

Observable Protective Activities of Quercetin on Aluminum Chloride-Induced Testicular Toxicity in Adult Male Wistar Rat

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ABSTRACT

Background: Aluminum chloride (AlCl₃) present in many manufactured consumable is considered as a toxic element. **Aim:** Our study evaluates the toxic effects induced by AlCl₃ on the testes as well as the therapeutic tendency of Quercetin (QUE) agent as an antioxidant. **Setting and Design:** In the department of Anatomy of Medical School. **Methods and Materials:** Thirty-two male Wistar rats weighing approximately 170 ± 10 g were assigned into four groups with eight each, fed with rat chow and water ad-libitum. Group A served as control and was given distilled water throughout; Group B was given only QUE (200 mg/kg body weight) for 21 days; Group C was given only AlCl₃ (300 mg/kg body weight) for 14 days; and Group D was given AlCl₃ (300 mg/kg body weight) for 14 days followed with QUE (200 mg/kg body weight) for 21 days. Substance administrations were done orally. **Statistical analysis:** One-way analysis of variance was used to analyze the data, in GraphPad Prism 6.0 being the statistical software. **Results:** AlCl₃ significantly reduced the relative organ (testes) weight, correlating the decrease in sperm count, sperm motility and sperm viability. Furthermore, there was a decrease in luteinizing hormone with an increase in follicle-stimulating hormone which accounted for a significant reduction in testosterone level that plays a great role in spermatogenesis, following AlCl₃ treatment. The cytoarchitecture of the testes showed degenerative changes in the seminiferous tubules and leydin cells, nitric oxide synthases immunoreactivity was intense in the seminiferous epithelium of rat in Group C. **Conclusion:** These suggest that QUE antioxidant property could reverse the decrease in sperm status, hormonal effects, and functional deficit induced by aluminum chloride on the testes of Wistar rats.

KEYWORDS: Aluminum chloride, neurotoxicity, quercetin, sperm status, testicular tissues damage

INTRODUCTION

Aluminum (Al) is one of the most widely distributed metals in the environment and the third most abundant element in the earth's crust. In the environment, Al exists in only one oxidation state (+3) and does not undergo oxidation reduction reactions.^[1] Humans can be exposed to Al through the consumption of food items, drinking water, and inhalation of ambient air.^[2] Varieties of medicines where Al compounds are being used include buffered

aspirins, phosphate binders, antacids, and vaccines as well as in consumer products such as first-aid antibiotic, antiperspirants, antiseptics, and food additives.^[14,15] Concomitantly, there has been an increased incidence of exposure of the general population to Al, which can cause serious effects on various systems of the body.^[3]

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Al is extensively used in daily life, such as in pharmaceuticals, in water treatment process, in utensils, in food additives and in consumer products, resulting to easy exposure to human beings.^[1] Al is mainly found in cosmetics, herbs, yellow cheese, salt, corn, spices, tea, Al ware, and containers.^[1] Environmental pollution containing the different Al compounds exposes people to higher than the normal levels of Al in their lifetime.^[25] Especially, matters that are distributed by cement-producing factories have a very high volume of Al and resident or populations residing in such vicinity are exposed to the pollution.^[4,5]

However, great interest is currently being attributed to Quercetin (QUE), a well-known natural occurring polyphenol products belonging to the family of flavonoids.^[7] It is known for their health beneficial effects even long before establishing their biochemical properties, and it is found in many foods, including tea, fruits, vegetables, and wine, which can be orally absorbed by humans.^[6] Furthermore, as a nutritional enhancement, QUE is used in treating inflammation, diabetes, cancer, asthma, obesity, and cardiovascular diseases.^[26] QUE therapy, targets the cell cycle, cellular substrates regulating apoptosis, growth arrest and inflammatory activities in a living organism.^[8] Thus, QUE has a wide range of reported biologic effects, including antioxidant, anti-hypertensive, antimicrobial, and antiprotozoan activities.^[9]

The consumption of Al in a very high volume will lead to the accumulation of Al in the various organs of the body, in both humans experiment and animal studies. Al effect has been associated with testicular tissues damage,^[10] histological alteration of the testes, spermatogenesis worsening,^[11] interruption in sex hormone secretion, development of free radicals and modifications in antioxidant enzymes,^[12] and biochemical changes in testes and other accessory reproductive organs.^[13] These are some of the aspects suggested that Al exposure causes adverse impact on male reproduction.

