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# Mitigative Effects of L-Arginine and N-Acetyl Cysteine against Cisplatin-Induced Testicular Dysfunction and Toxicity through the Regulation of Antioxidant, Anti-inflammatory, and Antiapoptotic Markers: Role of miR-155 and miR-34c Expression

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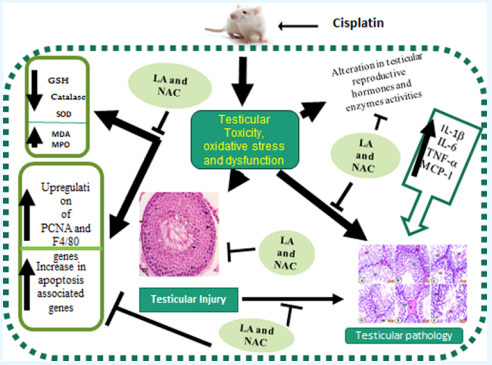
**ABSTRACT:** Testicular dysfunction is a common adverse effect of cisplatin (CIS) administration as a chemotherapeutic drug. The current study has outlined the role of micro-RNAs (miR-155 and 34c) in CIS-induced testicular dysfunction and evaluated the protective effect of N-acetyl cysteine (NAC) and/or L-arginine (LA). Seven groups of Albino rats were used for this study. The control (C) group received physiological saline; the CIS group was injected CIS (7 mg/kg IP, once) on day 21 of the experiment; the NAC group was administered NAC (150 mg/kg intragastric, for 28 days); and the LA group was injected LA (50 mg/kg IP, for 28 days). NAC+CIS, LA+CIS, and NAC+LA+CIS groups received the above regime. CIS significantly reduced serum testosterone, LH, and FSH concentrations with decline of testicular enzyme activities. CIS caused significant elevation in testicular oxidative-stress biomarkers, inflammation-associated cytokines, and apoptosis markers, along with overexpression of miR-155 and low miR-34c expression. Additionally, marked testicular degenerative changes were observed in the examined histological section; a significant decrease in the expression of PCNA with significant increase in expressions of F4/80 and BAX was confirmed. The administration of NAC or LA upregulated testicular functions and improved histopathological and immunohistochemical changes as well as miRNA expression compared with the CIS-administered group. Rats receiving both NAC and LA showed a more significant ameliorative effect compared with groups receiving NAC or LA alone. In conclusion, NAC or LA showed an ameliorative effect against CIS-induced testicular toxicity and dysfunction through the regulation of antioxidant, anti-inflammatory, and antiapoptotic markers and via modulating miR-155 and miR-34c expression.




## 1. INTRODUCTION

Cisplatin (CIS) is a common medication for different organs suffering from cancer.[1] However, CIS administration as anticancer agent has been associated with serious adverse effects in vital organs like the brain,[2] heart,[3,4] liver,[5] and kidney.[6] In addition, the male gonad is affected during CIS therapy. In testicular dysfunction, a degenerative disease, spermatogenesis and testosterone synthesis are both impaired.[7] Several studies have recorded CIS-induced testicular dysfunction including degeneration and apoptosis of spermatogenic cells and impairment of steroidogenic functions of Leydig cells,[8−11] which are attributed to overproduction of the reactive oxygen species (ROS)[12] and reduction in antioxidant activities.[13] Male infertility occurred after CIS exposure as a side effect of CIS therapy;[14] therefore, critical attention is directed to testicular protection against the harmful impact of CIS administration.

It has been shown that the mechanism by which cisplatin affects testis function includes reduction in epididymal sperm concentration to the level of azoospermia, decrease in sperm motility, and viability.[15] In parallel, cisplatin treatment caused a decrease in the level of serum testosterone. Testosterone promotes protein synthesis in all spermatogenic cells, so it plays a significant role in the formation of sperm.[15] Moreover, cisplatin lowered the immune/inflammatory status and stimulated apoptosis and oxidative stress biomarkers.[16] The accumulation of cisplatin in mitochondria leads to excess ROS production, and disturbance in the mitochondria respiratory

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chain triggers oxidative stress and apoptotic processes in testicular tissues.[17]

Several natural products and compounds, such as lycopene, melatonin, taurine, L-carnitine, and bilobetin, which have antioxidant and anti-inflammatory properties, were administered to protect against CIS-induced testicular toxicity..[8,10,11,14,18] L-arginine (LA) is a semiessential amino acid, not essential for health, but it becomes essential and needed in cases of inflammation, childhood, and adolescence.[19] It is naturally present in poultry, red meat, fish, and eggs. A recent study reported that LA can improve male reproductive fertility;[20] it is also involved in several vital biochemical pathways.[21,22] The antioxidant capacities of LA have been proven; it is considered a nitric oxide (NO) precursor[23] and is important for the biosynthesis of glutathione.[21] N-acetyl cysteine (NAC) formed from L-cysteine is considered a glutathione (GSH) precursor.[22,24] NAC has anti-inflammatory and antioxidant activities due to its thiol group.[25] Recently, NAC has been recommended as an excellent choice to deal with male reproductive toxicity.[26,27] The powerful antioxidant and anti-inflammatory characteristics of both LA and NAC make them potential protective agents for ROS-related disorders.

miRNA is a noncoding RNA of 18−24 nucleotides.[28] It is considered a novel biomarker due to its high cell-type specificity.[29] Some miRNAs have key regulatory roles in many cellular processes of the male reproductive system, including androgen signaling regulation,[30] spermatogenesis,[31,32] and expression of cell proliferation and oncogenic pathways.[33] Also, certain miRNAs act as inflammatory mediators, so alteration of their expression can induce apoptosis, uncontrolled cell proliferation, oxidative stress, inflammation, and cancer.[34]

Among these miRNAs, miR-155, which is controlled by proinflammatory reactions,[35] is considered a component of the macrophage response to inflammatory mediators and is thought to be a part of how macrophages react to inflammatory substances. miR-34c is commonly expressed in the spermatogenic cells[36] and its downregulation causes male infertility.[37,38]

The purpose of the current study was to examine the potential mitigative impacts of LA and/or NAC supplementation on CIS-induced testicular damage via investigation of testicular function tests, oxidative stress indicators, cytokines, miR-155 and 34c expression, histopathological alterations, and immunohistochemical expression of F4/80, BAX, and PCNA.

