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Reproductive and paternal mediated developmental toxicity of Benzo(a)Pyrene in adult male Wistar rats

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SUMMARY

In this study, we evaluated reproductive toxic effects of benzo(a)pyrene (BaP) in adult male Wistar rats. Rats received intra-peritoneal injections containing BaP at a dose of 1, 10 or 100 µg/kg bw on alternate days for 60 days and analyzed for fertility. Control and experimental male rats were cohabited with control females and sperm positive females were analyzed for paternal-mediated reproductive and developmental toxicity. Dose dependent reduction in litter size and crown rump length was apparent in pups sired by experimental males. The developmental land marks were comparable among pups in all groups except delay in testis descent and vaginal opening which were delayed in experimental pups. After completion of fertility studies, rats were sacrificed and analyzed for reproductive endpoints. The relative weights of testes, caput epididymis, cauda epididymis, seminal vesicle and prostate gland were decreased significantly in BaP exposed animals. Daily sperm production and epididymal sperm count, motility and viability decreased significantly in a dose dependent manner in BaP treated rats. Also, there was a dose dependent decrease in the testicular steroidogenic enzyme activities. Additionally, serum testosterone levels were decreased in BaP treated animals. *In silico* studies revealed the binding affinity of BaP with simulated StAR protein at the hydrophobic tunnel region. It can be concluded that chronic exposure to sub-lethal doses of BaP affects steroidogenesis and spermatogenesis resulting in reduced fertility in adult male rats.

Key words: Benzo(a)pyrene, StAR, steroidogenesis, testosterone, spermatogenesis, fertility

INTRODUCTION

Poly aromatic hydrocarbons (PAHs) are a gamut of toxic, lipophilic and endocrine disrupting chemicals that are widely distributed in the environment. Benzo(a)pyrene (BaP) is a prototypical representative of the PAHs, mainly produced from the incomplete combustion of fossil fuels, wood and other organic materials.[1] Significant amount of BaP is also found in tobacco smoke, diesel exhaust, charcoal-broiled foods and industrial waste by-products[2,3] and accumulate in human tissues to toxic levels within a short time.[4] Experimental studies involving adult rats and mice have established that exposure to BaP results in significant decrease in testicular and epididymal weights, testicular DNA synthesis and sperm counts.[5,6] A significant decrease in the sperm progressive motility and disruption in the reproductive hormonal profile was observed in adult rats exposed to BaP through inhalation.[7-9] Furthermore, reduced litter size, diminished fertility and gonad deformity were also observed in mice exposed to BaP.[10]

In spite of the growing knowledge on the adverse reproductive effects of BaP, it is relatively unclear whether or not; BaP has the same effects on male reproductive activities as it relates to androgen synthesis. Leydig cells are the prime testosterone-producing cells in interstitial compartment of the mammalian testis, support spermatogenesis in seminiferous tubules. The rate limiting step in steroidogenesis is the delivery of cholesterol molecule into the mitochondria. It is well known from previous studies that inter-membrane of mitochondria is impermeable to hydrophobic compounds including cholesterol.[11] The transportation of cholesterol into mitochondria is a complex process involving an interaction between the steroidogenic acute regulatory protein (StAR) and cholesterol.[12] Any disruption in cholesterol transport (by StAR) results in decreased steroidogenesis and thereby spermatogenesis.

It is reported earlier that the metabolism of BaP occurs in mitochondria of different tissues including testis[13]. Further, BaP and other PAHs share structural similarity with cholesterol. Although there is association between environmental PAH exposures and increased incidence of male reproductive toxicity in laboratory animals[7] and humans,[14] little attention has been focused on the effect of BaP on paternal mediated reproductive toxicity. The long-term goal of this study is to understand the mechanism of BaP action on male reproduction, especially in steroidogenesis. The present study was, therefore, designed to investigate the effects of BaP on reproduction in male rats using the following reproductive endpoints: (1) weights of reproductive organs, (2) quality and quantity of sperm, (3) serum testosterone levels, (4) steroidogenesis, (5) fertility related parameters, and (6) developmental milestones in pups sired by BaP exposed male rats. In addition, this study broadened its scope by including *in silico* studies to evaluate the interaction between BaP and simulated StAR protein that has not been studied so far.

MATERIALS AND METHODS

Procurement and maintenance of experimental animals

Healthy male rats of Wistar strain with a body weight ranging from 150 to 160 g (70 days old) were obtained from an authorized vendor (Sri Venkateswara Traders, Bengaluru, India). Upon arrival, rats were housed in polypropylene cages (18" x 10" x 8") containing sterilized paddy husk as bedding material, and provided filtered tap water and standard rodent feed (purchased from Sai Durga Agencies, Bengaluru, India) *ad libitum*. Animals were maintained in a well-controlled laboratory facility (temperature 22-25°C; 12:12 hr light:dark cycle, humidity 50 ± 5%). The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India. The

experiments were also reviewed and approved by the Institutional Animal Ethical resolution No: 57/2012/(i)/a/CPCSEA/IAEC/SVU/PSR- KPR dt.08-07-2012.

Chemicals

BaP (≥96% purity by HPLC), dehydroepiandrosterone, androstenedione, NADPH and NAD were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Trypan blue, fructose and sodium citrate were purchased from Himedia Laboratories Private Limited, Mumbai, India. Sodium carbonate, sodium pyrophosphate, formalin, Tris-HCl, Triton X-100 and glutaraldehyde were purchased from Merck, Mumbai, India.

