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AKADÉMIAI KIADÓ

Chlorogenic acid alleviated testicular inflammation and apoptosis in tunicamycin induced endoplasmic reticulum stress

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ABSTRACT

Purpose: Chlorogenic acid (CA) is a polyphenolic compound, found in many herbs and foods including coffee, berries and potatoes. Anti-inflammatory, anti-oxidant, anti-cancer and anti-apoptotic effects of CA have been proven in many tissues. Testicular inflammation and apoptosis are essential factors in male infertility that could result from endoplasmic reticulum (ER) stress. ER stress leads to unfolding and misfolding of nascent proteins and thereby provokes cellular inflammatory and apoptotic pathways. This study was designed to assess the effects of CA on ER stress-induced testis inflammation and apoptosis. *Methods:* To do this, male mice were divided into six groups. The control, vehicle and CA groups received saline, DMSO and 50 mg kg^{-1} CA. Tunicamycin (TM (was injected to induce ER stress (TM group). In the CA20-TM and CA50-TM groups, 20 mg kg^{-1} CA and 50 mg kg^{-1} CA were administered one hour before TM injection. After thirty hours, animals were sacrificed and testes were removed. Hematoxylin & eosin staining, ELISA assay and real-time PCR were performed. *Results:* CA administration significantly downregulated gene expression of TNFα, IL6, P53, Bax/Bcl2 ratio and caspase3. It also reduced testis levels of ALP, NF-κB, TNFα and caspse3. Finally, CA relieved structural changes in seminiferous tubules. *Conclusions:* This study demonstrated that the positive effects of CA on the attenuation of ER-stress induced inflammation and apoptosis might be due to the inhibition of NF-κB and thereby suppression of inflammatory and apoptotic pathways.

KEYWORDS

apoptosis, chlorogenic acid, ER stress, inflammation, NF-κB, testis

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INTRODUCTION

Infertility is a common and serious problem worldwide [1]. Statistics show that 72.4 million couples suffer from infertility problems and 40–50% are infertile [1, 2]. One of the important factors in men's infertility is testicular dysfunction could result from inflammation and apoptosis in the testis tissue [3]. Various factors such as diabetes, hyperlipidemia, viral infections, varicocele, toxins, and drugs could provide testicular inflammation and apoptosis [3, 4]. In all these, endoplasmic reticulum (ER) Stress is one of the main factors in the pathogenesis of these disorders which provides inflammation and apoptosis in the testes [5] Under ER stress status glucose-regulated protein-78 (GRP78) as the principal ER stress chaperon is activated and then releases three stress sensors in the ER membrane; including inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and the PKR-like endoplasmic reticulum kinase (PERK) which they launch a cascade of inflammatory and apoptotic pathways [6, 7]. ER stress also leads to the accumulation of unfolded or misfolded proteins in the endoplasmic reticulum lumen. These proteins can then produce and activate inflammatory cytokines and apoptotic enzymes in the testicular tissue [8]. ER stress in the testis is associated with spermatogenesis disorder, blood-testis barrier impairment, and germ cell depletion [9]. Studies have reported that upregulation of PERK and IRE1 activates NF-κB, JAK1/STAT1, elf2, and TLRs signaling pathways and increases inflammatory cytokine levels [10, 11]. ER stress also promotes P53, Bax, and caspases expressions and induces testicular apoptosis [12]. Tunicamycin (TM) is a bacterial nucleoside antibiotic, which induces pharmacological ER stress and increases gene expression of GRP78, IRE1, PERK, and ATF6 [13–15]. TM challenge is prevalently used to study ER stress pathways in cells and animals [13, 15–17].

Chlorogenic acid (CA) is a phenolic compound of the Hydroxycinnamic acid family, which is found in the ordinary diet, especially in large amounts in green coffee, potato, and apple [18–20]. The chemical structure of this compound contains Caffeic acid and Quinic acid, so is called 5-O-caffeoylquinic acid (5-CQA) [18]. CA is a nutritional additive against many microorganisms such as bacteria, yeasts, fungi, viruses, and amoebae. CA also has a powerful antioxidant activity, especially in preventing lipid oxidation [21, 22]. Previous studies have shown that CA attenuates inflammation and toxicity in the liver and kidney tissues by reducing TNFα, TLR4, NFκB, and IL1, 6 and 10 [21, 23]. Moreover, CA can downregulate CHOP, Bax, cytochrome C, caspase 3, and 9 and inhibit apoptosis in the liver, kidney, and lung [21, 24].

Due to the importance of testicular inflammation and apoptosis in male infertility and the notable effects of Chlorogenic acid on alleviating them, in the present study, we aim to evaluate the effect of chlorogenic acid in reducing TM-induced inflammation and apoptosis, focusing on the role of NF-κB as inflammation and apoptosis cross point in male mice.