## 2. MATERIALS AND METHODS

**2.1. Chemicals, Natural Products, and Reagents.** CIS, NAC, and LA were obtained from EIMC Pharmaceuticals Co. (Cairo, Egypt); Mena Pharm Co. (Cairo, Egypt); and Sigma-Aldrich Chemical Co. (St. Louis), respectively. Kits for tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Cat. MBS700574), interleukin-6 (IL-6) (Cat. MBS2701082), interleukin-1$\beta$ (IL-1$\beta$) (Cat. MBS711191), and interleukin-10 (IL-10) (Cat. MBS2020828), monocyte chemotactic protein-1 (MCP-1) (Cat. MBS2701125), follicle-stimulating hormones (FSH) (Cat. MBS2502190), testosterone (Cat. MBS702057), and luteinizing hormone (LH) (Cat. MBS2509833) were imported from Mybiosource Inc. (CA) through Biodiagnostic Company, Dokki, Giza. Meanwhile, miRNeasy extraction kit (Cat. ID: 217,084) for miR-155 and miR-34c was purchased from Qiagen (Germany, GmbH). All other kits were bought from Biodiagnostic Co. (Cairo, Egypt).

**2.2. Animals.** Thirty-five male Albino rats (weighing 200 ± 20 g) were obtained from Animal House, Faculty of Veterinary Medicine, Cairo University, Egypt. Rats were acclimated for 1 week prior to the current experiment. Rats were fed a standard balanced diet ad libitum and housed in metal cages at 23 ± 2 °C and light (12 h dark/light cycles). The Research Ethics Board at the Faculty of Veterinary Medicine, Benha University, Egypt looked over the study protocol and methods and gave their approval (Approval No. BUFVTM 15-04-23).

**2.3. Animal Groups and Treatment schedule.** Separate cages were used to randomly divide the rats into seven groups with five rats. The first group (C) served as control animals and received physiological saline intragastric 1 mL/kg/day; the second group (CIS) was injected with a single dose of cisplatin 7 mg/kg b.wt. IP on day 21 of the experiment;[39] the third group (NAC) received N-acetylcysteine at a dose of 150 mg/kg b.wt. intragastric;[40] the fourth group was injected L-arginine (LA) at a dose of 50 mg/kg b.wt IP;[19] the fifth group (NAC + CIS); sixth group (LA + CIS); and seventh group (NAC + LA + CIS). NAC and LA were administered daily for a continuous 28 days.

**2.4. Sampling.** Blood samples were collected 24 h after the last dose, from the retro orbital venous plexus of all rats in plain vacutainer tubes. Sera were separated by centrifugation of blood at 5000 rpm for 10 min and kept at −20 °C until use for biochemical analysis.

Rats were immediately sacrificed under light anesthesia (ketamine-xylazine mixture, 0.15 mL/100 g BW/IP) after blood collection. The testes were freshly excised and blotted on filter paper and then divided into 3 parts. The first part was preserved in 10% neutral buffered formalin for histopathological and immunohistochemical studies. The second part was homogenized in saline and preserved at −20 °C for measurement of oxidative stress/antioxidant parameters. The third part was stored at −80 °C for miRNA assessment.

**2.5. Assessment of Testicular Function Tests.** Serum samples were used to estimate the enzymatic activities of alkaline phosphatase (ALP), glucose-6-phosphate dehydrogenase (G6PDH), acid phosphatase (ACP), and lactate dehydrogenase (LDH) depending on previously reported methods.[41−44] Also, testosterone, LH and FSH hormone levels were determined using the ELISA kit according to the manufacturer's instruction manual.

**2.6. Assessment of Cytokines.** Serum IL-6, IL-10, TNF$\alpha$, IL-1$\beta$, and MCP-1 were assayed using commercially available ELISA kits as described in the instruction manual associated with each kit. All kits were imported from Mybiosource Inc. (CA) through the Biodiagnostic company Dikki, Giza.

**2.7. Measurements of Oxidative-Antioxidant Parameters.** Homogenates from testes were used to measure the activities of MDA, MPO, SOD, CAT, and GSH. Those oxidative stress biomarkers were measured as described before.[45−49]

**2.8. Assessment of Testicular microRNAs (miR-155 and miR-34c).** The miRNeasy extraction kit (Qiagen, Germany, GmbH) was used for RNA extraction as described by manufacturer's protocol. Extracted RNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Applied Biosystem). Real-time qPCR amplification and analysis were performed using Applied Biosystem real-time PCR system software. Amplification was done using


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SYBR Green Master Mix (*Applied Biosystems*). The expression of each miRNA was adjusted to that of the endogenous control gene RNU6B. The relative expression in each group was computed as $^{2-\Delta}$Ct. Table 1 lists the primer sequences for miR-155 and miR-34c.

**Table 1. Primer Sequence for the Measured miRNAs**

| gene | primer sequence | accession No. |
|---|---|---|
| miR-155 | forward: 5′-TAA TGC TAA TCG TGA TAG GGGTT-3′ | NR_132107.1 |
| | reverse: 5′- CAC CGT ACC CTG TTA ATG CT-3′ | |
| miR-34c | forward: 5′-AGT TAC TAG GCA GTG TAG-3′ | XM_017596027.3 |
| | reverse: 5′-TCT TTT TAC CTG GCC GTG T-3′ | |
| RNU6B | forward primer: 5′-CTC GCT TCG GCA GCACA-3′ | XM_063264310.1 |
| | reverse primer: 5′-AAC GCT TCA CGA ATT TGC GT-3′ | |

**2.9. Testicular Histopathology.** Testicular specimens were fixed in 10% neutral buffered formalin for 72 h, dehydrated in ascending grades of ethyl alcohol, cleared in xylene for 3 successive times, and then embedded in paraffin wax. Paraffin sections at 5 $\mu$m were cut and exposed to H&E stain according to Bancroft et al.[50] Based on Meyerholz and Beck,[51] a semiquantitative scoring method was used to grade the histopathological changes in the testes. Five random fields at 40X were blindly checked using the Leica DM3000 imaging system. The score for each parameter ranged from 0 to 4, where 0 indicated normal, 1 indicated <25% affected, 2 indicated 25−50% affected, 3 indicated 50−75% affected, and 4 indicated >75% affected.

**2.10. Immunohistochemical Examination.** Paraffin sections at 5 $\mu$m were deparaffinized, rehydrated, and exposed to 3% $H_2O_2$ in methanol for 10 min. Next, sections were incubated overnight with mouse monoclonal F4/80 antibody at 4 °C to detect macrophages (Cat. Sc-377009, Santa Cruz Biotechnology Inc., CA, 1:150 dilution), mouse monoclonal PCNA antibody (Cat. ABIN1724759, DAKO, Glostrup, Denmark, dilution 1:100), and polyclonal rabbit Bax antibody (Cat. ABIN3020683, DAKO Corporation Carpinteria, CA, dilution 1:40). The immunohistochemistry method was carried out as outlined before.[52] After incubation with the primary antibodies, the sections were incubated with the secondary antibody for 30 min. A commercial ABC system (Santa Cruz Biotech, San Diego, CA) was used for visualization of the reactions. The sections were then subjected to diaminobenzidine (DAB) for 5 min, then counterstained with hematoxylin.