Therefore, this study evaluate the toxic effects induced by aluminum chloride (AlCl₃) on the testes as well as the therapeutic tendency of QUE agent as an antioxidant, remedying the toxic damage caused by AlCl₃.

RESEARCH METHODOLOGY

Rat procurement, care, and ethical approval

Thirty-two adult male Wistar rats weighing about 170 ± 10 g were gotten from the University animal house and used for this study. The rats were housed in the Institutional Animal Holding Facility under suitable environmental condition, in standard seized plastic cages and fed with normal pelletized chow with free

access to water ad libitum throughout the duration of experiment. All protocol and treatment procedures were done according to the Institutional Animal Care and Use Committee Guideline and as approved by the Research Ethical Committee of the University with approval number BUHREC022/19, which is in line with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NRC, 2010).^[31]

Treatment schedule

Experimental animals were split into four groups (labelled A-D) of eight animals each in order to avoid overcrowding. Administration started after 2 weeks of adaptation to the environment, Group A served as control and was given distilled water throughout, Group B was given only QUE (200 mg/kg body weight) for 21 days according to Tripathi *et al.*^[32] Group C was given only AlCl₃ (300 mg/kg body weight) for 14 days according to Amjad and Umesalma,^[33] and Group D was given AlCl₃ (300 mg/kg body weight) for 14 days followed with QUE (200 mg/kg body weight) for 21 days [Table 1]. Thus, all substance administrations were done orally and were continued within 35 days, as well as all activities involving the use, handling, treatment, and management of the experimental animals were carried out in compliance with ethics and standard institutional research practices.

Procurement of aluminium chloride and quercetin

AlCl₃ and QUE were purchased from Sigma-Aldrich, USA. All enzyme-link immunosorbent assay (ELISA) kits were purchased from Melson Medical Corporation Limited, China and the antibody for nitric oxide synthases (iNOS) immunohistochemistry was obtained from Fischer Scientific, USA.

Tissue preparations

24 h after the treatment period for each group, the rats were sacrificed by cervical dislocation and the abdominal region toward the thoracic cavity was dissected to harvested the testes and have easy access to the rat heart. Blood was collected from the left ventricle of experimental animal heart with capillary tubes into a heparinized bottle and immediately preserved in a cooler filled with ice to prevent coagulation and used for hormonal analysis. Two animals from each group were perfused with 10% formal saline, and their testes

Table 1: Experimental animal grouping

Groups (n=8)	Administration (mg/kg)	Dosage
A	Distilled water	0.5 (ml)
B	Quercetin	200 (mg/kg)
C	AlCl ₃	300 (mg/kg)
D	AlCl ₃ + quercetin	300 + 200 (mg/kg)

AlCl₃=Aluminium chloride

were carefully excised using scalpel and forceps and preserved/fixated in 10% formalin for histological and immunohistological demonstrations while the others animal (remaining 4 rats) in the same group were not perfused and used for sperm quality/morphology assay. Histological and immunohistological slides prepared were viewed using LEICA DM 750 microscope with a digital camera connected to a computer.

Collection of semen

The testicles and epididymis were exposed through a lower abdominal incision. The left and right caudal part of the epididymis was excised from the body of the testes; sperm cells from the caudal were released into a petri dish containing normal saline.

Sperm viability

It was immediately assessed and recorded after the tissue isolation as described earlier.^[34] Briefly, a mixture of semen, eosin and nigrosin was made by mixing one drop of semen with two drops of 1% eosin followed by three drops of 10% nigrosin solution. Within 30 s of making mixture, a smear was made on a microscope slide, air dried and examined under oil immersion ($\times 1000$) using a light microscope. The percentage of sperm viability was calculated using the number of live (white/unstained) sperm over total number of dead (pink/red) sperm cells. A minimum number of 200 spermatozoa were scored per slide.