METHODS AND MATERIALS

Reagents

Tunicamycin and chlorogenic acid were purchased from Sigma-Aldrich. Tunicamycin was solved in DMSO and chlorogenic acid was solved in saline.

EXPERIMENTAL PROCEDURE

In this study, 36 C57bl/6 male mice were used (weighing 25–27 gr). Animals were placed in a room temperature-controlled at a 12:12 light/dark cycle and had free access to food and water. All procedures were in accordance with the Guidelines for Animal Care and Use at the Qom University of Medical Sciences (IR.MUQ.AEC.1400.004). The study groups were as follows:

1. Saline group: the animals that received a dose of saline as the control (intraperitoneally (*ip)*, 0.1 cc).
2. Vehicle group: the animals that received DMSO as TM solvent (0.1 cc, *ip*).
3. CA group: the animals that were injected with chlorogenic acid (50 mg kg^{-1}) to identify possible toxic effects (0.1 cc, *ip*) [17].
4. TM group: the animals that were injected with a dose of 2 μg g^{-1} body weight TM to induce ER Stress (0.1 cc, *ip*) [18].
5. CA20-TM group: the mice that received chlorogenic acid (20 mg kg^{-1}) one hour before TM injection (0.1 cc, *ip*) [13].
6. CA50-TM group: the mice that received chlorogenic acid (50 mg kg^{-1}) one hour before TM injection [0.1 cc, *ip*].

Thirty hours after the TM injection, the animals were anesthetized with sodium pentobarbital (35 mg kg^{-1}, *ip*) and laparotomy were performed. One testis tissue was removed and placed in a freezer at −80 °C to assess inflammatory and apoptotic indexes. Another testicular tissue was placed in a 10% paraformaldehyde solution to assess histopathological parameters.

HISTOLOGICAL ASSESSMENT

After dehydration and washing of the testis tissues, they were embedded in paraffin. Five μm thick sections were then obtained for hematoxylin and eosin (H&E) staining and evaluated under a light microscope (Olympus BH-2, Tokyo, Japan) by an expert blinded histologist.

REAL-TIME RT-PCR

According to the manufacturer's instructions, total RNA was extracted from frozen tissue samples using Trizol (Yekta Tajhiz, Iran). Briefly, 1 mL of ice cold Trizol solution was added to homogenized samples. After 5 min incubation at room temperature, 200 μL of chloroform were added. The mixture was then incubated on ice and centrifuged at 12,000 rpm at 4 °C for 15 min. The aqueous phase was dissected and an equal volume of isopropanol was added, followed by incubation and centrifugation. Afterwards, the supernatant was discarded and 75% ethanol was added and the mixture was centrifuged at 4 °C for 8 min at 7,500 rpm. Finally, the pellets were allowed to dry at room temperature and dissolved in 50 μL of DEPC treated water. The quantity and purity of the RNA samples were measured by a Nanodrop spectrophotometer (Thermo Scientific, USA). Complementary DNAs (cDNA) were made from mRNA templates for qRT-PCR using the qRT-PCR using the cDNA synthesis kit (Yekta Tajhiz, Iran). Briefly, at first, RNA-primer mixture was prepared as follows: 5 μg of total RNA and 1 μL of oligo d(T) primer solution were dissolved in DEPC treated water and diluted to 10 μL. The mixture was incubated at 65 °C for 5 min. and chilled on ice. cDNA

synthesis mixture was then prepared as follows: 0.5 µL of MMLV reverse transcriptase, 2 µL of 10X buffer MMLV, 0.5 µL of RNase inhibitor and dNTP were mixed in DEPC treated water. cDNA mixture was added to RNA-primer mixture. Afterwards, the mixture obtained was centrifuged and incubated at 42 °C for 60 min. The reaction was terminated by incubation at 85 °C and the product was cooled and stored. The synthesized cDNAs were used as templates for real-time PCR. For real-time quantification, a 20 µL reaction mixture consisting of 10 µL of 2 × SYBR Green master mix (Biofact, Korea), 1 µL of template, 1 µL of forward and 1 µL of reverse specific primers, and 8 µL of DEPC of treated water was prepared. The initial denaturing temperature was 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s, 59 °C for 20 s, and 72 °C for 30. Glyceraldehyde-3-pPhosphate dehydrogenase (GAPDH) was used as an internal control. Melting curve analysis was conducted at temperatures between 60 and 95 °C. The quantitation of data was performed using the comparative 2-ΔΔCt method. Primers for the genes studied were designed by primer3 software (v. 0.4.0) (http://primer3.ut.ee) and were based on sequences found in the ensemble. Primer sequence homology and total gene specificity were confirmed with BLAST analysis (www.ncbi.nlm.nih.gov/blast).

