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Pro-apoptotic and oxidative effects of artemisinin and l-carnitine on testes

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We evaluated the effects of artemisinin (ART) and L-carnitine (LC) on apoptosis and redox status of testicular tissue. Sixty mature male laboratory mice were randomly divided into six groups as follows. Control (saline + corn oil); Art50 (therapeutic ART) (50 mg/kg ART); Art250 (toxic ART) (250 mg/kg ART); Art50+L-car (370 mg/kg LC + 50 mg/kg ART); Art250+L-car (370 mg/kg LC + 250 mg/kg ART); and L-car (370 mg/kg LC). Drugs were given orally for one week. Therapeutic ART reduced the expression of the *Bax* without significant effect on *Bcl2*. Co-administration of LC and therapeutic ART reduced the expression of *Bax* and increased the expression of *Bcl2*. ART increased glutathione (GSH) and peroxidase activity. LC alone or together with ART also increased the GSH levels. Unlike the group receiving toxic ART, the number of apoptotic cells in the germinal epithelium was only slightly (~8%) higher than the control in the groups receiving therapeutic ART. Co-administration of LC and therapeutic ART reduced the number of apoptotic cells to the control level. Therapeutic ART not only did not induce apoptosis and oxidative stress but together with LC increased the antioxidant power of the testis by increasing the GSH level.

Keywords: Artemisinin, l-carnitine, *Bax*, *Bcl2*, Peroxidase, Glutathione

Artemisinin (ART) is a natural anti-malaria agent extracted from the plant *Artemisia annua*[1]. ART and its derivatives contain a peroxide bridge that reacts with the iron and generates carbon-centered free radicals and reactive oxygen species (ROS)[2]. There is promising evidence that ART, beyond its anti-malaria effects, also has effective anti-cancer properties. ART appears to exert anti-cancer effects by inducing cell cycle arrest, promoting cell death (apoptosis, ferroptosis, and autophagy), and inhibiting cell metastasis[3]. However, the toxic properties of ART on cancer/infected cells may also affect normal, vulnerable cells. Embryotoxicity, genotoxicity, cardiotoxicity, hematotoxicity, and reproductive toxicity are frequently reported as toxic effects of ART in many experimental and clinical studies[4]. The toxicity of ART and its derivatives on the male reproductive system are reported possibly through sperm abnormalities[5], hormonal imbalance[6], redox imbalance[7], and structural injuries[8]. However, there are reports that ART at therapeutic doses may have no significant deleterious effects[9,10].

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Antioxidant therapy is a potential option to reduce ROS and improve male fertility[11]. L-carnitine (LC) is an amino acid derivative provided primarily from exogenous and, to some extent, endogenous sources. LC primarily mediates the transport of long-chain fatty acids (LCFAs) into the mitochondria. LC protects membrane lipids, proteins, and DNA against oxidative damage due to its antioxidant properties[12]. Normally, LC has a high concentration in testicular tissue and positively affects sperm production and maturation[13]. LC by its antioxidant and anti-apoptotic properties can improve male fertility[14]. Clinical studies have shown that LC improved sperm motility, concentration, and morphology either alone or combined with L-acetyl-carnitine[15,16]. Considering the conflicting reports about ART's side effects and the potential of LC in protecting against oxidative damage, we investigated the effects of treatment with ART and LC on apoptosis and redox status of the testis.

Material and Methods

Experimental design and sampling

After environmental adaptation, 60 healthy adult male laboratory mice were randomly divided into six groups. Control (saline + corn oil); group Art50 (50 mg/kg ART); group Art250 (250 mg/kg ART); group Art50+L-car (370 mg/kg LC + 50 mg/kg ART);

group Art250+L-car (370 mg/kg LC + 250 mg/kg ART); and group L-car (370 mg/kg LC). The animals were kept under controlled natural photoperiod of about a 12h light-dark cycles and water and a standard diet were provided for them *ad libitum*. All steps were carried out following the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and guidelines of the Bioethics Committee of University of Tabriz on the care and maintenance of laboratory animals.

ART dissolved in corn oil. Therapeutic (50 mg/kg) and toxic (250 mg/kg) doses of ART were chosen according to the recommendation for the ART mono-therapy[17,18]. LC dissolved in normal saline. The dose of LC in humans was converted to an amount for mice (370 mg/kg)[19]. ART and LC were administered orally for one week.

Twenty-four hours after the last administration, we euthanized the animals and removed the testes. The left testes were fixed in 10% formalin solution for the TUNEL assay. The right testes were frozen for biochemical and gene expression studies.

