ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Ameliorative Effect of Ferulic Acid on Acrylamide Induced Inflammation and Oxidative Damage in Rat Testes.

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ABSTRACT

Acrylamide (ACR) is a reproductive toxic compound that has been formed during the heating process through the interactions of amino acids. ACR also significantly increased oxidative stress and depletion of antioxidants. Ferulic acid (FA) protects cells from oxidative stress through enhancement cellular antioxidant defense. The present study carried out to investigate the protective role of FA treatment on ACR-induced oxidative changes in rat testis tissues. Thirty-two adult male albino rats were divided into four groups as control (C), FA (20 mg/kg, p.o.), ACR (15 mg/kg, p.o.) and FA+ACR groups. The results showed that, administration of ACR caused a significant decrease ($P<0.05$) in testosterone (TS) and total antioxidant capacity (TAC) levels in serum but lipid peroxidation (as malondialdehyde, MDA) was increased. FA treatment significantly ($P< 0.05$) ameliorated the previous parameters. 8-hydroxydeoxyguanosine (8-OHdG), protein carbonyl (PC) and MDA levels ($P<0.05$) increased in ACR treated rats and this was attenuated significantly ($P<0.05$) by FA. Glutathione-S-transferase (GST) activity and glutathione (GSH) content were decreased after ACR administration. Co-administration of FA with ACR significantly reversed the oxidative damage in rat testes. Interleukin 6 (IL-6), cyclooxygenase 2 (COX-2) and inducible nitric oxide (iNOS) gene expressions were increased in ACR group, whereas FA improved. Moreover, in ACR group, tumor necrosis factor-α (TNF-α) and nitric oxide (NO) contents increased in the rat testes. Furthermore, gene expression and activity of (cytochrome P450 2E1) CYT 2E1 was increased in rats treated with ACR, whereas, FA attenuated. Therefore, ferulic acid had a protective role against ACR-induce DNA damage, inflammation and oxidative stress in rat testes.

Keywords: acrylamide, ferulic acid, inflammation, oxidative damage, DNA damage, antioxidant enzymes.

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ISSN: 0975-8585

INTRODUCTION

Acrylamide formed by a reaction between asparagine and reducing sugars through Maillard reaction in many starch-rich foods [1,2]. Acrylamide had reproductive toxicity in mice including abnormal morphology of sperms and testicular damages such as vacuolation and swelling of the round spermatids [3]. The administration of acrylamide significantly elevated malondialdehyde level and significantly reduced the level of reduced glutathione and the activities of glutathione-S-transferase, glutathione peroxidase and glutathione reductase in testis and epididymis. In addition, acrylamide significantly reduced serum total testosterone and progesterone levels [4]. Acrylamide exerted significant reductions of mating, fertility, and pregnancy indices in male rats [5]. Acrylamide is metabolized by CYT 2E1 produces glycidamide (the epoxide metabolite) which has a higher mutagenic potential than acrylamide and can form DNA adducts causing DNA damage [6]. Glycidamide binding to spermatid protamines causes dominant lethality of gonadal cells and morphological anbnormalities of sperms [7]. Mustafa [8] showed that exposure to acrylamide produced degeneration of germ cells, numerous multinucleated giant cells with sloughed seminiferous epithelium, and vacuolation in-between the germ cells. Nixon et al [9] demonstrated that chronic exposure to acrylamide generates DNA damage at doses equivalent to human exposures in male germ cells of mice.

Ferulic acid [(E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid)] is a common polyphenolic compound most abundant in vegetables and belongs to the phenolic acid group. FA had antioxidant and anti-inflammatory properties *in vitro* [10]. FA reduced protein oxidation and lipid peroxidation in female rat livers treated with CCl_4 [11]. Moreover, FA administration inhibited the expression of endothelial and inducible nitric oxide synthase in rat cortical neurons [12]. Ferulic acid had protective effects against various diseases such as cancer, diabetes, and neurodegenerative diseases [12,13]. Co-administration of green tea extract with the acrylamide was significantly ameliorated testosterone hormone level in serum [14]. The aim of present study is to investigate the ameliorative effect of ferulic acid on acrylamide-induced oxidative stress, inflammation and DNA damage in rat's testes.

MATERIALS AND METHODS

Chemicals

Acrylamide, glutathione (GSH), thiobarbituric acid (TBA) 2,2-dinitrophenyl hydrazine (DNPH), guanidine hydrochloride, Ferulic acid and Griess reagent were purchased from Sigma Chemical Company, USA. All other chemicals were of analytical grades.