**2.11. Statistical Analysis.** The statistical analysis was performed using SPSS (SPSS Inc., Chicago, Illinois). All data points are represented as mean values ± SE. Differences between groups were analyzed using ANOVA (analysis of variance) and post hoc Tukey's tests. $P$ values <0.05 were regarded as significant.

## 3. RESULTS

**3.1. Serum Testicular Function Tests.** CIS-treated groups showed a clear significant decrease in ALP, ACP, G6PDH, and LDH activities along with significant decrease in testosterone, FSH, and LH concentrations compared with control rats. On the other side, NAC+CIS, LA+CIS, and NAC +LA+CIS groups showed a significant elevation in ALP, ACP, G6PDH, and LDH activities besides significant enhancement of testosterone, FSH, and LH concentrations compared with CIS-treated groups. Meanwhile, groups administered NAC or LA only revealed nonsignificant changes in the previous parameters (Tables 2 and 3).

**Table 3. Serum Testicular Function Tests (Hormones) in Different Treated Groups[a]**

| parameters group | testosterone (ng/mL) | FSH (ng/mL) | LH (ng/mL) |
|---|---|---|---|
| control | 4.03 ± 0.99$^{d}$ | 2.73 ± 0.67$^{d}$ | 2.53 ± 0.81$^{d}$ |
| CIS | 1.73 ± 0.84$^{a}$ | 1.03 ± 0.57$^{a}$ | 0.99 ± 0.68$^{a}$ |
| NAC | 3.98 ± 0.87$^{d}$ | 2.69 ± 0.61$^{d}$ | 2.50 ± 0.57$^{d}$ |
| LA | 4.01 ± 0.99$^{d}$ | 2.71 ± 0.59$^{d}$ | 2.51 ± 0.61$^{d}$ |
| NAC+CIS | 3.06 ± 0.73$^{b}$ | 1.85 ± 0.65$^{b}$ | 1.53 ± 0.73$^{b}$ |
| LA+CIS | 3.08 ± 0.66$^{b}$ | 1.83 ± 0.87$^{b}$ | 1.58 ± 0.57$^{b}$ |
| NAC+LA+CIS | 3.51 ± 3.74$^{c}$ | 2.21 ± 2.77$^{c}$ | 2.11 ± 0.61$^{c}$ |

[a]Values are means ± SE for 5 different rats per each experiment. Different superscript letters showed significant difference ($P$ < 0.05) within the same column. CIS: cisplatin; NAC: N-acetyl cysteine; LA: L-arginine.

**3.2. Cytokines' Findings.** Serum levels of IL-6, TNF$\alpha$, IL-1$\beta$, and MCP-1 revealed significant increases with decline in IL-10 level in CIS-injected rats relative to control rats. But these cytokines showed a significant decline in their levels except IL-10, exhibiting a significant increase in groups pretreated by NAC, LA, and both NAC+LA compared to the CIS-treated group. Meanwhile, IL-6, IL-10 TNF$\alpha$, IL-1$\beta$, and MCP-1 levels showed nonsignificant alterations in NAC- or LA-administered groups compared to the control group (Table 4).

**3.3. Oxidative Stress/Antioxidant Parameters.** The CIS-treated group exhibited significant increase in MDA and MPO levels along with significant decrease in SOD, CAT

**Table 2. Serum Testicular Function Tests (Enzymes) in Different Treated Groups[a]**

| parameters group | ALP (U/L) | ACP (U/L) | G6PDH (U/L) | LDH (U/L) |
|---|---|---|---|---|
| control | 261.62 ± 3.84$^{d}$ | 45.46 ± 1.53$^{d}$ | 67.08 ± 2.12$^{d}$ | 48.03 ± 1.37$^{d}$ |
| CIS | 143.43 ± 4.02$^{a}$ | 20.11 ± 1.94$^{a}$ | 30.41 ± 2.34$^{a}$ | 19.53 ± 1.45$^{a}$ |
| NAC | 259.76 ± 3.92$^{d}$ | 43.08 ± 1.64$^{d}$ | 65.88 ± 1.94$^{d}$ | 47.91 ± 1.14$^{d}$ |
| LA | 260.82 ± 3.94$^{d}$ | 44.32 ± 1.71$^{d}$ | 66.32 ± 2.14$^{d}$ | 46.86 ± 1.39$^{d}$ |
| NAC+CIS | 183.22 ± 4.13$^{b}$ | 31.67 ± 1.54$^{b}$ | 46.08 ± 2.24$^{b}$ | 25.45 ± 1.54$^{b}$ |
| LA+CIS | 186.35 ± 3.76$^{b}$ | 30.74 ± 1.59$^{b}$ | 45.91 ± 1.99$^{b}$ | 26.02 ± 1.21$^{b}$ |
| NAC+LA+CIS | 210.54 ± 3.89$^{c}$ | 38.12 ± 1.79$^{c}$ | 56.08 ± 2.21$^{c}$ | 35.08 ± 1.32$^{c}$ |

[a]Values are means ± SE for 5 different rats per experiment. Different superscript letters show significant differences ($P$ < 0.05) within the same column. CIS: cisplatin; NAC: N-acetyl cysteine; LA: L-arginine.



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**Table 4. Cytokine Levels in Different Treated Groups**[a]

| parameters group | TNF$\alpha$ (pg/mL) | IL-1$\beta$ (pg/mL) | IL-6 (pg/mL) | IL-10 (pg/mL) | MCP-1 (pg/mL) |
|---|---|---|---|---|---|
| control | 25.57 ± 1.43$^{a}$ | 34.03 ± 1.72$^{a}$ | 60.46 ± 2.07$^{a}$ | 24.52 ± 2.07$^{d}$ | 83.32 ± 2.27$^{a}$ |
| CIS | 111.22 ± 1.55$^{d}$ | 114.67 ± 1.57$^{d}$ | 129.23 ± 2.11$^{d}$ | 9.42 ± 1.95$^{a}$ | 168.94 ± 2.18$^{d}$ |
| NAC | 24.93 ± 1.47$^{a}$ | 33.97 ± 1.46$^{a}$ | 60.21 ± 2.09$^{a}$ | 24.02 ± 2.03$^{d}$ | 82.69 ± 2.32$^{a}$ |
| LA | 25.23 ± 1.53$^{a}$ | 34.01 ± 1.57$^{a}$ | 59.99 ± 2.27$^{a}$ | 24.11 ± 2.01$^{d}$ | 83.02 ± 2.24$^{a}$ |
| NAC+CIS | 80.87 ± 1.37$^{c}$ | 82.33 ± 1.43$^{c}$ | 85.19 ± 2.37$^{c}$ | 15.61 ± 1.99$^{b}$ | 109.51 ± 2.17$^{c}$ |
| LA+CIS | 81.33 ± 1.51$^{c}$ | 83.07 ± 1.47$^{c}$ | 86.93 ± 2.17$^{c}$ | 16.02 ± 2.07$^{b}$ | 108.48 ± 2.11$^{c}$ |
| NAC+LA+CIS | 50.13 ± 1.48$^{b}$ | 49.91 ± 1.16$^{b}$ | 51.63 ± 2.32$^{b}$ | 20.01 ± 2.03$^{c}$ | 93.32 ± 2.03$^{b}$ |

[a]Values are means ± SE for 5 different rats per each experiment. Different superscript letters show significant differences ($P < 0.05$) within the same column. CIS: cisplatin; NAC: N-acetyl cysteine; LA: L-arginine.