RNA isolation and cDNA synthesis

Total testicular RNA was extracted by TriPure based on the manufacturer's guide (Roche, Canada). The quality and quantity of isolated RNA was analyzed by gel electrophoresis and nanodrop (Eppendorf, Germany) spectrophotometry. RNA samples with an A260/A280 ratio of 1.8–2 were used for cDNA synthesis. Treatment with DNase I was done to remove possible RNA contaminants according to the guide of the manufacturer (Vivantis, Malaysia). A RocketScript RT PreMix kit (Bioneer Corporation, South Korea) was applied for cDNA synthesis based on the manufacturer's protocol.

Real time quantitative PCR (qPCR)

The expression of *Bax* and *Bcl2* in the testicular tissue was evaluated by qPCR using a master mix for SYBR Green I (Jena Bioscience, Germany) on a Lightcycler detection system (Roche, USA). The relative expression of the target genes was quantified based on the comparative $2^{-\Delta\Delta Ct}$ method using the *GAPDH* as a normalizer. The primers used in qPCR were: *GAPDH* 5′AAGGGCTCATGACCACAGTC3′; 5′ACTTGGCAGGTTTCTCCAGG3′, *Bax* 5′ CCACC AGCTCTGAACAGATC3′; 5′CAGCTTCTTGGTGG ACGCAT3′, *Bcl2* 5′GGATAACGGAGGCTGGGAT G3′; 5′GCAGGTTTGTCGACCTCACT3′. Reactions were performed in triplicate in mixtures containing 10 µl qPCR master mix (2×), 0.175 µM of each primer, 2 µl cDNA, and final volume of 20 µl. The cycling condition was as 94 °C for 3 min followed by 45 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. A reaction without cDNA and another reaction with RNA were included in each run as controls.

Detection of apoptotic cells

Five sections (5 µm thickness) were prepared from each testicular sample. Apoptosis detection was carried out by TUNEL assay using a commercial kit according to the manufacturer (Roche, Mannheim, Germany) guide. The apoptotic index was defined as a percentage of TUNEL-positive cells per all cells.

Assessment of testicular redox status

Samples were homogenized and centrifuged. The supernatants were used to measure the peroxidase (POX) activity and GSH concentration. The protein concentration was determined by Lowry's method, using BSA (Merck) as the standard.

The POX assay was based on the changes in the absorbance at 460 nm upon oxidation of o-dianisidine in the presence of H_2O_2[20]. The amount of POX required for oxidation of 1 µmol of o-dianisidine per min was defined as one unit.

The CSH assay was done using Ellman's reagent[21]. In this method the absorbance at 412 nm changes due to the TNB formation upon the reaction of GSH with DTNB.

Statistical analysis

SPSS 16.0 software package (SPSS) was used for data analysis. All data were presented as mean ± SD. The mean of variables were compared between groups by One-way ANOVA. *P* values of <0.05 were considered significant.

Results

Apoptosis in the testicular tissue

The effect of ART and LC on the apoptosis in the testicular tissue was evaluated by *Bax* and *Bcl2* expression analyses (Fig. 1) and TUNEL assay (Fig. 2). ART suppressed the expression of *Bax* in testicular tissue at the therapeutic and toxic doses by 1.6-and 1.3-fold, respectively ($P<0.05$). LC, along with a therapeutic dose of ART, decreased the expression of *Bax* by 1.5-fold. LC, with a toxic dose of ART, increased the expression of *Bax* by 1.4-fold ($P<0.05$) (Fig. 1A). Neither the therapeutic dose nor

