Original Article

Effect of the combined administration of vitamin-E and 5-aminosalicylic acid on acrylamide-induced testicular toxicity

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Received 21 January 2017; revised 28 February 2017; accepted 5 March 2017; Available online 23 May 2017

Objective:

This study aimed to evaluate the comparative protective antioxidant effect of 5-aminosalicylic acid (5-ASA) and vitamin-E against acrylamide (ACR)-induced testicular toxicity in rats.

Methods:

This study was performed at King Fahad Medical Research Centre, Jeddah, KSA. A total of 49 adult Wistar rats (250 ± 20 g) that were 60 days old were divided into seven groups (control, ACR alone, ACR + 5-ASA, ACR + Vitamin-E, ACR + 5-ASA + Vitamin-E, Vitamin-E alone, 5-ASA alone). Acrylamide [45 mg/kg (bw)/day] and vitamin-E [200 mg/kg (bw)/day] were gavaged orally, and 5-ASA [25 mg/kg (bw)/day] were injected intra-peritoneally for five consecutive days after one day of observation. Rats were sacrificed by cervical dislocation. Histopathology of the testes, enzyme linked immunosorbent assay (ELISA) of testosterone, the lactate dehydrogenase (LDH) assay and antigenemia, and histological examination of the testis were performed.

Results:

Rats treated with ACR showed signs of aggression and rough coats, with reduced food and water intake. ACR treated rats showed histopathological changes in the form of a sloughed seminiferous epithelium in the tubular lumen with no multinucleated giant cells. Shrinkage of seminiferous tubules with widening of the interstitial space was also observed with atrophy and the shedding of normal mucosa. Our results indicated

Abstract

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Results: Rats treated with ACR showed signs of aggression and rough coats, with reduced food and water intake. ACR treated rats showed histopathological changes in the form of a sloughed seminiferous epithelium in the tubular lumen with no multinucleated giant cells. Shrinkage of seminiferous tubules with widening of the interstitial space was also observed with atrophy and the shedding of normal mucosa. Our results indicated
that maximum protection was conveyed by the combined antioxidant effect of vitamin-E and 5-ASA on testicular histopathology.

**Conclusion:** We conclude that acrylamide-induced degeneration of seminiferous tubules can be partially reversed by the administration of 5-ASA and vitamin-E and suggests restricting exposure to ACR.

**Keywords:** 5-ASA; Acrylamide; Seminiferous tubules; Testicular atrophy; Vitamin-E

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**Introduction**

Acrylamide (ACR) is a highly toxic chemical that is widely used in the manufacturing of polyacrylamides, which have a wide range of industrial applications, including the production of dyes, paper, plastics and the treatment of water. The workplace environment can be considered dangerous where exposure to acrylamide occurs via different routes, including direct contact with the toxic substance itself and inhalation through ACR contaminated airways. Apart from occupational exposure, a major route of acrylamide toxicity to humans is via foods that are heated to temperatures above 120 °C. ACR as a food toxicant is found in carbohydrate rich foods with low protein, including fried potatoes, potato chips, coffee and cereals that are cooked under high temperature where the Maillard reaction occurs between asparagine amino acids and glucose, producing acrylamide. ACR was declared a "potential human carcinogen" in 1994 by the International Agency for Research on Cancer. This finding was supported by the Scientific Committee on Toxicity, Ecotoxicity and the Environment by explaining its connate toxic nature with adverse effects on the skin, digestive system, circulation, respiratory system, endocrine system, nervous system and reproductive system, particularly the testicles, in addition to its harmful carcinogenic impact. The chemical structure of acrylamide is shown in Figure 1.

Once absorbed by the body, ACR is metabolized by either of two chief pathways: glutathione conjugation and glycidamide epoxidation. It may either be conjugated by glutathione-S-transferase to N-acetyl cysteine or may react with cytochrome P2E1 (CYP2E1) to produce glycidamide. The toxicity of ACR is credited to the fact that it can be bio-transformed to its highly active metabolite, glycidamide (GA). This pathway is regulated by the enzyme cytochrome P450 E1 (CYP2E1). Glycidamide was found to be more harmful to deoxyribonucleic acid (DNA) and proteins compared to acrylamide. The metabolism of ACR by CYP450 E1 leads to the formation of free radicals [reactive oxygen species (ROS)], consequently initiating oxidative stress and tipping the balance between the production and destruction of ROS, thus expediting lipid peroxidation and DNA and protein alterations.