Bax: (F) AGACAGGGGCCTTTTTGCTA (R) AATTCGCCGGAGACACTCG
Bcl-2: (F) CTTTGAGTTCGGTGGGGTCA (R) AGTTCCACAAAGGCATCCCA
GAPDH: (F) TGGCCTTCCGTGTTCCTAC (R) GAGTTGCTGTTGAAGTCGCA
IL6: (F) TCTGAAGGACTCTGGCTTTG (R) GATGGATGCTACCAAACTGGA
TNFα: (F) AGGGTCTGGGCCATAGAACT (R) CCACCACGCTCTTCTGTCTAC
P53: (F) GCCATGGCCATCTACAAGAA (R) CTCGGGTGGCTCATAAGGTA
Caspase3: (F) AAGATACCGGTCGAGGCTGA (R) AAGGGACTGGATGAACCACG
GRP78: (F) TGTGTGTGAGACCAGAACCG (R) TAGGTGGTCCCCAAGTCGAT
IRE1: (F) CACTGCCTGAGACCTTGTTGT (R) TTAAAGTCCACTTGATGGAGCC
PERK: (F) AGTCCCTGCTCGAATCTTCCT (R) TCCCAAGGCAGAACAGATATACC

ELISA ASSAY

Briefly, 100 mg of the testis tissue was weighed, homogenized, and added 1 mL phosphate buffer. It then was centrifuged (3,000–4,000 rpm for 20 min) and supernatants were then collected, allocated, and kept at −80 °C. NFκB, TNFα, and caspase3 levels were performed using ELISA kits (ZellBio, Germany) according to the manufacturer's protocol. Optical density was finally read at 450 nm by an ELISA reader.

STATISTICAL ANALYSIS

Data normality was checked by the Kolmogorov–Smirnov test and presented as mean± SEM. To compare between groups, one-way analysis of variances (ANOVA) and *Tukey's* post hoc test were performed using SPSS. $P < 0.05$ was statistically considered significant.

RESULTS

CA reduced testicular ER stress markers in the TM-induced mice

First, we evaluated ER stress markers in the testis tissue. As expected, TM administration markedly increased GRP78 gene expression compared with the saline, vehicle and CA groups

($P < 0.001$). While it significantly decreased in the CA20-TM and CA50-TM groups compared with the TM group ($P < 0.001$). The CA20-TM and CA50-TM groups had a significant increase compared with the saline group ($P < 0.05$). The groups of the CA20-TM and CA50-TM didn't show any significant differences (Fig. 1A). PERK gene expression also significantly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$). However, it had a marked decrease in the CA20-TM and CA50-TM groups compared with the TM group ($P < 0.001$ and $P < 0.01$, respectively). The CA20-TM group had a marked increase compared with the saline group ($P < 0.05$) and CA50-TM group showed a marked increase compared with the saline, vehicle, CA and CA20-TM groups ($P < 0.01$). Moreover, in the CA50-TM group was seen a significant increase compared with the CA20-TM group ($P < 0.01$) (Fig. 1B). As the same way, gene expression of IRE1 significantly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$). While, it had a marked decrease in the CA20-TM and CA50-TM groups compared with the TM group ($P < 0.001$). The CA20-TM group also showed a