**Table 5. Oxidative Stress/Antioxidant Parameters in Different Treated Groups**[a]

| parameters group | MDA (nmol/mg. tissue) | MPO (U/g. tissue) | CAT (U/g. tissue) | SOD (U/g. tissue) | GSH (nmol/mg. tissue) |
|---|---|---|---|---|---|
| control | 4.35 ± 1.44$^{a}$ | 0.95 ± 1.34$^{a}$ | 26.43 ± 2.02$^{d}$ | 17.23 ± 2.07$^{d}$ | 53.32 ± 2.07$^{d}$ |
| CIS | 24.43 ± 1.54$^{d}$ | 9.49 ± 1.41$^{d}$ | 11.83 ± 2.07$^{a}$ | 5.23 ± 2.11$^{a}$ | 19.46 ± 2.05$^{a}$ |
| NAC | 4.33 ± 1.39$^{a}$ | 0.93 ± 1.29$^{a}$ | 25.93 ± 1.96$^{d}$ | 16.91 ± 2.09$^{d}$ | 52.92 ± 2.01$^{d}$ |
| LA | 4.31 ± 1.37$^{a}$ | 0.94 ± 1.32$^{a}$ | 26.07 ± 1.89$^{d}$ | 16.95 ± 2.07$^{d}$ | 53.72 ± 2.11$^{d}$ |
| NAC+CIS | 14.17 ± 1.45$^{c}$ | 5.07 ± 1.43$^{c}$ | 17.33 ± 2.11$^{b}$ | 8.99 ± 1.97$^{b}$ | 30.51 ± 1.99$^{b}$ |
| LA+CIS | 13.23 ± 1.31$^{c}$ | 5.11 ± 1.26$^{c}$ | 17.27 ± 1.99$^{b}$ | 9.03 ± 2.17$^{b}$ | 31.28 ± 2.12$^{b}$ |
| NAC+LA+CIS | 8.03 ± 2.08$^{b}$ | 2.83 ± 1.48$^{b}$ | 22.01 ± 1.76$^{c}$ | 12.73 ± 2.32$^{c}$ | 42.03 ± 2.03$^{c}$ |

[a]Values are means ± SE for 5 different rats per each experiment. Different superscript letters show significant difference ($P < 0.05$) within the same column. CIS: cisplatin; NAC: N-acetyl cysteine; LA: L-arginine.

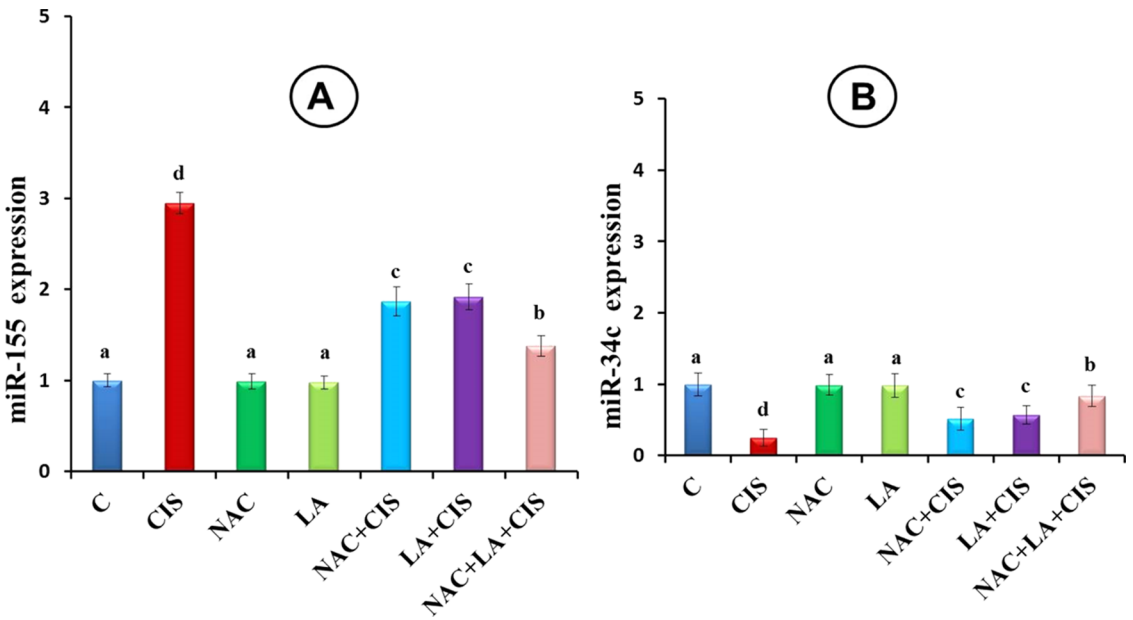


**Figure 1.** Testicular miR-155 (A) and miR-34c (B) expression in different treated groups, control (C), cisplatin (CIS), N-acetyl cysteine (NAC), L-arginine (LA), N-acetyl cysteine plus cisplatin (NAC+CIS), N-acetyl cysteine plus L-arginine (NAC+CIS), and N-acetyl cysteine plus L-arginine plus N-acetyl cysteine (NAC+LA+CIS). Different superscript letters (a, b, c, and d) show significant difference.

activities, and GSH level in testicular tissue compared to control rats. In the protective groups, there were significant reductions in MDA levels and MPO activities with significant increases in SOD, CAT, activities, and GSH level compared to the CIS-treated group. On the other hand, the groups treated by NAC or LA showed nonsignificant alterations of the examined parameters compared to the control group (Table 5).

**3.4. MicroRNA expression.** miR-155 revealed upregulation and miR-34c showed downregulation in their expressions in the CIS-treated group compared with the control. In contrast, groups pretreated by NAC and LA revealed significant amelioration in changes observed in miR-155 and miR-34c compared with CIS-injected rats. On the other hand, groups administered NAC or LA alone showed nonsignificant alterations in miR-155 and miR-34c expressions compared with the control group (Figure 1).