Experimental design and Necropsy

Forty healthy adult male rats were selected and randomly divided into four equal groups. The animals in group 1 served as control and intraperitoneally injected with 100 µL of 0.5% DMSO diluted in distilled water.[9] Rats in groups 2, 3 and 4 were injected with BaP 1, 10 and 100µg/Kg body weight respectively dissolved in 0.5% DMSO (volume of injection was 100 µL per animal), on alternative days through intra-peritoneal route for 60 days. The BaP levels that were used in our study (1-100 µg/kg bw/day) are environmentally relevant, because the estimated BaP intake for the human in the vicinity of hazardous waste sites is ranged from 20-800 ng/day.[15] The rationale for choosing 60-day experimental period was to evaluate the effect of BaP through a complete spermatogenic cycle, which takes approximately 55 days in Wistar rats.[16] The body weight of the animals was recorded in the beginning and at the end of treatment period. Food and water intake were recorded once in a week during the treatment period. After completion of the experimental period, rats were fasted overnight, weighed, and killed by cervical dislocation. Body weight, brain, liver, kidney, testes, epididymis (caput, corpus, cauda),

prostate gland, vas deferens, seminal vesicles and penis were weighed and used for the determination of tissue somatic indices (TSI).

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

Testes were used for determination of daily sperm production and enzyme assays, whereas the epididymis was used for sperm analysis.

Sperm parameters

Epididymal sperm analysis

Epididymal sperm were obtained by mincing cauda epididymis in physiological saline (0.9% NaCl in distilled water). The sperm were counted using a Neubauer Chamber, as described by Belsey *et al.*[17] The data was expressed as millions/ml. Progressive sperm motility was evaluated within 5 min following their isolation from cauda epididymis at 37°C by the method of Belsey *et al.*[17] and the data was expressed as percent motility. The viability of the sperm was determined using 1% trypan blue reagent, as described earlier by Talbot and Chacon.[18] Sperm membrane integrity was determined by exposing the sperm to hypo-osmotic solution and observed for tail coiling under the microscope (HOS tail coiling). The percent of HOS tail coiled sperm was determined following the method described by Jeyendran *et al.*[19]

Daily sperm production

Daily sperm production (DSP) was determined in the testis by the method of Blazak *et al.*[20] Briefly, testis was decapsulated and homogenized in 50ml of ice-cold 0.9% NaCl solution containing 0.01% Triton X-100 using a sterilized mortar and pestle. The homogenate was allowed to settle for 1 minute. After thorough mixing of each sample, the number of sperm heads

were counted using haemocytometer. The number of sperm produced per gram testicular tissue per day was calculated.

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 enzyme source. The activity levels of 3β hydroxysteroid dehydrogenase (3β HSD) (EC 1.1.1.51) and 17 β hydroxysteroid dehydrogenase (17β HSD) (EC 1.1.1.64) were determined by the method of Bergmeyer.[21] 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. Protein content in the enzyme source was estimated by the method of Lowry *et al.*[22] using bovine serum albumin as standard. The enzyme activities were expressed as nmol of NAD converted to NADH/mg protein/min (3β-HSD) or nmol of NADPH converted to NADP/mg protein/ min (17β-HSD).

Serum hormonal measurement

Radio Immuno Assay (RIA) for serum testosterone level determination was performed by the method of Rao *et al.*[23] The sensitivity of the assay was calculated as 0.002 ng and intra-assay variation was found to be 6.5%. Serum FSH and LH levels were determined according to the method of Lin *et al.*[24] Iodination of rFSH and rLH with ^{125}I was performed by the method of Greenwood *et al.*[25] using Chloramine-T as an oxidizing agent. The sensitivity of the assay was calculated as 0.008 ng for FSH and 0.006 ng for LH. The intra-assay variations were 5.8% and 6.9% for FSH and LH, respectively. All of the samples were run at the same time to avoid inter-assay variation.

Scanning Electron Microscopic (SEM) analysis of Sperm

Scanning Electron Microscope analysis of sperm was performed by the method of Lohiya *et al*.[26] In brief, spermatozoa were washed with phosphate buffer (0.01M, pH7.2) and then pelleted by centrifugation. The sperm pellets were fixed in 2.5% glutaraldehyde for 30 min and washed thrice in phosphate buffer followed by distilled water. A thin film of spermatozoa was smeared on a clean glass slide, air dried, then mounted on SEM stub, sputtered with gold particles, and observed under SEM (EVO MA 15).

Fertility studies

After completion of treatment period, each male from the control and experimental groups was transferred to a mating cage and cohabited with untreated female rats in proestrus on a 1:1 basis in the home cage of the male. The maximum duration of cohabitation was 6 days. Positive evidence of copulation was confirmed by the presence of vaginal plugs and/or sperm in vaginal smear taken each morning during cohabitation.[27] The conception time, the interval between the first day of cohabitation and the day of vaginal plug/or sperm in vaginal smear, was recorded for each female. Pregnant rats were moved into separate cages and housed individually and designated as day zero of gestation. The number of pregnant rats with each experimental or control group was recorded to determine fertility index. On the 6^{th} day of gestation, laparotomy was performed using 5 animals from each group to determine number of corpora lutea, and number of implantations. Also, celiotomy was performed on day 18^{th} of gestation to find out the number of fetuses and resorption sites.Conception time, mating index, fertility index, pre- and post-implantation loss was determined. Six pregnant rats from each group were allowed to deliver the pups.