Animals

Thirty-two adult male albino rats, weighing 180-200 g, were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR). They were maintained on standard pellet diet and tap water *ad libitum* and were kept in polycarbonate clean cages under a 12 h. light/dark cycle and room temperature 22-24 °C. Rats were acclimatized for two week prior to experimental use. This study was approved by the Ethics Committee, NODCAR.

Grouping of animals

Rats were divided into four equal groups (n=8) and treatment was given as follows:

- 1-Control Group treated with saline for 5 weeks.
- 2-FA Group treated with FA for 5 weeks (20 mg/kg, p.o) [15].
- 3-ACR Group treated with ACR for 4 weeks (15 mg/kg, p.o.) [16].
- 4-FA+ACR Group treated with FA (20 mg/kg, p.o) one week prior to ACR (15 mg/kg, p.o. for 4 weeks).

Blood samples were collected and kept without anticoagulant at room temperature for 1 h, then centrifuged at 3000 rpm/30 min and the separated serum was used for estimation of TS, TAC and MDA. TS was determined by ELISA Method (Biocheck, USA). MDA was estimated according to Ohkawa et al. [17] whereas TAC was measured using commercial available kit.

Preparation of testis homogenates

Rats were sacrificed by cervical dislocation to obtain the testes. The obtained testes were homogenized in ice-cold phosphate buffered saline (PBS; pH 7.4) with a Potter-Elvehjem glass homogenizer to prepare 10 % w/v homogenate. The homogenates were centrifuged at 10,000×g for 20 min at 4°C (**Sigma-3K30, Germany**) to obtain the supernatant.

Determination of NO level

Nitrite was estimated using Greiss reagent and served as an indicator of NO production. Nitrite concentration was calculated using a standard curve for sodium nitrite [18].

Determination of PC level

Levels of PC were determined according to Floor and Wetzel [19].

Determination of GST activity

The activity of GST was calculated according to Habig et al. [20].

Determination of MDA level

MDA, as a marker of lipid peroxidation, was determined according to Ohkawa et al. [17].

Determination of GSH level

GSH levels were assayed in tissue homogenates according to the method of Ellman [21]. Concentrations were calculated by using GSH calibration curve and results were expressed as µg/mg protein.

Determination of CYT 2E1 activity

CYT 2E1 activity was measured in testes microsomal fraction as described by Chang et al. [22].

Determination TNF-α and 8-OHdG levels

Levels of TNF-α (Assaypro, USA) and 8-OHdG (SunLong, China) were quantified using ELISA kits according to the manufacturer's instructions and guidelines.

Real Time PCR for quantitative expression of IL-6, iNOS, COX-2 and CYT 2E1

The mRNA expression level was quantified by qRT–PCR (Real time PCR). 1 µg of the total RNA from each sample were used for cDNA synthesis by reverse transcription using First Strand cDNA Synthesis Kit (Qiagene). The cDNA was subsequently amplified with the Syber green Master Kit (Qiagen) in a 48-well plate using the Step One instrument (Applied Biosystem, Foster City, CA, USA) as follows: 10 minutes at 95 °C for enzyme activation followed by 40 cycles of 15 seconds at 95 °C, 20 seconds at 55°C and 30 second at 72°C for the amplification step. Changes in the expression of target gene were measured relative to the mean critical threshold (CT) values of GAPDH housekeeping gene, by the ΔΔCt method.

The total protein content

The total protein content of testis was determined according to Lowry et al. [23].

Statistical analysis

The values were expressed as the mean±SE for the eight rats in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the statistical package for social sciences (SPSS) software package for Windows (version 13.0). Post hoc testing was performed for intergroup

ISSN: 0975-8585

comparisons using the least significant difference (LSD) test. A value corresponding to P<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Ferulic acid is a derivative of cinnamic acid with molecular formula $C_{10}H_{10}O_4$. FA is a common polyphenolic compound most abundant in vegetables, especially artichokes and eggplants. In addition, it is commonly found in seeds of plant such as rice, wheat and oats. The molecular mechanisms of ACR reproductive toxicity could be due to alkylation of SH groups in the sperm nucleus and tail, depletion of GSH, or DNA damage in the testis [24].