Thus, compounds showing antioxidative properties could be used as effective protective agents against ACR-induced toxicity. Vitamin-E is one such compound known for high antioxidant properties. It has a high capacity for protection against free radical formation and can reduce peroxidative chain reactions. Several hypotheses have been made with regards to the effectiveness of 5-aminosalicylic acid (5-ASA) against reactive oxygen species (ROS) formation. One study shows that it plays a dominant role as an antioxidant as well as an anti-inflammatory compound in vivo. 5-ASA could be developed as a potential curative agent for ACR-induced renal toxicity, either in combination with vitamin-E or alone. The protective effect of 5-ASA relies on the blockage of two compounds: prostaglandin synthase and lipoxygenase enzymes. A study reported that 5-ASA induced overall improvement both physiologically and biochemically against the toxic damage caused by ACR.

Though many studies have found that acrylamide induces testicular toxicity in rats, there is a lack of data suggesting that the use of antioxidant compounds would effectively confer a protective effect against such toxicity. Hence, the aim of our study was to evaluate the testicular toxicity of ACR and compare the antioxidant effects of both vitamin-E and 5-ASA on ACR-induced testicular toxicity.

**Materials and Methods**

**General materials**

Plus one acrylamide (PAGE) grade with purity >99.95 was purchased from Pharmacia Biotech (Uppsala, Sweden), 5-ASA 95%, Vitamin-E (DL-α-tocopherol acetate) and > 98% high performance liquid chromatography (HPLC) were purchased from Sigma–Aldrich (Steinheim-Germany). Testosterone kits were purchased from ALPCO Diagnostics (Windham, USA). Unless otherwise mentioned, all other chemicals and materials of molecular biology grade were
purchased from BHD laboratory supplies (Analar®, England).

**Animals and dosage formulation**

A total of 49 adult male virgin Wister rats (250 ± 20 gm) that were 60 days old on arrival were procured from King Fahad Centre for Medical Research (KFMRC) in Jeddah, KSA, and were housed four per polycarbonate cage with wood shavings as bedding. Animals were kept in a controlled environment at 22 ± 2 °C and relative humidity of 40–65% with 12 h/12 h light/dark cycles throughout the experimental period. The rats were fed laboratory chow. Tap water in plastic bottles with steel sipper tubes were used to provide an *ad libitum* supply of water to rats.

All animal care procedures and treatments were performed at KFMRC, Jeddah, KSA with the approval of the Unit of Biomedical Ethics, King Abdulaziz University (KAU), Medical College, Jeddah, KSA in accordance with the guidelines of the KAU, which follow the national and international laws and National Institute of Health (NIH) policies (National Institute of Health Guiding Principles in the Care and Use of Animals). Animals were allowed to acclimatize in the experimental environment for 3 days before dosing initiation. The animals were randomly divided into 7 groups (n = 7 each). Groups consisted of one control group, four acrylamide treated groups (ACR alone, ACR + 5-ASA, ACR + vitamin-E and ACR + 5-ASA + vitamin-E) and two groups for vitamin-E and 5-ASA alone.

The dose of acrylamide used was 45 mg/kg (bw)/day for 5 consecutive days, which was selected as an effective dose for inducing ACR toxicity after a comprehensive literature review. The dosing solutions were freshly prepared daily using distilled water. Rats were given ACR by oral gavage using a metallic curved-ball ended needle (Size PS-18). The control group was gavaged with 1 ml of distilled water. Rats were treated with vitamin-E at a dose of 200 mg/kg (bw)/day by oral gavage. The dose selected for vitamin-E was previously reported in the literature to show a protective effect on endosulfan-induced reproductive toxicity in rats. In the 5-ASA treated group, rats were intra-peritoneally injected with a dose [25 mg/kg (bw)/day] of 5-ASA for 5 consecutive days. All animals were weighed and observed for mortality or any behavioural changes once per day during the dosing and recovery period.

After 5 days of treatment, 2 ml of blood was collected from the retro-orbital sinus in plain tubes. Blood samples were centrifuged at 3200 g for 10 min. After a recovery period of one day post ACR cessation, animals were sacrificed by cervical dislocation and their right testis and right cauda epididymis were isolated for further experimental evaluation.

**Histopathology**

The testes were fixed in bouin solution for 24 h.