Analysis of pups

Pups were identified by paw tattoo and monitored daily for clinical signs of toxicity until completion of lactation. The litter size (number of pups delivered), body weight of pups, crown rump length (length from head to rump), anogenital distances for both male and female (distance from anus to genitalia) was recorded on day 1. The developmental landmarks viz., pinna detachment (separation of closed flip), lower incisor eruption (the first appearance of lower incisors), upper incisor eruption (the first appearance of upper incisors), eye slit formation (occurrence of closed eye lids), eye opening (when both eye lids were completely separated), development of fur (the appearance of thin layer of fur over the body), testes descent (testes descent into the scrotal sac) and vaginal opening were also determined by an investigator that was blinded to treatment.

Molecular simulation

The crystal structure of StAR protein of human was down loaded from Protein Data Bank (PDBID: 3POL) and water molecules were removed. The 3POLwas simulated using NAMD 2.9 software. CHARM and the total number of atoms force-field was used for lipid and protein along with the TIP3P model for water[28] and total number of atoms are 17517. Initially, energy minimization was carried out for side chains, backbone of protein and solvent to remove bad contacts. Equilibration was run with 500,000 steps and simulation began for 10 ns respectively. Integrated motion time step of 2fs was computed using multiple time step algorithms.[29,30] Short-range non-bonded interactions were computed every two-time step, and long range electrostatic forces were computed every four-time steps. The pair list of the non-bonded interactions was computed with a pair list distance of 14.0 Å^{o}. Short range interactions were defined as Vander

Waals and electrostatic interactions within 12 Àº. Long range electrostatic interactions were taken in an account using Partial Mesh Ewald (PME) approach. Pressure was maintained at 1 atm using the Langevin piston and temperature at 300k was controlled using Langevin dynamics. Covalent interactions between hydrogen and heavy atoms were constrained using SHAKE/RATTLE algorithm. Finally, the graph was drawn by taking root mean square deviation (RMSD) of structures generated during minimization and equilibration methods on Y-axis with time in ns on X-axis. Structure with least RMSD difference between the structures generated was used for further studies. All hydrogen atoms were included during the calculation.

Docking studies

Molecular docking studies were performed with BaP and cholesterol against simulated StAR protein (PDB ID: 3POL) using PyRx virtual screening software, which uses Auto Dock Vina 4.0.[31] The molecular interactions between StAR protein and ligands were interpreted using PyMOL.[32]

Statistical analysis

The data were statistically analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey test using SPSS 16.0. The data were expressed as mean ± S.D. and '*p*' value < 0.05 was considered significant. All statistical tests were performed using Statistical Package for Social Sciences (SPSS Inc., Chertsey, UK).

RESULTS

General toxicity

The rats were checked for overall appearance, body position, activeness, co-ordination or gait and behavior. No significant differences in body weight gain (Table 1), food and water

intake, lacrimation, respiration, vocalization, urination, postural or gait abnormalities were found in any of the control and experimental rats. All the animals were apparently normal and no unusual behavior (*viz.,* head searching, head flicking, licking, biting, circling, self-mutilation and walking back-wards) were observed in any of the rats. BaP treatment (1, 10 and 100 µg/kg bw) for 60 days did not show any clinical signs of toxicity or none of the rats were excluded from the study.

Organ weight profile

BaP treatment significantly reduced the relative weights of testis ($F = 52.285$; df = 3, 28; $p < 0.0001$), cauda epididymis ($F = 23.333$; df = 3, 28; $p < 0.0001$), caput epididymis ($F = 17.462$; df = 3, 28; $p < 0.0001$), seminal vesicles ($F = 64.711$; df = 3, 28; $p < 0.0001$) and prostate gland ($F = 26.519$; df = 3, 28; $p < 0.0001$) when compared to control rats (Table 2). On the other hand, no significant difference was observed in the relative weights of corpus epididymis, vas deferens and penis of BaP exposed rats when compared to control rats (Table 2). Relative weights of brain, liver and kidney are comparable among different groups (data not shown).

Spermatology

The daily sperm production per gram of testicular parenchyma ($F = 82.092$; df = 3, 28; $p < 0.0001$), epididymal sperm reserves ($F = 141.57$; df = 3, 28; $p < 0.0001$), percentage of motile sperm ($F = 273.81$; df = 3, 28; $p < 0.0001$), viable sperm ($F = 126.93$; df = 3, 28; $p < 0.0001$), and HOS tail coiled sperm ($F = 105.84$; df = 3, 28; $p < 0.0001$) were significantly decreased in a dose dependent manner in BaP treated rats (Table 3). No significant morphological abnormalities were observed in control rat sperm (Fig. 1A). The most commonly observed

abnormalities are misshapen head, sperm with double head, tail coiled, tail looped and tail serrated sperm (Fig. 1B-J). The sperm with head abnormalities in control, 1, 10, and 100 µg BaP treated groups are 2.17 ± 1.17%, 5.33 ± 1.04%, 8.17 ± 0.98%, and 12.5 ± 0.84 % respectively. The sperm with tail abnormalities in control, 1, 10, and 100 µg BaP treated animals are 1.67 ± 0.52%, 3.67 ± 0.82%, 5.83 ± 0.75%, and 10.17 ± 1.17% respectively.