Sperm motility

Cell motility was recorded and evaluated immediately after tissue isolation. A drop of sperm cells was dropped on a glass slide, covered with slip and then examined under a light microscope. The percentage of sperm motility was calculated using the total number of sperms moving into 100 divided by the total number of sperms counted.

Sperm count

Sperm count was done under the microscope using improved hemocytometer. Sperm cells were counted in five diagonal large Thomas squares and the concentration of sperm in cells was calculated using this formula: Number of sperm counted \times dilution factor/volume \times 1000 = sperm/ml.

Biochemical assay

The levels of reproductive hormones (follicle-stimulating hormone [FSH], luteinizing hormone [LH], and testosterone [T]) were quantified using competitive enzyme immunoassay technique utilizing a polyclonal anti-LH, anti-FSH, and anti-T. Assay procedures were carried out through ELISA at 450 nm using the microplate readers. The analysis was done according to the manufacturer's instruction in ELISA kits.

Statistical analysis

In this research, all data obtained from each group were presented as a group data and analyzed using the one-way analysis of variance with Graph Pad Prism® software (Version 6.1) being the statistical tool. The results were expressed as mean \pm standard error of mean in a tabular form. Newman-Keuls *post hoc* test was used to compare the means thereby identifying differences. The confidence interval was placed at 95% such that in all cases a value of $P < 0.05$ was considered significant.

STUDY RESULTS

Assessment of sperm status

The results of epididymal sperm analysis of control and all treated groups are summarized in Table 1. Sperm count which entails counting the number of sperm in a sample of semen is used as a measure of determining male infertility. As observed in this study, $AlCl_3$ treatment (Group C) significantly deteriorated the level of sperm count, sperm motility, and sperm viability, which plays a vital role in determining male infertility. However, there was an obvious but not significant improvement in Group D ($AlCl_3$ + QUE) when compared to Group C ($AlCl_3$). Thus, this suggest that, QUE has a great tendency in ameliorating the decrease in sperm count, sperm motility, and sperm viability accounted for by the toxic effect of accumulated $AlCl_3$ in the body [Figure 1 and Table 2].

Quercetin ameliorate aluminum chloride -induced hormonal alteration

LH is co-secreted along with FSH by the gonadotrophin cells in the adenohypophysis, which stimulates T release by the Leydig cells of the testes in the male reproductive system. Following $AlCl_3$ treatment (Group C), this study documented a notable reduction in LH with an increase in FSH levels, which accounted for a significant reduction in T level when compared with control (Group A). However, there was an obvious increase in LH and T with a decrease in FSH as seen in Group D ($AlCl_3$ + QUE) when compared to Group C ($AlCl_3$) [Figure 2 and Table 3].

Table 2: The effect of quercetin on sperm morphology in aluminium chloride-induced testicular damage in westar rats

Groups	Sperm count	Sperm motility	Sperm viability
A	74.20 \pm 3.235	54.00 \pm 2.449	74.00 \pm 1.871
B	73.40 \pm 2.956	42.00 \pm 3.742	65.00 \pm 5.099
C	51.70 \pm 2.769*#	21.00 \pm 7.810*#	40.00 \pm 7.071*#
D	53.60 \pm 5.896	30.00 \pm 5.477	50.00 \pm 9.434

*Significantly different from control, #Significantly different from quercetin ($P < 0.005$)