**3.5. Histopathological Findings.** Using H&E stain, testicular sections from control, LA, and NAC groups showed normal histoarchitecture of interstitial tissue and seminiferous tubules (Figure 2A–C). Testicular sections from the CIS group exhibited various significant degenerative changes including an irregular and thickened basement membrane (Figure 2D), vacuolated spermatogonia (Figure 2D–F),


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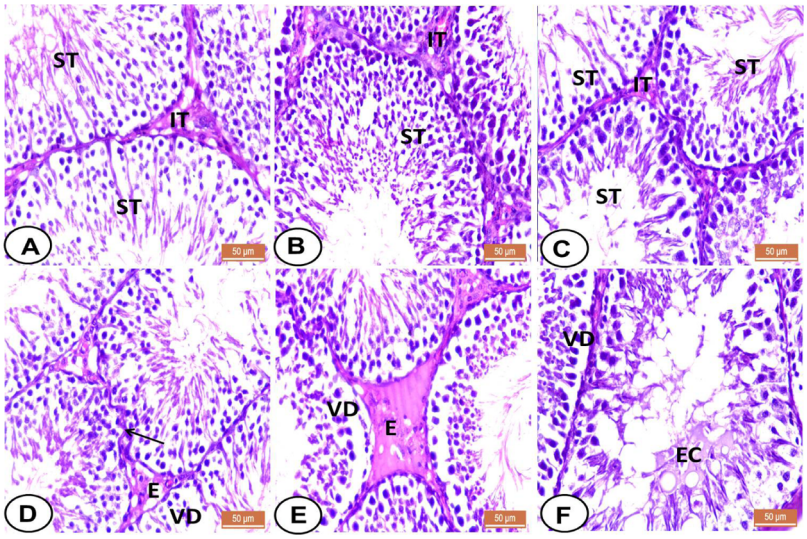


**Figure 2.** H&E-stained testicular sections of control, LA, NAC, and CIS groups. (A, B, and C) Control, LA, and NAC groups showed normal seminiferous tubules (ST) and normal interstitial tissue (IT). (D-F) CIS-treated rats showed irregular and thickened basal lamina (arrow), vacuolization of spermatogonia (VD), and exfoliation of germ cells into the lumen (EC) as well as interstitial edema (E). Scale bars = 50 $\mu$m.

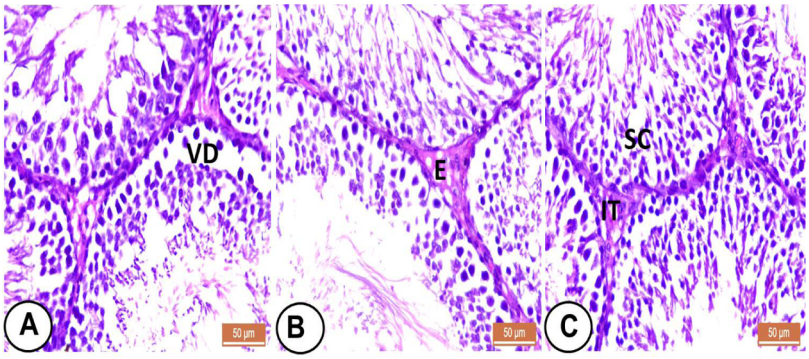


**Figure 3.** (A, B, and C) Histological sections of testes from LA+CIS, NAC+CIS, and NAC+LA+CIS groups, respectively. A and B showed an improvement of the degenerative changes compared to CIS-exposed rats, but with slight interstitial edema (E) and vacuolated spermatogonia (VD). C showed restoration of the normal testicular architecture of normal spermatogenic cells (SC) and interstitial tissue (IT). H&E stain, scale bars = 50 $\mu$m.

**Table 6. Semiquantitative Scoring of Histopathological Changes Induced by CIS in the Testes**[a]

| parameters group | thickened basement membranes | vacuolated spermatogonia | intraluminal exfoliated cells | interstitial edema |
|---|---|---|---|---|
| control | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] |
| CIS | 3.53 ± 0.03[d] | 3.68 ± 1.26[d] | 3.22 ± 1.51[d] | 3.46 ± 1.22[d] |
| NAC | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] |
| LA | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] | 0.00 ± 0.00[a] |
| NAC+CIS | 2.67 ± 1.40[c] | 2.26 ± 1.03[c] | 2.33 ± 1.11[c] | 2.63 ± 1.05[c] |
| LA+CIS | 2.30 ± 1.31[c] | 2.49 ± 1.14[c] | 2.27 ± 1.09[c] | 2.57 ± 0.31[c] |
| NAC+LA+CIS | 0.88 ± 1.08[b] | 1.13 ± 1.42[b] | 0.65 ± 0.18[b] | 0.93 ± 0.06[b] |

[a]All values are expressed as the mean ± SE. Superscript letters within the same columns are significant ($P < 0.05$). CIS: cisplatin; NAC: N-acetyl cysteine; LA: L-arginine.

exfoliated cells into the lumen (Figure 2F), and interstitial edema (Figure 2E). However, testicular sections from LA +CIS- and NAC+CIS-treated groups significantly demonstrated less degenerative changes compared to CIS-exposed rats with the persistence of vacuolated spermatogonia (Figure 3A) and slight interstitial edema (Figure 3B). Meanwhile, NAC +LA+CIS groups revealed significant restoration of the testicular histoarchitecture, which was seen in the form of normal germ cells and interstitium (Figure 3C). The scoring of histopathological changes induced by CIS in the testes of different groups is shown in Table 6.

### 3.6. Immunohistochemical Expression of F4/80.

Testicular sections from the CIS group revealed significantly numerous F4/80-positive interstitial macrophages (Figure 4D) compared to the scarce number of F4/80-positive cells in the interstitium of testicular sections of control, LA, and NAC groups (Figure 4A−C). However, LA+CIS, NAC+CIS, and NAC+LA+CIS groups revealed moderate expressions for F4/80 (Figure 4E−G) compared with the CIS group.