Testicular steroidogenic enzymes and serum hormones

A significant and dose-dependent decrease in the activity levels of 3β- (F = 178.73; df = 3, 28; $p < 0.0001$) and 17β- HSD (F = 108.50; df = 3, 28; $p < 0.0001$) was observed in the testis of rats exposed to BaP (Table 4). Serum testosterone levels were also decreased significantly (F = 35.717; df = 3, 28; $p < 0.0001$) in BaP treated rats (Table 5). On the contrary, serum LH (F = 27.006; df = 3, 28; $p < 0.0001$) and FSH (F = 28.184; df = 3, 28; $p < 0.0001$) levels were significantly elevated in BaP exposed rats as compared to controls (Table 5).

Fertility studies

Rats in all groups exhibited signs of sexual motivation as soon as the female was introduced into mating cage; including grooming, licking the genitalia, chasing the female, and passing under each other's body. Although the visual observations on male sexual behavior are not quantified, they made several attempts to mount the female. Even though all females cohabited with control males and males exposed to BaP had copulatory plugs and showed implantations (mating index 100% and fertility index 100%); a significant difference was observed in the conception time (F = 17.020; df = 3, 28; $p < 0.0001$) (interval between the day of cohabitation and the day of presence of vaginal plug or presence of sperm in vaginal smear) of treated male rats when compared to controls (Fig. 2). The mean number of corpora lutea was

comparable in all groups. The mean number of implantations per rat mated with 1, 10 and 100 µg BaP exposed males was 9.75 ± 0.5, 8.67 ± 0.58 and 6.5 ± 0.71, respectively, in contrast to 11.67 ± 0.58 in the control group. The mean pre-implantation loss (difference between the number of corpora lutea and the number of implantation sites expressed as per number of corpora lutea) and post-implantation loss (difference between the number of implantation sites and the number of live fetuses expressed as per number of implantation sites) was increased in a dose-dependent manner in females mated with BaP treated males as compared to controls (Table 6). The mean number of live fetuses per rat was 11.33 ± 0.58, 9.33 ± 0.58, 7.5 ± 0.71, and 5.5 ± 0.71 in the control, 1, 10, and 100 µg groups, respectively (Table 6). The number of live fetuses decreased significantly in a dose-dependent manner after BaP treatment ($F = 118.46$; $df = 3, 28$; $p < 0.0001$).

Developmental landmarks

Number of pups delivered to females mated with BaP treated males were significantly ($F = 106.43$; $df = 3, 28$; $p < 0.0001$) reduced in a dose dependent manner when compared to the number of pups delivered by females mated with control males (Table 7). The body weights determined on postnatal day 1 and were significantly decreased in pups delivered to females mated with 100 µg BaP exposed male rats, whereas, bodyweights are comparable in pups delivered by females mated with control or 1 and 10 µg BaP exposed male rats. Crown rump length (PND 1) in pups of BaP treated groups were reduced in a dose-dependent manner ($F = 52.289$; $df = 3, 28$; $p < 0.0001$) when compared to pups of control group. The anogenital distance (males and females), time required for lower and upper incisors eruption, pinna unfolding, eye slit formation, fur development and eye opening in pups of BaP treated rats was not significantly

different from control pups, whereas the time (days) taken to descend testes ($F = 83.347$; $df = 3, 28$; $p < 0.0001$) into scrotal sac and opening of vagina ($F = 141.21$; $df = 3, 28$; $p < 0.0001$) was significantly delayed in experimental pups when compared to control pups (Table 7).

Molecular simulation

The crystal structure of StAR (PDB: 3POL) was refined by molecular dynamics simulation using NAMD software and the trajectory graph was drawn between the RMSD of Cα trace and time (ns) (Fig. 3). The RMSD was stable around 4 ns and then shuttered up to 10 ns respectively. Simulated structure (Fig. 4B) was compared with the crystal structure generated by SPDBV software suite (Fig. 4A) (http://www.expasy.org/spdbv). Both, simulated StAR and crystal structure possess 3 α-helices.

Docking studies

The binding affinity of BaP with simulated StAR protein (PDB ID: 3POL) was greater (-9.9Kcal/mol) than its natural ligand cholesterol (-6.9Kcal/mol). BaP interacts with Asn148, Asn150, Val151, Lys169, Ala171, Ala172, Glu173, Ala174,Gly179, Arg182, Phe184, Gly201, Ala218, His220, Try241, Leu243, Ile245, Leu247, Val259, Leu260, Gln262 and Thr263 of hydrophobic tunnel of StAR (Fig. 5B), which are also involved in binding of single cholesterol molecule (Fig. 5A).

Discussion

In the present study, we used three dose regimens of BaP to evaluate its toxic effects on the following reproductive end points: weights of reproductive organs, spermatogenesis, steroidogenesis, reproductive hormones, fertility and developmental milestones of pups sired by BaP exposed male rats.

