Original Article

Protective Effects of 5-Aminosalicylic Acid on Acrylamide Toxicity in the Testis and Blood Leukocytes of the Rat

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ABSTRACT

Objectives: Acrylamide (AA) has many applications in the chemical industry. It has been shown to be a reproductive toxicant in animals and is associated with risk of cancer. The objective of this study was to investigate the protective effect of 5-aminosalicylic acid (5-ASA) against AA induced testicular and geno-toxicity.

Design: Experimental study

Setting: King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

Intervention: Animals were orally gavaged with AA at a dose of 45 mg/kg/day for five consecutive days and 5-ASA was injected concomitantly at two different doses, 25 and 50 mg/kg/day.

Main Outcome Measures: Effect on epididymal sperm count, on histological changes in the testis, on COMET assay in blood leukocytes, on serum testosterone level and on CYP2E1 expression in liver and testis (S9) fractions

Results: COMET assay undertaken on blood leukocytes showed geno-toxicity in the form of COMET cells with increased tail movement, while ELISA of serum testosterone showed severe reduction in testosterone level, which was reversed by concomitant 5-ASA treatment. ELISA of CYP2E1 showed a two-fold higher concentration in control liver S9 when compared to control testis S9. 5-ASA (50 mg/kg) induced the level of liver CYP2E1, potentially increasing AA metabolism and clearance. Light microscopy examination showed multinucleated giant cells and tubular atrophy in the testis after AA treatment.

Conclusion: At the used dose, AA caused toxic effects in male rat that can be reduced by concomitant treatment with 5-ASA, which might be considered as an antidote to AA toxicity in victims of AA poisoning.

KEY WORDS: acrylamide, 5-ASA, COMETs, CYP2E1, testosterone

INTRODUCTION

Acrylamide (AA) is an important compound in the production of polyacrylamide which is used in a variety of industries. Polyacrylamides are used in sewage treatment and potable water treatment and purification, paper industry, petroleum industry to enhance oil recovery and greatly used for chromatography and electrophoresis in experimental research. AA monomer is also used in the production of grouts and soil stabilizers[1].

Acrylamide has also been considered as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC)[2]. It is well known as a neurotoxicant after human and animal exposure. In rat and mice studies, the no observable effect level (NOEL) for neurotoxic effect has been reported to be between 0.2-10 mg/kg body wt/day, and this is far above dietary exposure[3]. It has also been shown to elicit reproductive toxicity in laboratory animals.

In a recent study conducted by Kermanl-Alghoraishi *et al*[4], AA has been reported to affect the membrane integrity of epididymal spermatozoa in mice. It also decreased sperm vitality and caused abnormal motility in a sub-chronic study conducted by Song *et al*[5]. In rat, AA was shown to affect the normal development of the sperm, significantly affect hind limb motor coordination and directly damage Leydig cells. Several lines of evidence suggest that AA biological activity is

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mainly due to adduction with protein, while its primary activity with DNA is alkylation, mainly due to its active metabolite glycidamide[6]. The two main metabolic pathways that have been elucidated for AA metabolism are glutathione conjugation[7,8] and epoxidation to glycidamide (GA)[8,9]. Many studies have reported on the role of cytochrome P450 in the metabolism of AA, in particular cytochrome P450 2E1[10,11]. Recently, human CYP2E1 was reported to mediate the formation of glycidamide from AA[12]. AA was proposed to cause the release of free radicals, which decrease the oxidative defense system in the cell, leading to the development of cancer[13,14]. Taken together, it is therefore important to identify protective applications against the actions of AA, both to prevent immediate and long-term damage from low- level environmental exposures and in victims of AA poisoning. 5-ASA has been reported previously to have a potent antioxidant and anti-inflammatory activity which were proposed to be due to inhibition of prostaglandin synthase and/ or lipoxygenase activities[15]. In addition, ASA has been shown to improve semen characteristics and restore fertility in patients with ulcerative colitis who suffered from infertility after long-term treatment with sulfasalazine (SSZ)[16]. Also, pretreatment of endosulfan treated rats with 5-ASA, significantly reduced sperm shape abnormalities, with histopathological analysis of seminiferous tubules and Leydig cells indicating significant protection from endosulfan induced tissue damage[17]. Taken together, these two last pieces of data are strongly supportive that 5-ASA is able to protect the testes from ROS-mediated damage, as occurs during AA toxicity. It was therefore hypothesized that 5-ASA could have a protective role in AA-induced reproductive toxicity in male rats. Therefore, the major aim of this study was to investigate the potential protective effects of 5-ASA on AA-induced testicular and geno-toxicity.

MATERIALS AND METHODS

General Materials

Plus One™ AA (PAGE grade, purity > 99.95 %, < 0.05% impurities of acrylic acid) was obtained from Pharmacia Biotech (Upsala, Sweden) and 5-ASA 95% was obtained from Sigma-Aldrich (Steinheim, Germany). Testosterone ELISA kit was purchased from Alpico Diagnostics™ (Windham, USA) and rat cytochrome P450 2E1 ELISA kit was obtained from USCNLIFE™ (Wuhan, China). All other chemicals and materials were purchased from BHD laboratory supplies (Analar®, England) and were of molecular biology grade.

Animals and Dosage Formulation

This was an experimental study in which a total of 30 virgin male Sprague-Dawley rats were used. Rats were purchased from King Fahad Medical Research Center (KFMRC) in Jeddah, KSA, and allowed to acclimatize in the experimental environment for three days before dosing initiation. They were housed 4-5 per polycarbonate cage with wood shaving as bedding. Animals were maintained under controlled environment at 22 ± 2°C and relative humidity of 40 - 65% and 12 hrs / 12 hrs light / dark cycles throughout the experimental period. The rats were fed laboratory chow, which was supplied by Grain Silos and Flour Mills Organization (Jeddah, KSA). Tap water in plastic bottles with steel sipper tubes were used for an adlibitum supply of water.

Rats were six weeks old and weighed 203 - 231 gm. The dose of AA used to produce testicular damage was 45 mg/kg/day; although this dose was above the daily dietary exposure (of AA) it was reported previously to induce a testicular toxicity with minimal neuro-behavioral changes. It was effective in eliciting a strong testicular and geno-toxic response over a short period of time to be able to study the mechanism of AA-induced reproductive toxicity and to investigate the protective effect of 5-ASA. The dosing solutions were freshly prepared daily using distilled water. 5-ASA was injected at two different doses, 25 and 50 mg/kg, as a freshly prepared suspension in 10% gum acacia. The control group was gavaged with 1 ml of distilled water.

Study Design

The study was conducted with five treatment groups and one vehicle control group, with five randomly chosen animals in each group (n = 5). Animals were orally gavaged with 1 ml of 45 mg/kg acrylamide, using a metallic needle curved-ball ended (size PS-20) to induce testicular damage. Concomitantly rats were treated with 1 ml of 5-ASA *via* intraperitoneal injection. The rats were exposed to AA and 5- ASA for five consecutive days. All groups were observed for mortality or any behavioral changes once daily during the dosing period. Animal body weight, water and food consumption were measured twice during the experiment. After five days of exposure, 3 ml of blood was collected from retro-orbital sinus in plain tubes, and then the rats were killed by cervical dislocation under ether anesthesia. One milliliter of blood was collected in EDTA tube for COMET assay. The right testis of all animals were isolated and weighed for further experimental evaluation.

