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Testicular and epididymal toxicity induced by benzo(a)pyrene, alcohol, and their combination in Wistar rats

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SUMMARY

Alcoholism and cigarette smoking are pervasive problems that have been implicated in human health. In this study, independent and combinative toxicities of alcohol and benzo(a)pyrene (BaP) were tested for reproductive toxicity in rats. Male Wistar rats were exposed to BaP (100 µg/kg body weight) on alternative days and alcohol (2 g/kg body weight/day) daily, either individually or in combination for 60 days. Exposure to BaP or alcohol significantly decreased fertility index, reduced number of implantations associated with elevated pre- and post-implantation losses. The relative weights of testes, epididymis, seminal vesicles, and prostate gland were significantly decreased in BaP or alcohol administered rats. Exposure to BaP or alcohol significantly decreased daily sperm production, sperm density, motile, viable, HOS-tail swelled sperm, testicular 3β- and 17β-hydroxysteroid dehydrogenase activity levels, mRNA levels of steroidogenic acute regulatory protein, and serum testosterone levels. Further, *in silico* studies revealed binding of BaP at the hydrophobic tunnel of StAR protein. Additional studies disclosed stable interaction of BaP with amide group of ASN150 and hydroxyl group of THR263 by forming three hydrogen bonds. Our results also showed that treatment of rats with BaP or alcohol caused a marked increase in levels of superoxide anions, hydrogen peroxides, lipid peroxidation in testis and epididymis. Conversely, glutathione levels, and activity levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase in testis, as well as in epididymis decreased significantly in the experimental rats. Under the same conditions, increased fragmented DNA levels were observed in sperm. The results of the present study indicate that exposure to BaP or alcohol adversely affected male reproductive functions which may be at least in part due to, androgen deficiency and/or oxidative stress-related mechanisms. Consistently, present results also showed higher reproductive toxicity upon exposure to

combinations of BaP and alcohol than their individual treatments. Therefore, this combination was classified as additive and synergistic responses of the BaP and alcohol.

Keywords: Benzo(a)pyrene, alcohol, steroidogenesis, spermatogenesis, oxidative stress, DNA damage.

Introduction

Approximately 15% of the sexually active population is affected by clinical infertility and in which male factor accounts for about 20-70%.[1,2] The cause of deterioration in reproductive health may be attributed to occupational exposure to some of the environmental persistent chemicals, metals etc. The association of life style factors such as tobacco smoking, alcohol consumption, use of modern electronic gadgets etc. has also been shown to adversely affect reproductive health. Tobacco and alcohol are the most addictive substances and tobacco smoking and alcohol consumption are often seen together in the society.

According to estimates, 35% of reproductively active males smoke.[1] Cigarette smoke contains more than 4000 toxic chemicals including BaP.[1] BaP, an ideal environment pollutant and well acknowledged member of polycyclic aromatic hydrocarbons (PAHs), is the one which is present both in mainstream and sidestream smoke.[3] Although, cigarette smoking is a common source of human exposure to BaP, other important sources include incomplete combustion of fossil fuel, automobile exhaust, industrial emissions, forest fires, air pollution, diet (barbecued foods and cereal grains), charcoal boiled foods, soot, and coal tar .[3] BaP binds to the cytosolic aryl hydrocarbon receptor (AHR), translocates to nucleus and induces the expression of aryl hydrocarbon hydroxylases (cytochrome P450s).[4] Costa *et al*.[5] also reported that cytochrome P450 enzymes metabolize BaP in liver, gill, and intestine of Nile tilapia. Biotransformation of BaP occurs oxidatively by cytochrome P450 enzymes and leads to the formation of reactive metabolites such as BaP-7,8-dihydrodiol 9,10-epoxide and quinones. The reactive intermediates can generate reactive oxygen species (ROS),[6] and cause DNA damage.[7] It is also reported that the accumulation of BaP metabolites have been detected in male reproductive system, particularly in the testis and epididymis of treated animals.[8] BaP exposure

results in oxidative stress as evidenced by increased ROS generation and decreased antioxidant defense system in lungs, liver, and brain tissues of rats.[9] Exposure to BaP also results in DNA damage at all stages of spermatogenesis in human sperm using comet assay.[10] Ramesh *et al*.[11] reported that exposure to BaP is responsible for reduced testicular and epididymal function in rats. Mice exposed to BaP experienced a significant decrease in concentration of germ cells, percentages of motility, viability, and normal morphological spermatozoa.[12] Further, it has also been reported that BaP is effective in reducing the levels of testosterone possibly by targeting StAR protein.[13,14]

Consumption of alcoholic beverages has been an integral part in many cultures since beginning of ancient history and also considered as lifestyle in association with fun, recreation, and other evening activities. Alcohol abuse has been related to more than 200 disease and injury conditions in individuals, including neuropsychiatric problems, cardiovascular diseases, gastrointestinal diseases, intentional and unintentional injuries, cancer, diabetes mellitus, fetal alcohol syndrome, infectious diseases, and reproductive disorders, and contribute to alcoholism-related morbidity and mortality.[15,16] Chia *et al*.[17] reported that about 42% of men with infertility cases consume alcohol. About 25-80% of males with infertility record high levels of reactive oxygen species (ROS).[18,19] Oxidative stress occurs when the production of ROS overwhelms the intrinsic anti-oxidant defenses. Ethanol administration caused an elevation in malondialadehyde and nitric oxide levels associated with diminished activity levels of superoxide dismutase, catalase, and glutathione peroxidase in testis of rat.[20] Alcohol consumption and oxidative stress have also been linked to DNA damage.[21] Alcohol intoxication is associated with reduced testicular weight,[22,23] morphological deterioration in testis,[24] diminished steroidogenic enzyme activities,[25] testosterone deficiency,[23] testicular atrophy,[26] and sexual dysfunction.[27] It is effective

in reducing the quality and quantity of sperm with increased morphologically abnormal sperm.[23,28,29] Arco *et al*.[30] showed that ethanol exposure results in altered expression of proteins in epididymis thereby results in diminished fertilizing ability of spermatozoa.[31]

Previously, we have demonstrated that BaP and alcohol exposure results in compromised reproductive health in adult male rats.[14,23,29] Although the individual evidences addressing the adverse reproductive outcomes resulting from exposures to BaP and alcohol have been demonstrated, the studies pertaining to combined effect of both in relation to male reproductive health are sparse. It is reasonable to suspect, that at times, humans are simultaneously exposed to both alcohol and BaP, this study focuses on the effect of alcohol and BaP, alcohol separately and combined on reproduction in male rats.

Materials and methods

Procurement and maintenance of animals

Male Wistar albino rats with a body weight of 190±10 g (90 days old) were purchased from an authorized vendor (Sri Ragavendra Enterprises, Bengaluru, India). Rats were housed (four per cage) in clean polypropylene cages (18" x 10" x 8") containing paddy husk as bedding material and were provided with standard rodent chow (obtained from Sai Durga Agencies, Bengaluru, India) and tap water *ad libitum*. Rats were acclimatized (temperature 22-25°C; 12:12 hr light:dark cycle) for one week before being used for experimentation. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India,[32] Ministry of Social Justice and Empowerment, Government of India. All the procedures were approved by the Institutional Animal Ethical Committee (Regd. No. 438/01/a/CPCSEA/dt.17.07.2001), and having resolution No: 57/2012/(i)/a/CPCSEA/IAEC/SVU/PSR- KPR dt.08-07-2012. During the study, maximum

care was taken to minimize animal suffering, and in addition, the number of rats was kept at a minimum. The study is planned and organized as completely double blind.