BaP at the selected doses for a period of 60 days did not cause any significant change in the body weight, food and water intake indicating no overt general toxicity. Exposure to BaP significantly reduced the weights of testis, epididymis, seminal vesicle and prostate gland which may be due to reduced availability of androgen in BaP treated rats. It is well recognized that testosterone is of paramount importance in the regulation of structural and functional integrity of reproductive organs.[33,34] Exposure to BaP significantly reduced daily sperm production, epididymal sperm count, sperm motility and sperm viability in a dose dependent manner. The sperm membrane integrity can be evidenced from HOS test, which also indicates deteriorated sperm membrane integrity in BaP treated rats. These results are in agreement with previous reports.[7-9,35-37] Impaired sperm motility[38] and deteriorated sperm membrane integrity[39] 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.

In the present study, the activity levels of testicular steroidogenic marker enzymes such as 3β HSD and 17β HSDs were significantly decreased in BaP treated rat testis in dose dependent manner. The data also reveal elevated levels of FSH and LH, associated with significantly reduced serum testosterone levels in rats exposed to BaP, indicating impaired steroidogenesis in BaP treated rats. Chung *et al.*[9] reported that long-term BaP exposure results in reduced serum and intra-testicular testosterone levels and increased serum LH concentration. Thus, the decrease in serum testosterone levels could be due to diminished responsiveness of Leydig cells to LH and/or direct inhibition of testosterone synthesis in rats exposed to BaP. The elevated serum FSH levels indicates an impairment of spermatogenesis in BaP treated rats and reflects germ cell loss or damage to Sertoli cells, thereby affecting the feedback regulation of

FSH secretion.[40] The lowered levels of serum testosterone with elevated FSH and LH also indicate an intact pituitary-testicular axis in BaP treated rats.

Another reproductive endpoint that was tested was the male's ability to sire offspring. Although all females that were cohabited with control or experimental males had a copulatory plug, 75% in 10 µg and 62.5% in 100 µg groups had implantations, suggesting that BaP treatment at a dose of 10 µg and above was effective in reducing male fertility. The conception time is also significantly increased in BaP treated rats. Additional observations of fewer pups per litter and high implantation loss rate in the BaP treated groups suggest compromised sperm fertility. Whether reduced fertility observed in BaP treated rats resulted from lower sperm numbers, altered sperm motility or depressed sexual desire cannot be determined from the present fertility data. Though there was a delay in conception time, the presence of copulatory plugs in all the females indicate sexual behavior is not compromised in BaP treated rats. Various studies have shown that BaP induced DNA damage at all stages of spermatogenesis and in the testis,[41-44] hence, we cannot exclude that the genetic integrity of spermatozoa of rat exposed to BaP may have been compromised which mediates post-implantation loss.

There were some effects on paternal BaP treatment on offspring development. In the present study, female and male pups born to 100 µg BaP exposed males had lower birth weights and smaller crown rump length than those born to controls. The current results do not reveal any major effects of paternal BaP exposure on the physical development of the offspring in pinna unfolding, eye slit formation, eye opening, upper- and lower incisor eruption, fur development, anogenital distance. Offspring of BaP rats showed delayed testis descent and vaginal opening and this might result from alteration in steroid hormone levels. Liang *et al*.[45] reported neonatal

exposure to BaP reduced serum testosterone levels in adult male rats. To our knowledge this is the first study to examine the paternal mediated developmental effects of BaP in rats.

Another interesting finding of this study was that, interaction of BaP with StAR. Earlier, Ramesh *et al.*[13] observed substantial accumulation of BaP and its metabolites in the testis of BaP exposed rats. It is also acknowledged that metabolism of BaP takes place in the mitochondria which ultimately results in dysfunction of testicular mitochondria.[46] Malfunctioning of mitochondria results in decreased production of pregnenolone, which is the precursor for all steroid hormones. Since mitochondrial membrane is impermeable to all hydrophobic compounds including cholesterol, we addressed the mechanism of transportation of BaP into mitochondria using *in silico* studies. Docking results showed that BaP binds to simulated structure of StAR with greater binding affinity (i.e.,-9.9 kcal/mol) than natural ligand i.e. cholesterol (i.e.,-6.9 kcal/mol). Both BaP and cholesterol interacts hydrophobically with the amino acids in hydrophobic tunnel of StAR. The interacting amino acids of BaP and cholesterol were also comparable. From these results it is clear that BaP competes with cholesterol to bind to StAR, there by affects cholesterol transport into mitochondria. Besides, Liang *et al.*[45] reported neonatal exposure to BaP decreases the levels of serum testosterone and histone H3K14 acetylation of the StAR promoter in the testis of adult SD rats. They also concluded such epigenetic regulation in the StAR promoter region results in decreased expression of StAR. Piecing these evidences together, it can be hypothesized that BaP affects both levels of StAR and transportation of cholesterol into mitochondria by StAR resulting in decreased androgen synthesis, which ultimately affects sperm quality, quantity thereby fertility. In the light of the above, it may be worthwhile to investigate in depth molecular mechanism of action of BaP.

In conclusion, this study provides compelling evidence of altered reproductive functions, including spermatogenesis, steroidogenesis and fertility in rats treated with BaP. Further, *in silico* studies suggests interaction of BaP with StAR which might result in reduced transport of cholesterol into mitochondria ultimately affects androgen synthesis in BaP treated rats. Additional *in vivo* experiments are warranted to confirm the above hypothesis.