All experiments were undertaken with the consent of the animal ethics committee in accordance with the guidelines set out by the Canadian Animal Care.

Methods

Preparation of hepatic and testicular S9 fractions

Preparation of liver and testes microsomal supernatant S9, was achieved according to the method of Loannides

and Parke[18]. Animals were sacrificed by cervical dislocation under ether anesthesia. The liver and testes were immediately excised and placed in tubes immersed in ice until sacrificing all animals, provided that all steps were carried out at 4°C and using cold solutions. Organs were rinsed immediately in ice-cold 1.15% (w/v) freshly prepared KCl to remove excess blood and were blotted dry before weighing. Organs were scissor minced in a small volume of KCl (less than the total required to produce 25% and 35% suspension) to produce coarse homogenate. After that the coarse homogenates were transferred to a motor driven potter Elvehjem homogenizer using a Teflon pestle (B. Braun Melsungen AG, Germany). The tissues were homogenized on ice keeping homogenization to the minimum required to produce a smooth homogenate. Small bursts were applied to prevent heat build-up. Extra KCl was added as required to allow full homogenization without exceeding the target suspension percentage. Then the homogenates were adjusted to a 25% suspension for the liver and 35% suspension for the testes by using ice-cold 1.15% (w/v) KCl. Then the homogenates were centrifuged at 9000 g for 20 min at 4°C using a high speed centrifuge (Sigma, 3K18-Germany) to remove debris, nuclei, mitochondria and lysosomes. The supernatant (S9, microsomal supernatant) was decanted and aliquots inserted into 1.5 ml tubes and stored at -80°C until required.

Protein concentration in (S9) was determined by Total Protein method (TP method) which is a modification of the biuret reaction[19]. The whole procedure was automatically performed using Dade Behring Dimension clinical chemistry system (USA).

Estimation of CYP2E1 concentration in testis and liver S9 fractions by ELISA

All reagents and standards were prepared and reconstituted according to the product protocol. After producing serial dilutions (10, 5, 2.5, 1.25, 0.625, and 0.312) from the undiluted standard (20 ng/ml), where the sample diluent serves as the zero standard and the undiluted standard serves as the highest concentration (20 ng/ml) of the CYP2E1. The undiluted standard is recombinant rat CYP2E1, produced by USCNLIFE™ Co. (China) from where the kit was purchased. 100 µl of all standards, and samples were loaded to the assay plate. After 2 h of incubation at 37° C, the liquid was removed from each well without washing. One hundred microliters of detection reagent-A working solution were added to each well and the plate was incubated for 1 h at 37 ºC. Then each well was aspirated and washed three times by the kit washing solution using an automatic microplate washer (ELX50, Biotek-USA). After this washing step, the plate was inverted and blotted against clean paper towels. Then 100 µl of detection reagent-B working solution were added to each well, covered with the plate sealer and incubated for another 1 h at 37 ºC. After a further five times aspiration / wash step for all wells, 90 µl of substrate solution was added to each well, the plate was covered and incubated for 30 minutes at 37 ºC under light protection. Finally 50 µl of stop solution were added to each well and the absorbance (optical density) of the stopped reaction mixture of all wells was measured at 450 nm using a multiwell microplate reader (ELX-800, Biotek-USA). For the results calculation the duplicate readings for each standard were averaged and the average zero standard optical density was subtracted from all results (standards and samples). A calibration curve was obtained by plotting the mean absorbance for each calibrator (X-axis) against the known concentrations (Y-axis) of rat CYP2E1and the best fit line through the points on the graph was determined by regression analysis using Microsoft Office, Excel-2007. Samples CYP2E1 concentrations were determined from the calibration curve in ng/ml. The measured data were analyzed by One-way Analysis of Variance (ANOVA) and a p-value of < 0.05 was used as the criterion for statistical significance.

Caudal Sperm Count: Two µl from each caudal tissue suspension (diluted 1:20) was taken, and sperm number was manually counted using a Makler Counting Chamber (Sefi Medical Instruments), 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 (20) and this represented their concentration in millions/ml of suspension. Counting was undertaken using a LEICA, DM 1000 light microscope at X 20 magnification.

Preparation of testis for histological examination: The organs were initially fixed in Boun's solution (75 ml saturated aqueous picric acid, 25 ml of 40% formaldehyde and 5 ml glacial acetic acid), for one hour and then removed for preliminary cutting. Tissues were further fixed for another 24h in 10% neutral buffered formalin[20].

Following fixation, tissues were then processed using standard laboratory procedures for histology. Briefly tissues were embedded in paraffin blocks, sectioned perpendicular to the longest axis of the testis at approximately 3-5µm thickness and stained with Hematoxylin & Eosin. Stained sections were mounted with dextran-plasticizer xylene (DPX) and were examined using light microscopy (LEICA, DM1000) at the indicated magnification and representative images were photographed with a Leica DC -180 camera.

Single cell gel electrophoresis (COMET assay): Alkaline comet assay was performed according to the protocol of Hartmann *et al*[21], and was analyzed using Loat's Comet Assay Software with extended dynamic range imaging (EDRI). A total of 2 µl of whole blood taken from the rats, initially collected in EDTA tubes,

was mixed with 100 µL of 1% (low melting point) agarose, after it was cooled to 37°C. Plating of 75µL of agarose mixture was performed in a dimmed light using Trevigen comet slides, which are specially treated to promote adherence of low melting point agarose, then slides were placed on ice for 8 minutes to allow agarose to solidify. After that, a gentle cell lysis was performed by immersing the slides in cold lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, pH 10 and 1% Triton X-100 and 10% DMSO added just before use) for 30 - 60 minutes at 4°C. The excess buffer was tapped off the slides and they were immersed in freshly prepared alkaline solution, (300 mM NaOH pellets and 1mM EDTA, pH 14 and allowed to cool before use) for 20 - 60 minutes at room temperature in the dark, to unwind the DNA and hydrolyze sites of damage, and then slides were removed from the alkaline solution with gentle removal of excess buffer as before. Slides were then transferred to a horizontal electrophoresis apparatus (CH420, UK) and alkaline solution added. Electrophoresis was conducted for 20 minutes at 1volt / cm, 300 mA, in an ice bath and under dim light to minimize light induced DNA breaks. Finally after gentle removal of excess electrophoresis solution, the slides were placed in Tris buffer (pH 7) for 10 minutes to neutralize alkali, and then immersed in absolute ethanol for five minutes to complete precipitation of DNA and accelerate dehydration. Slides were then drained, air dried, and then stained with 1µg/ml of ethidium bromide for five minutes, followed by washing for five minutes with distilled water. Analysis of comet tail was done by Loat`s single gel comet assay software with EDRI and observed with fluorescent microscope, Olympus, BX-51(Japan). Positive control used in this assay was glycidamide (from LKT laboratories, USA). It was reconstituted with distilled water. For preparation of negative control, control rat whole blood was used, alkaline comet assay was performed, and the average tail movement was calculated. For the preparation of different glycidamide dilutions (0.025 - 10mM), glycidamide was incubated with the whole rat blood for four hours at 37º C and then alkaline comet assay was performed, and analysis of comet tail was done. The percentage of cell viability and comet cells were calculated.