Chemicals

BaP (≥96% purity by HPLC), androstenedione (≥98% purity), dihydroepiandrosterone (98% purity), NAD (≥98% purity) and NADPH (97% purity), INT (95% purity), horse radish peroxidase (>90%), glutathione reductase (≥90% purity), reduced glutathione (≥98% purity), and oxidized glutathione (≥98% purity) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Dimethyl sulfoxide (purity ≥99%) (DMSO), malondialdehyde (purity ≥95%) (MDA), pyrogallol (purity ≥99%), were from Merck, Darmstadt, Germany. RNA isolation kit, and cDNA synthesis kit were from Fermentas (Lithuania, India). All other chemicals used in study were from HIMEDIA (Bangalore, India).

Experimental design

Animals were divided randomly into 4 groups (n=6 per group). The animals in group 1 were intraperitoneally injected with 100 µL of 0.5% DMSO diluted in distilled water and served as control. Rats in Group 2 were injected intraperitoneally with BaP (100 µg per kg body weight) dissolved in 0.5% DMSO (the volume of injection was 100 µL per animal), on alternative days for a period of 60 days. Animals in group 3 were administered orally with 40% alcohol at a dose of 2 g/kg body weight daily via orogastric tube for a period of 60 days. Animals in group 4 were treated with BaP and alcohol same as in groups 2 and 3.

During the experimental period, rats were observed daily for overall appearance and signs of toxicity such as postural abnormalities, unusual respiration, vocalization, salivation, head flicking, abnormal appearance of fur, compulsive biting, urination, circling and walking backward. Food and water intake were monitored once in a week throughout the experimental

period. Control and experimental rats were checked for fertility efficiency after the completion of treatment period. The initial body weight on the first day and the final body weight on the terminal day (prior to necropsy) of the experiment were determined and used to calculate body weight gain over the 60-day period of treatment.

Rats were fasted overnight before being euthanized by ether anesthesia and cervical dislocation on the day following the last treatment. After blood was collected, brain, liver, kidney, spleen, testes, epididymis (caput, corpus, and cauda), vas deferens, seminal vesicles, prostate gland, and penis were dissected out, and immediately weighed to the nearest milligram by using Shimadzu electronic balance (Model No: BL- 220H; Kyoto, Japan). Tissue somatic indices (TSI) were calculated by using the following formula:

TSI = [weight of the tissue (g) / body weight of the animal (g)] × 100

Testes were used for determination of daily sperm production and biochemical studies whereas epididymides were used for the determination of sperm analysis and biochemical studies. Further, sperm were also used for DNA analysis. Serum was separated by centrifugation for the determination of testosterone concentrations.

Sperm parameters

Daily sperm production (DSP)

DSP was determined by the procedure previously reported.[33] Testis was reweighed and the tunica albuginea was removed. Testis was homogenized in 0.9% sodium chloride solution containing 0.05% Triton X-100 solution using glass Teflon homogenizer. After staining by trypan blue, unbroken nuclei of elongated spermatids were counted on the haemocytometer. The number of sperm produced per gram of testicular tissue per day was obtained from the formula

of testicular spermatid numbers/6.1 days of sperm production period for rats. Its efficiency was DSP/testicular weight.

Epididymal sperm analysis

Sperm were collected from the right tail of the epididymis by mincing with scissors into 2.0 ml of M199 containing 0.5% fetal bovine serum. Suspensions were incubated at 37ºC for 15 min to release sperms and pippetted 20 times without bubbling. The sperm was counted using a Neubauer chamber, as described by Belsey *et al*.[34] The sperm sample was further diluted (1:3) with the M199 with Hank's salts and L-glutamine containing 0.5% fetal bovine serum and incubated for 5 min at 37ºC. The motility of 200 sperm was observed in each sample in a Neubauer hemocytometer, and expressed as percentage of the motile sperm of the total sperm counted as described by Belsey *et al*.[34] The ratio of live and dead spermatozoa was determined using 1% trypan blue by the method of Talbot and Chacon.[35] Unstained spermatozoa were considered as viable and stained were taken as dead. Sperm viability was expressed as percentage of unstained sperm of the total sperm counted. The membrane integrity of the sperm was determined by exposing the sperm to hypoosmotic medium and observed for swelled tails [hypoosmotic swelling (HOS)] under microscope, and the percent of tail swelled sperm was estimated by the method of Jeyendran *et al*.[36] To determine sperm morphological abnormalities, an aliquot of sperm suspension (50 µl) used for motility analysis was further diluted in 0.5 ml of M-199 medium containing 10 µl of 37% formalin. A 10 µl drop of the diluted formalized sperm sample was placed on a glass slide, cover slipped, and examined with a phase contrast microscope (400 X total magnification). About two hundred sperms from each animal were evaluated and classified as follows: normal, head defect, and tail defect.

Assay of testicular steroidogenic marker enzymes

The testicular tissue was homogenized in ice-cold Tris-HCl buffer (pH 6.8). The microsomal fraction was separated and used as the enzyme source. The activities of 3β- and 17β-hydroxysteroid dehydrogenases were measured by the method of Bergmeyer.[37] The reaction mixture in a volume of 2.0 mL contained: 100 mmol of sodium pyrophosphate buffer (pH 9.0) and 0.5 mmol cofactor (NAD for 3β-hydroxy steroid dehydrogenase (HSD) and NADPH for 17β-HSD), 0.08 mmol of substrate (dehydroepiandrosterone for 3β-HSD and androstenedione for 17β-HSD), and 100 mL of enzyme source. The reactions were carried out in a quartz cuvette of 1.0 cm path length at 23ºC. The absorbance at 340 nm was measured at an interval of 20 seconds for 3 minutes in an UV-visible spectrophotometer (Hitachi U-2001). The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration. The activities of the enzymes were expressed as nmol of NAD converted to NADH/mg protein/min for 3β-HSD or nmol of NADPH converted to NADP/mg protein/min for 17β-HSD.

Determination of serum testosterone concentrations

Serum testosterone concentrations were determined using an enzyme-linked immunosorbent assay kit (Oxford Biomedical Research Inc., Oxford, MI, USA).[33]The concentrations were calculated from a calibration curve using authentic testosterone. Cross-reactivity was 100% for both testosterone and 5a-dihydrotestosterone, 0.02–0.13% for estradiol and testosterone esters, and less than 0.01% for corticosterone and progesterone. The estimated limit of detection (sensitivity) was 2 pg/ml. The mean intra-assay and inter-assay variations were 2.4% and 6.7% respectively.

Determination of levels of StAR mRNAs by semi-quantitative RT-PCR

Testes were removed from animal and immediately processed for RNA isolation. Total RNA was extracted from testis according to manufacturer's protocol using RNA isolation kit (Fermentas, Lithuania). The purity and concentration of extracted RNA was measured using Nano Drop (NanoVue Plus Spectrophotometer, GE Healthcare Life Sciences, USA.), and 1 μg of total RNA was used for complementary DNA (cDNA) synthesis using RevertAid cDNA synthesis kit (Fermentas, Lithuania). The following primer sets were used to amplify StAR and GAPDH (internal control).

StAR

Forward: 5'TTGGGCATACTCAACAACCA3' (Gene bank accession No.NM031558)

Reverse: 5'ATGACACCGCTTTGCTCAG3'

GAPDH

Forward: 5'AGACAGCCGCATCTTCTTGT3' (Gene bank accession No. NM017008)

Reverse: 5'CTTGCCGTGGGTAGAGTCAT3'

All the primers were synthesized by Eurofins Genomics Pvt. Ltd., India. The amplification of synthesized cDNAs was performed by using a Peltier Thermal Cycler (Corbett model No.CG1-96). The PCR products were run on 1.8% agarose gels in TBE buffer and the relative intensities of the StAR, bands were normalized to the corresponding GAPDH band intensities.