**Processing of fixed sections**

Following fixation, tissues were then processed using standard laboratory procedures for histology. Tissues were briefly embedded in paraffin blocks, sectioned perpendicular to the longest axis of the testis at approximately 3–5 μm thickness and stained with haematoxylin & eosin (H & E). Stained sections were mounted with dextran-plasticizer xylene (DPX). The slides were examined for histological changes using light microscopy (Olympus BX51TF, Olympus Life Science Solutions, Shinjuku, Tokyo, Japan) at 10×, 20× and 40× magnifications and representative images were captured with an Olympus DP 72 camera.

**Biochemical analysis**

A. Testosterone ELISA (Enzyme Linked Immunosorbent Assay):

Blood from all groups was collected in plain tubes with red caps to obtain serum for testosterone hormonal analysis which was performed via an automated analysis system provided by Siemens Healthcare Diagnostics INC (ADVIA Centaur and ADVIA Centaur XP Immunoassay Systems), Tarrytown, New York, USA. The assay was conducted according to the standard manufacturer’s protocol for the analyser.

B. Lactate dehydrogenase (LDH) assay:

Lactate dehydrogenase (LDH) is commonly found in the cytoplasm within different mammalian bodies and can be easily evaluated by using quantitative data measurements obtained by the Dimension Vista® System and Flex® reagent cartridge (Siemens Healthcare Diagnostics INC, Tarrytown, New York, USA). The reaction took place within a 96 micro-well plate where all reagents are ready to use liquid solutions.

**Caudal sperm count**

Two μl from each caudal tissue suspension (diluted 1:20) was taken and the sperm number was manually counted using a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) in a strip of ten squares. In case of oligospermia, 3–4 strips were counted and their mean was used. The resultant number was multiplied by the dilution factor (10) to represent their concentration in millions/ml of suspension and then divided by gm of cauda. Counting was performed using a Leica-DM 1000 light microscope (Leica Microsystems, Wetzlar, Germany) at 20× magnification.

**Preparation of tissue suspension from cauda epididymis**

Tissue suspension from the right cauda was prepared according to the method employed by Sakamoto and Hashimoto. The right cauda epididymis from each rat was minced with scissors in 1 ml of 10% neutral buffered formalin [4% formaldehyde, 33 mM monosodium phosphate (NaH2PO4)]. The resulting suspension was filtered through a 340 μm stainless steel mesh to remove large tissue fragments. Smear samples were prepared on slides after staining the slides with 10% Rose Bengal sodium salt (C20H14Cl4Na2O5) solution for 15 min and were then examined for morphological abnormalities at 40× magnification using a Leica-DM 1000 light microscope. Representative sperm cells were photographed with a Leica DC-180 camera (Leica Microsystems, Wetzlar, Germany).
Statistical analysis

All statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) 16.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± 2SD. Differences among the groups were analysed by one-way analysis of variance (ANOVA) followed by the Tukey’s test as a post hoc for multiple comparisons. A p-value less than 0.05 was considered statistically significantly different.

Results

General observation

Rats treated with a dose of 45 mg/kg (bw)/day of ACR showed signs of aggression and rough coat, with an apparent reduction in food and water intake. Improvement in water and food intake was observed in the group given a concomitant treatment of ACR and 5-ASA. Rats in the control group showed no symptoms of illness or mortality during the experimental period. No mortality was reported among the ACR treated rat group or other groups.

Effect of acrylamide and antioxidants on body weight in rats

No statistically significant difference was detected among the groups compared to controls (Figure 2).

Effect of ACR and antioxidants on absolute cauda weights

No statistically significant difference was detected among the groups compared to controls (Figure 3).

Effects of ACR and antioxidants on serum testosterone level

As testosterone is produced within the Leydig cells of the testis, we examined the impact of ACR mediated toxicity on circulating testosterone levels and the protective effect of ASA and vitamin-E against ACR toxicity. No statistically significant changes were observed in serum testosterone levels in any of the experimental groups (Figure 4).

Effect of ACR and antioxidants on epididymal sperm count

No significant difference in epididymal sperm count was observed in any of the experimental rat groups (Figure 5).

Effect of ACR and antioxidants on serum lactate dehydrogenase (LDH) concentration

No significant difference (P > 0.05) in the lactate dehydrogenase serum concentration was observed in ACR treated or any antioxidant treated group of rats (Figure 6).