Present study showed that showed that ACR induced significant decrease (P<0.05) in TAC and TS levels of adult male rats treated with ACR alone compared with control group. However, ACR increased MDA level in rat serum compared with control group. On the other hand, FA+ACR group significantly (P<0.05) improved ACR effect on TAC, TS and MDA compared with ACR group (Table 1). Administration of ACR caused a significant reduction of serum testosterone level [14,4]. This significant reduction of testosterone may be a result of direct damage of ACR on the Leydig cells [25]. Co-administration of green tea extract with the acrylamide was significantly ameliorated testosterone hormone level in serum [14]. The results exerted that FA enhanced serum TAC in rats treated with ACR suggesting that FA effectively inhibited ACR toxicity.

Table 1: Effects of ferulic acid on levels of TS, TAC and MDA of rat serum treated with acrylamide.

Parameters	Control	FA	ACR	FA+ACR
TS (ng/ml)	3.63±0.05^a	3.56±0.07^a	1.50±0.04^b	2.96±0.21^c
TAC (mM/L)	1.81±0.13^a	1.96±0.09^b	0.38±0.04^c	1.02±0.11^d
MDA (nmol/ml)	5.55±0.05^a	5.30±0.09^a	13.59±0.27^b	7.66±0.48^c

Values are expressed as means±SE (n=8). Values on the same row not sharing the same superscript letters were significantly different (P<0.05).

Oxidative stress has been demonstrated to be a key mechanism in many ACR induced cell injuries and neurodegenerative diseases. The present study revealed that ACR induced marked enhancement in MDA in rat testes (Table 2, P<0.05), which is agreement with Abd El-Halim and Mohamed [26] who found that administration of ACR caused a significant enhancement in testes MDA level. In addition, Jiazhong et al. [27] mentioned that ACR is able to increase LPO by inducing oxidative stress with generation of free radicals.

Table 2: Effect of ferulic acid on oxidative stress markers (8-OHdG, MDA, PC and GSH levels and GST activity) of rat testes exposed to acrylamide

Parameters	Control	FA	ACR	FA+ACR
8-OHdG (ng/mg protein)	4.13±0.16^a	3.03±0.23^b	12.63±0.08^c	6.11±0.16^d
MDA (nmol/mgprotein)	0.40±0.03^a	0.30±0.04^a	1.70±0.05^b	0.83±0.05^c
PC (nmol /mgprotein)	22.72±1.20^a	20.87±0.81^a	34.62±0.81^b	26.68±1.27^c
GSH (µg/mg protein)	4.60± 0.21^a	5.08±0.16^a	1.66± 0.11^c	3.82± 0.17^d
GST (µmol/min/mgprotein)	122.52±1.91^a	116.46 ±2.78^a	73.12±3.86^b	102.20±3.47^a

Values are expressed as means±SE (n=8). 8-OHdG protein expression was determined using ELISA kits (8-OHdG was purchased from SunLong, China).Values on the same row not sharing the same superscript letters were significantly different (P<0.05).

In addition , Table 2 showed that ACR significantly (P<0.05) decreased GSH content in testes, which can be explained by the reaction of ACR with GSH, which in turn causes the depletion of GSH and the enhancement of LPO. These results are in agreement with Abd El-Halim and Mohamed [26] who found that administration of ACR caused a significant reduction in testes GSH level.

Our results showed that co-treatment of rats with FA ameliorated GSH depletion by ACR (Table 2). In consistence, Treatment of rats with lipoic acid caused an increase in GSH level with a decrease in LPO [28]. In Table 2, ACR exhibited increase in the protein carbonyl of rat testes which is in accordance with the results of Lakshmi et al. [29] who indicated that ACR administered rats showed increased levels of lipid peroxidative product, protein carbonyl content, hydroxyl radical and hydroperoxide. In addition, administrations of ACR form adduct with reduced glutathione and increased levels of lipid peroxidative (LPO) products and carbonyl content in brain [30]. The obtained data exerted that FA ameliorates the protein carbonyl content in rats treated with ACR (Table 2, $P<0.05$). FA reduced protein oxidation and lipid peroxidation induced by hydroxyl radicals [31] and by CCl_4 [11].