Testosterone ELISA assay: Direct quantitative determination of free testosterone in rat serum was performed by using an ELISA kit for competitive enzyme immunoassay. All reagents were prepared and / or reconstituted according to the product protocol. A total of 25 µl of each ready to use calibrators or standards with the following approximate concentrations (0, 0.35, 1.45, 7.2, 30, and 150 pg/ml - they contain free testosterone in a serum based buffer with a non-mercury preservative); the kit control (7.1pg/ml); and the serum samples were dispensed onto a 96-well anti-free testosterone antibody coated micro-well plate. Then 100 µl of the conjugate working solution (free testosterone horseradish peroxidase conjugate) was added into each well and gently mixed for 10 seconds followed by incubation at 37 ºC for one hour. Wells were then washed three times with wash buffer (buffer with a non-ionic detergent and a non-mercury preservative) using an automatic micro plate washer (ELX50, Biotek-USA) that dispensed 300 µl of wash solution per well per cycle. Excess wash solution was removed and the total of 150 µl of TMB substrate (tetramethyl benzidine and hydrogen peroxide in a DMSO-containing buffer) was then added to each well. After 10-15 minutes incubation at 37 ºC the reaction was stopped with the addition of 50 µl/well of stopping solution (1 M sulfuric acid). The optical density (450 nm) of each well was subsequently measured with multi-well micro-plate reader (ELX-800, Biotek-USA). The mean absorbance for each standard was calculated and calibration curve plotted on semi-log paper with the mean optical density on the Y-axis and the calibrator concentrations on the X-axis and the values of the unknown samples were read directly from the curve.

Statistical analysis: Differences between obtained values (mean ± SD) were compared by one-way analysis of variance (ANOVA), using Graph Pad Prism & Graph Pad Instat, followed by Tukey-Kramer multiple comparison test. A p-value less than 0.05 were used as the criterion for a statistically significant difference.

Ethical committee approval

The research was approved by the Biomedical Ethics Research committee in the Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

RESULTS

Effects of ASA on AA induced changes in epididymal sperm count

Rats treated with AA (45 mg/kg/day) showed significant reduction in sperm count in comparison to normal control group (Fig.1, $p < 0.05$), as was expected. However, no statistically significant change in epididymal sperm count was detected for rats treated with AA and both doses of ASA when compared to AA control group which suggested that both doses of ASA on this five days duration exposure could not protect rats from the toxic effects of AA on total sperm count. As indicated ASA (25 or 50 mg/kg) was administered by IP injection concomitantly with oral gavage of AA treatment, with gum acacia as a solvent.

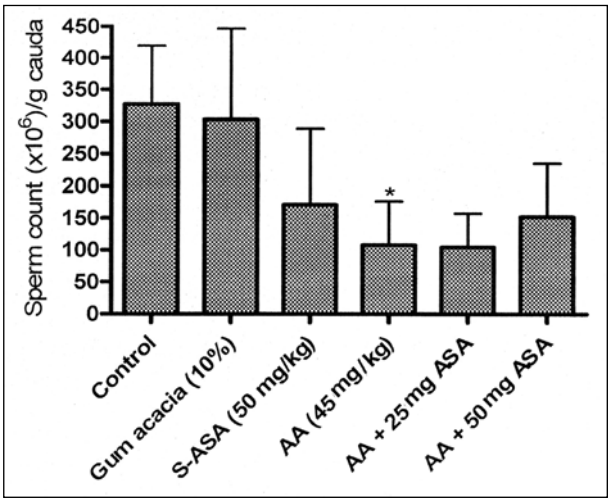
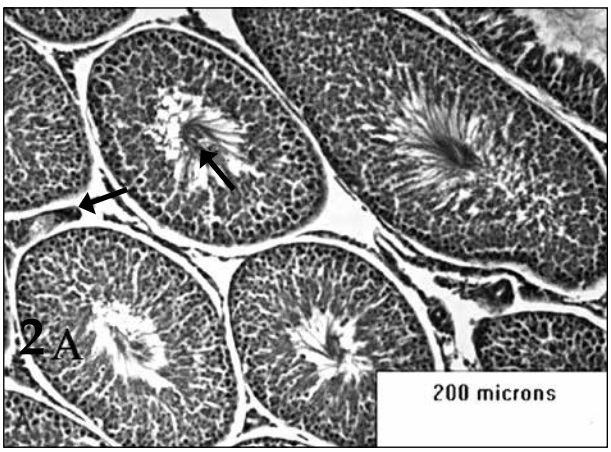


Fig. 1: Total sperm count per gram of cauda after AA and ASA treatment. (Data were expressed as mean ± SD, n = 5). The symbol represents statistical significance (ANOVA) from normal control: *, $p < 0.05$, followed by Tukey-Kramer multiple comparison test. The AA + ASA groups were compared to AA control.

Histopathology

In the control group seminiferous tubules and spermatogenesis appeared normal with apparent luminal sperm reserve. Also, there was a clear cellular differentiation starting with the spermatogonia from the basement membrane and ending at the lumen with the spermatozoa. In addition rats treated with 5-ASA and gum acacia showed normal testes, (Fig. 2A, B).



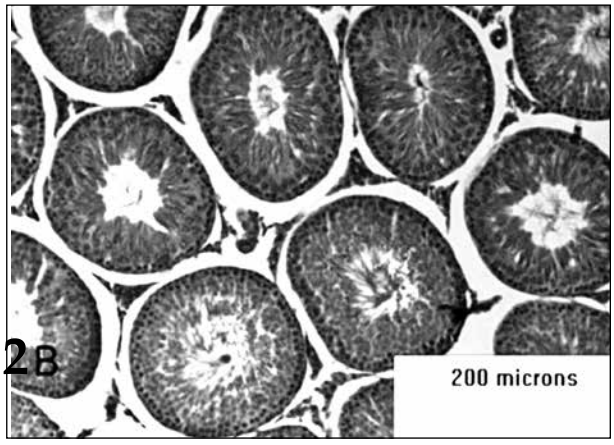
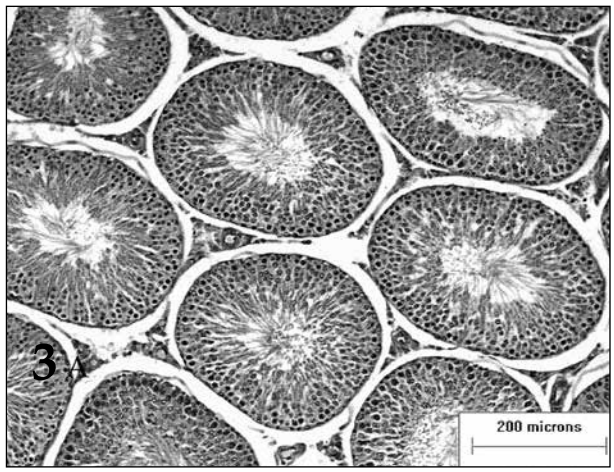


Fig. 2: Representative light microscopy of transverse sections of testes isolated from: (A) control, (B) 50 mg/kg ASA. Rats were treated for 5 consecutive days. All show testes with normal histology. Sections stained with H&E stain and viewed using light microscopy.