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

Figure1. Scanning electron microscopic pictures showing sperm from cauda epididymis of rats; A. normal sperm from control rats; B-J: sperm showing defects in morphology after BaP treatment; B: bent neck; C: misshaped head; D: curved head; E: double headed; F: coiled tail; G: looped tail; H: serrated tail; I and J: curve tail.

Figure 2. A. Uterus of control rat showing implantations (11.67 ± 0.58) on 6th day of pregnancy

B. Uterus of 6th day pregnant rat mated with male rat exposed to 1µg BaP/kg bw during embryonic development (number of implantations 9.75 ± 0.5).

C. Uterus of 6th day pregnant rat mated with male rat exposed to 10µg BaP/kg bw during embryonic development (number of implantations 8.67 ± 0.58).

D. Uterus of 6th day pregnant rat mated with male rat exposed to 100µg BaP/kg bw during embryonic development (number of implantations 6.5 ± 0.71).

Figure 3. Calculated RMSD graph of molecular dynamics simulations of StAR protein using NAMD software.

Figure 4. Crystal (PDB: 3POL) (A) and simulated structure StAR protein (B). The simulated structure is received by energy minimization and equilibrium over the last 500,000 runs with 10 ns of molecular dynamic simulation.

Figure 5. Binding site of StAR protein with cholesterol (A) and BaP (B).

Table 1. Effect of BaP treatment on body weight (g) of rats after sub-chronic exposure of 60 days

	Control	**1 µg**	**10 µg**	**100 µg**
Initial body weight	195.17±9.24	194.12±13.06	197.6±14.61	189.37±9.59
Final body weight	290.92±16.8	289.92±11.58	291.75±11.10	282.14±20.99
Weight gain	95.75±5.17	95.8±6.59	96.15±4.89	92.77±12.18

Values are mean ± S.D. of 10 individuals

Table 2. Effect of BaP on tissue somatic indices (g %) in rats

Parameter	Control	1 µg	10 µg	100 µg
Testes	$1.05^{a} \pm 0.06$	$1.02^{a,b} \pm 0.06$ (-2.86)	$0.91^{b} \pm 0.08$ (-13.33)	$0.71^{c} \pm 0.03$ (-32.38)
Cauda epididymis	$0.11^{a} \pm 0.01$	$0.10^{a,b} \pm 0.01$ (-9.09)	$0.09^{b} \pm 0.01$ (-18.18)	$0.07^{c} \pm 0.01$ (-36.36)
Caput epididymis	$0.99^{a} \pm 0.03$	$0.98^{a} \pm 0.03$ (-1.01)	$0.94^{b} \pm 0.02$ (-5.05)	$0.90^{c} \pm 0.03$ (-9.09)
Corpus epididymis	$0.06^{a} \pm 0.01$	$0.05^{a} \pm 0.01$ (-16.67)	$0.06^{a} \pm 0.01$ (0)	$0.05^{a} \pm 0.01$ (-16.67)
Seminal vesicles	$0.37^{a} \pm 0.03$	$0.35^{a} \pm 0.01$ (-5.40)	$0.29^{b} \pm 0.02$ (-21.62)	$0.25^{c} \pm 0.01$ (-32.43)
Prostate	$0.18^{a} \pm 0.03$	$0.17^{a} \pm 0.02$ (-5.55)	$0.12^{b} \pm 0.02$ (-11.11)	$0.10^{b} \pm 0.01$ (-44.44)
Vas deferens	$0.12^{a} \pm 0.03$	$0.11^{a} \pm 0.02$ (-8.33)	$0.10^{a} \pm 0.01$ (-16.67)	$0.09^{a} \pm 0.01$ (-25.00)
Penis	$0.12^{a} \pm 0.01$	$0.13^{a} \pm 0.01$ (8.33)	$0.11^{a} \pm 0.02$ (-8.33)	$0.11^{a} \pm 0.01$ (-8.33)

Values are mean ± S.D. of 10 individuals
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 $p < 0.01$.

Table 3. Effect of BaP on daily sperm production and epididymal sperm parameters in rats

Parameter	Control	1 µg	10 µg	100 µg
DSP (millions/g. testis)	$25.73^{a} \pm 2.34$	$21.77^{b} \pm 1.51$ (-15.39)	$18.74^{c} \pm 1.42$ (-27.17)	$12.78^{d} \pm 1.36$ (-50.33)
Sperm count (millions/ml)	$73.16^{a} \pm 3.23$	$58.13^{b} \pm 3.44$ (-20.54)	$52.63^{c} \pm 2.98$ (-28.06)	$35.76^{d} \pm 4.77$ (-51.12)
Motile sperm (%)	$76.66^{a} \pm 3.32$	$61.8^{b} \pm 2.31$ (-19.38)	$53.17^{c} \pm 2.48$ (-30.64)	$38.62^{d} \pm 2.67$ (-49.62)
Viable sperm (%)	$78.5^{a} \pm 2.88$	$65.17^{b} \pm 4.17$ (-16.98)	$55.5^{c} \pm 3.27$ (-29.30)	$44.5^{d} \pm 4.03$ (-43.31)
HOS tail coiled sperm (%)	$68.33^{a} \pm 3.01$	$50.83^{b} \pm 4.07$ (-25.61)	$42.5^{c} \pm 3.39$ (-37.80)	$35.76^{d} \pm 4.77$ (-47.66)

Values are mean ± S.D. of 10 individuals
Values in the parentheses are percent decrease from that of control.
Mean values with different superscripts in a row differ significantly from each other at $p < 0.01$.