The present results in Table 2 showed that ACR induced significant ($P<0.05$) increase in 8-OHdG (a marker for DNA damage). It has shown that ACR induced reproductive toxicity such as reduced litter size [7] and DNA strand breaks [32]. In addition, ACR induced DNA damage which measured by comet assay in rat brain and testes [33] or by 8-OHdG in liver, brain and kidney [34]. Co-treatment of rats with FA decreased the level of 8-OHdG compared with ACR group (Table 2). The results are in agreement with Hirose et al. [35] who reported that FA reduced DNA single-strand breakage and disruption of biological membranes. Furthermore, the administration of blueberry anthocyanins extract reduced decreased the DNA damage in lymphocyte and liver cells [36].

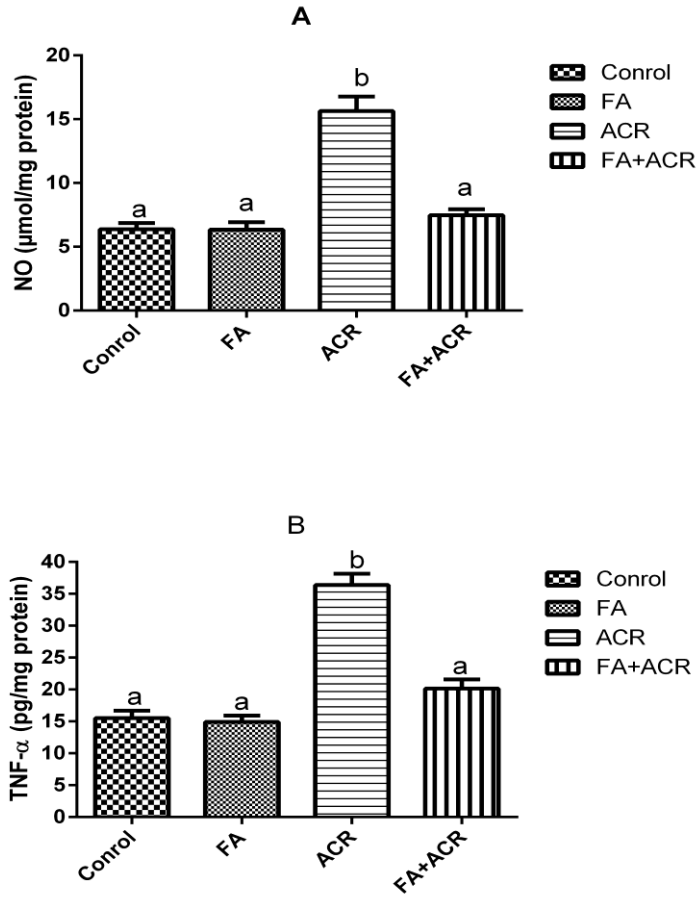

Figure 1: Effect of ferulic acid (20 mg/kg) on inflammation biomarkers, (A) NO content and (B) TNF-α protein expression of rat testes exposed to acrylamide.

Values are expressed as means±SE (n=8). TNF-α protein expression was determined using ELISA kits (TNF-α was purchased from Assaypro, USA). NO; nitric oxide, TNF-α; tumor necrosis alpha. Values on the same row not sharing the same superscript letters were significantly different ($P<0.05$).

ISSN: 0975-8585

In present study, we have found that ACR induced increase significantly (P<0.05) in protein expression of TNF-α and NO (Fig 1) along with increase in gene expression of iNOS, IL-6 and COX-2 (Fig 2), as markers of inflammation. These findings are in agreement with Santhanasabapathy et al. [37] who indicated that ACR stimulated increase in levels of pro-inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β) and iNOS. Moreover, ACR activated the transcriptional factors NFκB leads to the transcription of inflammatory genes as well as iNOS leading to NO production [38]. ACR enhanced interleukin 1 beta, interleukin 6, and tumor necrosis factor α in rat liver, kidney and brain [34]. Nitric oxide is a key mediator in many physiological processes including smooth muscle relaxation, neurotransmission and inhibition of platelet aggregation [39]. Nitric oxide levels in testes tissue was altered in ACR treated group. It is known that, NO may show either cytoprotective or cytotoxic effects and the elevated NO level has the ability to induce γ-glutamyl cycle [40].