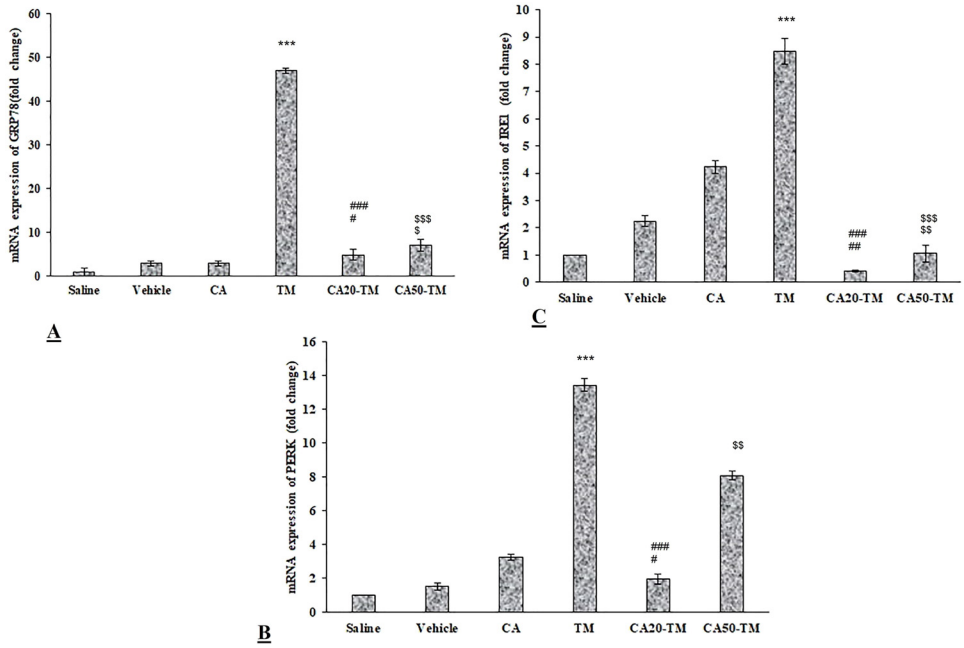

Fig. 1. **A**. mRNA expressions of GRP78 in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group, $\#P < 0.05$ compared with the saline group, $\$\$\$P < 0.001$ compared with the TM group and $\$P < 0.05$ compared with the saline group (one-way ANOVA followed by Tukey's post hoc test) **B**. mRNA expressions of PERK in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group and $\#P < 0.05$ compared with the saline group, $\$\$P < 0.01$ compared with the TM, CA20-TM, saline, vehicle and CA groups **C**. mRNA expressions of IRE1 in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group and $\#P < 0.05$ compared with the vehicle and CA groups, $\$\$\$P < 0.001$ compared with the TM group and $\$\$P < 0.01$ compared with the vehicle and CA groups

significant decrease compared with the CA groups ($P < 0.05$) and the CA50-TM group had a marked decrease compared with the vehicle and CA groups ($P < 0.01$) and a marked increase compared with the CA20-TM group ($P < 0.05$) (Fig. 1C).

CA improved histological evaluations in the TM-induced mice

In this experiment, H&E staining results revealed the normal architecture of testis tissue in the saline and vehicle groups. Germinal cell depletion and their dissociation and inflammation were observed in the TM-exposed testes. Whereas, 20 mg CA administration could improve the seminiferous structure and attenuate testicular inflammation (Fig. 2A). More analysis showed that tubular diameter significantly decreased in the TM group compared with the saline group ($P < 0.05$). While, it remarkedly increased in the CA20-TM compared with the TM, vehicle, CA and CA50-TM groups ($P < 0.01$) (Fig. 2B). Luminal diameter also significantly decreased in the TM group compared with the saline group ($P < 0.05$) and CA20-TM group showed a marked increase compared with the TM, vehicle, CA and CA50-TM groups ($P < 0.01$ (Fig. 2C). Moreover, germinal epithelial height had a remarkable decrease in the TM group compared with the saline, vehicle and CA groups ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively), however, germinal epithelial height significantly increased in the CA20-TM group compared with the TM, CA and CA50-TM groups ($P < 0.01$) (Fig. 2D). As an important point, in the CA50-TM group, CA administration could not provide a significant increment in the germinal epithelial height, luminal and tubular diameter of the testis tissue

CA attenuated testicular inflammation in the TM-induced mice

Inflammation is one of the most features in tissue injuries and inhibition of inflammation efficaciously diminishes tissue damage. In our study, gene expression of TNFα markedly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively) and both 20 mg kg^{-1} and 50 mg kg^{-1} of CA administration significantly decreased these expressions compared with the TM and CA groups ($P < 0.001$). There were no significant differences between CA20-TM and CA50-TM groups (Fig. 3A). As the same way, testis TNFα level significantly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively), However, both 20 mg kg^{-1} and 50 mg kg^{-1} of CA administration significantly decreased TNFα level compared with the TM group ($P < 0.01$). The CA20-TM and CA50-TM groups were significantly seen TNFα levels lower than saline and vehicle groups ($P < 0.05$). Nevertheless, there were no marked differences between CA20-TM and CA50-TM groups (Fig. 3B). ELISA findings also showed a marked increase of NFκB level in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$, $P < 0.01$ and $P < 0.01$ respectively), while NFκB significantly decreased in the CA20-TM and CA50-TM groups ($P < 0.05$. There were no marked differences between CA20-TM and CA50-TM groups (Fig. 3C).

Gene expression of IL6 also markedly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$). Although, both 20 mg kg^{-1} and 50 mg kg^{-1} of CA administration significantly decreased IL6 expression compared with the TM group ($P < 0.001$). Here, there was not seen any remarkable differences between 20 mg kg^{-1} and 50 mg kg^{-1} of CA (Fig. 3D). Similarly, testis ALP level markedly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$, $P < 0.001$ and $P < 0.01$,