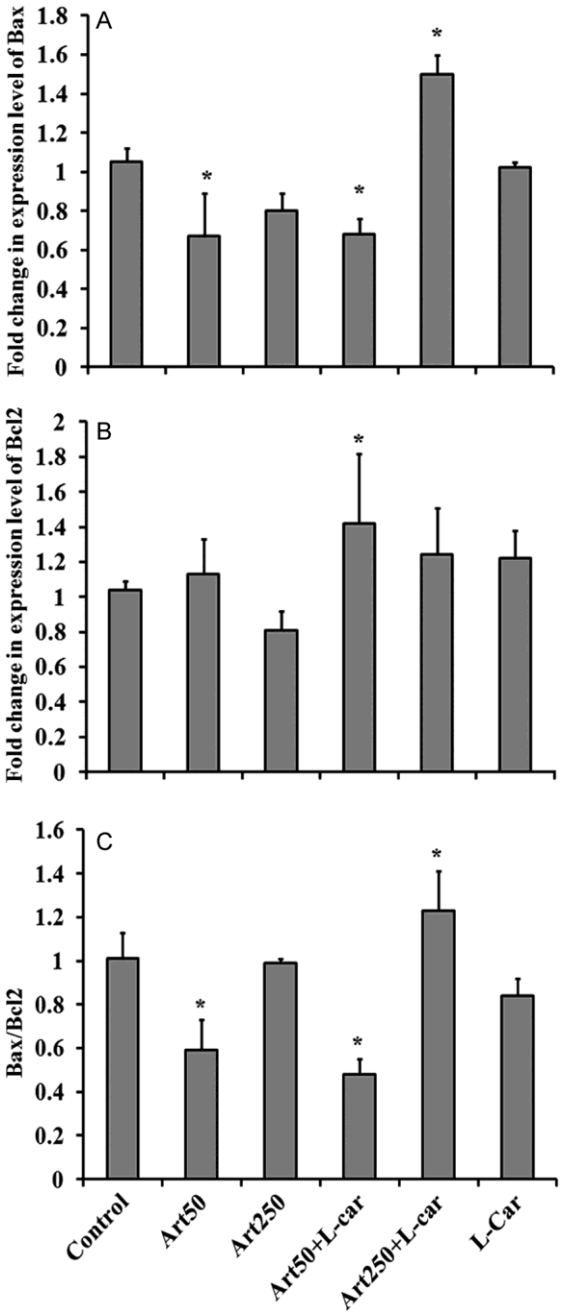

Fig. 1 — Apoptosis-related gene expression levels in the testicular tissue among experimental groups. (A) *Bax*, (B) *Bcl2*, (C) the *Bax*/*Bcl2* ratio. Asterisks at the top of the columns mean a significant difference with control ($P < 0.05$).

the toxic dose of the ART had any significant effects on the expression of *Bcl2*. LC + a therapeutic dose of ART increased the expression level of *Bcl2* by 1.4-fold ($P<0.05$) (Fig. 1B). The therapeutic dose of ART, both alone or combined with LC, reduced the *Bax*/*Bcl2* by 1.7 and 2.1-fold, respectively ($P<0.05$) (Fig. 1C). Therapeutic and toxic doses of ART increased the number of TUNEL-positive nuclei in the germinal epithelium ($P<0.01$) by about 8% and 29%, respectively. Co-administration of LC with ART lowered the number of TUNEL-positive nuclei. The highest number of TUNEL-positive nuclei was observed in the group receiving a toxic dose of ART, and the lowest number in the group receiving LC alone (Fig. 2A & 2B).

The redox status of the testicular tissue

The POX (Fig. 3A) and GSH (Fig. 3B) were assayed to find whether ART and LC affect the redox state of testicular tissue. ART and LC together with toxic ART increased ($P<0.05$) the POX activity by about 17% - 20%. LC alone or together with ART, and toxic ART, increased the GSH levels in the testicular tissue ($P<0.05$). The therapeutic ART also increased the GSH level (~26%), but this effect was not statistically significant ($P=0.7$).

Discussion

To shed more light on the molecular mechanism of the potential testicular toxicity of ART, this study aimed to evaluate the effects of ART and LC on the expression of *Bax* and *Bcl2* genes, apoptosis, POX activity, and the GSH level in testicular tissue. Therapeutic ART suppressed the expression of the *Bax*. Co-administration of LC and a therapeutic ART reduced the expression of *Bax* and increased the expression of *Bcl2*. Unlike the group receiving toxic ART, the number of apoptotic cells in the germinal epithelium in the group receiving therapeutic ART was only slightly (~8%) higher than the control. Co-administration of LC and therapeutic ART reduced the number of apoptotic cells. ART and LC increased the GSH levels. ART also increased the POX activity.

Artemisinin-based therapy is highly effective and generally regarded as a safe treatment for malaria[22]. However, there are conflicting reports about the toxicity of ART and its mechanism on different organs. Some studies have shown that ART has pro-apoptotic effects. ART triggers apoptosis inside cancer cells by increasing the ROS levels, disturbance in mitochondrial membrane potential, activation of pro-apoptotic proteins, inhibition of anti-apoptotic proteins, formation of membrane bubbles, and activation of caspase[23]. ART extract activated the caspase-3 and increased the *Bax* expression in human colorectal cancer HCT-116 cells[24]. ART and dihydroartemisinin aggravated the palmitic acid-induced apoptosis and