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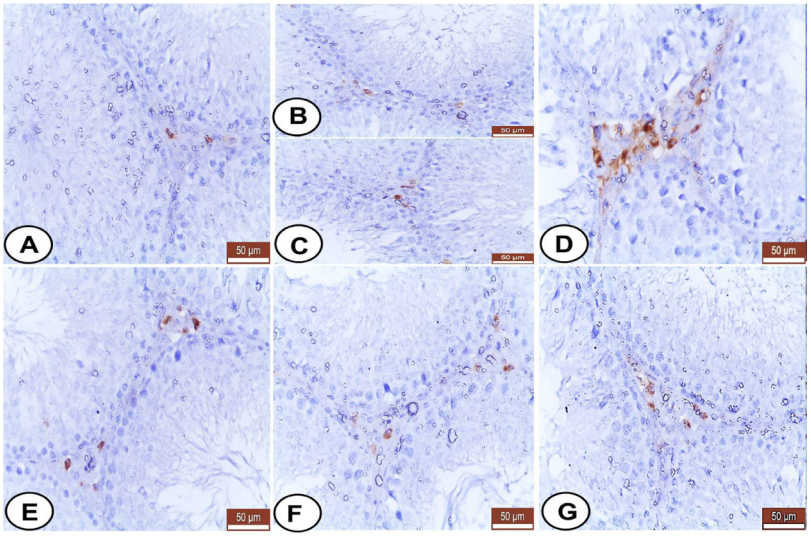


**Figure 4.** Immunohistochemical staining of F4/80 in testicular sections from all of the examined groups. (A, B and C) LA and NAC groups showed scarce number of positive cells in the interstitium. (D) CIS group revealed numerous positive interstitial cells. (E−G) LA+CIS, NAC+CIS, and NAC+LA+CIS groups showed moderate immunoreaction in comparison to the CIS group. Scale bars = 50 $\mu$m.

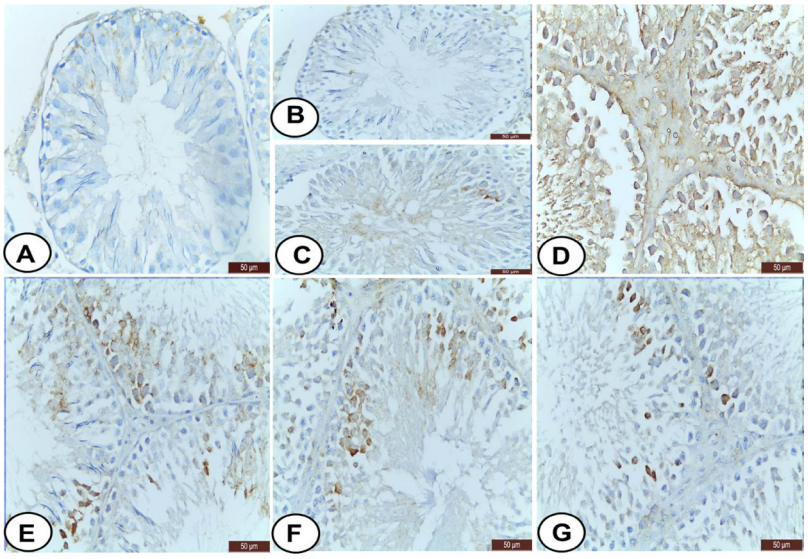


**Figure 5.** Immunohistochemical staining of BAX in testicular sections of all examined groups. A, B and C; LA, and NAC groups showed scarce number of positive spermatogenic cells. D; CIS group revealed numerous positive spermatogenic cells. E-G; LA+CIS, NAC+CIS, and NAC+LA +CIS groups respectively showed moderate immunoreaction in comparison to CIS group. Scale bars = 50 $\mu$m.

**3.7. Immunohistochemical Expression of BAX.** Spermatogenic cells in testicular sections of the CIS-injected group showed significantly numerous BAX-positive cells (Figure 5D) compared to the scarce number of BAX-positive cells in the spermatogenic cells from control, LA, and NAC groups (Figure 5A−C). However, LA+CIS, NAC+CIS, and NAC +LA+CIS groups revealed moderate expressions for BAX (Figure 5E−G) compared to CIS rats.

**3.8. Immunohistochemical Expression of PCNA.** Testicular sections from CIS-receiving rats revealed significantly weak PCNA-positive spermatogonia only (Figure 6D) compared to strong PCNA-positive spermatogenic cells from control, LA, and NAC groups (Figure 6A−C). However, LA +CIS, NAC+CIS, and NAC+LA+CIS groups revealed moderate expressions for PCNA in spermatogenic cells (Figure 6E−G) compared to the CIS group.

## 4. DISCUSSION

Testicular damage or dysfunction is a common adverse effect of chemotherapeutic drugs like CIS. This study estimated several hormonal, biochemical, histological, and immunohistochemical parameters and miRNAs' expression that were associated with testicular oxidative stress and toxicity. Moreover, the protective effect of NAC and/or LA was explored as well, and the potential role of both miR-155 and 34c in CIS-induced testicular dysfunction was confirmed. CIS administration resulted in testicular toxicity including ROS formation, damage of DNA that subsequently led to germ epithelial damage and apoptosis, as well as inflammation.[53]

Testosterone is important for controlling spermatogenesis; LH promotes testosterone synthesis, while FSH activates spermatogenesis by binding with Sertoli cells.[54] The CIS-treated group exhibited significant decreases in testosterone,


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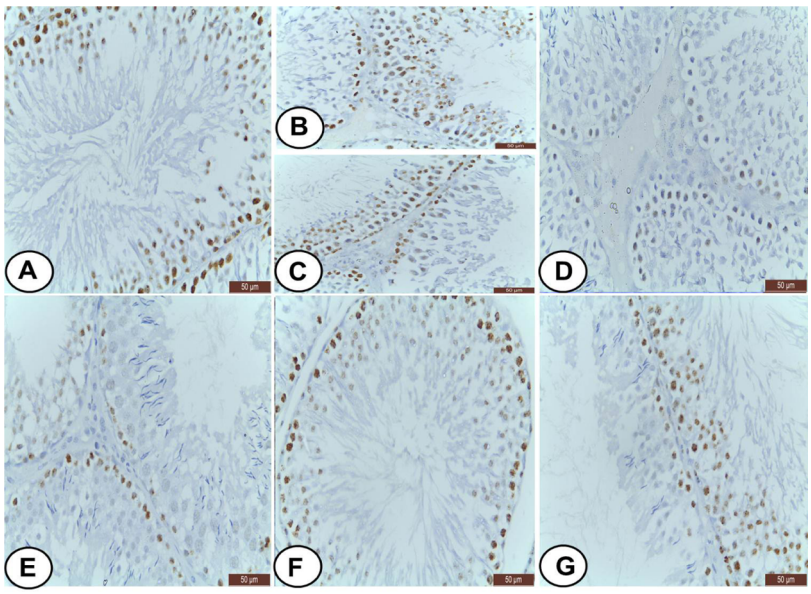


**Figure 6.** Immunohistochemical staining of PCNA in testicular sections from all examined groups. (A, B and C) LA and NAC groups showed strong positive spermatogenic cells. (D) The CIS group exhibited weak reaction in spermatogonia only. (E−G) LA+CIS, NAC+CIS, and NAC+LA +CIS groups, respectively, showed moderate immunoreaction in comparison to the CIS group. Scale bars = 50 $\mu$m.