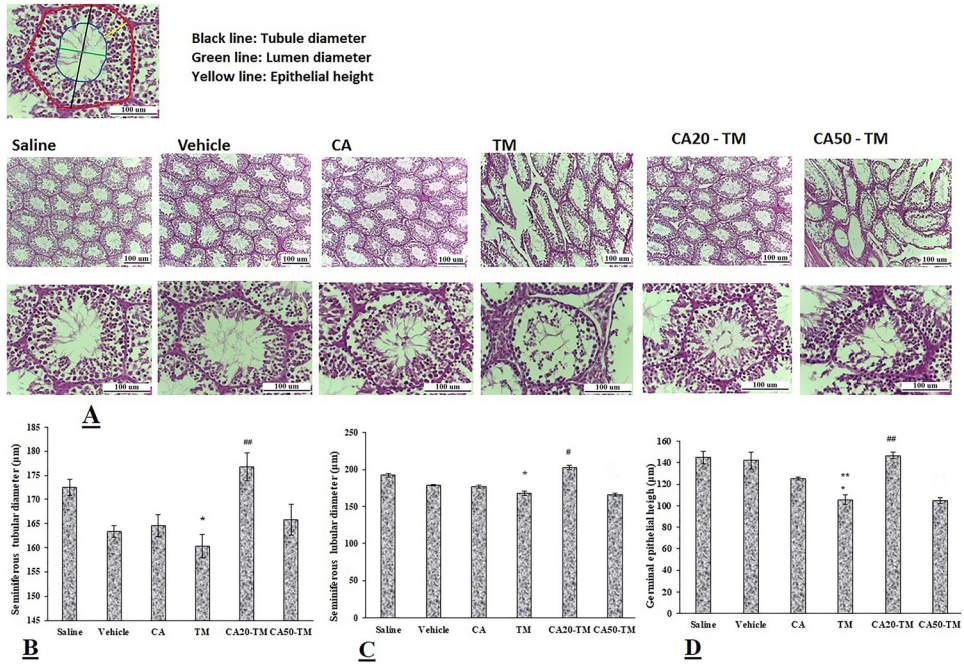

Fig. 2. **A**. Saline: representative normal structure, Vehicle: representative normal structure, CA: indicative a little textural chaos, TM: germinal cell depletion, dissociation and inflammation, CA20-TM: improvement of cell depletion, dissociation and inflammation in the testis and CA50-TM: a little structural changes and inflammation (× 200) **B**. The luminal diameter of the seminiferous tubules in different groups. (Mean ± SEM, $N = 6$), $^{*}P < 0.05$ compared with the saline group, $\#\#P < 0.01$ compared with the TM, vehicle, CA and CA50-TM groups, (one-way ANOVA followed by Tukey's post hoc test) **C**. The tubular diameter of the seminiferous tubules in different groups. (Mean ± SEM, $N = 6$), $^{*}P < 0.05$ compared with the saline group, $\#\#P < 0.01$ compared with the TM, vehicle, CA and CA50-TM groups, (one-way ANOVA followed by Tukey's post hoc test) **D**. The Epithelial height of the seminiferous tubules in different groups. (Mean ± SEM, $N = 6$), $^{*}P < 0.05$ compared with the saline, vehicle and CA groups, $\#\#P < 0.01$ compared with the TM, CA and CA50-TM groups, (one-way ANOVA followed by Tukey's post hoc test)

respectively), and CA administration lowered it significantly in both groups compared with TM and CA groups ($P < 0.001$), Again, there were no marked differences between CA20-TM and CA50-TM groups (Fig. 4).

CA prevented testicular apoptosis in the TM-induced mice

Apoptosis is one of the crucial aspects of ER stress. As presented in Fig. 5A, gene expression of P53 showed a significant increase in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$) whereas, its expression markedly decreased after CA administration ($P < 0.001$). 20 mg CA also had more decrease than 50 mg CA ($P < 0.01$). 20 mg CA was significantly seen decrease compared with saline, vehicle and CA groups ($P < 0.01$). As the same way, Bax/Bcl2 expression ratio significantly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$);