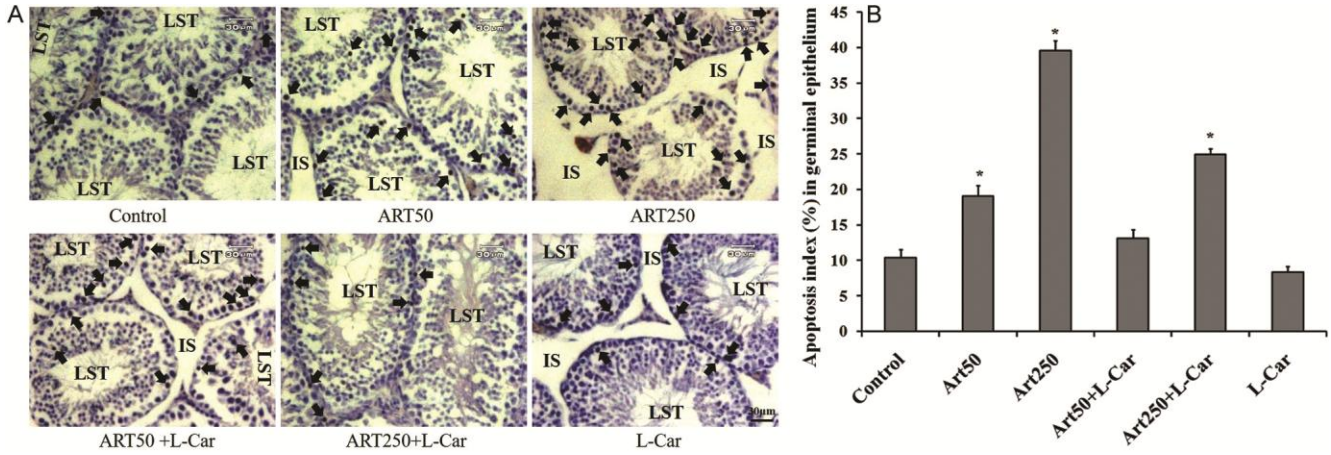

Fig. 2 — Apoptosis detection in the seminiferous tubules among experimental groups. (A) Light micrographs of the seminiferous tubules. The nuclei of TUNEL-positive cells were stained dark brown (black arrow). Artemisinin increased the TUNEL-positive nuclei. LST: lumen of seminiferous tubule; IS: interstitial space. (B) Apoptosis index in germinal epithelium among experimental groups. Asterisks at the top of the columns mean a significant difference with control ($P < 0.05$)

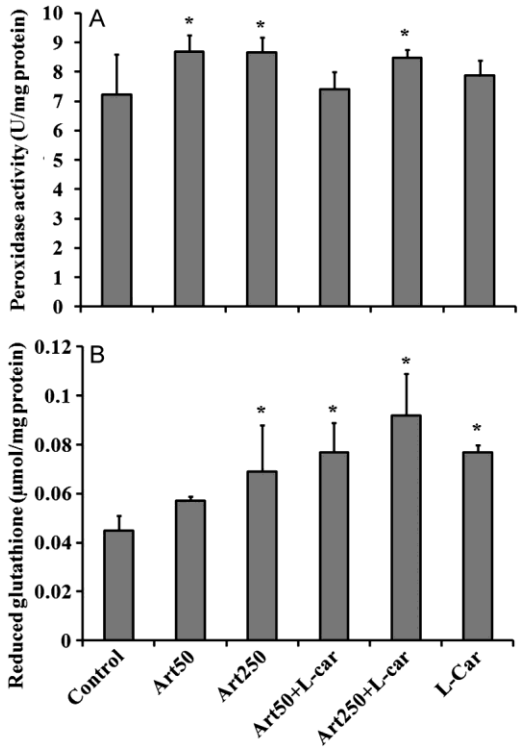

Fig. 3 — The redox indices in the testicular tissue among experimental groups. (A) Peroxidase activity, and (B) glutathione level. Asterisks at the top of the columns mean a significant difference with control ($P < 0.05$).

endoplasmic reticulum stress in β-cells[25]. On the other hand, other studies indicate the anti-apoptotic effects of ART. ART reduced the apoptosis caused by H_2O_2 in RGC-5 cells by about 60% through a reduction in the *Bax* expression[26]. Hydroalcoholic extract of *Artemisia* reduced apoptosis in testicular tissue of rats treated with a high-fat diet and streptozotocin by suppressing the *Bax* and inducing the *Bcl2*[27]. Yan *et al*. showed that ART decreased caspase-3 expression and protected the SH-SY5Y cells from apoptosis induced by methylphenylpyridinium[28]. In the present study, ART reduced the expression of *Bax* in testicular tissue by about 1.5-fold. The therapeutic dose of ART also had no significant effect on *Bcl2* expression. These results may indicate the anti-apoptotic effects of the therapeutic ART. Although ART increased the number of apoptotic cells in the germinal epithelium, this index was only about 8% higher than the control in the groups receiving the therapeutic ART. In addition to late-phase apoptotic DNA fragments, the TUNEL assay also detects the breaks caused by damaging factors like toxins, drugs, and their metabolites[29]. Therefore, an increase in the number of TUNEL-positive cells does not simply mean an increase in apoptosis. In testicular tissue, the sensitivity of various cells to apoptosis is different, and the cells in the germinal epithelium are more sensitive to apoptosis[30,31]. The proliferating and developing cells are more susceptible to apoptosis[32]. Induction of cell death may be required to remove defective cells resulting from errors during normal division of germ cells[33]. So, the increase in TUNEL-positive cells could be attributed to some extent to the compensatory proliferation in germinal epithelium due to the treatment with ART.