Biochemical estimations in testis, and different regions (caput, corpus, and cauda) of epididymis

A 10% (w/v) homogenate of tissues was prepared in normal saline with the help of motor driven glass-Teflon homogenizer and centrifuged at 800 g for 20 min at 4^{o}C. The supernatant was used for biochemical assays. The levels of superoxide anion,[38] hydrogen,[39] lipid

peroxidation,[40] reduced glutathione,[41] and activity levels of superoxide dismutase,[42] catalase,[43] glutathione peroxidase,[44] glutathione reductase,[45] were determined in the supernatants. Protein content in the enzyme source was determined by the method of Lowry *et al*.[46]

Determination of sperm DNA quality

The integrity of sperm DNA was determined by alkaline comet assay.[47] Sperm samples collected from cauda epididymis were processed immediately. Sperm samples embedded in low melting agarose were submitted to cell lysis, DNA unwinding, electrophoresis and neutralization and then were dehydrated in absolute ethanol, dried, stored at room temperature. Two slides were made from each animal. Before microscopic evaluation, the slides were rehydrated and stained with ethidium bromide. Images of nucleoids from a fluorescence microscope (Zeiss company, Germany) were captured with Cool SNAP® Pro color digital camera. One hundred nucleoids were scored from each slide. The nucleoid length (μm) was determined by Image Pro® plus software.

Fertility studies

After completion of treatment period, control and experimental male rats were cohabited with untreated normal cyclic female rats (1:2) in pro-estrus stage for 6 days. Females were checked twice daily for the presence of vaginal plugs, and vaginal washings were evaluated for the presence of sperm everyday morning.[48] Females with positive plugs and/or sperm were separated from males. Six pregnant rats from each group were laparotomized on 6th day of pregnancy. Both uteri were removed and examined for the number of implantations. In addition, both ovaries were removed and the number of corpora lutea was counted. Remaining six pregnant rats were sacrificed on day 18 of pregnancy and the number of fetuses and resorption sites in uteri was counted. Data were analyzed to determine the conception time (the interval

between the first day of cohabitation and the day of plug and/or sperm in females), mating index (number of sperm-positive females/number of pairings), fertility index (number of pregnant females/number of pairings), pre- implantation loss (number of corpora lutea-number of implantations/number of corpora lutea) X 100) and post-implantation loss (number of implantations-number of live fetuses/number of implantations) X 100).

Molecular dynamics simulation of StAR protein-BaP complex

The crystal structure of StAR protein of humans (PDB ID: 3POL) was retrieved from Protein Data Bank (http://www.rcsb.org/pdb). In order to prepare the model structure for docking studies, it is loaded into Molecular Operating Environment (MOE) modeling software and all water molecules and hetero atoms were removed. The structure was protonated at an implicit solvated environment (Born salvation model) performed at a temperature of 300K, pH of 7, polar hydrogens were added and with a salt concentration value of 0.1. A non-bonded cut off value of 8-10A^{o} was applied to the Leonard-Jones terms. After protonation, energy minimization was done in MMFF94x force field at a gradient cut off value of 0.05. The BaP (ligand) was taken from PUB CHEM and docked into the StAR protein. Docking procedure was followed using the standard protocol implemented in MOE 2008.10. After the successful docking process, the best energy conformation of StAR-BaP complex was subjected to molecular dynamics simulation for a period of 10 nanoseconds (ns) by setting temperature to 300 K for a heat time of 10 ps. The time step was considered as 0.001, and the temperature relaxation time was set to 0.2 ps. The position, velocity, and acceleration were saved for every 0.5 ps.

Statistical analysis of data

The data were statistically analyzed using one-way Analysis of Variance (ANOVA) followed by the Tukey test using the Statistical Package for Social Sciences (SPSS) version 16.0.

The data were expressed as mean ± standard deviation. The limit of significance was set at $p < 0.05$.

Results

No significant changes were observed in overall appearance, body position, co-ordination or gait, activity, lacrimation and vocalization. No significant ($p > 0.05$) differences were observed in food and water intake (data not shown) of experimental rats when compared to rats in control group.

Body weight and weights of tissues

No Significant ($p > 0.05$) difference was observed in the body weight gain of experimental rats when compared to control rats (Table 1). The relative weights of brain, liver, kidney, spleen, vas deferens and penis in experimental rats were comparable to control rats (Table 2). Conversely, a significant decrease in relative weights of testes ($F = 91.176$; df = 3, 20; $p < 0.001$), caput epididymis ($F = 22.242$; df = 3, 20; $p < 0.001$), corpus epididymis ($F = 4.000$; df = 3, 20; $p = 0.022$), cauda epididymis ($F = 12.500$; df = 3, 20; $p < 0.001$), seminal vesicles ($F = 81.357$; df = 3, 20; $p < 0.001$) and prostate gland ($F = 21.429$; df = 3, 20; $p < 0.001$) was observed in experimental animals when compared to control animals (Table 2). Additional decrease in relative weights of testis (-28.12% and -29.59% compared to BaP and alcohol alone treated rats), caput epididymis (-6.31% and -8.25% compared to BaP and alcohol alone treated rats), corpus epididymis (-16.67% and -16.67% compared to BaP and alcohol alone treated rats), cauda epididymis (-22.22% and -30.00% compared to BaP and alcohol alone treated rats), seminal vesicle (-20.00% and -29.41% compared to BaP and alcohol alone treated rats), and prostate gland (-23.08% and -28.57% compared to BaP and alcohol alone treated rats) was

observed in rats treated with both BaP and alcohol, when compared to either BaP or alcohol alone treated rats (Table 2).

Spermatology

Daily sperm production (F = 136.92; df = 3, 20; $p < 0.001$) and epididymal sperm reserves (F = 359.07; df = 3, 20; $p < 0.001$), motile (F = 250.70; df = 3, 20; $p < 0.001$), viable (F = 170.88; df = 3, 20; $p < 0.001$) and HOS-tail swelled (F = 401.70; df = 3, 20; $p < 0.001$) sperm in BaP or alcohol treated rats were significantly decreased, with more number of head (F = 127.62; df = 3, 20; $p < 0.001$) and tail (F = 112.02; df = 3, 20; $p < 0.001$) abnormal sperms (Table 3). Further decline in the daily sperm production (-28.36% and -31.59% compared to BaP and alcohol alone treated rats), sperm density (-41.43% and -43.18% compared to BaP and alcohol alone treated rats), motile sperm (-24.10% and -25.58% compared to BaP and alcohol alone treated rats), viable sperm (-32.27% and -35.44% compared to BaP and alcohol alone treated rats), HOS-tail swelled sperm (-22.90% and -26.67% compared to BaP and alcohol alone treated rats) was observed in rats co-administered with BaP and alcohol when compared to either BaP or alcohol treated rats (Table 3).

The sperm nucleoid length showed a significant (F = 194.08; df = 3, 20; $p < 0.001$) increase in BaP and/or alcohol treated rats. The sperm nucleoid length of control, BaP, and alcohol treated rat sperm was 10.09 ± 1.30, 62.64 ± 7.07, and 70.64 ± 7.26 μm respectively, which was further increased (98.39% Vs BaP; 75.92% Vs alcohol) to 124.27 ± 12.87 μm in combinatorial treatment of both BaP and alcohol (Table 3; Fig. 1).

Steroidogenesis and testosterone levels

The activities of 3β- (F = 97.226; df = 3, 20; $p < 0.001$) and 17β- (F = 76.793; df = 3, 20; $p < 0.001$) HSDs (Table 4), and levels of StAR mRNA in the testes were significantly decreased

in rats exposed to BaP or alcohol (Fig. 2). The levels of serum testosterone were also significantly (F = 100.58; df = 3, 20; $p < 0.001$) reduced in rats exposed to BaP or alcohol when compared to control rats (Table 4). Co-administration BaP and alcohol resulted in further decrease in activities of 3β-HSD (-44.54% and -47.53% compared to BaP and alcohol alone treated rats), 17β-HSD (-31.89% and -41.87% compared to BaP and alcohol alone treated rats), expression levels of StAR mRNA, and serum hormonal levels of testosterone (-38.92% and -42.70% compared to BaP and alcohol alone treated rats) when compared to that of BaP or alcohol exposed rats.