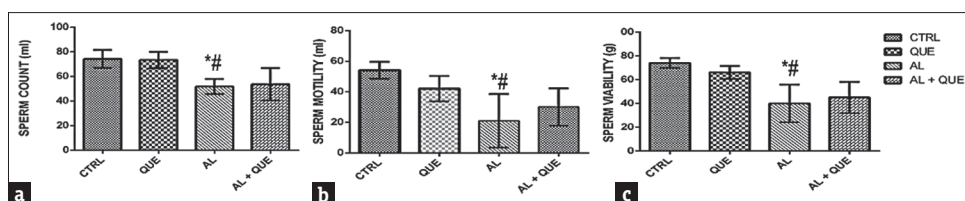


Figure 1: Bar chart showing the effect of quercetin on sperm morphology in aluminium chloride (alcl3) induced testicular damage in wistar rats. [a] sperm count; [b] sperm motility; [c] sperm viability. *Significantly different from control; # significantly different from quercetin (p < 0.005)

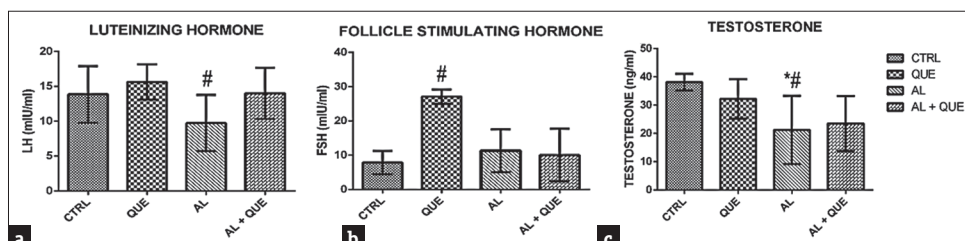


Figure 2: Bar chart showing the effect of quercetin on luteinizing hormone, follicle stimulating hormone and testosterone in aluminium chloride induced testicular damage in wistar rats. [a] lh level; [b] fsh level; [c] testosterone level. *Significantly different from control; # Significantly different from Quercetin (P < 0.005). FSH = Follicle stimulating hormone, LH = Luteinizing hormone

Histomorphological examination of testicular tissue

Histological staining of tissues using Hematoxylin and Eosin, help in recognize precise cell modes and mechanisms that contributes to physiological alterations. In this study, testicular section of control rats and QUE treated rat showed normal testicular architecture consisting of regular and highly organized seminiferous tubules with full spermatogenesis and typical interstitial connective tissue (black arrow head), with slight clusters of Leydig cells in interstitial tissue (blue arrow). Rat in treated with AlCl₃ revealed hypertrophy and hyperplasia of seminiferous tubules with degeneration in the different stages of the seminiferous tubules (black >). Furthermore, clusters of leydig cells in enlarge lumen (yellow arrow) and hyperplastic interstitial tissue with edema (yellow star) were observed [Figure 3a]. However, QUE is known for its antioxidant efficacy which was able to ameliorate the toxic effect of AlCl₃. In AlCl₃ + QUE treated group, testicular section revealed restoration of the normal architecture of the seminiferous tubules which were similar to those of control group (yellow arrow head), with small clusters of leydig cells in interstitial tissue (blue arrow).

The histopathological demonstration of testicular tissue through the expression of Gordon and Sweet (G and S) in this study, exhibited normal morphology of interstitial cells (black arrow) likewise seminiferous tubule with thick germinal epithelium containing proliferating germ cells and lumen filled with spermatids (yellow star), both in control and QUE treated rat. AlCl₃ treated rat showed wide

Table 3: Effect of quercetin on luteinizing hormone, follicle stimulating hormone, and testosterone in aluminum chloride-induced testicular damage in Wistar rats

Groups	LH	FSH	Testosterone
A	13.85±2.028	7.875±1.702	38.13±1.475
B	15.65±1.258	27.13±1.050	32.23±3.478
C	9.725±2.016 [#]	11.30±3.106 [#]	21.25±6.037* [#]
D	14.00±1.833	10.08±3.844	23.53±4.869

*Significantly different from control, #Significantly different from quercetin (P<0.005). FSH=Follicle stimulating hormone, LH=Luteinizing

interstitial space (yellow arrow) and equivalent proportion of normal lumen filled with spermatids and disruption of spermatogenesis, characterized by dilated tubular lumen (blue arrow), which indicates structural alteration in the testicular tissue architecture. However, AlCl₃ + QUE treated group presents normal morphology of interstitial cells (black arrow) and slight spermatogenesis disruption, characterized by increase tubular lumen (blue arrow) with light shedding of germinal epithelium (red arrow), as shown in Figure 3b.