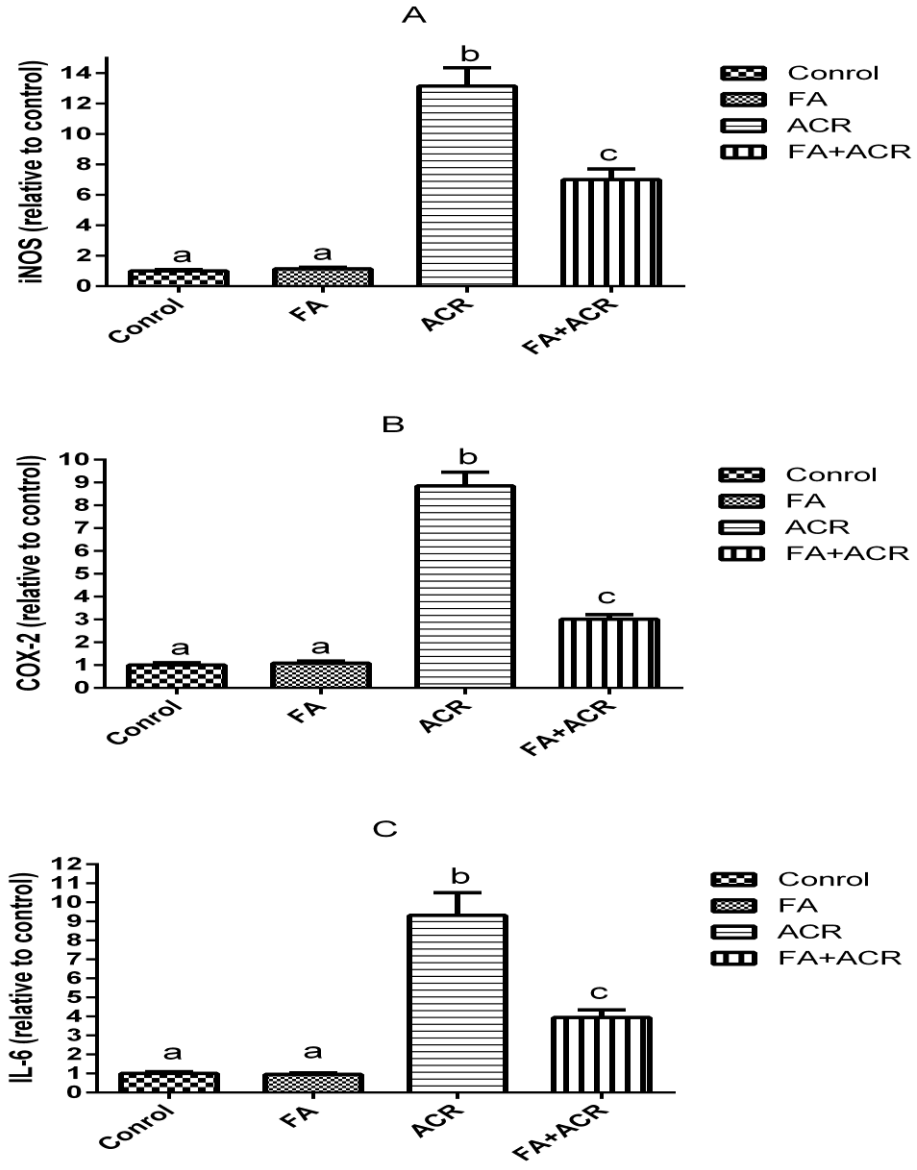

Figure 2: Effect of ferulic acid (20 mg/kg) on inflammation biomarkers, (A) iNOS, (B) COX-2 and (C) IL-6 gene expression) of rat testes exposed to acrylamide.

Data are expressed as fold change (relative to control group). Using reverse transcriptase, cDNA was synthesized from 1 µg total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing primers for IL-6, COX-2, iNOS and GAPDH. Each reaction contained cDNA derived from 1 µg total RNA. Three replicates of each reaction were performed. Values on the same row not sharing the same superscript letters were significantly different (P<0.05).

Combined treatment with FA and ACR resulted in a significant improvement in protein expression of TNF-α and NO (Fig 1) as well as increase in gene expression of iNOS, IL-6 and COX-2(Fig 2). FA administration inhibited the expression of endothelial and inducible nitric oxide synthase in rat cortical neurons [12]. Morin hydrate a natural polyphenolic compound, protected mice from oxidative stress, hepatic inflammation induced by ACR [41]. In addition, thymoquinone, a main active constituent of the volatile oil extracted from the black seed (*Nigella sativa* L.), reduced MDA and NO levels and elevated GSH in rat testes treated with ACR [42].