Antioxidant parameters

The levels of superoxide anion (F = 70.834 in testis, F = 76.044 in caput, F = 64.951 in corpus, and F = 53.457 in cauda epididymis; df = 3, 20; $p < 0.001$), hydrogen peroxide (F = 66.636 in testis, F = 62.077 in caput, F = 78.363 in corpus, and F = 82.151 in cauda epididymis; df = 3, 20; $p < 0.001$), and lipid peroxidation (F = 63.933 in testis, F = 76.158 in caput, F = 55.013 in corpus, and F = 64.941 in cauda epididymis; df = 3, 20; $p < 0.001$) were significantly elevated in testis, caput, corpus, and cauda epididymal regions of BaP or alcohol treated rats (Tables 5-8). Further, co-administration of BaP and alcohol resulted in a further increase in production of superoxide anion (28.39% and 22.08% in the testis; 30.74% and 25.74% in caput epididymis; 30.03% and 25.02% in corpus epididymis; 29.72% and 22.85% in cauda epididymis compared to BaP and alcohol alone treated rats in testis), hydrogen peroxide (32.05% and 27.71% in the testis; 25.42% and 20.39% in caput epididymis; 18.50% and 29.63% in corpus epididymis; 25.95% and 19.80% in cauda epididymis, compared to BaP and alcohol alone treated rats), and lipid peroxidation (23.58% and 41.87% in the testis, 40.02% and 41.79% in caput epididymis; 20.65% and 23.46% in corpus epididymis; 16.18% and 14.99% in cauda

epididymis, compared to BaP and alcohol alone treated rats) when compared to BaP or alcohol treated rats. Conversely, the level of natural antioxidant glutathione was significantly reduced in testis (F = 52.357; df = 3, 20; $p < 0.001$), caput epididymis (F = 84.618; df = 3, 20; $p < 0.001$), corpus epididymis (F = 79.795; df = 3, 20; $p < 0.001$), and cauda epididymis (F = 52.964; df = 3, 20; $p < 0.001$) of BaP or alcohol exposed rats. Additional reduction in glutathione level was observed in testis (-19.14% and -16.79% compared to BaP and alcohol alone treated rats), caput epididymis (-21.65% and -20.82% compared to BaP and alcohol alone treated rats), corpus epididymis (-25.61% and -27.22% compared to BaP and alcohol alone treated rats), and cauda epididymis (-15.59% and -16.13% compared to BaP and alcohol alone treated rats) of rats co-administered with BaP and alcohol.

The activity levels of antioxidant enzymes such as superoxide dismutase (F = 141.99 for testis; F = 52.720 for caput epididymis; F = 80.426 for corpus epididymis; and F = 83.639 for cauda epididymis; df = 3, 20; $p < 0.001$), catalase (F = 57.698 for testis; F = 86.224 for caput epididymis; F = 99.054 for corpus epididymis; and F = 35.761 for cauda epididymis; df = 3, 20; $p < 0.001$), glutathione peroxidase (F = 66.849 for testis; F = 102.83 for caput epididymis; F = 61.866 for corpus epididymis; and F = 88.575 for cauda epididymis; df = 3, 20; $p < 0.001$), glutathione reductase (F = 77.349 for testis; F = 59.749 for caput epididymis; F = 55.034 for corpus epididymis; and F = 45.468 for cauda epididymis; df = 3, 20; $p < 0.001$) were significantly decreased in the testis, caput epididymis, corpus epididymis, and cauda epididymis of BaP or alcohol administered rats when compared with the control rats (Tables 5-8). Co-administration of BaP and alcohol resulted in further decrease in the activity levels superoxide dismutase (-49.02% and -40.46% compared to BaP and alcohol alone treated rats in testis, -24.60% and -12.84% compared to BaP and alcohol alone treated rats in caput peididymis, -

44.56% and -45.45% compared to BaP and alcohol alone treated rats in corpus epididymis, and -32.67% and -19.05% compared to BaP and alcohol alone treated rats in cauda epididymis), catalase (-21.14% and -24.22% compared to BaP and alcohol alone treated rats in testis; -19.72% and -24.83% compared to BaP and alcohol alone treated rats in caput epididymis; -14.26% and -15.37% compared to BaP and alcohol alone treated rats in corpus epididymis, and -17.83% and -20.77% compared to BaP and alcohol alone treated rats in cauda epididymis), glutathione peroxidase (-21.40% and -18.62% compared to BaP and alcohol alone treated rats in testis; -21.68% and -21.93% compared to BaP and alcohol alone treated rats in caput epididymis, -17.98% and -19.87% compared to BaP and alcohol alone treated rats in corpus epididymis; and -28.38% and -24.18% compared to BaP and alcohol alone treated rats in cauda epididymis) and glutathione reductase (-23.66% and -20.28% compared to BaP and alcohol alone treated rats in testis; -21.85% and -13.01% compared to BaP and alcohol alone treated rats in caput epididymis; -17.38% and -14.16% compared to BaP and alcohol alone treated rats in corpus epididymis; and -22.73% and -19.24% compared to BaP and alcohol alone treated rats in cauda epididymis) when compared to BaP or alcohol alone treated rats.

Fertility studies

All females mated with males in the control, BaP, alcohol, or BaP+alcohol groups had copulatory plugs (mating index 100%). All females in the control group had implantations. In contrast, implantations were present in eight of twelve in BaP group, nine of twelve in alcohol group and seven of twelve in BaP+alcohol group. The numbers of corpora lutea are comparable among all groups. Significant delay in conception time (F =64.707; df = 3, 38; $p < 0.001$) was observed in females mated with BaP or alcohol treated males when compared with controls. Mean number of implantations (F =236.66; df = 3, 20; $p < 0.001$), and mean number of live

foetuses (F = 296.82; df = 3, 20; $p < 0.001$) per dam decreased in females mated with BaP or alcohol treated rats. Conversely, pre- and post-implantation losses increased in females mated with BaP or alcohol treated males. All the fertility related parameters (delay in conception time: 64.10% and 57.54% compared to BaP and alcohol alone treated rats; mean number of implantations: -31.93% and -34.60% compared to BaP and alcohol alone treated rats; mean number of live fetuses: -31.59% and -35.08% compared to BaP and alcohol alone treated rats) further deteriorated in females mated with males treated with both BaP and alcohol (Table 9).

Docking studies

The StAR-BaP complex showed stabilized energy (Fig. 3) and root mean square deviation (RMSD) (Fig. 4) at 2500 kcal/mol and 4.5 A^o respectively and stable throughout 10 ns molecular dynamics simulation. BaP interacts with ASN150, ALA172, GLU173, ASN177, VAL179, LEU247, VAL259, GLN262 and THR263 of the hydrophobic tunnel of StAR (Fig. 5), with a binding affinity of -11.98 kcal mol^{-1}. Further, three stable hydrogen bond interactions were observed between BaP and StAR protein; one H-bond with amide group of ASN150 having bond length 3.1, and another two H-bonds with hydroxyl functional group of THR263 having bond lengths 2.3 and 2.7 (Fig. 3), whereas, molecular docking studies showed no interaction of either alcohol or its metabolite (acetaldehyde) with the hydrophobic tunnel of StAR protein. Further, binding affinity of alcohol and acetaldehyde with StAR was -2.7 and -2.5 kcal mol^{-1} respectively, which is negligible.