FSH, and LH concentrations compared to the control group. Such findings were in accordance with earlier reports.[55−57] The decline of these hormones' levels may be owing to the excess free radical formation induced by CIS that causes severe damage to Sertoli and Leydig cells; also, the inflammation induced by CIS can disturb the hypophyseal gonadal hormone axis.[58−60] Additionally, CIS interferes with LH receptors and inhibits mobilization of cholesterol to mitochondrial cytochrome P450 so that it inhibits the initial steps of testosterone production.[61]

It has been shown that methotrexate (MTX) as a radiotherapeutic medication has impacts on different organs of the body, with more effects on the liver, kidney, and testis. In the testis, MTX impairs fertility and decreases spermatogenesis, testicular cellular redox status, and reproductive dysfunction, which were ameliorated by allicin and lycopene.[62]

CIS-treated groups showed a significant decrease in ACP, ALP, LDH, and G6PDH activities. This result partially agreed with others[57] that reported that CIS treatment reduced testicular ALP activity. ALP is necessary for spermatogenic mitosis and transport of glucose. ACP is located in the lysosomes of Leydig cells and is important for removal of unnecessary sperm cells.[63] Decreases in ACP and ALP activities reflect a decline in phosphatase activity in the nucleus of the spermatocytes during spermatogenesis. Therefore, ALP and ACP activities in the testes may be useful indicators of spermatogenic function.[64] The reduction in ALP and ACP activities in CIS-treated rats suggested degeneration of testicular tissue, which may be a result of the suppression of testosterone and lysosomal function that is induced by ROS formation.

LDH is expressed in spermatogenic, Leydig, and Sertoli cells. It plays an important role in production of energy by providing lactate to developing germ cells.[63] The noticed decline in activity of testicular LDH may be reflecting the interference of testicular energy metabolism.[65] G6PDH is considered one of the key enzymes of the testicular tissue, but the reduction in its activity indicates germ cell depletion and impairment of Leydig cell's function.[66] Additionally, G6PDH is related to glutathione metabolism, and reduced activity of this enzyme increases oxidative stress, inducing cellular death.[67]

Serum levels of IL-1$\beta$, TNF$\alpha$, IL-6, and MCP-1 revealed significant increases along with a decline in IL-10 level in CIS-treated rats compared with the control group. The present findings agree with an earlier study[68] that reported that CIS can stimulate inflammatory cells and consequently magnifies the inflammatory response through the control of TNF and IL-6 release, resulting in testicular damage. Also, others[69] found that CIS elevates IL-6 and IL-1$\beta$ levels, and decreases IL-10 levels. Yet others[70] revealed that CIS upregulates IL-6, IL1-$\beta$, and TNF-$\alpha$ secretion in the kidney as a consequence of stimulation of the inflammatory cascade.

CIS elevates intracellular ROS, resulting in activation of NF-$\kappa$B transcription[71] and increases in inflammatory mediators such as TNF-$\alpha$, which promotes the generation of other cytokines such as IL-1$\beta$, IL-6, and MCP-1.[72] Also, Kang et al.[73] reported an elevation in MCP-1 expression. MCP-1 is a chemokine regulating migration and infiltration of monocytes/macrophages.[74] Additionally, the decline in IL-10 induced by CIS was confirmed by others.[75]

The CIS-treated group exhibited a significant elevation in MDA with reduction in CAT, SOD activities and GSH level in testicular tissue compared with control rats as reported by us and others.[10,39,76−78] The increased MPO activity is related to inflammation and production of excessive free radical and oxidative stress,[79,80] so it can be used as an inflammatory marker of the testicular tissue of the CIS group.

According to the present results, the decline in GSH levels and CAT, SOD activities in the CIS-administered group may be attributed to the excessive production of superoxide anion and hydroxyl radicals[81] as well as decreasing antioxidant enzymes[76] resulting in impairment of testicular antioxidant defense systems.

Some miRNAs are increased during oxidative stress and inflammatory responses, which contribute to degenerative diseases.[82] Among these miRNAs, miR-155, which is regulated


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by preinflammatory reactions,[35] and its overexpression are associated with NF-κB transcriptional regulation, which was increased by TNF-α.[83] Additionally, miR-155 upregulation was reported to inhibit the SIRT1 (sirutin1) pathway,[84] which plays an important role in cell proliferation and differentiation;[85] also, SIRT1 suppresses the activity of Bax.[86] miR-155 is considered a part of the macrophage primary response to various types of inflammatory mediators, so miR-155 was revealed to be a component of inflammatory response.[38] The current findings showed that increased TNF-α causes significant elevation in miR-155 expression, which is in accordance with Guo et al.[87] However, administration of NAC and/or LA reduced miR-155 expression, which may contribute to TNF-α reduction, suggesting an anti-inflammatory effect for NAC and/or LA.

miR-34c plays roles in spermatogenesis and its downregulation causes male infertility.[37] Besides, it prevents apoptosis of spermatogenic cells[88] as it regulates NOTCH1 and Nanos-2, which are key regulators of spermatogenic cell differentiation. Also, miR-34c downregulation in prostate cancer suppresses tumor migration and invasion.[89] The present results showed a significant downregulation in miR-34c expression in CIS-injected rats compared to control. The findings from this study are in harmony with others[90] who reported that miR-34c is downregulated in doxorubicin-treated rats. Pretreatment with NAC and/or LA reversed the effect of CIS on miR-34c.

Oxidative stress (OS) is the main factor in tissue destruction. OS occurs when there is an imbalance between the body's capacity to counteract or repair the damaging effects of reactive oxygen species (ROS) and the synthesis of these molecules. By damaging spermatozoa, testicular function, and Leydig cells, ROS can cause infertility. It seems that testicular oxidative stress, caused by hormone alterations and sperm production deficiencies, is a distinguishing feature of male infertility.[91]

Interestingly, pretreatment with NAC and/or LA with CIS revealed an enhancement in testicular function parameters. This improvement suggests that NAC and LA can maintain cell membrane integrity or mitigate the regeneration of damaged cells. NAC, the acetylated version of the amino acid L-cysteine, is a thiol-based antioxidant having a direct antioxidant activity and free thiol groups in its structure, which easily permeate cells due to their molecular structure.[92] NAC has a protective effect against CIS-induced testicular dysfunction, which may be owing to its antioxidant properties and its ability to scavenge free radicals[93] via enhancing the cellular GSH level as NAC is a precursor for glutathione[24] and subsequently protects against lipid peroxidation.[94] Additionally, NAC increased IL-10 levels as it possesses a critical anti-inflammatory effect through cyclooxygenase enzyme inhibition.[95] It has been confirmed that NAC has a protective impact against testicular damage and dysfunction.[26,96]