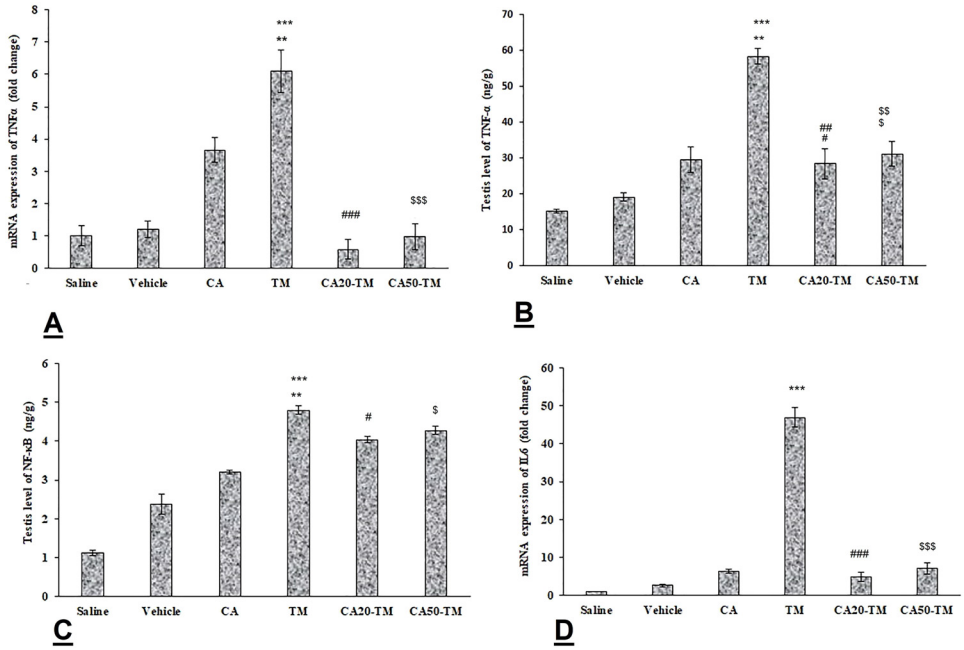

Fig. 3. **A**. mRNA expressions of TNFα in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline and vehicle groups, $^{**}P < 0.01$ compared with the CA group, $\#\#\#P < 0.001$ compared with the TM and CA groups, $\$\$\$P < 0.001$ compared with the TM and CA groups (one-way ANOVA followed by Tukey's post hoc test) **B**. testis level of TNFα in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline and vehicle groups, $^{**}P < 0.01$ compared with the CA group, $\#\#P < 0.01$ compared with the TM group and $\#P < 0.05$ compared with the saline and vehicle groups, $\$\$P < 0.01$ compared with the TM, $\$P < 0.05$ compared with the saline and vehicle groups **C**. testis level of NFκB in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline group, $^{**}P < 0.01$ compared with the vehicle and CA groups, $\#P < 0.05$ compared with the TM group, $\$P < 0.05$ compared with the TM group **D**. mRNA expressions of IL6 in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group and $\$\$\$P < 0.001$ compared with the TM

meanwhile in the CA20-TM and CA50-TM groups significantly declined compared with the TM group ($P < 0.001$). Again, 20 mg CA showed better results than 50 mg CA, saline, vehicle and CA groups ($P < 0.01$) (Fig. 5B). Caspase3 gene expression markedly increased in the TM group compared with the saline, vehicle and CA groups ($P < 0.001$) and the TM-CA20 and CA50-TM groups showed a significant decrease compared with the TM group ($P < 0.001$ and $P < 0.01$, respectively). 20 mg CA also, had a marked decrease compared with the 50 mg CA, saline, vehicle and CA groups ($P < 0.01$) (Fig. 5C). Similarly, ELISA analysis of caspase3 level indicated that TM expression strikingly increased caspase3 concentration compared with the saline, vehicle and CA groups ($P < 0.05$), however administration of CA clearly lowered caspase3 level in the CA20-TM and CA50-TM groups ($P < 0.01$). There was not seen any remarkable differences between CA20-TM and CA50-TM groups, however, they had a significant decrease compared with the control groups ($P < 0.05$) (Fig. 5D).

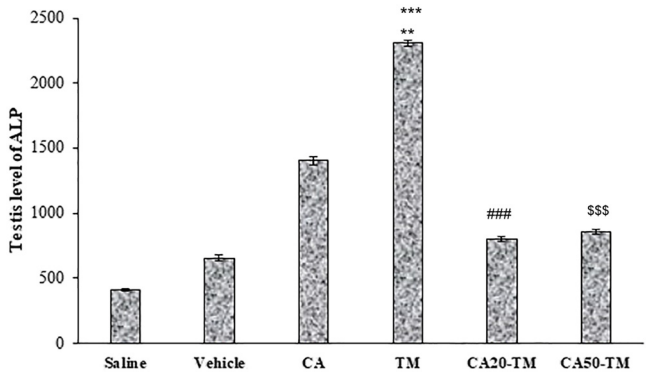

Fig. 4. ALP concentration in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline and vehicle groups and $^{**}P < 0.01$ compared with the CA group, $\#\#\#P < 0.001$ compared with the TM group and $\$\$\$P < 0.001$ compared with the TM group (one-way ANOVA followed by Tukey's post hoc test)

DISCUSSION

In the present study, we revealed that CA could decrease GRP78, IRE1, and PERK gene expression as ER stress chaperones. It also downregulated expressions of TNFα, IL6, P53, Bax/Bcl2 ratio and caspase3. Moreover, CA administration could decline ALP, NFκB, TNFα, and caspase3 levels and improve histological indexes in the testis. To the best of our knowledge, this is the first report that evaluates the effects of CA on testicular inflammation and apoptosis resulting from TM challenge in mice. CA is abundantly available in a typical diet and is famous for its unique effects against oxidative stress, inflammation, apoptosis, and even carcinoma [18, 23, 25–28].