As (Fig. 3A) from control rat testis showed normal testis histology, evidence of morphological changes in the testicular histology was observed in the AA-treated rats, including germ cell degeneration and atrophy to the seminiferous epithelium of rat testis, with disruption in the normal looking appearance of the testis. In addition, a reduction in the luminal sperm reserve was observed, and in many tubules vacuolations were observed in-between cells of seminiferous tubule (Fig. 3B). Co-treatment of rats with AA and 25 mg/kg 5-ASA, did not completely remove the acrylamide-induced testicular changes, but the observed damage



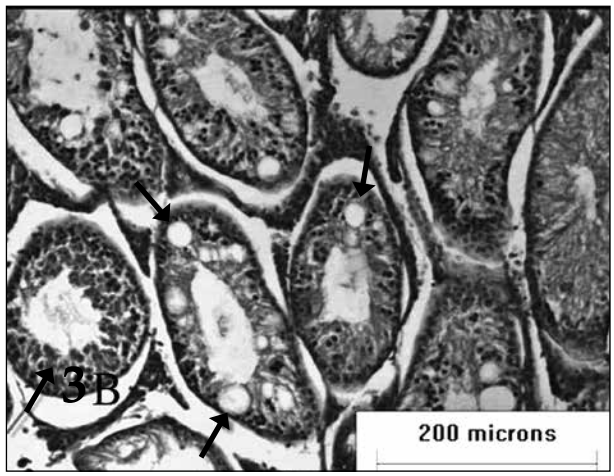


Fig. 3: Representative light microscopy of transverse sections for general architecture of testes, indicating the AA toxicity and the protective effect of ASA, isolated from control, AA and ASA treated rats.(A) Shows testis isolated from control rat (B) Shows testis of AA (45 mg/kg)treated rat, with disruption in normal histological appearance of the tubules with germ cell degeneration and reduction in sperm reserve with multiple vacuoles of different size (see arrows).

was generally less, with a significantly less damage to the germ cell population in most of the tubules, although there were still areas of damage (Fig. 3C).

Interestingly, AA-treated rats exposed to a dose of 50 mg/kg 5-ASA, appeared to be almost completely protected from the AA-mediated toxicity, with nearly normal histological appearance of the testis. Normal spermatogenesis was observed, with no multinucleated giant cells, no vacuolations, and no tubular atrophy (Fig. 3D).

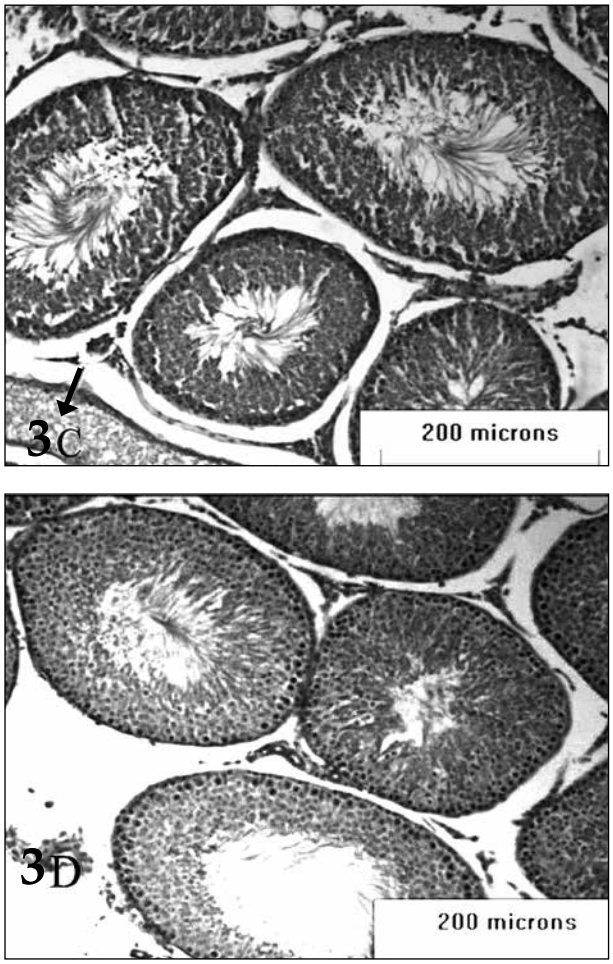


Fig 3 : (C) Shows the mild protective effect of ASA at a dose of 25 mg/kg on the testicular toxicity caused by AA, note the slight restoration of normal histological structure of the testis with increased sperm reserve in the lumen of the tubule with some residual damage in the form of atrophy and germ cell degeneration, (D) Shows the strong protective effect of ASA at a dose of 50 mg/kg on the testicular toxicity caused by AA, the tubules almost appearing normal with normal histology and restoration of luminal sperm reserve with normal germ cells. Sections stained with H&E stain and viewed using light microscopy.

As shown in (Fig. 4A) from control rat testis, Leydig cells showed normal histology and color intensity, However with acrylamide treatment (Fig. 4B) Leydig cells showed severe atrophy and degeneration with reduction in size, numbers of cells and color intensity. Importantly, at the dose of 25 mgl kg lday ASA, (Fig. 4C) and at the dose of 50 mglkglday (Fig. 4D) of ASA, appeared to strongly prevent the Leydig cell