There are two primarily competing pathways to metabolize ACR: oxidation by CYT 2E1 to form GA (activation) and conjugation by glutathione-S-transferase (GST) with reduced glutathione (detoxification). The GST assay was suggested as a useful tool for biomonitoring oxidative stress [43]. It is well known that GST enzymes are involved in the detoxification of many compounds that are toxic to humans. The activity of GST is able to catalyze the conjugation of GSH with ACR for detoxification [44]. Our results showed that the GST activity significantly ($P<0.05$) reduced in ACR group, while FA significantly ($P<0.05$) improved this effect in rats treated with ACR compared to ACR group (Table 2). It has been shown that, the activity and protein expression of GST markedly reduced in ACR group indicating that the balance of redox system was broken down [36]. FA significantly reduced the GST activity in rat testes. Furthermore, gene expression and activity of CYT 2E1 was increased in rats treated with ACR, whereas, FA attenuated (Fig 3). CYT 2E1 the primary enzyme is responsible for conversion of acrylamide to its epoxide, glycidamide leading to the formation of glycidamide-DNA and haemoglobin adducts [45]. FA significantly improved the CYT 2E1 gene expression (Fig 3).

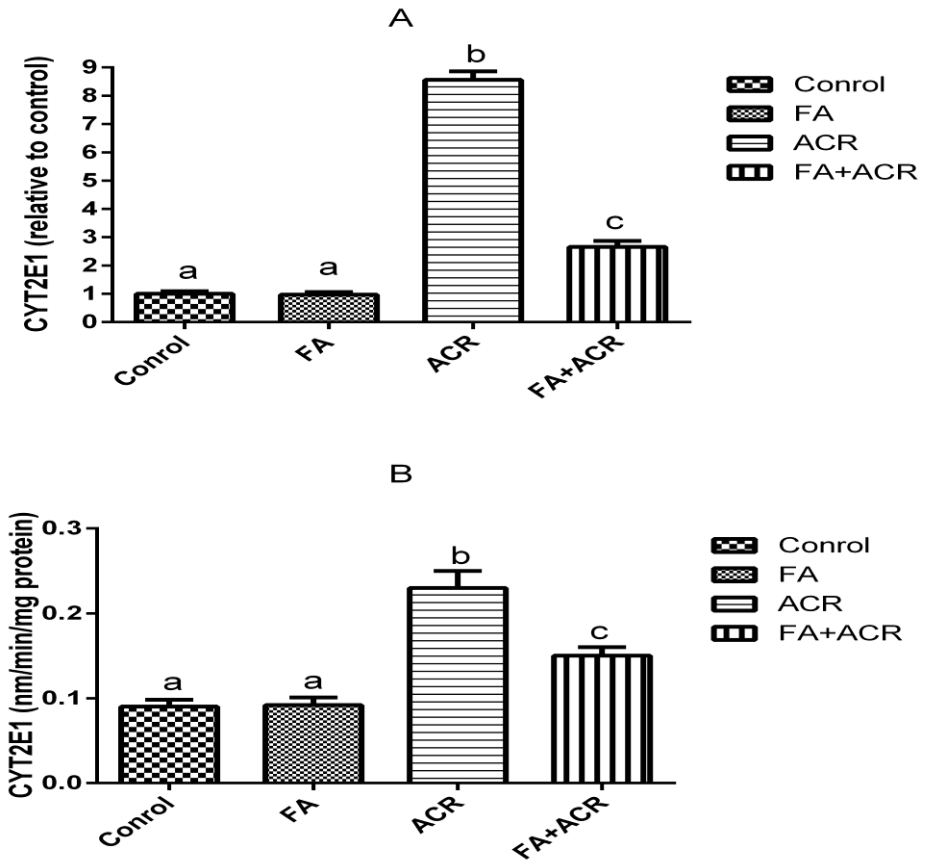

Figure 3: Effect of ferulic acid (20 mg/kg) on (A) CYT 2E1 gene expression and (B) CYT 2E1 activity of rat testes exposed to acrylamide.

Data are fold change (relative to control group) for CYT 2E1 gene expression or expressed as means±SE (n=8) for CYT 2E1 activity. Using reverse transcriptase, cDNA was synthesized from 1 µg total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing primers for CYT 2E1 and GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Three replicates of each reaction were performed. Values on the same row not sharing the same superscript letters were significantly different ($P<0.05$).

In Conclusion, Ferulic acid is effective in preventing ACR-induced reproductive toxicity, oxidative stress, inflammation and DNA damage in rat testes. Protective effect of Ferulic acid on ACR might be due to the recovery of the redox system and inhibition of the CYT 2E1 enzyme.

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