Table 4. Effect of BaP on 3β- and 17β- HSD activity levels in the testis of rats

Enzyme	Control	1µg	10µg	100µg
3β-HSD (n moles of NAD converted to NADH/mg. protein/min)	$33.97^{a} \pm 2.12$	$30.04^{b} \pm 2.83$ (-11.57)	$20.74^{c} \pm 1.73$ (-38.95)	$11.97^{d} \pm 1.37$ (-64.76)
17β-HSD (n moles of NADPH converted to NADP/mg. protein/min)	$26.43^{a} \pm 2.53$	$21.92^{b} \pm 1.64$ (-17.06)	$17.52^{c} \pm 2.30$ (-33.71)	$9.23^{d} \pm 1.21$ (-65.08)

Values are mean ± S.D. of 10 individuals
Values in the parentheses are percent decrease 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 BaP on serum testosterone, FSH and LH levels in rats

Hormone	Control	1 µg	10 µg	100 µg
Testosterone (ng/mL)	$8.09^{a} \pm 1.02$	$6.17^{b} \pm 0.78$ (-23.73)	$5.14^{b,c} \pm 0.78$ (-36.46)	$4.02^{c} \pm 0.65$ (-50.31)
FSH (ng/mL)	$5.19^{a} \pm 1.93$	$8.83^{b} \pm 1.38$ (70.13)	$9.98^{b,c} \pm 1.09$ (92.29)	$11.56^{c} \pm 1.23$ (122.74)
LH (ng/mL)	$2.92^{a} \pm 0.28$	$3.70^{a,b} \pm 0.40$ (26.71)	$3.93^{b} \pm 0.62$ (34.59)	$5.63^{c} \pm 0.96$ (92.81)

Values are mean ± S.D. of 10 individuals
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 $p < 0.01$.

Table 6. Effect of BaP on reproductive performance in male rats

Parameters	Control	1 µg	10 µg	100 µg
Conception time (days) [$]	1.33[a] ± 0.52	1.5[a] ± 0.75 (12.78)	2.12[a] ± 0.83 (59.4)	4.12[b] ± 1.25 (209.77)
Mating index (%)	100(16/16)	100(16/16)	100(16/16)	100(16/16)
Fertility index (%)	100(16/16)	100(16/16)	75.0(12/16)	62.5(10/16)
No. of corpora lutea/rat[#]	13.75[a] ± 0.95	14.0[a] ± 0.82 (1.82)	13.25[a] ± 0.96 (-3.64)	13.5[a] ± 0.58 (-1.82)
No. of implantations/rat[#]	11.67[a] ± 0.58	9.75[b] ± 0.5 (-16.45)	8.67[c] ± 0.58 (-25.71)	6.5[d] ± 0.71 (-44.30)
Pre- implantation loss (%)	15.13	30.36	34.57	51.85
No. of live fetuses/rat[#]	11.33[a] ± 0.58	9.33[b] ± 0.58 (-17.65)	7.5[c] ± 0.71 (-33.80)	5.5[d] ± 0.71 (-51.46)
No. of resorptions/rat[#]	0.0[a] ± 0.0	0.67[b] ± 0.58	1.67[c] ± 0.58	3.25[d] ± 0.5
Post- implantation loss (%)	2.91	4.31	13.49	15.38

Values are mean ± S.D; [$]n = 16; [#]n = 5
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 $p < 0.01$.

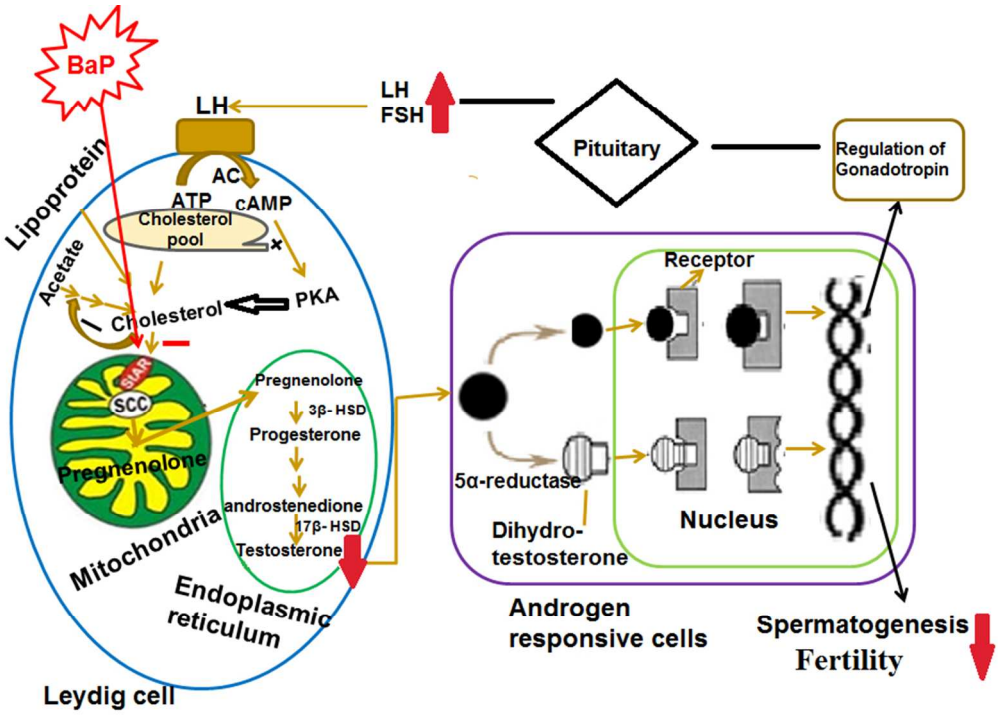
Table 7. Effect of BaP on developmental milestones in F_1 pups.