Discussion

Considering the reproductive-toxic potential of BaP and alcohol as well as their co-exposure especially, alcohol consumption and cigarette smoking go in hand-in-hand in modern society, the present study was conducted to investigate the reproductive toxicity effects in male

Wistar rats exposed to BaP and alcohol taken individually or combined. In the present study, the animals were administered with BaP and/or alcohol at the doses below No Observed Effect Level (NOEL) in order to evaluate if low doses of BaP and/or alcohol induce reproductive toxicity in rats. In the present study, rats were given 2.0 g alcohol/kg bw/day, corresponding to the consumption of 2 L of wine (10% alcohol) or 0.35 L (half bottle) of whiskey (40% alcohol) by a man of 70 kg body weight or 100 µg BaP/kg body weight, equivalent to BaP consumed by workers in coke ovens during occupation (42 µg BaP/m^3 in coke ovens), assuming that a worker inhales 9.6 m^3 of air per 8 hour workday, weighs 70 kg, works 5 days per.[49] Animals were administered with test chemicals for 60 days in order to evaluate their effects through a complete spermatogenic cycle which takes 55 days in Wistar rats.[50]

In the present study, exposure of rats to BaP or alcohol did not affect body weight gain, indicating that administration of BaP or alcohol did not change the metabolic activity of the animals. Jeng *et al*.[51] and Himabindu *et al*.[29] also reported that rats exposed to BaP and alcohol didn't alter body weight gain. The relative weights of brain, liver, and kidney in experimental rats were comparable with those of control rats indicating that the general metabolic condition of the animals was within normal range. Conversely, the relative weights of testes, epididymis, seminal vesicles, and prostate were significantly decreased in experimental rats when compared to respective relative weights of control rats. Similar results were reported earlier in rats exposed to either BaP[14] or alcohol.[23,29]

Testis weight is largely depending on the mass of the differentiated spermatogenic cells and requires continuous androgenic stimulation for its normal growth and function.[52] The observed reduction in the weight of the testes could be due to the reduction in the number of Leydig cells and germ cells as well as impaired Leydig cell LH responsiveness in experimental

rats. The reduction in epididymis weight may be due to reduced sperm density. The observed reduction in the weights of testes and accessory organs also reflects reduced bioavailability and/or production of androgen in the experimental rats. The weights of accessory sex organs (vas deferens, seminal vesicles and ventral prostate) also depend on bioavailability and/or production of androgen in animals. In the present study, circulatory levels of testosterone decreased significantly in rats treated with alcohol or BaP, indicating reproductive tissues are more sensitive to the toxic effects of alcohol or BaP toxicity when compared to brain, liver and kidney.

The present work has shown that exposure of rats to BaP or alcohol reduced testicular daily sperm production, epididymal total, motile and viable sperm. There was a statistically significant reduction in the numbers of swelled sperm exposed to hypo-osmotic solution in the rats exposed to BaP or alcohol compared to the control group. Conversely, exposure of rats to BaP or alcohol resulted in more number of sperm with morphological abnormalities. Similar decrease in the quality and quantity of sperm were reported in rats exposed to BaP [53-55] or alcohol.[26,56] The production and maturation is critical for the normal development and functioning of spermatozoa. The reduction in production, maturation, function and survival of sperm in experimental rats is may be due to its direct effect on sperm in testis or indirectly by affecting sperm function in epididymis, indicating BaP and/or alcohol are inimical to male fertility. The impaired sperm motility and deteriorated sperm membrane integrity may result in infertility due to the failure of sperm to reach the site of fertilization as well as loss of their ability to penetrate zona-pellucida.[57] The reduced level of serum testosterone diminishes epididymal functioning of synthesis and release of proteins thereby reduces epididymal sperm maturation.[58]

In the present study, the levels of superoxide anion, hydrogen peroxide and lipid peroxidation were significantly increased, with a significant decrease in the levels of reduced glutathione, activity levels of superoxide dismutase, catalase, glutathione peroxide, and glutathione reductase in different parts of epididymis and testes of BaP and/or alcohol administered rats. The antioxidant enzyme superoxide dismutase has been shown to accelerate the dismutation of the superoxide anion into hydrogen peroxide.[61] Catalase allows degradation of hydrogen peroxide into water and oxygen [62] and the glutathione peroxidase/reductase system has been shown to catalyse the degradation of hydrogen peroxide and lipid hydroperoxide by using reduced glutathione.[63] The reduction in the activities of antioxidant enzymes and increase in hydrogen peroxide and lipid peroxidation could reflect the adverse effects of BaP and/or alcohol on the antioxidant system in testes and epididymides. It is also reported that[55] and alcohol[64] induces oxidative stress by elevating the levels of ROS and by reducing the antioxidant defense system. It has been stated that generation of oxygen free radicals impairs normal sperm functions and thereby causes male infertility.[65] Aitken and Roman[66] also emphasized that cigarette smoking together with alcohol consumption disrupts testicular function through induction of ROS and also by concomitant disruption of testicular antioxidant enzymes. Further, it is also evident that excess production ROS in testes and epididymis leads to DNA damage in sperm.[67] The data also revealed exposure of rats to BaP and/or alcohol resulted in severe damage of sperm DNA. These results are in agreement with earlier studies, stating that DNA damage in sperm after exposure to BaP[68] or alcohol.[44,69] Thus, we cannot exclude that the genetic integrity of spermatozoa exposed to BaP or alcohol may have been compromised. It is established that sperm DNA integrity is strongly correlated with fertility outcome in males.[70]

The data also reveal statistically significant decrease in the activity levels of 3β- and 17β-HSD in the testes, associated with significantly reduced serum testosterone levels in rats exposed to BaP or alcohol, indicating a probable inhibition of androgen synthesis in experimental rats. Furthermore, there was a statistically significant reduction in the mRNA levels of StAR in the testes of rats exposed to BaP or alcohol. Decreased testicular steroidogenic enzyme activity levels and reduced StAR mRNA levels were reported after exposure to BaP[13,14,59] or alcohol.[60]

The final parameter studied was the effect of BaP or alcohol treatment on fertility related parameters and their relationships, if any, with testicular and epididymal disorders. Although all females that were cohabited with control or experimental males had a copulatory plug, but 66.67% in BaP, 75.0% in alcohol and 58.33% in BaP+alcohol group had implantations, suggesting that BaP and/or alcohol were effective in reducing male fertility. Though there is a delay in conception time females mated with BaP and/or alcohol treated rats, the presence of copulatory plugs or sperm in vaginal washings in females mated with these males suggests that sexual behavior is not compromised. Further, decrease in number of implantations and foetuses associated with increased pre- and post-implantations observed in females mated with experimental males suggests that reduced fertility probably resulted from lower sperm numbers, altered sperm motility and compromised sperm DNA integrity.

The mechanism(s) of action of BaP and alcohol is identical and showed additive effect in suppressing steroidogenesis and spermatogenesis, inducing oxidative toxicity in testis and epididymis and in reducing fertility in rats co-administered with BaP+alcohol. The only exception is interaction of BaP and alcohol with StAR. Previously, using *in silico* studies we showed that BaP hinders cholesterol transport into mitochondria by binding to StAR protein.[14] In extension to the previous study, molecular dynamics simulation of StAR-BaP complex showed

that complex was stable throughout 10 ns by showing strong binding affinity towards amino acid residues (ASN150, ALA172, GLU173, ASN177, VAL179, LEU247, VAL259, GLN262 and THR263) in hydrophobic tunnel of StAR. Further, three hydrogen-bond interactions, one with the amide group of ASN150 and two with hydroxyl functional groups of THR263 with BaP indicates StAR-BaP complex is more rigid. The greater binding affinity of BaP with StAR indicates disturbance in transportation of cholesterol into mitochondria, thus affects biosynthesis of testosterone in BaP treated rats. Conversely, alcohol interaction with StAR is very fragile, indicating alcohol is not interfering with cholesterol transport into mitochondria. Thus, we cannot exclude that the reduced steroidogenesis in BaP+alcohol treated rats may have been due to both additive (reduction in StAR mRNA, steroidogenic enzyme activities by both BaP and alcohol) and synergistic (hindrance of cholesterol transport by BaP) effect.

