mean±SE of ten mice in each group. *p<0.05.

At 900 ppm of $NaNO_3$, a significant increase in abnormal sperm involving head (2.41±0.20) and tail (6.32±0.24) regions was noticed (P<0.05). Also, NaNO3 significantly decreased the serum testosterone levels (1.54+0.28) in the experimental group compared with control (3.98±0.38, p<0.001). The levels of serum FSH was significantly higher in $NaNO_3$ group (4.01±0.54) compared with the control group (2.99±0.63, p<0.001). The LH levels of the control group and $NaNO_3$ group were (2.67±0. 37) and (5.47±1.25) respectively which were significantly different (p<0.001) (TABLE 3).

TABLE 3 : Effect of nitrate exposure on sperm abnormalities and biochemical assays of mice treated for 35 days

	Parameters	Control	Garlic oil	$NaNo_3$	$NaNO_3$+ GO
Sperm abnormalities	Head	1.79±0.28	1.68±0.31	2.41±0.20*	1.56±0.27
	Tail	3.67±0.38	3.56±0.32	6.32±0.24*	4.01±0.37
Hormonal assays	Testosterone ng/ml	3.98±0.38	4.19±0.15	1.54±0.28*	3.44±0.08
	LH (ng/ml)	2.99±0.63	2.80±0.61	4.01±0.54*	3.01±0.49
	FSH (ng/ml)	2.67±0.37	2.68±0.25	5.47±1.25*	2.72±0.31

Mean±SE of ten mice in each group. *p<0.05.

HISTOLOGICAL EVALUATIONS OF TESTIS TISSUE IN DIFFERENT GROUPS

Histological evaluations of testis tissue in different groups were done in the sections stained with hematoxylin and eosin.

In group I, seminiferous tubules were richly populated and gave healthy appearance (Figure 1A). There is thin basement membrane. All the cells of the spermatogenic series could be identified in the tubules. Lumen could easily be delineated in almost all the tubules, and majority of them were occupied by mature spermatozoa. In group II, the nearly histological feature of the testis was the same as in the control group.

In group III animals, which were poisoned with $NaNO_3$, the blood vessels were dilated and congested (Figure 1B & C). A majority of seminiferous tubules were shrunken and had a wavy outline. The basement membrane was thickened and hyalinized. Debris of shed cells occupied most of the lumen of the seminiferous tubules. Most of the tubules contained spermatogonia and spermatocytes, which were large in size and contained darkly stained nuclei. In some cells, the nuclear membranes had been ruptured and were accompanied by fragmentation of nucleus (karyorrhexis). Some of the tubules contained only spermatogonia, which were scanty in number, bigger in size, and had eccentrically placed dark nuclei. The interstitial cells of Leydig were