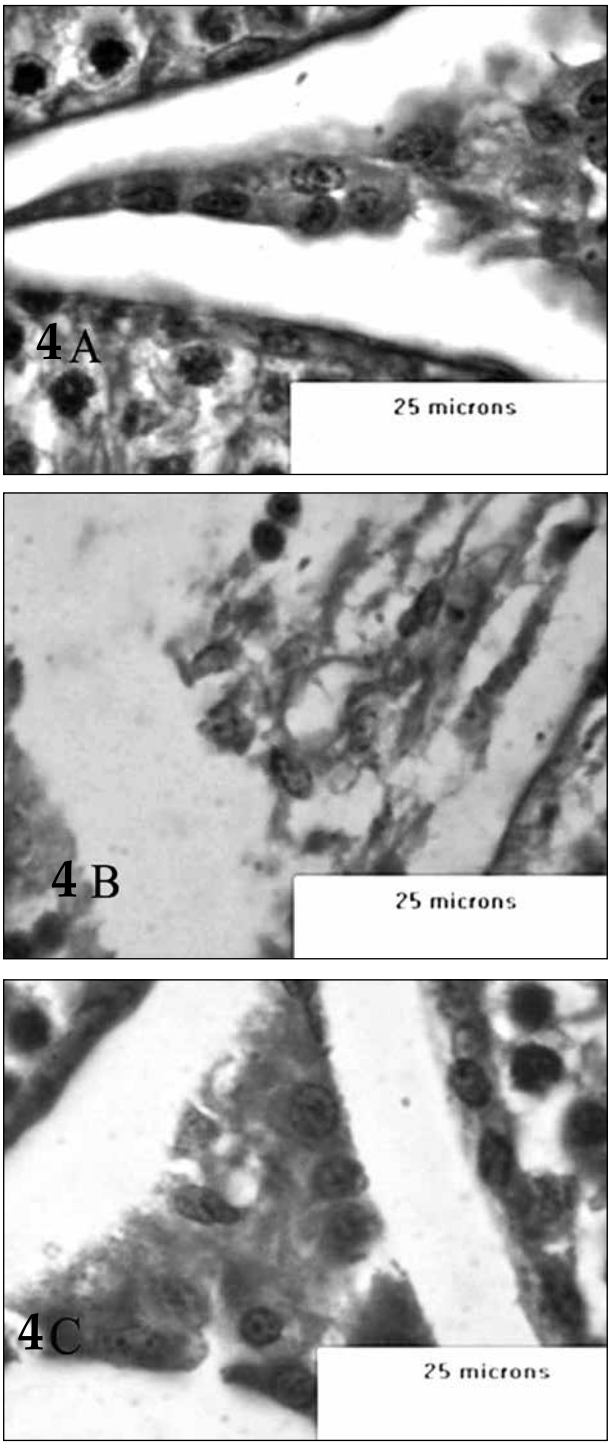


Fig. 4: Representative light microscopy of transverse sections for Leydig cells of the testes, indicating the AA toxicity and the protective effect of ASA on these cells. (A) Shows Leydig cells from control rat testis, with normal histology and color intensity, (B) Shows Leydig cells from AA (45 mg/kg) treated rat testis with severe atrophy and reduction in color intensity, **(**C) Shows Leydig cells from rat treated with AA and ASA at a dose of 25 mg/kg, indicating the protective action of ASA on AA induced toxicity on Leydig cells. Note the normal appearance of cells with increase in cells number and color intensity.

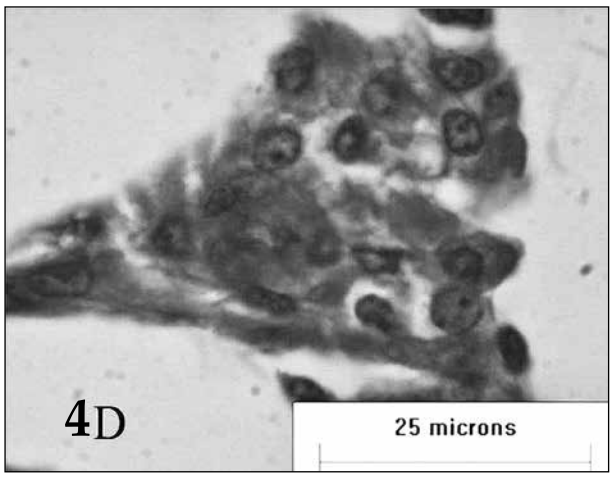


Fig 4 (D) Shows Leydig cells from rat treated with AA and ASA at a dose of 50 mg/kg, indicating the protective action of ASA at higher dose level. Note the normal appearance of cells with increase in cells number. Sections stained with H&E stain and viewed using light microscopy.

COMET assay in peripheral blood leukocytes

In the control group, group treated with 5-ASA, and gum acacia group the measured tail movement was (0.00 - 0.001) (Fig. 5A, B, C), which is consistent with a very low level of DNA damage. In comparison, the group treated with 45 mg/kg AA (Fig. 6A) demonstrated 8.3% olive shape COMET cells, (tail movement 37.5 - 44), indicative of massive levels of DNA damage. Co-treatment with 25 mg/kg ASA (Fig. 6B), demonstrated 8.2% COMET cells and dramatically reduced the tail movement of the detected COMET to (0.8 - 1.0), with no further protection observed with the higher ASA dose treatment (50 mg/kg, Fig. 6C).

Effects of ASA on AA induced changes in serum testosterone level

As expected, a significant reduction in blood testosterone level was detected in rats treated with 45 mg/kg AA when compared to the normal control group,

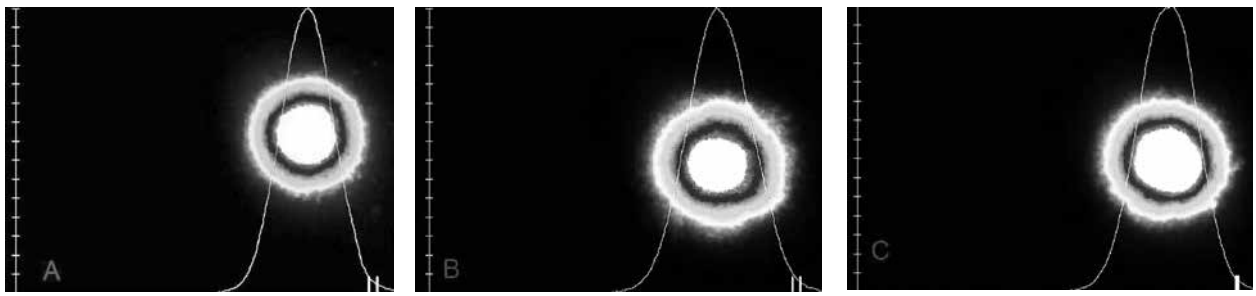


Fig. 5: Photographic Images of leukocytes from (A) control, (B) gum acacia (10%) and (C) ASA treated rats, subjected to single cell gell electrophoresis and subsequently analyzed by Image Analysis System. Tail Movement = 0.00 - 0.01.

atrophy detected with acrylamide treatment, because Leydig cells appeared normal in these groups of rats.

It should be noted that at both doses of ASA protection was not complete, as some of the tubules still appeared abnormal compared to control. However, the protective effect of the 50 mg/kg of ASA was generally, more clear than with the dose of 25 mg/kg ASA. However, recovery at both doses of 5-ASA was not complete at this five-days duration of experiment.

with no statistically significant differences detected between the control group and AA + ASA treated group. Further, there was a statistically significant increase in the level of circulating testosterone hormone among the AA plus ASA (25 or 50 mg/kg) treated rats when compared to AA control group (Fig. 7). Once again, ASA co-treatment appeared to prevent AA toxicity. This suggests that the suppressive effect of AA on circulating testosterone levels can be ablated by concomitant ASA treatment.