It was observed both in control and QUE treated rat that, the expression of iNOS immunoreactivities revealed negative against iNOS reaction in the seminiferous tubules cells. Although AlCl₃ treated rat exhibited strong positive expression of iNOS in seminiferous tubules, while the group treated with AlCl₃ + QUE showed a negative iNOS reaction in seminiferous tubules and Leydig’s cells had negative reactions against iNOS [Figure 3c].

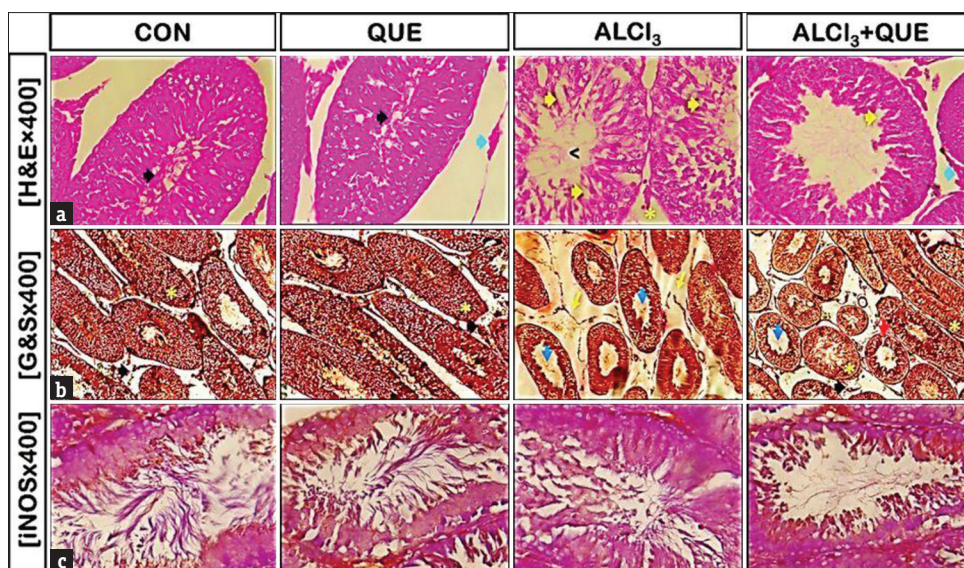


Figure 3: Representative micrographs showing: (a) The general histo-architecture of the testis stained with H and E; (b) Gordon and Sweet; (c) Nitric oxide synthases immunoreactivities. CON = Control, QUE = Quercetin, $AlCl_3$ = Aluminium chloride

DISCUSSION

Al is a heavy metal and a trivalent cation that has the ability to interfere with the absorption and utilization of certain basic nutrients, as well as reproductive function, either directly or in combination with some endogenous substances. Testicular injury in rats, as well as weight loss in the testis and other reproductive organs, has been linked to free radical development mediated by $AlCl_3$. Therefore, our study evaluates the toxic effects induced by $AlCl_3$ on the testes as well as the therapeutic tendency of QUE agent as an antioxidant.

Indices of sperm activity

The state of spermatozoon in the male organism is very necessary to achieve fertilization, as viability and motility plays critical roles in enabling the sperm to ascend the female reproductive tract into the site of fertilization. Treatment of male rats with $AlCl_3$ in this study resulted to a chronic testicular toxicity, which have been reported earlier by Yousef *et al.* and Yousef and Salama.^[11,16] According to Yousef *et al.*, $AlCl_3$ decreased sperm quality *in vivo* and *in vitro*, causing substantial reductions in ejaculate volume, sperm concentration, total motile sperm per ejaculate, total sperm content, sperm motility, packed sperm volume, normal and live sperm, and an increase in the number of dead and abnormal sperm cells. Equally, Yousef and Salama^[16] and Hala *et al.*^[17] documented in their study that, exposure of male Wister rats to $AlCl_3$ significantly decreased sperm motility as well as the sperm concentration of treated rat, and also increased sperm death and abnormal sperm when compared to control group. Thus, the findings of this study's sperm analysis