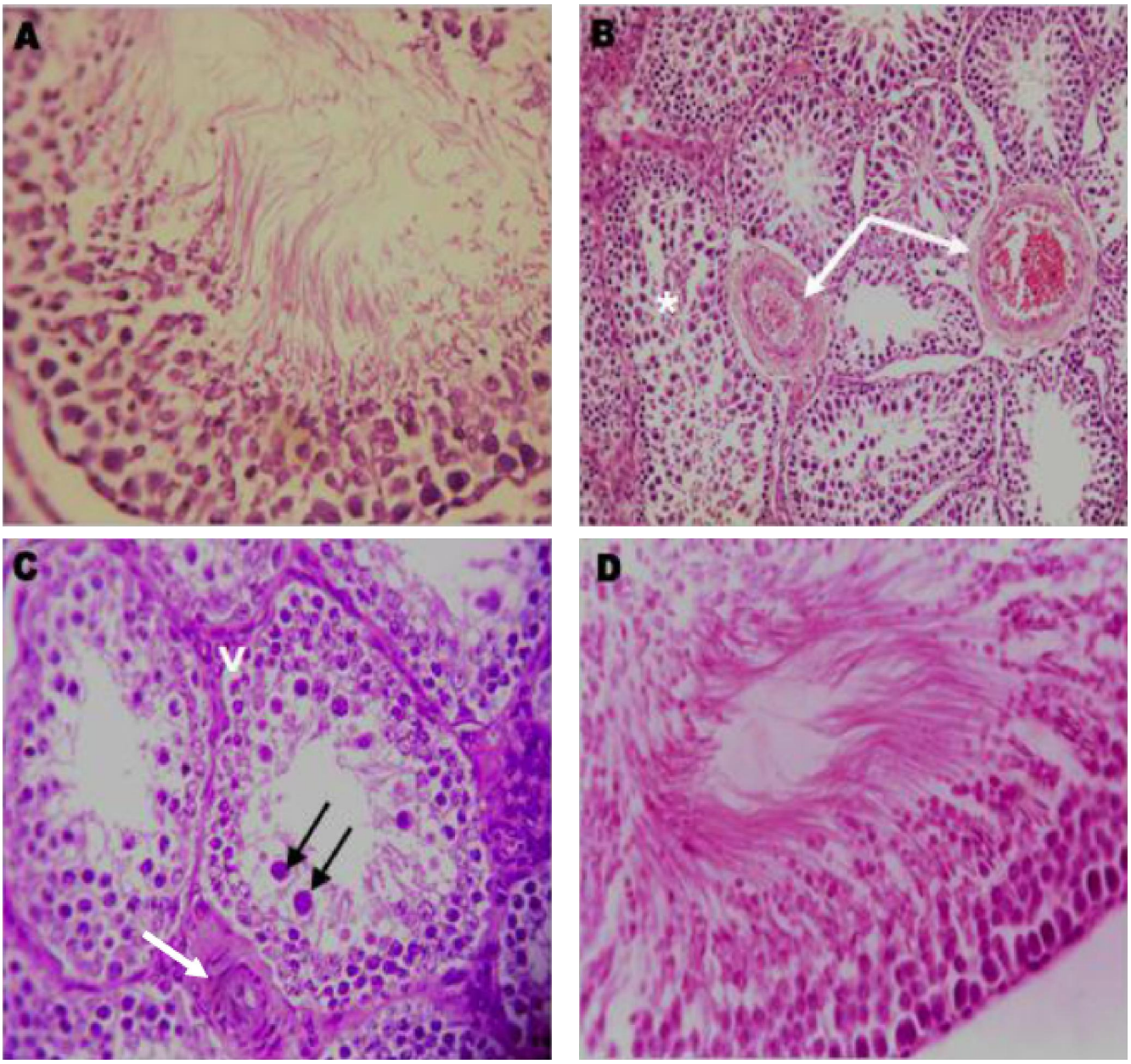

Figure 1 : Testicular section of control mice which show normal spermatogenesis. Note the normal cell arrangement in the seminiferous tubule (A). Testicular sections of mice treated with $NaNO_3$. Note the large, and congested dilated interstitial blood vessels (white arrows). Note the severe degenerations in the seminiferous tubules and intercellular disassociations of germ cells. *, Sloughing of germ cells into tubular lumen; V, vacuolization in Sertoli cells, giant cells (black arrows) (B & C). Section of testis of group treated with $NaNO_3$ pulse garlic oil, showing normal seminiferous tubules more or less as control (D).

also reduced in number and their characteristic tendency of clumping together to form groups was also reduced. All these features were suggestive of atrophy of the testes.

In groups IV which were treated with $NaNO_3$ plus GO, the recovery features were shown when compared with $NaNO_3$ group (Figure 1E). Recovery includes an accumulation of increased spermatozoa in the luminal areas, normal seminiferous tubules, and thin basement membrane along with partial amelioration of sodium nitrate-induced changes.

FLOW CYTOMETRY ANALYSIS

The mean percentages of haploid, diploid, and tetraploid populations in different experimental groups are

Regular Paper

listed in TABLE 4. These data also are demonstrated graphically in Figure 2. The proportions of diploid and tetraploid cells decreased in $NaNO_3$ treatment, but showed an increase after treatment with garlic oil.

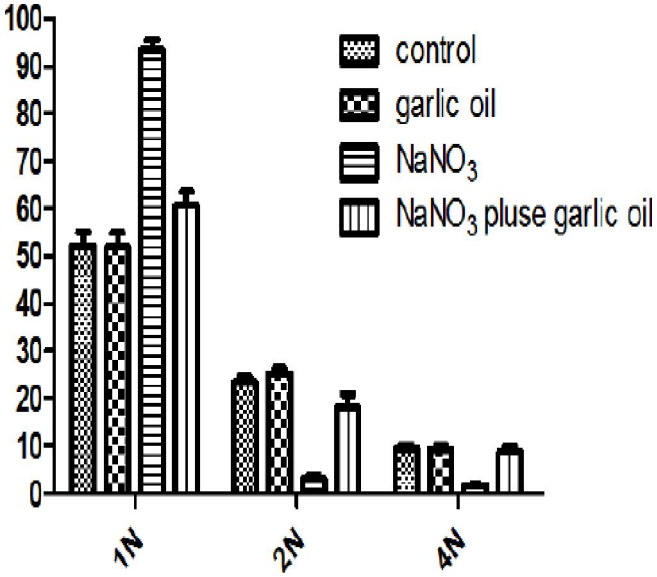

Figure 2 : Relative proportions of cells with haploid, diploid, and tetraploid states of DNA ploidy in the testicular cell suspensions of mice in different experimental groups.

TABLE 4 : DNA flow cytometry analysis of the testes in different experimental groups.

Parameters	Control	Garlic oil (GO)	$NaNO_3$	$NaNO_3$ +GO
1N (Haploid)	52.01±2.99	51.88±2.98	93.45± 2.09*	60.52± 3.07
2N (Diploid)	23.34±1.08	25.29±1.09	2.99± 0.83*	18.12± 2.84
4N (Tetraploid)	9.45±0.65	9.21±0.78	1.76± 0.08*	8.79± 1.04

Mean±SE of ten mice in each group. *p<0.05.