Our findings showed that CA administration before the TM challenge decreased GRP78, IRE1, and PERK gene expression. TM blocks N-linked glycosylation of proteins and is frequently used to induce ER stress in animal researches [29]. Under the ER stress conditions, GRP78 is activated and releases IRE1 and PERK as ER stress transmembrane sensors into the cytosol. Now, they start to activate many intracellular pathways, such as inflammation, lipogenesis, oxidative stress, and apoptosis [30, 31]. Previous studies have shown that CA reduced GRP78, IRE1, and PERK expressions and alleviated ER in the heart, lung, and liver [21, 23, 24, 32–34]. Tatar et al. [17] reported administration of melatonin against TM challenge decreased GRP78 in the rat's testicular tissue and ameliorated ER stress. CA also improved testicular torsion/detortion damages by reducing GRP78, AFT6, and CHOP expressions [35]. Our findings are consistent with previous studies and seem that CA could downregulate ER stress indexes and attenuate ER stress in the testicular tissue.

Our histological results revealed that TM injection caused broad destruction in the testes with a reduction in seminiferous tubular and luminal diameters and epithelial height; although administration of CA increased tubular and luminal diameters and epithelial height in the seminiferous tubules and improved testicular damage. Several documents demonstrated that some agents could improve testicular injuries after drug and toxins exposure [36–38]. Also, a report by Owumi and his team [39] showed that CA treatment improved the regeneration of

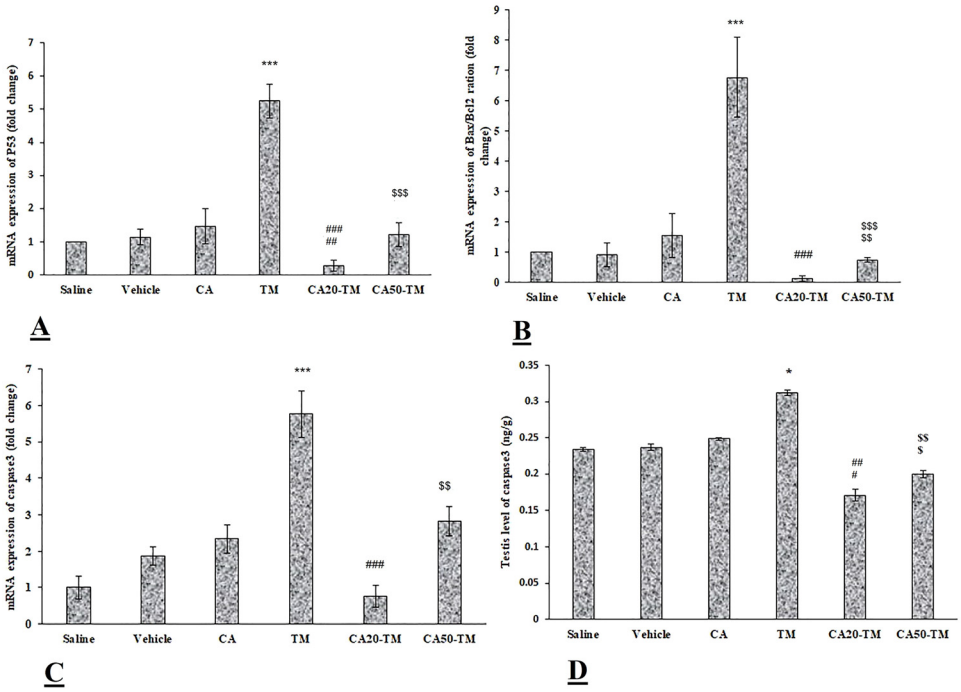

Fig. 5. **A**. mRNA expressions of p53 in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group, $\#\#P < 0.01$ compared with the saline, vehicle, CA20-TM and CA groups, $\$\$\$P < 0.001$ compared with the TM group (one-way ANOVA followed by Tukey's post hoc test) **B**. mRNA expressions of bax/bcl2 ratio in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group, $\$\$\$P < 0.001$ compared with the TM group and $\$\$P < 0.01$ compared with the CA20-TM group, **C**. mRNA expressions of caspase3 in different groups. (Mean ± SEM, $N = 6$), $^{***}P < 0.001$ compared with the saline, vehicle and CA groups, $\#\#\#P < 0.001$ compared with the TM group, $\$\$P < 0.01$ compared with the TM and CA20-TM groups **D**. testis level of caspase3 in different groups. (Mean ± SEM, $N = 6$), $^{*}P < 0.05$ compared with the saline, vehicle and CA groups, $\#\#P < 0.01$ compared with the TM group, $\#P < 0.05$ compared with the saline, vehicle and CA groups, $\$\$P < 0.01$ compared with the TM group, $\$P < 0.05$ compared with the saline, vehicle and CA groups

spermatogonia and spermatocyte cells after tamoxifen-induced testis injury. In another study, CA could restore spermatogenic cell degeneration and pathological changes in the testes of arsenic-exposed mice [40]. These results are compatible with our findings and confirm the CA positive effects on the decrement of ER stress-induced testicular injuries. Surprisingly, 20 mg kg^{-1} CA had better result than 50 mg kg^{-1} CA. In this regard, Du et al. [41] have stated that a high dose of CA caused structural damage in the ileum villi and villus congestion and it seems that a high dose of CA may reverse the positive effects of CA.