back up reports of $AlCl_3$'s reproductive toxicity, showing that the oral administration of $AlCl_3$ caused a substantial decrease in sperm count, sperm motility, and sperm viability as compared to the control and $AlCl_3$ + QUE treated groups (Group D). This implies that exposure to $AlCl_3$ can hamper spermatogenesis, especially as a result of increased reactive oxygen species (ROS) activity and or oxidative stress. Increased development of ROS in the testis is said to alter tissue physiology and cause DNA damage, potentially jeopardizing male reproductive capacity.^[35]

Further, the observed decreases in sperm motility and viability may have been caused by an alteration in antioxidant system, depletion in cyclic adenosine monophosphate (cAMP), and an increase in nitric oxide (NO) development caused by $AlCl_3$ treatment. Furthermore, LH stimulates the Leydig interstitial cells to secrete T. Depletion in LH and T levels in the present study, both of which are essential for spermatogenesis, may explain the lower sperm count observed in $AlCl_3$ treated rats [Table 2]. Previous studies have reported decreased sperm motility and viability to correlate with high concentrations of $AlCl_3$ in spermatozoa likewise in seminal plasma.^[28,29] However, in rats given $AlCl_3$ + QUE (Group D), 200 mg/kg of QUE treatment showed an obvious but not significant improvement in sperm count, motility, and viability. This indicates that treating rats with 200 mg/kg of QUE could mitigate $AlCl_3$ negative effects on sperm count, motility, and viability. QUE, a well-known natural occurring polyphenol product for its health beneficial effects, has been documented in treating oxidative stress-related diseases,^[36] inflammation, diabetes,^[37] cancer,^[38] asthma,

obesity, and cardiovascular diseases, by targeting the cell cycle, controlling/regulating apoptosis, oxidative stress, and inflammatory activities in a living organism. Therefore, the antioxidant properties of QUE have a great tendency in antagonizing or ameliorating the deteriorated sperm status accounted for by the toxic effect of $AlCl_3$ on the testis of rat, resulting from the accumulation of $AlCl_3$ in the body.

Indices of hormonal activity

The function of reproductive hormones is very essential and complicated, and it is associated with the moderation of spermatogenesis as well as sperm development in an organism.^[27] LH is co-secreted along with FSH by the gonadotrophin cells in the adenohypophysis, which stimulates T release by the Leydig cells of the testes in the male reproductive system, thus an alteration in sperm quality in most case is traceable to the hormonal activities. From our study results, there was a high depletion in LH levels of rats treated with $AlCl_3$ (Group C [9.725 ± 2.016]) when compared to the control (Group-A [13.85 ± 2.028]) and Group D (14.00 ± 1.833). Furthermore, the level of FSH in rats treated with $AlCl_3$ (Group C [11.30 ± 3.106]) was observed to increase slightly over control (7.875 ± 1.702) and Group D (10.08 ± 3.844) but significantly reduced when compared to QUE treated rats (Group B [27.13 ± 1.050]). Thus, the reduction in FSH and LH levels could be related with the calcium channel blocking effect of Al, leading to impaired secretion of gonadotrophins from the hypothalamus as Ca^{2+} ions are important for gonadotrophin-releasing hormone (GnRH) secretion in the hypothalamus.^[12] Diminished secretion of GnRH might be responsible for decreased FSH and LH levels.^[12] Corresponding to LH finding above, there was an obvious significant reduction in T levels of rats treated with $AlCl_3$ (Group C [21.25 ± 6.037]) when compared to the control (38.13 ± 1.475) and Group B (32.23 ± 3.478), with an increase in Group D (23.53 ± 4.869) when compared with Group C [Table 3]. The reduction in semen quality of $AlCl_3$ treated rats can be relatively correlated with $AlCl_3$ induced reduction in T.^[19]