The protective effects of LC in various tissues have been investigated. LC showed anti-apoptotic effects against atrazine-induced hepatotoxicity in rats by

reducing *Bax* and *caspase-3* expression and increasing *Bcl2* expression[34]. LC decreased and increased the expression of *Bax* and *Bcl2* in the hearts of Imatinib-treated rats, respectively[35]. Long-term treatment with LC increased LC concentration and *Bcl2* expression in testicular tissue and decreased *Bax* and *caspase-3* expressions[36]. Examination of apoptosis in germinal epithelium showed that LC reduced the number of TUNEL-positive cells in diabetic rats[37]. LC reduced the number of TUNEL-positive sperms in rats treated by formalin[38]. In the present study, in line with the previous studies, LC decreased the number of TUNEL-positive cells and increased the expression of *Bcl2*. These results confirm the *Bcl2* induction as a cytoprotective mechanism of LC to combat apoptosis. Interestingly, contrary to previous reports in the present study, LC together with toxic ART increased the expression of *Bax*. Treating rats with propionyl LC after injury reduced the relative volume of the aortic intima, increased *Bax* expression, and decreased NFκB without altering *Bcl2* levels. These effects were different in other aorta layers so that propionyl LC did not change the expression of *Bax* and *Bcl2* in the media[39]. According to Park *et al*., LC increased the *Bax* and *Bcl2* expression and sensitized TRAIL-resistant cancer cells[40]. LC supplementation (250 or 500 mg/kg of diet) did not affect the *Bak/Bcl2* ratio in the testicular tissue of breeder roosters[41]. Therefore, the antioxidant/prooxidant and anti-apoptotic/pro-apoptotic effects of carnitines may vary depending on the dosing, cell type, and intracellular conditions.

Reduced glutathione (GSH) is the major thiol (-SH)-containing peptide at a millimolar concentration in most living cells. GSH is oxidized to disulfide-form (GSSG) by the enzyme glutathione peroxidase (GPX) upon dissipation of H_2O_2[42]. The GSSG/GSH couple is considered the main cellular redox buffer, and the GSH level is a marker of the cell redox status[43]. Alteration in the cell redox status is one of the critical events proposed for the mechanism of ART and LC actions. ART reduced the ROS generation by H_2O_2 in RGC-5 cells[26]. The extract of *Artemisia* protected PC12 cells from H_2O_2 by preventing ROS generation and GSH depletion[44]. Hydroalcoholic extract of *Artemisia* decreased the malondialdehyde (MDA) and nitric oxide (NO) levels and increased the GSH and antioxidant enzyme levels in testicular tissue of rats treated with high-fat diets and streptozotocin[27]. ART prevented the ROS and MDA elevation in cells treated with methylphenylpyridinium and improved the superoxide dismutase (SOD) and GSH levels[28]. These findings confirm the elevation in the GSH levels and POX activity observed in the present study following seven days of treatment with ART. LC prevented the development of fatty liver and oxidative stress in mouse[45]. LC improved oxidative stress induced by Imatinib by reducing inflammation, MDA, and NO, and increasing SOD activity and GSH levels in heart tissue[35]. The increasing effect of LC on GSH levels has also been shown in various tissues[46,47]. These reports are consistent with an increase in the GSH level of testicular tissue observed in the present study. LC increased the expression and function of enzymes involved in GSH synthesis[48,49]. It is well accepted in the literature that GSH level and antioxidant enzymes are conversely associated with apoptosis[50].

Conclusion

The therapeutic level of ART reduced the expression of Bax and increased the GPX activity and GSH levels. The therapeutic ART alone or in combination with LC did not induce apoptosis and oxidative stress in testicular tissue but also increased the antioxidant power of testicular tissue. Further studies can elucidate the pre- and post-treatment effects of LC. The details of the ART's effects on various mechanisms of apoptosis require future investigations.

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Conflict of interest

Authors declare that there is no conflict of interest.

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