Testes histopathology

Some evidences of histological changes in the testes were observed in rats treated with ACR when compared with controls. In control rats, seminiferous tubules, spermatogenesis and the epididymis appeared normal with a regular defined basement membrane. The general cellular arrangement of the seminiferous tubules with an apparent luminal sperm reserve, and the appearance of Leydig cells in the rat testis was also normal (Figure 7A and B). However, rats treated with ACR showed minor changes in the form of sloughed seminiferous epithelium in the tubular lumen with no multinucleated giant cells. The capsule also appeared normal. Shrinkage of seminiferous tubules with widening of the interstitial space was also noted with atrophy and shedding of normal mucosa (Figure 8A). There was disruption in the normal appearance of the testis (Figure 8B). Multiple vacuoles appeared in between the cells of the seminiferous tubules (Figure 8C). However, Leydig cells appeared very mildly affected with no major effect on luminal sperm reserve (Figure 8D). Surprisingly, rats treated with ACR and a dose of (25 mg/kg (bw)/day) 5-ASA showed incomplete protection with improvement in

Figure 2: Effect of acrylamide (ACR) and antioxidants on rat body weight change at the end of the observation period.

Figure 3: Absolute cauda weights after acrylamide (ACR) and antioxidant (5-aminosalicylic acid and vitamin-E) treatment in rats.
the general histological organization of the testis, with no areas of atrophy and a normal appearance of Leydig cells (Figure 9A). However, rats treated with ACR and vitamin-E (200 mg/kg (bw)/day) only showed no protection compared to 5-ASA with some areas of tubular atrophy and Leydig cell degeneration (Figure 9B and C). Maximum protection from ACR-induced damage was observed in groups treated with both antioxidants, vitamin-E and 5-ASA, as seen in Figure 10A, while rats treated with vitamin-E alone showed some abnormal histology in the form of multiple vacuolations and mildly atrophied Leydig cells (Figure 10B). The rats treated with 5-ASA alone had normal histology (Figure 10C).

**Effect on sperm morphology in the cauda epididymis**

In the control rats, sperm appeared with a normal head and tail (Figure 11A), but rats treated with ACR alone showed many sperm with cut heads. In rats treated with ACR and 5-ASA, little difference in sperm morphology was observed compared to controls (Figure 11B). However, in the group of rats treated with ACR and Vitamin-E, some abnormalities in sperm morphology, such as the absence of sperm heads, could be seen (Figure 11C).

**Discussion**

The aim of the current study was to investigate and compare the antioxidant effects of both vitamin-E and 5-ASA on acrylamide induced testicular toxicity in male rats. Our results indicated that maximum protection was obtained by the combined effect of both antioxidants on testicular histopathology, followed by 5-ASA alone. Moreover, vitamin-E alone showed an inability to protect against ACR-induced testicular toxicity.

Our present study revealed that there was no statistically significant difference among all experimental groups, including the acrylamide treated group (45 mg/kg (bw)/day), compared to controls with regards to body weight changes. However, we did observe some reduction in food intake in rats treated with ACR. Similarly, many other investigators have reported that acrylamide has no influence on the body weight of treated animals.

In contrast to our results, acrylamide has been reported by many investigators to decrease the body weight of treated animals. For example, in a study conducted by Yang et al., there was a significant reduction in rat body weight at doses of 45 and 60 mg/kg (bw)/day compared to the control group, following the oral gavage of ACR for five consecutive days. Furthermore, the body weight gain after the 5 day ACR treatment period and at the end of 72-h recovery period was decreased significantly at those doses. In a study by Sakamoto et al., in which prepubertal and adult male mice received a single oral dose (150 or 100 mg/kg (bw)/day)
of ACR, the mice showed a significant reduction in body weight for 5 and 3 days, respectively, following treatment. Furthermore, Rajeh et al.\textsuperscript{6} reported a severe reduction in rat body weight after acrylamide treatment. This loss of body weight could be attributed the reduced appetite of rats which was observed with acrylamide treatment.