DISCUSSION

Various methods are used by toxicologists and pathologists to detect the testicular toxicity in men or laboratory animals. Some of the common parameters include organ weights, hormonal analysis, semen analysis, dominant lethal assays, enumeration of elongated spermatids in the testicular homogenate, and morphometry[31]. Recent revision of regulatory guidelines for reproductive, fertility, and developmental toxicity studies have emphasized the importance of histopathology and flow cytometry as a sensitive and early indicator of spermatogenic disturbances.

We investigated the efficacy of garlic, which is considered both a traditional natural medicine and an edible vegetable, against the toxicological disorders induced by sodium nitrate using a mice model. Treatment with sodium nitrate in this study significantly decreased sperm count in the experimental mice, which is in agreement with previous findings by[38]. They reported that reduced sperm motility and increased secondary abnormalities of the spermatozoa due to damaged membrane integrity occurred following nitrate treatment in bulls. Similarly, decreased sperm count, sperm motility and increased abnormal spermatozoa as well as altered activity of marker testicular enzymes such as 17-β hydroxysteroid dehydrogenase (17-β HSD) and fi-glutamil transpeptidase (fi-GT) were reported in nitrate-treated mice[25]. The results agreed with the finding that spermatozoa are capable of producing free radicals and that loss of sperm function in certain cases of infertility is associated with excessive activity of the free radical generating system[10]. In the present study, garlic oil administration to sodium nitrate – treated mice produced an appreciable increase in the sperm count up to that of the control level. Thus, it appears that garlic oil counteracted the spermicidal effects of spermatozoa-generated free radicals and enhanced the antioxidant capacity of the several antioxidant factors contained in the spermatozoa and seminal plasma.

In regard to testosterone, LH and FSH level studies, the result of the present work is contradictory to previous findings that nitrates depress Leydig's cell function and inhibit steroidogenesis[13]. Evidence of tolerance and adaptation to nitrate toxicity has been reported in subchronic and chronic studies[4], which may explain the disparity between the finding of this study and those of the previous ones. This requires further investigation.

Components of garlic oil appear able to counteract the sodium nitrate-induced production of aggressive oxidants or to impair the mechanisms by which these oxidants damage key molecules within tissues. The antioxidant properties of garlic were attributed to glutamyl cysteine, steroidal glycosides, essential oil, flavonoids, lectins, prostaglandins, fructan, pectin, vitamins, fatty acids, phospholipids and essential amino acids, all being known to be efficient radical scavengers[3]. The observed reduction in the level of lipid peroxidation in garlic-treated animals is in part due to its ability to scavenge the hydroxyl- and peroxyl- radicals[28]. Different

protective pathways were evoked to explain the beneficial effect of garlic components on histological testicular tissues. Where, the large, and congested dilated interstitial blood vessels, the severe degenerations in the seminiferous tubules and intercellular disassociations of germ cells, Sloughing of germ cells into tubular lumen, vacuolization in Sertoli cells and giant cells were disappeared in section of testis of group treated with $NaNO_3$ pulse garlic oil, showing normal seminiferous tubules more or less as control.

Flow cytometry can provide a rapid way to investigate germinal epithelium integrity and to perform a quantitative analysis of spermatogenesis[18], and has been used in a number of laboratories to examine spermatogenesis in men[30] and animals[9]. Testes with normal spermatogenesis show a characteristic DNA distribution pattern with three peaks. Representing the major portion were 1N cells (spermatozoa, spermatids), followed by 2N cells (spermatogonial cells, secondary spermatocytes, Sertoli cells, Leydig cells, connective tissue cells), and 4N cells (primary spermatocytes)[30]. In the present studies, we performed qualitative examination of germ cell quantitative examination by flow cytometry. Flow cytometry demonstrated that proportions of diploid and tetraploid cells were decreased by $NaNO_3$ toxicity, but diploid and tetraploid cells were increased after treatment with garlic oil. This result coincided with histopathologic observations. Thus, simultaneous qualitative observation by histopathologic examination and quantitative examination of DNA ploidy by flow cytometry of seminiferous tubules were useful in the detection of testicular toxicity.

In conclusion, garlic treatments appear able to cure the sodium nitrate-induced several histopathological changes in mice testis, reduced cauda epididymis weight, reduced the number of sperm count in cauda epididymis and decreased the epithelial thickness of seminiferous tubules, and other biochemical assays. Also, our results suggest that adult mice exposure to $NaNO_3$ (900 mg/kg) induces spermatogenic apoptosis through increasing oxidative stress in male mice. However, the precise mechanism(s) by which $NaNO_3$ affects spermatogenesis may be more complex.

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Regular Paper

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