LA is the precursor for nitric oxide. When the need for arginine increases, as in infection and inflammation, it becomes essential. Therefore, LA is a semiessential amino acid.[97] It is included in the biosynthesis of polyamides such as spermine, spermidine, and putrescine, which are essential for cell growth and differentiation[97] as well as spermatogenesis.[98] Because of its antioxidant effect, LA is important for glutathione synthesis, as a precursor of NO, its scavenge-free radical,[21] as a lipid peroxidation inhibitor, and as a prooxidant enzyme inhibitor; additionally, it is important for energy metabolism in the tissues of nerves, muscles, and testes.[99] L-Arginine restores testosterone levels toward normalcy and this finding agreed with others.[19] Also, LA attenuates oxidative stress by reducing lipid peroxidation (MDA) along with a significant enhancement in antioxidant enzymes (SOD and CAT), which is attributed to its free radical scavenging properties and suppression of the oxidation process.[100] LA reduced serum levels of TNFα and IL-1β due to its direct anti-inflammatory action that reduces inflammatory cytokine production, as well as an indirect action that may be owing to its enhancement of NO production that resulted in decrease in leukocyte adhesion.[101]

The current histopathological and immunohistochemical findings matched and confirmed the biochemical results and miRNA expression. Administration of CIS induced significant testicular degenerative changes. An irregular and thickened basement membrane reported in seminiferous tubules was detected, which also matched with others.[7] Degeneration and vacuolization of spermatogonia were recorded,[13] as well as exfoliation and shedding of germ cells into the lumen; in addition, interstitial edema was reported.[10,102] The common pathways for CIS-induced testicular toxicity are via DNA damage and apoptosis of spermatogenic cells, and impairment of Leydig cell function,[11] which are attributed to the overproduction of ROS.[12] However, LA or NAC supplementation can alleviate these testicular damages induced by CIS and improve the histological features of the seminiferous tubules. Combined administration of LA plus NAC significantly exhibited a better histological result against CIS testicular toxicity. Recent studies[19,103] have confirmed the testicular protective effect of both LA and NAC against toxic agents.

F4/80 is a marker for tissue macrophages including hepatic Kupffer cells, splenic macrophages, brain microglia, and cutaneous Langerhans cells. In this study, CIS treatment showed an increase of F4/80-positive interstitial macrophages. This supports that testicular macrophages increase the response to inflammatory stimuli,[104] leading to impairment of testicular function. Activated macrophages damage the testicular microenvironment,[105] causing inflammation and disruption of spermatogenesis.[106] However, a scarce positive number in the interstitium of testicular sections from control, LA, and NAC groups was detected. The present results revealed that the protective groups administered LA, NAC, and NAC+LA showed restored normal number of F4/80-positive cells.

The present study explores the impact of NAC and/or LA on CIS-induced apoptosis and cell proliferation profiles in the testes of rats. Apoptosis plays a critical role in tissue homeostasis via removing damaged cells. Meanwhile, spermatogenic cell apoptosis will result in impairment of spermatogenesis.[107] On the other hand, the process of spermatogenesis is a complex cycle of proliferating cells. Proliferating cell nuclear antigen (PCNA) is a nuclear polypeptide involved in DNA replication and repair.[108] Therefore, we aimed to examine PCNA to assess the degree of spermatogenesis.

The present study revealed that CIS increases the immunohistochemical expression of spermatogenic cells for Bax and decreases them for PCNA, indicating an increase of apoptosis and a decrease of the proliferation processes of spermatogenic cells, which coincide with others.[102,109] The reduced proliferation of spermatogenic cells in the present study may be owing to the CIS causing low testosterone levels,


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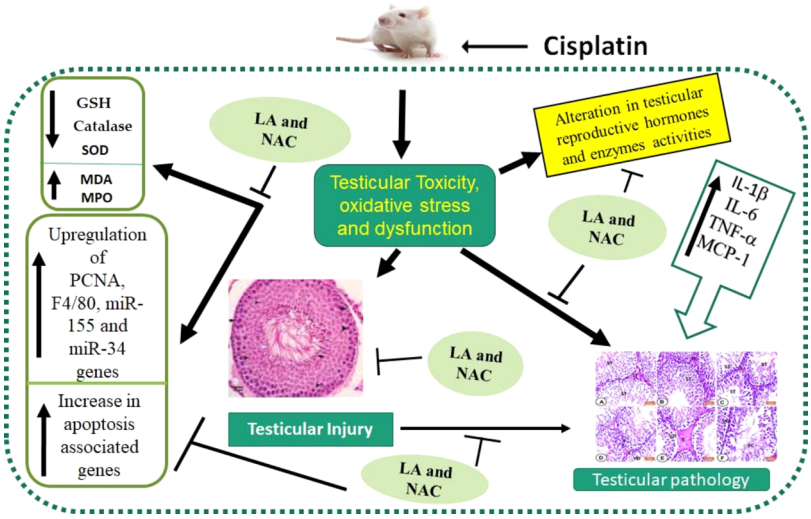


**Figure 7.** Collective graph for the inhibitory effect of L-arginine and n-acetyl cysteine against cisplatin-induced testicular dysfunction, oxidative stress, and alterations in gene expression. NA: n-acetyl cysteine; LA: L-arginine.

which in turn causes reduction of spermatogenesis;[109] however, the increased apoptosis may be owing to the excess of ROS production and translocation of Bax from the cytoplasm to the perinuclear site.[110]

Meanwhile, rats coadministered LA, NAC, or NAC+LA plus CIS revealed noticeably increased PCNA and decreased Bax immunohistochemical expression compared to the CIS group. Therefore, LA and NAC could stimulate spermatogenic cell proliferation and suppress their apoptosis, resuming the normal process of spermatogenesis, which in turn regenerates testicular germ cells. The present study revealed that the NAC+LA+CIS group showed immunohistochemical expression of BAX and PCNA nearly similar to that of the control group, indicating that the administration of combined LA and NAC is preferred for best results against CIS-induced testicular dysfunction.

## 5. CONCLUSIONS

The current findings are based on a 28-day in vivo study in Albino rats. It concluded that administration of CIS causes impairment in testicular histoarchitecture, spermatogenesis and cell proliferation, steroidogenesis, and expression of miR-155 and miR-34c via induction of cellular oxidative stress, inflammation, and apoptosis. However, the combination of NAC and LA effectively mitigated CIS-induced testicular dysfunction, confirming their antioxidant, anti-inflammatory, and antiapoptotic properties via modulating miR-155 and miR-34c expression. Therefore, future studies are needed to test the direct effects of NAC and LA against male infertility in experimental animal models. The collective effects of LA and NAC against cisplatin-induced testicular degeneration are shown in Figure 7.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

The current data are available upon request.

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Preparation, writing, data analysis, and revising paper contents were equally carried by all authors.


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### Notes

The authors declare no competing financial interest.

**Ethical Statement** All the experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the care and use of laboratory animals. All steps were followed to minimize the suffering of the experimental animals.



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