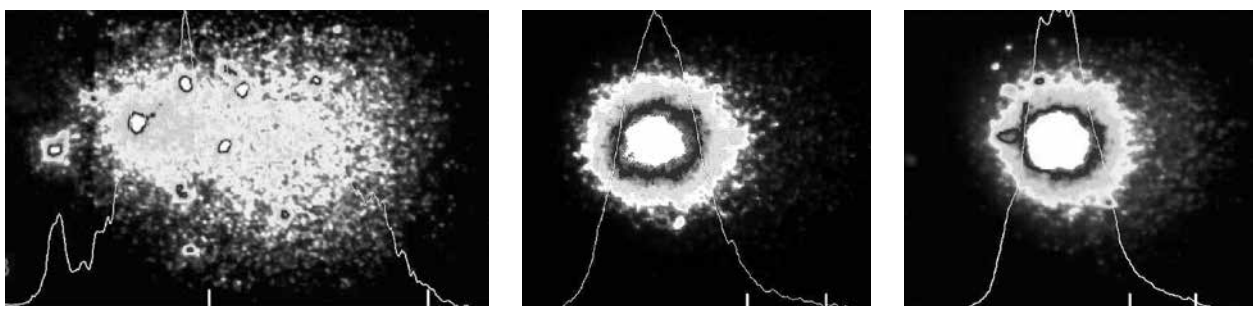


Fig. 6: Photographic images of leukocytes subjected to single cell gell electrophoresis and subsequently analyzed by Image Analysis System. (A) rats treated with 45 mg/kg AA, Tail movement (37.5 - 44), (B) rats treated with 25 mg/kg ASA, Tail movement (0.8 - 1.0), and (C) rats treated with 50 mg/kg ASA, Tail movement (0.8 - 1.1) .

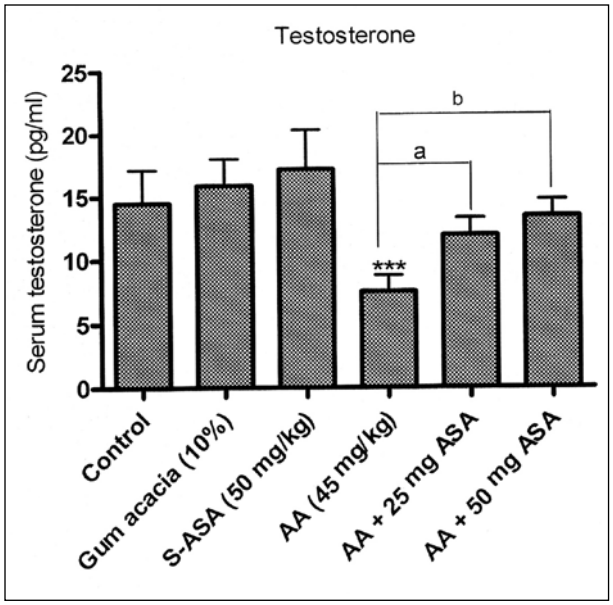


Fig. 7: Modulatory effect of ASA on serum testosterone concentration in AA-treated rats. Data were expressed as mean ± SD, n = 5. The symbol represents statistical significance (ANOVA) from normal control: ***, $p < 0.001$, followed by Tukey-Kramer multiple comparison test. The letter (a) represents statistical significance (ANOVA) from AA control, $p < 0.01$ and (b) represents statistical significance (ANOVA) from AA control, $p < 0.001$.

Effect of AA and ASA on CYP2E1 expression in liver and testis S9 fractions

CYP2E1 has been linked to the mechanism of AA-mediated toxicity, due to both the ability of this enzyme to readily become uncoupled and produce ROS and the CYP2E1-catalysed metabolism of AA to the reactive molecule glycidamide[22,23]. A calibration curve was constructed from mean absorbance versus concentration, using the least square regression equation with correlation coefficients (r^2) routinely ~ greater than 0.999. As shown in (Fig. 8A), CYP2E1 is expressed in the S9 fraction from control rats liver at a higher concentration (0.44 ng/mg protein) than in the control testis (0.074 ng/mg protein) (Fig. 8B), which is perhaps not surprising, given the important role of the liver in xenobiotic metabolism. However, whereas, AA caused a significant reduction in CYP2E1 ($p < 0.01$) levels in the liver when compared to the normal control, the impact of AA- treatment was the reverse in the testes when compared to the control, with CYP2E1 concentrations increased up to two-fold when compared to the normal control in the testis. It should be noted that the decrease in CYP2E1 levels in the liver was not due to liver weight changes, because no significant difference was detected in liver weight between all groups, with mean liver weight of 9.77gm (Fig. 9).

In the liver, co-treatment of AA-treated rats with ASA at a dose of 25 mg/kg/day for five days does not show any significant difference when compared to AA control group . However, at a dose of 50 mg/kg ASA there was a significant increase ($p < 0.01$) in the level of CYP2E1 when compared to AA control group, which indicates the strong reversing action of this higher dose of ASA on acrylamide effect on CYP2E1 level in the liver, with increasing rate of AA metabolism. This

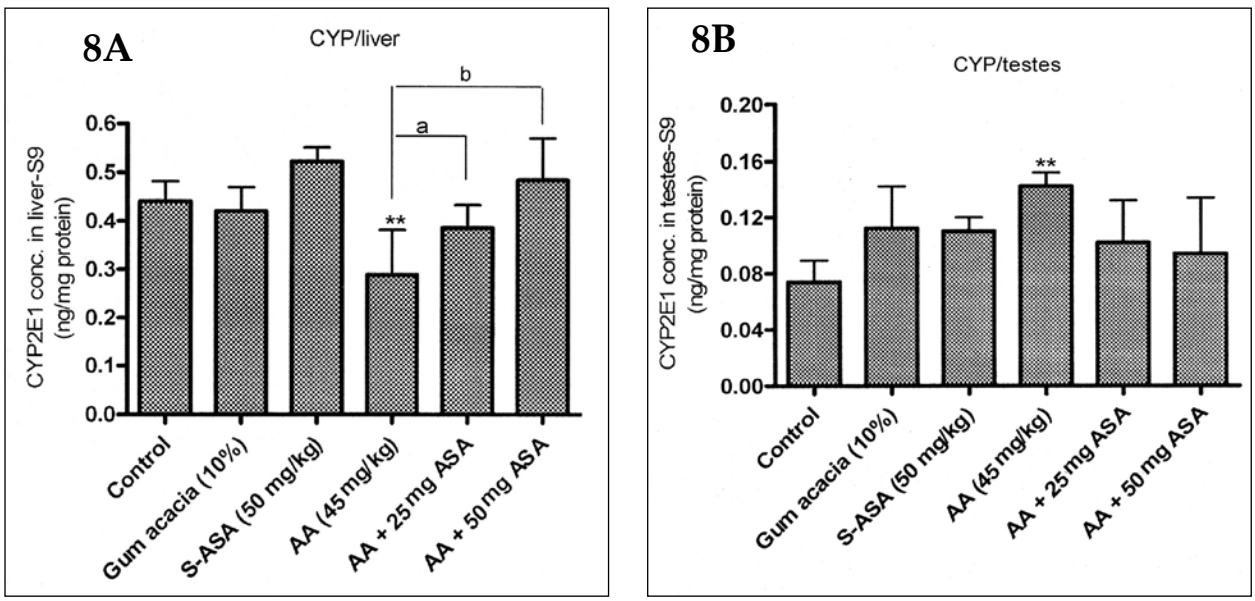


Fig. 8: CYP2E1 concentration in liver (A) and testis (B) S9, following AA and ASA treatment in rat, as detected by ELISA 3h after the last doses. Acrylamide was administered by oral gavage at a dose of 45 mg/kg/ day for 5 consecutive days to adult male rats. Data are expressed as mean ± SD, n = 5). The symbol represents statistical significance (ANOVA) from normal control: **, $p < 0.01$, followed by Tukey-Kramer multiple comparison test. The letter (a) represents no statistical significance from AA control and (b) represents statistical significance (ANOVA) from AA control, $p < 0.01$.