As testosterone is produced within the Leydig cells of the testis, we next examined the impact of ACR-mediated toxicity on circulating testosterone levels and the ability of 5-ASA and vitamin-E to protect against ACR toxicity. The result of the current study showed no statistically significant difference among all groups in comparison to controls. In contrast, other studies reported a significant reduction in the serum testosterone concentration after acrylamide exposure.\textsuperscript{9,32} This reduction was mainly attributed to severe atrophy in Leydig cells caused by acrylamide treatment. Moreover, Rajeh et al.\textsuperscript{9} reported a significant increase in the serum testosterone concentration in acrylamide treated rats after injection with 5-ASA indicating its protective antioxidant effect. This was probably due to the difference in the duration of exposure. As in other studies, longer acrylamide exposure might induce more testicular toxicity, thus causing severe atrophy that may affect Leydig cells, which are responsible for testosterone production.\textsuperscript{33} It has been

![Figure 7](image1.png)

**Figure 7:** Light microscopy of transverse sections of testes isolated from control rats. (A) Normal testis histology at 10× magnification; (B) Normal appearance of Leydig cells (asterisk) at 40× magnification. Sections were stained with H & E.

![Figure 8](image2.png)

**Figure 8:** Light microscopy of transverse sections of testes isolated from acrylamide (ACR) treated rats. (A) Shrinkage of seminiferous tubules at 10× magnification; (B) Atrophy of tubules (arrow) with mucosal shedding (asterisk) at 40× magnification; (C) Multiple vacuoles between cells in tubules (arrows) at 10× magnification; (D) Mildly affected Leydig cells (arrow) at 20× magnification. Sections were stained with H & E.
**Figure 9:** Light microscopy of transverse sections of testes isolated from acrylamide (ACR) + 5-aminosalicylic acid (5-ASA) and ACR + vitamin-E treated rats. (A) Normal histology in testis isolated from rats treated with ACR + 5-ASA; (B) Partial atrophy of Leydig cells from rats treated with ACR + vitamin-E (arrow) at 10× magnification; (C) Atrophy of tubules from rats treated with ACR + vitamin-E (arrow) at 10× magnification. Sections were stained with H & E.

**Figure 10:** Light microscopy of transverse sections of testes isolated from (A) acrylamide (ACR) + vitamin-E + 5-aminosalicylic (5-ASA) (B) vitamin-E (C) 5-ASA treated rats. (A) Testis isolated from rats treated with ACR + 5-ASA + vitamin-E show good protection against ACR induced testicular toxicity; (B) Multiple vacuoles (arrow) between germ cells with an effect on Leydig cells after vitamin-E treatment [200 mg/kg (bw)/day] alone; (C) Normal histology in rat testis after 5-ASA injection alone. Sections were stained with H & E.
reported that many other polycyclic aromatic hydrocarbons are metabolically activated to active metabolites in rat Leydig cells. Moreover, the use of different rat strains could also be related to acrylamide toxicity, tolerance and resistance, in addition to the number of recovery days, all of which could explain how toxicity can take some time to optimally generate a negative impact.

In spite of the insignificant effect of acrylamide or antioxidants on serum testosterone levels in the current study, we further investigated the effect of acrylamide on sperm count per cauda. Our results indicated that acrylamide (45 mg/kg (bw)/day) for 5 consecutive days led to no significant effect on sperm count between all groups compared to controls. This result was expected, as there was no significant effect on testosterone in this study which may affect the events of spermatogenesis in the seminiferous tubules and consequently the total sperm count per cauda. The result of the current study was consistent with studies in which there was no significant effect of acrylamide on either epididymal sperm motility or concentration in rat testis after oral administration of ACR at doses of 5, 15, 30, 45 or 60 mg/kg (bw)/day for 5 days.

In contrast to our result, a study reported a significant reduction in sperm count per cauda in acrylamide treated rats with the same dose (45 mg/kg (bw)/day) for 5 days. However, there was no significant difference from the ACR control group at both doses of 5-ASA. Other studies have shown a significant reduction in testosterone after acrylamide exposure in rats. A possible interpretation for the reduction in sperm count in the cauda epididymis observed with ACR treatment might be due to a dominant lethal effect of ACR on germ cells, mainly at the stages of late spermatid and early spermatozoa formation.

Considering the above findings, we suggest that an experiment be conducted with different rat strains with body weights that do not exceed 230 g as excess weight may affect resistance to acrylamide toxicity. Furthermore, the impact of acrylamide toxicity is inversely proportional to animal body weight; the more damage acrylamide causes, the lower the body weight which in turn weakens animal resistance.

The determination of the lactate dehydrogenase (LDH) concentration indicates how much the liver was damaged. In the present study, no significant difference among all groups was detected compared to controls. In contrast, a study reported a highly significant increase in the LDH level in a group of Sprague Dawley (SD) rats injected with a dose of ACR equal to 40 mg/kg (bw)/day for one month. This increase in LDH level indicates overall tissue damage.