Conclusions

Therefore, the present study indicates that exposure to BaP or alcohol may pose a danger to the male reproduction by affecting spermatogenesis, steroidogenesis, inducing oxidative toxicity in testis and epididymis, and by reducing fertility efficiency. Further, induction of oxidative stress in the testis and epididymis is a major factor in the aetiology of BaP or alcohol-mediated reproductive toxicity. On the other hand, *in silico* studies revealed interaction of BaP with StAR thereby hinders cholesterol transport into mitochondria that leads to reduced testosterone biosynthesis. Co-administration of BaP and alcohol showed an increase in the reproductive toxic responses when compared to BaP and alcohol alone. The interactive effect of BaP and alcohol in inducing oxidative toxicity in testis and epididymis, decreasing sperm density and deteriorating sperm quality and suppressing fertility can be described as additive. Conversely, decrease in steroidogenesis in BaP+alcohol treated rats can be illustrated as both

additive and synergistic. Only the combination of two chemicals was considered herein. It may happen that several congeners of these chemicals prevail in the environment and act simultaneously on the reproductive system. Thus additional studies targeted at investigating the effect of simultaneous exposure to multiple chemicals will thus provide information that can be used to better characterize the potential human health risks associated with chronic exposure to environmental, occupational and dietary chemicals. Extrapolation of rat data to human is always difficult. However, it should be noted that the doses used in the present study are relevant to the doses exposed in the contemporary society.

Guidelines for ethical approval

Authors declare that the experiments were consistent with the guidelines and principles of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India and approved by the Institutional Animal Ethical Committee at S.V. University, Tirupati, India (vide No. IAEC/No- 438/01/a/CPCSEA) with a resolution No: 57/2012/(i)/a/CPCSEA/IAEC/SVU/PSR- KPR dt.08-07-2012.

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Conflict of interest

The author(s) declare that no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1. Fluorescence microscope-derived pictures of sperm nucleoid length of (a) control rats, (b) rats given BaP, (c) rats given alcohol, (d) rats given BaP + alcohol measured by alkaline comet assay. For each sample, 100 sperm in two separate slides were scored. Marked increase in tail length was observed in all experimental groups.

Figure 2. The mRNA levels of StAR in testis of rat, obtained after normalizing with GAPDH mRNA levels. M: Marker; C: Control; B: Benzo(a)pyrene (BaP); A: alcohol; B+A: BaP + alcohol. Each bar is mean ± standard deviation of six rats. Bars with different superscripts differ significantly at $p < 0.05$

Figure 3. Total energy plot of the complex StAR-BaP that was subjected to 10 ns molecular dynamics simulation.

Figure 4. The root mean square deviation (RMSD) graph of the complex StAR-BaP that was subjected to 10 ns molecular dynamics simulation.

Figure 5. Interaction of BaP at the hydrophobic tunnel of StAR protein.

Table 1. Benzo(a)pyrene (BaP) and/or alcohol induced changes on body weights (g) of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Initial body weight	207.00[a] ± 7.79	212.75[a] ± 9.10 (2.78)	195.58[a] ± 14.48 (-5.52)	192.42[a] ± 12.33 (-7.04)
Final body weight	332.06[a] ± 8.21	331.06[a] ± 9.47 (-0.30)	315.70[a] ± 14.59 (-4.93)	308.05[a] ± 13.29 (-7.23)
Weight gain	125.06[a] ± 14.29	118.31[a] ± 9.47 (-5.40)	120.12[a] ± 9.09 (-3.95)	115.63[a] ± 9.31 (-7.54)

Values are mean ± S.D. of 6 individuals for each treatment group
Values in parenthesis are percent change from that of control
Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 2. Benzo(a)pyrene (BaP) and/or alcohol induced changes on tissue somatic indices (g%) of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Brain	$0.64^{a} \pm 0.02$	$0.62^{a} \pm 0.02$ (-3.12)	$0.62^{a} \pm 0.02$ (-3.12)	$0.63^{a} \pm 0.04$ (-2.34)
Liver	$3.26^{a} \pm 0.22$	$2.97^{a} \pm 0.31$ (-8.89)	$3.15^{a} \pm 0.12$ (-3.37)	$3.18^{a} \pm 0.11$ (-2.45)
Kidney	$0.64^{a} \pm 0.03$	$0.61^{a} \pm 0.03$ (-4.69)	$0.61^{a} \pm 0.02$ (-4.69)	$0.62^{a} \pm 0.04$ (-3.12)
Spleen	$0.41^{a} \pm 0.03$	$0.38^{a} \pm 0.04$ (-7.32)	$0.39^{a} \pm 0.02$ (-4.88)	$0.38^{a} \pm 0.05$ (-7.32)
Testis	$1.12^{a} \pm 0.04$	$0.96^{b} \pm 0.04$ (-14.28)	$0.98^{b} \pm 0.02$ (-12.50)	$0.69^{c} \pm 0.07$ (-38.39)
Caput epididymis	$1.14^{a} \pm 0.10$	$0.95^{b} \pm 0.02$ (-16.67)	$0.97^{b} \pm 0.02$ (-14.91)	$0.89^{b} \pm 0.04$ (-21.93)
Corpus epididymis	$0.07^{a} \pm 0.01$	$0.06^{ab} \pm 0.01$ (-14.28)	$0.06^{ab} \pm 0.01$ (-14.28)	$0.05^{b} \pm 0.01$ (-28.57)
Cauda epididymis	$0.13^{a} \pm 0.03$	$0.09^{bc} \pm 0.01$ (-30.77)	$0.10^{b} \pm 0.01$ (-23.08)	$0.07^{c} \pm 0.01$ (-46.15)
Vas deferens	$0.09^{a} \pm 0.02$	$0.10^{a} \pm 0.01$ (11.11)	$0.11^{a} \pm 0.02$ (11.11)	$0.09^{a} \pm 0.01$ (0)
Seminal Vesicles	$0.47^{a} \pm 0.04$	$0.30^{b} \pm 0.02$ (-36.17)	$0.34^{b} \pm 0.02$ (-27.66)	$0.24^{c} \pm 0.02$ (-48.94)
Prostate	$0.16^{a} \pm 0.02$	$0.13^{b} \pm 0.01$ (-18.75)	$0.14^{ab} \pm 0.01$ (-12.50)	$0.10^{c} \pm 0.01$ (-37.50)
Penis	$0.11^{a} \pm 0.02$	$0.12^{a} \pm 0.02$ (9.09)	$0.12^{a} \pm 0.01$ (9.09)	$0.11^{a} \pm 0.01$ (0)

Values are mean ± S.D. of 6 individuals for each treatment group
Values in parenthesis are percent change from that of control
Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 3. Effect of benzo(a)pyrene (BaP) and/or alcohol on sperm production in testis and sperm quantity and quality in cauda epididymis of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
DSP (millions/g testis)	$19.78^{a} \pm 2.11$	$9.31^{b} \pm 0.69$ (-52.93)	$9.75^{b} \pm 0.81$ (-50.71)	$6.67^{c} \pm 0.49$ (-66.28)
Sperm count (millions/mL)	$74.66^{a} \pm 3.34$	$41.20^{b} \pm 2.82$ (-44.82)	$42.47^{b} \pm 2.67$ (-43.11)	$24.13^{c} \pm 1.90$ (-67.68)
Motile sperm (%)	$75.83^{a} \pm 4.44$	$41.50^{b} \pm 1.87$ (-45.27)	$42.33^{b} \pm 2.58$ (-44.18)	$31.50^{c} \pm 2.43$ (-58.46)
Viable sperm (%)	$75.67^{a} \pm 5.24$	$47.50^{b} \pm 2.43$ (-37.23)	$49.83^{b} \pm 2.32$ (-34.15)	$32.17^{c} \pm 2.64$ (-57.50)
HOS-tail swelled sperm (%)	$71.83^{a} \pm 3.06$	$35.67^{b} \pm 2.06$ (-35.67)	$37.50^{c} \pm 1.87$ (-50.34)	$27.50^{d} \pm 2.43$ (-61.71)
Head abnormality (%)	$3.33^{a} \pm 0.82$	$10.50^{b} \pm 1.29$ (215.31)	$11.33^{b} \pm 0.82$ (240.24)	$14.83^{c} \pm 1.17$ (345.34)
Tail abnormality (%)	$2.67^{a} \pm 0.52$	$9.25^{b} \pm 0.96$ (246.44)	$10.33^{b} \pm 1.21$ (286.89)	$12.83^{c} \pm 1.17$ (380.52)
Sperm nucleoid length (μm)	$10.09^{a} \pm 1.30$	$62.64^{b} \pm 7.07$ (520.81)	$70.64^{b} \pm 7.26$ (600.10)	$124.27^{c} \pm 12.87$ (1131.61)