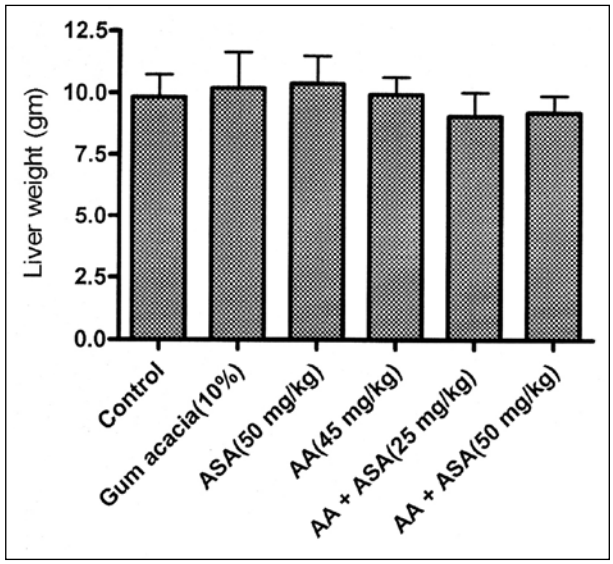


Fig. 9: Mean liver weight of control, AA and ASA treated rats Acrylamide was administered by oral gavage at a dose of 45 mg/ kg/ day for 5 consecutive days to adult male rats. (Data expressed as mean ± SD, n = 5).

is again consistent with the hypothesis that ASA can protect the rat body from the toxic effects of AA, and expands this to include other organs than the testes, such as the liver.

In the testis, AA exposure caused induction of CYP2E1($p < 0.01$) when compared to normal control. In addition, exposing AA treated rats to two different doses of ASA does not show any significant difference from AA control group in the testis.

DISCUSSION

In this study the potential role of the antioxidant 5-ASA in protection of AA- induced reproductive toxicity following a sub-acute exposure to AA was evaluated in rats. Concomitant treatment of 5-ASA with acrylamide was effective in preventing AA-mediated suppression in the blood testosterone level at both examined doses of ASA. In addition, ASA co-treatment improved the histological appearance of the testis, reduced the tail movement in the detected COMET cells in the blood, and suppressed CYP2E1 induction in the liver.

The result of this study indicate that, while a significant reduction was found between the 45 mg/ kg AA group and the normal control with respect to sperm count per cauda, there was no significant difference from AA control group at both tested ASA doses. The result of the current study was consistent with the observed effects of ASA in ulcerative colitis patients[24], which reported a reduction in total sperm count in patients suffering from ulcerative colitis. After changing treatment from sulphasalazine to 5-ASA, some improvement in spermatozoa function was noted, resulting in an improvement of fertility in those patients. Further O'Marain *et al*[25] reported that 13 slow acetylator patients had significantly lower sperm counts (6.3×10^6) and motility than 9-fast acetylator patients (41.2×10^6). Importantly, these data also support the idea that the protective effect of ASA against testicular toxins is applicable to both humans and the model system used herein (*i.e.*, rats).

In contrast, other investigators[17] reported a significant increase in total sperm count at (25 and 50 mg/kg) of 5-ASA as compared with endosulfan (7.5 mg/kg) alone, which was documented previously to cause a reduction in total sperm count. However, in the previous study, 5-ASA at a dose of 25 mg/kg for 10 days was reported to improve sperm counts more than at a dose of 50 mg/kg without complete recovery. This improvement in sperm count after a high dose of 5-ASA could be due to the established antiprostaglandin-like activity of ASA, as was reported by Moskov *et al*, and indeed ASA has been suggested as a potential treatment in some cases of unexplained oligospermia, as sperm improvement has been observed after antiprostaglandin therapy[26]. In the current study the reason for the lack of significant effect of ASA on AA-treated rats with respect to sperm count / cauda, could be due to dose, or short duration of exposure to ASA.

A striking feature of this study was the effect of 45 mg/kg AA on serum testosterone concentrations when compared to the normal control. In the current study, blood was taken 24h after the last dose, and the result showed that there was a very significant reduction in blood testosterone level following treatment with 45 mg/kg AA. The reduction in serum testosterone following AA exposure is consistent with the reports of Yang *et al*, who demonstrated a significant reduction in testosterone concentration by using radioimmunoassay, in sera of AA-treated rats at a dose of 30, 45, and 60 mg/kg/day for five days followed by three days of observation[27]. Moreover, testosterone concentration in the culture medium of Leydig cells after incubation for 24h, decreased significantly in all AA-treated groups, indicating that testosterone reduction was due to influence of acrylamide, presumably caused by the observed dose-dependent Leydig cell death[27]. As a result of increased Leydig cell death, testosterone level in the testis is likely to be decreased, resulting in a reduction in spermatogenesis. The result of the current study further showed that both doses of ASA can cause significant increase in the level of circulating testosterone level when compared to the AA control, which indicated that 5-ASA was successful at antagonizing the toxic effect of AA in reducing testosterone concentration in serum. In contrast, another study reported that 5-ASA could cause a reduction in the level of testosterone, when 7.5 mg/kg endosulfan was given to the rats for 10 days, during which testosterone was significantly increased,

but this was not recovered following an additional 10 days treatment with 25 mg/kg ASA. It was stated by the authors that the mechanism by which 5-ASA causes a reduction in testosterone level is not clear, although it is not clear if this is really a case of ASA causing a reduction, or failing to prevent the endosulfan-mediated reduction[17]. The powerful antioxidant capacity of 5-ASA exerted on the Leydig cells, could be the cause of its influence on testosterone level, as it was reported previously that Leydig cell atrophy and death could be the cause of testosterone reduction in AA-treated rats[27].

Further[28,29], it was reported that 7, 12-dimethylbenz(a)anthracene and many other polycyclic aromatic hydrocarbons are metabolically activated to active metabolites in rat Leydig cells. Also, CYP2E1 is documented to have a role in the metabolic activation of various toxicants and carcinogens such as benzene, styrene, acrylonitrile, vinyl carbamate and many other halogenated hydrocarbon compounds[30]. Taken together, these data suggest that CYP2E1 expressed in Leydig cells, will potentiate the toxicity of these compounds with increasing their active toxic metabolites with excessive release of free radicals, causing increased oxidative stress in the Leydig cells. Hence, the antioxidant activity of ASA might improve the Leydig cell toxicity after AA treatment leading to increased testosterone level.