Deterioration in 17-ketosteroid reductase enzyme activity, important in converting androstenedione to T could also be potential reason for T depletion, following exposure to Al.^[21] However, the significant decline in the levels of serum T in $AlCl_3$ treated rat with the observed alteration in LH and FSH levels following $AlCl_3$ treatment reflects disturbances in the functions of the anterior pituitary as well as the testicular Leydig cells. Our findings are strengthened by that of Yakubu *et al.*^[18] who also reported that oral administration of $AlCl_3$ result

in a significant decrease in the serum LH and T levels of treated rats, but the controversy in Yakubu *et al.*^[18] study with this present study is that, their study documented a significant decrease in FSH levels of $AlCl_3$ treated rats while this present study observed an increase in $AlCl_3$ treated rats when compared to various study control rats. This controversy may be due to the difference in environment as well as the experimental duration. Guo *et al.*^[19] also reported Al intoxication to induce the production of NO which might be a suppressor of T synthesis in an organism. Correspondingly, Dobashi *et al.*^[20] also observed that the inhibitory effect of NO on Leydig cells may suppress T synthesis. Based on our findings, QUE has a great tendency in ameliorating the impaired sperm LH, FSH, and T level resulting from the toxic effect of $AlCl_3$ on the testis.

Cytoarchitecture

Histological staining of tissues using Hematoxylin and Eosin, help in recognize precise cell modes and mechanisms that contributes to physiological alterations. In this study, the histological demonstration showed that the control testes (Group-A) are surrounded by normal testicular architecture consisting of regular and highly organized seminiferous tubules with full spermatogenesis and typical interstitial connective tissue, while rat treated with (Group-C) revealed hypertrophy and hyperplasia of seminiferous tubules with degeneration in the different stages of the seminiferous tubules, likewise clusters of Leydig cells in enlarge lumen and hyperplastic interstitial tissue with edema. Histological demonstration in this study is corresponding to Afeefy *et al.*^[22] and Falana *et al.*,^[23] finding who reported structural alteration following treatment with $AlCl_3$. Furthermore, the histopathological demonstrate of testicular tissue via the expression of G and S staining of both control (Group-A) and Group-B exhibited normal morphology of interstitial cells likewise seminiferous tubule with thick germinal epithelium containing proliferating germ cells and lumen filled with spermatids. However, $AlCl_3$ treated rat (Group C) showed wide interstitial space and equivalent proportion of normal lumen filled with spermatids and disruption of spermatogenesis, which are characterized by dilated tubular lumen, suggesting structural alteration in testicular tissue architecture. The result of this study is very similar to previous findings.^[16] In $AlCl_3$ + QUE treated rat (Group-D), testicular section revealed restoration of the normal architecture of the seminiferous tubules which were similar to those of control group.

Furthermore, based on our immunohistological expression of iNOS, it was observed both in control and QUE treated rat (Group-B) that, the expression of iNOS

immunoreactivities revealed negative against iNOS reaction in the seminiferous tubules cells. Although $AlCl_3$ treated rat (Group-C) exhibited strong positive expression of iNOS in seminiferous tubules, while the group treated with $AlCl_3$ + QUE (Group-D) showed a negative iNOS reaction in seminiferous tubules and Leydig's cells had negative reactions against iNOS. A study also showed that iNOS may play a functional role in spermatogenesis through apoptosis having in mind that iNOS produces large amounts of NO as a defense mechanism.^[24,30]

CONCLUSION AND RECOMMENDATION

In summary, QUE has antioxidant property among other essential benefits which was able to a great extent reverse the decrease in sperm status, hormonal effects, and functional deficit induced by aluminum chloride in Wistar rats' model. However, we also noticed that the efficacy of QUE in amelioration $AlCl_3$ -induced testicular damage was minimal, which may be as a result of the treatment dose or duration of QUE administration in this study, and we will recommend further studies to look into it, likewise adopting *in silico* approach in optimizing quercetine derivative and selecting lead novel compounds that could be more effective.

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Conflicts of interest

There are no conflicts of interest.

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