Values are mean ± S.D. of 6 individuals for each treatment group

Values in parenthesis are percent change from that of control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 4. Effect of benzo(a)pyrene (BaP) and/or alcohol on 3β- and 17β-HSD activity levels in the testis and serum levels of testosterone in adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
3β-HSD (n moles of NAD converted to NADH/mg protein/min)	$23.31^{a} \pm 1.36$	$13.20^{b} \pm 1.60$ (-43.37)	$13.95^{b} \pm 2.04$ (-40.15)	$7.32^{c} \pm 1.49$ (-68.60)
173β-HSD (n moles of NADPH converted to NADP/mg protein/min	$16.98^{a} \pm 1.38$	$8.56^{b} \pm 1.42$ (-49.59)	$10.03^{b} \pm 1.37$ (-40.93)	$5.83^{c} \pm 1.12$ (-65.66)
Testosterone (ng mL^{-1})	$2.88^{a} \pm 0.26$	$1.67^{b} \pm 0.18$ (-42.01)	$1.78^{b} \pm 0.15$ (-38.19)	$1.02^{c} \pm 0.14$ (-64.59)

Values are mean ± S.D. of 6 individuals for each treatment group
Values in parenthesis are percent change from that of control
Mean values with different superscripts in a row differ significantly from each other at $p < 0.01$

Table 5. Effect of benzo(a)pyrene (BaP) and/or alcohol on pro- and anti-oxidant system in the testis of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Superoxide anion (n moles/mg protein/min)	$7.09^{a} \pm 0.46$	$10.85^{b} \pm 0.85$ (53.03)	$11.41^{b} \pm 0.79$ (60.93)	$13.93^{c} \pm 1.07$ (96.47)
Hydrogen peroxide (n moles/mg protein/min)	$15.55^{a} \pm 1.65$	$22.93^{b} \pm 1.62$ (47.46)	$23.71^{b} \pm 1.89$ (52.47)	$30.28^{c} \pm 2.04$ (94.73)
Lipid peroxidation (µ moles malondialdehyde/g tissue)	$12.11^{a} \pm 1.12$	$18.70^{b} \pm 1.65$ (54.42)	$16.29^{c} \pm 1.05$ (34.52)	$23.11^{d} \pm 1.69$ (90.83)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	$5.54^{a} \pm 0.52$	$3.06^{b} \pm 0.32$ (-44.76)	$2.62^{b} \pm 0.28$ (-52.71)	$1.56^{c} \pm 0.17$ (-71.84)
Catalase (n moles H_2O_2 metabolized/mg protein/min)	$21.01^{a} \pm 2.06$	$13.53^{b} \pm 1.12$ (-35.60)	$14.08^{b} \pm 0.96$ (-32.98)	$10.67^{c} \pm 1.26$ (-49.21)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	$43.43^{a} \pm 3.09$	$28.69^{b} \pm 2.66$ (-33.94)	$27.71^{b} \pm 2.65$ (-36.20)	$22.55^{c} \pm 2.29$ (-48.08)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	$44.66^{a} \pm 3.69$	$29.92^{b} \pm 2.56$ (-33.00)	$28.65^{b} \pm 1.96$ (-35.85)	$22.84^{c} \pm 1.66$ (-48.86)
Reduced glutathione (µ moles/g tissue)	$49.74^{a} \pm 5.45$	$31.81^{b} \pm 3.08$ (-36.05)	$30.91^{bc} \pm 2.28$ (-37.86)	$25.72^{c} \pm 2.44$ (-48.29)

Values are mean ± S.D. of 6 individuals for each treatment group

Values in parenthesis are percent change from that of control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 6. Effect of benzo(a)pyrene (BaP) and/or alcohol on pro- and anti-oxidant system in the caput epididymis of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Superoxide anion (n moles/mg protein/min)	$7.29^{a} \pm 0.49$	$11.06^{b} \pm 0.79$ (51.71)	$11.50^{b} \pm 0.85$ (57.75)	$14.46^{c} \pm 1.07$ (98.35)
Hydrogen peroxide (n moles/mg protein/min)	$14.27^{a} \pm 1.10$	$20.81^{b} \pm 1.35$ (45.83)	$21.68^{b} \pm 1.33$ (51.93)	$26.10^{c} \pm 2.10$ (82.90)
Lipid peroxidation (µ moles malondialdehyde/g tissue)	$7.14^{a} \pm 0.46$	$11.22^{b} \pm 0.68$ (57.14)	$11.08^{b} \pm 0.58$ (55.18)	$15.71^{c} \pm 1.69$ (120.03)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	$4.08^{a} \pm 0.43$	$2.52^{b} \pm 0.28$ (-38.23)	$2.18^{bc} \pm 0.29$ (-46.57)	$1.90^{c} \pm 0.29$ (-53.43)
Catalase (n moles H_2O_2 metabolized/mg protein/min)	$8.62^{a} \pm 0.89$	$4.26^{bc} \pm 0.53$ (-50.58)	$4.55^{b} \pm 0.57$ (-47.21)	$3.42^{c} \pm 0.32$ (-60.32)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	$25.86^{a} \pm 1.85$	$16.14^{b} \pm 0.94$ (-37.59)	$16.19^{b} \pm 1.43$ (-37.39)	$12.64^{c} \pm 1.09$ (-51.12)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	27.27 ± 1.94^{a}	$18.99^{b} \pm 2.19$ (-30.36)	$17.06^{bc} \pm 1.20$ (-37.44)	$14.84^{c} \pm 1.35$ (-45.58)
Reduced glutathione (µ moles/g tissue)	$24.76^{a} \pm 1.67$	$15.10^{b} \pm 1.38$ (-39.01)	$14.94^{b} \pm 1.17$ (-39.66)	$11.83^{c} \pm 1.69$ (-52.22)

Values are mean ± S.D. of 6 individuals for each treatment group

Values in parenthesis are percent change from that of control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 7. Effect of benzo(a)pyrene (BaP) and/or alcohol on pro- and anti-oxidant system in the corpus epididymis of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Superoxide anion (n moles/mg protein/min)	$8.23^{a} \pm 0.56$	$11.22^{b} \pm 0.61$ (36.33)	$11.67^{b} \pm 0.72$ (41.80)	$14.59^{c} \pm 1.14$ (77.28)
Hydrogen peroxide (n moles/mg protein/min)	$13.18^{a} \pm 1.23$	$21.89^{b} \pm 1.88$ (66.08)	$20.01^{b} \pm 1.07$ (51.82)	$25.94^{c} \pm 1.58$ (96.81)
Lipid peroxidation (µ moles malondialdehyde/g tissue)	$4.19^{a} \pm 0.29$	$6.15^{b} \pm 0.33$ (46.78)	$6.01^{b} \pm 0.44$ (43.44)	$7.42^{c} \pm 0.62$ (77.09)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	$3.40^{a} \pm 0.35$	$1.84^{b} \pm 0.23$ (-45.88)	$1.87^{b} \pm 0.30$ (-45.00)	$1.02^{c} \pm 0.17$ (-70.00)
Catalase (n moles H_2O_2 metabolized/mg protein/min)	$10.94^{a} \pm 1.18$	$5.33^{b} \pm 0.46$ (-51.28)	$5.40^{b} \pm 0.55$ (-50.64)	$4.57^{b} \pm 0.44$ (-58.23)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	$27.87^{a} \pm 2.20$	$19.13^{b} \pm 1.13$ (-31.36)	$19.58^{b} \pm 1.69$ (-29.74)	$15.69^{c} \pm 1.18$ (-43.70)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	$35.76^{a} \pm 1.96$	$26.35^{b} \pm 2.13$ (-26.31)	$25.36^{b} \pm 2.24$ (-29.08)	$21.77^{c} \pm 1.46$ (-39.12)
Reduced glutathione (µ moles/g tissue)	$30.09^{a} \pm 2.35$	$18.90^{b} \pm 1.70$ (-37.19)	$19.32^{b} \pm 1.48$ (-35.79)	$14.06^{c} \pm 1.78$ (-53.27)

Values are depicted as mean ± S.D. of 6 individuals for each treatment group

Values in parenthesis are percent change from that of control

Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$.