Testicular inflammation is one of the most current reasons for male infertility. ER stress and disturbances in nascent protein formation trigger many inflammatory pathways in the cells. Inflammation resulting from ER stress also is the pathological basis of many diseases, including

diabetes, inflammatory bowel disease, cancer, non-alcoholic steatohepatitis, renal failure, and even testicular injuries [5, 6, 11, 29, 42, 43]. Indeed IRE1, through activation of XBP1, JNK, and TRAF2 pathways, leads to NF-κB hyperactivity, then NF-κB upregulates inflammatory cytokines such as TNFα, IL1, and IL6 and inflammation is induced [11]. In addition, PERK another ER stress marker, raise NF-κB expression via several pathways inclusive of eIF2α, ERK, Nrf2, and JAK/STAT and thereby enhances TNFα, IL1, and IL6 levels, and finally inflammation is provided [10, 11, 42]. As seen, NF-κB is a major transcriptional factor in this context and its upregulation activates many inflammatory ways. Obtained results from this research indicated that pretreatment with CA lowered testis NF-κB level downregulated IL6 and decreased TNFα in the level of gene expression and testicular tissue. Testis ALP level also reduced after CA administration. CA is identified as an anti-inflammatory agent and its positive effects on the inhibition of NF-κB activity and inflammatory cytokines have been evidenced in several studies [21, 25]. In this regard, documents have shown that CA attenuated IL1, IL10, and TNFα in animal epididymis and testis after exposure to arsenic and tamoxifen [39, 40]. Our findings are congruent with these studies and it seems that CA lowers NF-κB levels through the diminution of IRE1 and PERK and ameliorates ER stress. Decreased levels of NF-κB next lead to downregulation of IL6 and TNFα and testicular inflammation is alleviated.

Cell death or apoptosis is an important result of ER stress in the cells. In turn, the release of PERK stimulates CHOP/NF-κB and eIF2α/NF-κB pathways [7], On the other hand, activation of IRE1 leads to the potentiation of TRAF2/NF-κB pathway [11]. NF-κB as a key transcriptional factor in apoptosis promotion now upregulates proapoptotic factors i.e. Bax and Bak and downregulates antiapoptotic factors such as Bcl2 and Bcl-Xl [3, 44]. Finally, an increased proapoptotic/antiapoptotic factors ratio leads to cytochrome C release and caspase enzyme activation especially caspase3, and apoptosis is caused. In another pathway, NF-κB can increase P53 expression in ER stress terms, then Bax expression increases, Bcl2 expression decreases, and apoptosis induces [45]. Our findings illustrated that the P53, Bax/Bcl2 ratio, and the caspase3 were downregulated and the caspase3 level decreased after CA pretreatment. Many documents have demonstrated notable effects of CA on apoptosis suppression in the liver, lung, brain, and kidney [21, 23, 24, 33]. In line with these studies, El-Khadragy and his team [40] have reported that CA treatment decreased Bax and caspase3 expression and increased Bcl2 expression, and improved testicular damage induced by arsenic. CA co-administration also suppressed testicular apoptosis through the reduction of caspase3 activity after tamoxifen-induced reproductive dysfunction [39]. Collected evidence confirms our data and it appears that attenuating effects of CA on testis apoptosis result from the decrement of ER stress, downregulation of PERK and IRE1 expressions, and then the decrease of NF-κB. NF-κB next, downregulates P53 and Bax and upregulates Bcl2, and thereby caspase3 level declines and apoptosis was inhibited.

CONCLUSION

The present study demonstrated that CA ameliorated TM-induced ER stress and thereby attenuated testicular inflammation and apoptosis and finally improved testis injuries. In this among, NF-κB played the cross-point role between inflammation and apoptosis. This indicates another considerable role for CA in restoring ER stress-induced testicular injury.

Conflict of interest statement: There was no Conflict of interest.

Author contributions: Tahereh Komeili –Movahhed performed PCR and ELISA assays, Fatemeh Heidari performed histological evaluations and Azam Moslehi designed this study, performed animal experiments and wrote manuscript. All authors approved the manuscript.

ABBREVIATIONS

AFT6	Activating transcription factor 6
ALP	Alkaline Phosphatase
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-Xl	B-cell lymphoma-extra-large
Bcl2	B-cell lymphoma 2
CA	Chlorogenic acid
CHOP	C/EBP homologous protein
elf2	E74-like factor 2
ER stress	Endoplasmic reticulum
JAK1/STAT1	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor erythroid-derived factor 2
TLRs	Toll like receptors
TM	Tunicamycin
TNFα	Tumor necrosis factor alpha
TRAF2	TNF Receptor Associated Factor 2
XBP1	X-Box Binding Protein 1

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