The result of the current study indicated that AA at a dose level of 45 mg/kg causes histopathological changes in the testis of the rats. These include tubule disruption, reduction in the luminal sperm reserve, shedding of normal germinal epithelium in the lumen of the seminiferous tubules, maturation arrest in some tubules and multinucleated giant cells with vacuolations in between inner cells of the tubules. Histopathological changes in rat testis after AA treatment were well-documented previously by Yang *et al*[27,31] and the results of this work supports these studies. AA treated rats co-exposed to 25 mg/kg ASA showed signs of reparation with significant increase in germ cell population in most of the tubules, compared to AA alone, although the recovery was far from complete. This result is in agreement with Jaiswal *et al*, study[17]. When male rats were exposed to endosulfan, together with a dose of 25 mg/kg of 5-ASA in a preventive study the rats showed signs of recovery and improvement in spermatogenesis. In the current study, the dose of 50 mg/kg of 5-ASA was much better in reducing the signs of AA-induced toxicity than with the dose of 25 mg/kg of 5-ASA, although at both dose levels of 5-ASA the recovery was not complete. This protective action of 5-ASA on AA induced histopathological changes might be due its antioxidant power to inhibit oxidative damage which depends on its ability to scavenge free radicals and by acting as a chain-breaking antioxidant with interference with the initiation and progression of lipid peroxidation[17]. Its antiprostaglandin property might improve signs of the accompanying inflammatory process as well. As a result, an improvement in intratubular testosterone levels could result, leading to an improvement on spermatogenesis. Another important factor that improves testosterone levels is the effect of 5-ASA on Leydig cell atrophy. AA causes atrophy of these cells with reduction in serum testosterone level. 5-ASA prevents Leydig cell atrophy and hence maintains normal testosterone level which results in normal spermatogenesis.

This work showed the tremendous effect of 5-ASA on COMETs produced by AA in blood lymphocytes. ASA co-treatment results in dramatic reduction in COMETs tail movement, which indicates a reduction in genotoxic damage. Our hypothesis was that AA and not glycidamide is responsible for reproductive and genotoxicity. For this reason, any compound that causes increase in AA metabolism will improve the level of genotoxicity produced by AA. In this study 5-ASA has been shown to exert an induction effect on CYP2E1 expression, which results in an improvement in the level of genotoxicity when compared to the group that received AA alone. Moreover, as free radicals produced by AA may impair DNA repair[32],oxidation is an important mechanism of cell damage, which initiates a chain-reaction of lipid peroxidation that will spread through the membrane causing cleavage of unsaturated fatty acids and alteration of integral protein function leading to cell dysfunction and death[33]. 5-ASA acts as a free radical scavenger[34], which characterizes its powerful antioxidant property, and leads to the beneficial effects observed when it is concomitantly given with AA.

A novel finding of the current study was the effect of 5-ASA on CYP2E1 induction in liver and testis. The results showed that CYP2E1 is normally present in the testes (0.074 ng/mg protein) with lower amount than the liver (0.44 ng/mg protein); further, this study demonstrated for the first time, that 5-ASA at the dose of 50 mg/kg caused a significant elevation (induction) of CYP2E1 in the liver when compared to AA control. Also AA caused a significant reduction in enzyme level when compared to the normal control, while in the testis AA caused significant increase in CYP2E1 level when compared to the normal control. These findings were highly consistent with the report of Jiang[35], who identified the presence of CYP2E1 mRNA in rat prostate and testis, by reverse transcription PCR, southern blotting and DNA sequencing. From the immunoblotting result, P4502E1 appears to be present in very low amounts in the testis. The contents of P450 2E1 in testicular microsomal fractions were determined to be 0.12 pmol /mg protein which increases > 2-fold (0.25 pmol /mg protein) after pyridine treatment, which

was used as an enzyme inducer. It is well documented that the expression of CYP2E1 is highest in the liver, mainly found in the endoplasmic reticulum. However it is present in small amounts in non–hepatic tissues such as kidney, nasal mucosa, lung, ovaries, testis, small intestine, colon, umbilical vein endothelial cells, lymphocytes and the brain[36]. This finding is of considerable potential importance, because it probably explains to a large extent the genotoxic insult produced in rat testis following AA treatment. While the physiological significance of P450 2E1 in testis remains unclear, the induction of this enzyme in testis might have important implications in testicular toxicity and function[17]. CYP2E1 metabolizes a wide variety of chemicals with different structures such as small and hydrophobic compounds, including potential carcinogens[36]. For this reason, the presence and inducibility of CYP2E1 in the testis may be of significance in the bioactivation of environmental chemicals to genotoxic metabolites. Based on the well-known and documented role of CYP2E1 in epoxidation of AA to its active metabolite glycidamide[10,23], as a consequence of the presence of CYP2E1 in testis, despite the apparently low level of expression, enhanced P4502E1 mediated metabolic activation in testes by exposure to inducers that are environmental pollutants such as AA, may influence adverse effects on spermatogenesis and hence on reproduction[35]. The result of the present study hints that glycidamide is not only formed mainly in the liver by CYP2E1 and transferred by the blood to testis, but it also formed locally in the testis in small amounts.

However, during this study the concomitant treatment of AA-treated rats with the dose of 50 mg/kg 5-ASA, higher level of CYP2E1 was detected in the liver when compared to AA control, which indicated that 5-ASA can induce CYP2E1 in the liver and (might be due to protein stabilization) as a consequence, increases rate of AA metabolism with less ROS formation. Due to antioxidant property of ASA, the formed glycidamide will further be metabolized to inactive form and then excreted causing less genotoxicity and less damaging effect. This was clearly observed in this study on COMETs produced by AA in peripheral blood lymphocytes, and histopathology in the testes. In contrast, in the group exposed to AA alone, they showed significant reduction in CYP2E1 level in liver. The interpretation is that as the level of CYP2E1 in the liver is not impressively high compared to some other P450 isozymes[36], this large dose of AA over five days of treatment might cause partial depletion of this enzyme with significant reduction in its level in the liver. Another explanation is that AA itself may cause inhibition of the enzyme it induced and this needs further investigations. However in the testis, normally the amount of the expressed CYP2E1 is less than that in the liver. Therefore, it remains high because the major part of the given AA is metabolized by CYP2E1 in the liver and the small part of AA transferred to testis acts as an enzyme inducer but it does not cause depletion of the enzyme as in the liver. The mechanism by which 5-ASA exerts its effect in inducing CYP2E1in the liver is not entirely clear at the moment. However, it seems likely that the antioxidant property of 5-ASA is the reason behind the reduced toxicity seen after ASA treatment. Induction of CYP2E1 is also very effective in generating reactive oxygen intermediates such as superoxide radical and H_2O_2[37]. Thus, ASA seems to cause induction of CYP2E1 and hence increased AA metabolism with minimum ROS formation that reduces the AA testicular and genotoxicity.

CONCLUSION

5-ASA has been shown to protect partially or completely AA-treated rats from the severe testicular and genotoxicity resulting from AA treatment. Both doses of 5-ASA were effective in reducing COMETs in peripheral blood leukocytes. The most striking result of this study was the ability of 5-ASA to cause induction of CYP2E1 in liver and the effect of 5-ASA in reversing atrophy of Leydig cells in the testis. This impacts eventually, on the testosterone level. Further studies on AA and 5-ASA are needed to explore further molecular mechanisms involved in 5-ASA protection against AA testicular toxicity in rats.

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