Table 8. Effect of benzo(a)pyrene (BaP) and/or alcohol on pro- and anti-oxidant system in the cauda epididymis of adult male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Superoxide anion (n moles/mg protein/min)	$8.15^{a} \pm 0.61$	$11.81^{b} \pm 1.02$ (44.91)	$12.47^{b} \pm 0.85$ (53.01)	$15.32^{c} \pm 1.33$ (87.97)
Hydrogen peroxide (n moles/mg protein/min)	$15.72^{a} \pm 0.79$	$21.04^{b} \pm 1.04$ (33.84)	$22.12^{b} \pm 1.30$ (40.71)	$26.50^{c} \pm 1.53$ (68.57)
Lipid peroxidation (μ moles malondialdehyde/g tissue)	$12.29^{a} \pm 1.07$	$19.34^{b} \pm 1.47$ (57.36)	$19.54^{b} \pm 1.15$ (58.99)	$22.47^{c} \pm 1.51$ (82.83)
Superoxide dismutase (n moles pyrogallol oxidized/mg protein/min)	$5.31^{a} \pm 0.58$	$3.03^{b} \pm 0.28$ (-42.94)	$2.52^{bc} \pm 0.32$ (-52.54)	$2.04^{c} \pm 0.29$ (-61.58)
Catalase (n moles H_2O_2 metabolized/mg protein/min)	$13.87^{a} \pm 0.94$	$9.98^{b} \pm 1.05$ (-28.05)	$10.35^{b} \pm 1.01$ (-25.38)	$8.20^{c} \pm 0.88$ (-40.88)
Glutathione peroxidase (n moles NADPH oxidized/mg protein/min)	$33.26^{a} \pm 2.43$	$22.55^{b} \pm 1.69$ (-32.20)	$21.30^{b} \pm 1.80$ (-64.05)	$16.15^{c} \pm 1.41$ (-51.44)
Glutathione reductase (n moles NADPH oxidized/mg protein/min)	$29.27^{a} \pm 2.63$	$21.07^{b} \pm 1.57$ (-28.01)	$20.16^{b} \pm 1.68$ (-31.12)	$16.28^{c} \pm 1.88$ (-44.38)
Reduced glutathione (μ moles/g tissue)	$34.70^{a} \pm 2.32$	$24.89^{b} \pm 1.87$ (-28.27)	$25.05^{b} \pm 1.71$ (-27.81)	$21.01^{c} \pm 1.90$ (-39.45)

Values are depicted as mean ± S.D. of 6 individuals for each treatment group
Values in parenthesis are percent change from that of control
Mean values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 9. Effect of benzo(a)pyrene (BaP) and/or alcohol on reproductive performance in male rats

Parameter	Control	BaP	Alcohol	BaP + Alcohol
Conception time (days) [$]	1.50[a] ± 0.55	3.12[b] ± 0.64 (108.00)	3.25[b] ± 0.71 (116.67)	5.12[c] ± 0.64 (241.33)
Mating index (%)	100(12/12)	100(12/12)	100(12/12)	100(12/12)
Fertility index (%)	100(12/12)	66.67(8/12)	75.0(9/12)	58.33(7/12)
No. of corpora lutea/rat[#]	15.33[a] ± 0.58	14.67[a] ± 1.53 (-4.30)	15.33[a] ± 0.58 (0)	14.67[a] ± 1.15 (-4.30)
No. of implantations/rat[#]	14.33[a] ± 0.58	8.33[b] ± 0.58 (-45.66)	8.67[b] ± 0.58 (-43.44)	5.67[c] ± 0.58 (-63.01)
Pre- implantation loss (%)	6.52	43.22	43.44	61.35
No. of live fetuses/rat[#]	13.67[a] ± 0.58	6.33[b] ± 0.58 (-55.83)	6.67[b] ± 0.58 (-53.45)	4.33[c] ± 0.58 (-69.78)
No. of resorptions/rat[#]	0[a]	2.75[b] ± 0.50	2.50[b] ± 0.58	3.75[c] ± 0.5
Post- implantation loss (%)	4.60	24.01	23.07	23.63

Values are mean ± S.D; [$]n = 12; [#]n = 6

Values in the parentheses are percent change from that of control.

Mean values with different superscripts in a row differ significantly from each other at $p < 0.001$

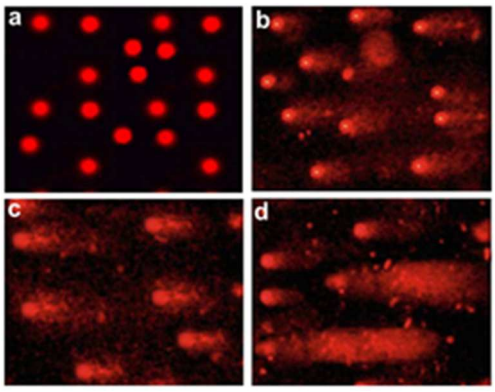

Figure 1. Fluorescence microscope-derived pictures of sperm nucleoid length of (a) control rats, (b) rats given BaP, (c) rats given alcohol, (d) rats given BaP + alcohol measured by alkaline comet assay. For each sample, 100 sperm in two separate slides were scored. Marked increase in tail length was observed in all experimental groups.
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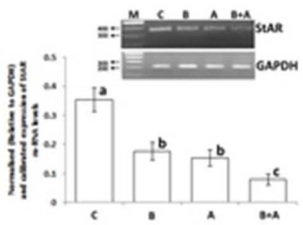

Figure 2. The mRNA levels of StAR in testis of rat, obtained after normalizing with GAPDH mRNA levels. M: Marker; C: Control; B: Benzo(a)pyrene (BaP); A: alcohol; B+A: BaP + alcohol. Each bar is mean ± standard deviation of six rats. Bars with different superscripts differ significantly at $p < 0.05$
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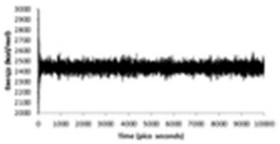

Figure 3. Total energy plot of the complex StAR-BaP that was subjected to 10 ns molecular dynamics simulation.
5x2mm (600 x 600 DPI)

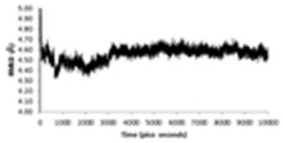

Figure 4. The root mean square deviation (RMSD) graph of the complex StAR-BaP that was subjected to 10 ns molecular dynamics simulation.
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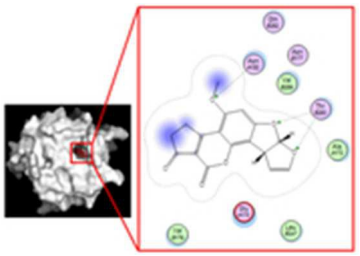
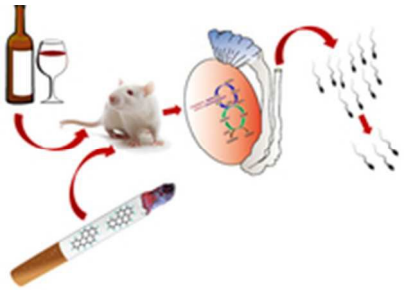


Figure 5. Interaction of BaP at the hydrophobic tunnel of StAR protein.
7x5mm (600 x 600 DPI)



Graphical Abstract
8x6mm (600 x 600 DPI)