Acrylamide has been reported by many investigators to produce characteristic histopathological changes in the rat. In the current study, rats treated with 45 mg/kg (bw)/day ACR showed minor changes in testis histology in the form of sloughed seminiferous epithelium in the tubular lumen with no multinucleated giant cells, shrinkage of seminiferous tubules with widening of the interstitial space, atrophy and shedding of the normal mucosa with disruption in the normal appearance of the testis. Multiple vacuoles appeared in between cells in the seminiferous tubules with no major effect on luminal sperm reserve. The maximum protection observed in this study on testis histology was in the ACR treated group that was administered both antioxidants, vitamin-E and 5-ASA. While ACR treated rats administered vitamin-E alone showed some abnormal histology in the
form of multiple vacuolations and mild atrophy of Leydig cells, some abnormalities in sperm shape such as cut heads indicated the ineffectiveness of vitamin-E alone as an antioxidant against ACR-induced testicular toxicity.

One important factor to consider as an explanation for the detachment or sloughing of germ cells and the resultant tubal atrophy after ACR treatment is that ACR might affect the adhesive contact between sertoli and germ cells. It was reported that a novel gene (flamingo1) encoding a cell adhesion protein (cadherin) was expressed in rat testis and was likely a sertoli cell product. Therefore, flamingo1 could be a target of ACR testicular injury and requires further investigation and evaluation. Again, in contrast to our results, the characteristic multinucleated giant cells alongside signs of acrylamide toxicity in the testicular lumen including atrophy were reported in several other studies.

Therefore, this protective action of 5-ASA on acrylamide induced-histopathological changes might be due the ability inhibit oxidative damage. This protection conferred by 5-ASA in turn relies on its competence to scavenge free radicals and by acting as a chain breaking antioxidant in lipid peroxidation. Furthermore, its anti-prostaglandin property might reduce the signs of the accompanied inflammation. ROS are known to be involved in the metabolism of prostaglandins, so the scavenging of ROS produces the anti-inflammatory effects of 5-ASA. 5-ASA acts by triggering a class of nuclear receptors, the γ-form of peroxisome proliferator-activated receptors, which are involved in the control of inflammation, cell proliferation, apoptosis and metabolic function. Vitamin-E can ameliorate the reproductive toxicity of some toxins such as sodium arsenite in rats. However, it has been reported previously that vitamin-E is not completely able to protect the testis or reverse testicular toxicity after ACR exposure. Nevertheless, it was reported that it can protect testis after cessation of acrylamide treatment but not during active administration as it induced more rapid recovery, in agreement with our current study. The result of the last study was in agreement with our result.

The limitations of this study are: (a) It took almost a month to obtain ethical approval before starting our experiment. (b) Due to the large number of animals, we could not perform the sperm count on the same day of rat sacrifice, but we preserved these animals in 10% normal saline to perform counting on the next day.

Conclusion

This study concludes that acrylamide induces degeneration of seminiferous tubules which can be partially reversed by co-treatment of acrylamide treated rats by both 5-ASA and vitamin-E. The maximal protection against the ACR induced damage on testicular histopathology is exhibited by the combined antioxidant effect of vitamin-E and 5-ASA, followed by 5-ASA alone. Furthermore, vitamin-E alone shows inability to protect against ACR-induced testicular toxicity. Based on the literature available and the current study the protective action of 5-ASA on acrylamide induced-histopathological changes might be due its proficiency to inhibit oxidative damage. Further investigations are required to elucidate the molecular basis of the antioxidant role of 5-ASA and vitamin-E against ACR induced testicular toxicity. We recommend restriction of ACR exposure either occupationally or in food containing product.

Funding

This research did not receive any specific grants from funding agencies in the public, commercial or not-for-profit sectors.

Author contributions

Both the authors actively contributed to the study. NAR conceptualized the study design. DK performed the experiments under the supervision of NAR. DK prepared the draft manuscript and NAR revised it.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

We thank the Unit of Biomedical Ethics, King Abdulaziz University (KAU) Medical College, Jeddah, KSA for approving the use of animals and the experimental design. We also thank King Fahad Medical Research Centre (KFMRC), Jeddah, KSA for providing the laboratory space in which to conduct our experiments.

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