Parameter	Control	1 µg	10 µg	100 µg
Pups/rat[$]	10.67[a] ± 0.58	8.67[b] ± 0.58 (-18.74)	7.33[c] ± 0.58 (-31.30)	5.67[d] ± 0.58 (-46.86)
Body weight of pups[#] (g)	6.21[a] ± 0.13	6.20[a] ± 0.13 (-0.16)	6.09[a] ± 0.12 (-1.93)	5.71[b] ± 0.21 (-8.05)
Crown rump length[#] (cm)	4.9[a] ± 0.16	4.64[b] ± 0.11 (-5.31)	4.46[c] ± 0.11 (-8.98)	4.18[d] ± 0.08 (-14.69)
Anogenital distance (male)[@] (cm)	0.35[a] ± 0.05	0.35[a] ± 0.05 (0.0)	0.33[a] ± 0.03 (-5.71)	0.33[a] ± 0.03 (-5.71)
Anogenital distance (female)[@] (cm)	0.17[a] ± 0.02	0.18[a] ± 0.03 (5.88)	0.17[a] ± 0.03 (0.0)	0.17[a] ± 0.03 (0.0)
Lower incisor eruption[#] (days)	3.0[a] ± 0.71	3.2[a] ± 0.45 (6.67)	3.2[a] ± 0.45 (6.67)	3.3[a] ± 0.55 (10.00)
Upper incisor eruption[#] (days)	7.6[a] ± 0.55	7.8[a] ± 0.84 (2.63)	8.0[a] ± 0.71 (5.26)	8.2[a] ± 0.84 (7.89)
Pinna unfolding[#] (days)	2.8[a] ± 0.45	2.8[a] ± 0.45 (0.0)	3.0[a] ± 0.71 (7.14)	3.0[a] ± 0.45 (7.14)
Eye slit formation[#] (days)	3.6[a] ± 0.55	4.2[a] ± 0.45 (16.67)	3.8[a] ± 0.45 (5.55)	4.0[a] ± 0.55 (11.11)
Fur development[#] (days)	5.2[a] ± 0.45	5.6[a] ± 0.89 (7.69)	5.2[a] ± 0.45 (0.0)	5.8[a] ± 0.84 (11.54)
Eye opening[#] (days)	15.0[a] ± 0.71	15.2[a] ± 0.84 (1.33)	15.4[a] ± 0.55 (2.67)	15.8[a] ± 0.84 (5.33)
Testes descent[@] (days)	21.6[a] ± 0.55	22.4[a] ± 0.55 (3.70)	24.6[b] ± 0.89 (13.89)	26.8[c] ± 0.84 (24.08)
Vaginal opening[@] (days)	32[a] ± 0.71	32.2[a] ± 0.84 (0.62)	36[b] ± 0.71 (12.5)	38.8[c] ± 0.84 (21.25)

Values are mean ± S.D. [$]n = 6; [#]n = 30; [@]n = 15

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 $p < 0.01$.



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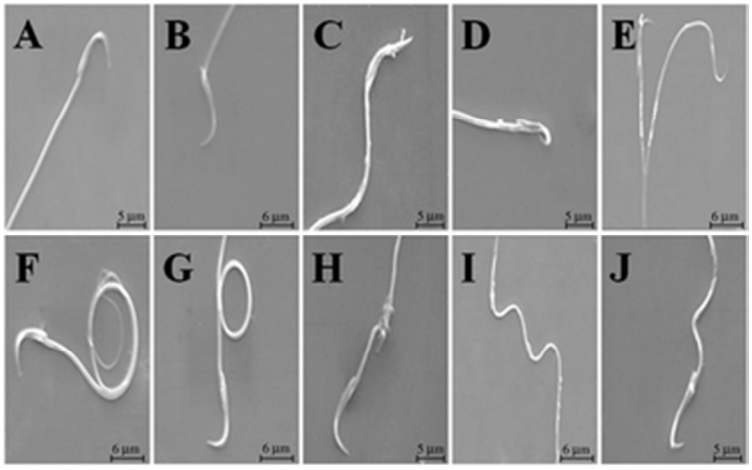

Scanning electron microscopic pictures showing sperm from cauda epididymis of rats;
A. normal sperm from control rats; B-J: sperm showing defects in morphology after BaP treatment; B: bent neck; C: misshaped head; D: curved head; E: double headed; F: coiled tail; G: looped tail; H: serrated tail; I and J: curve tail.

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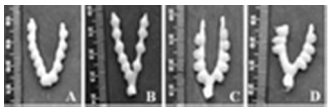

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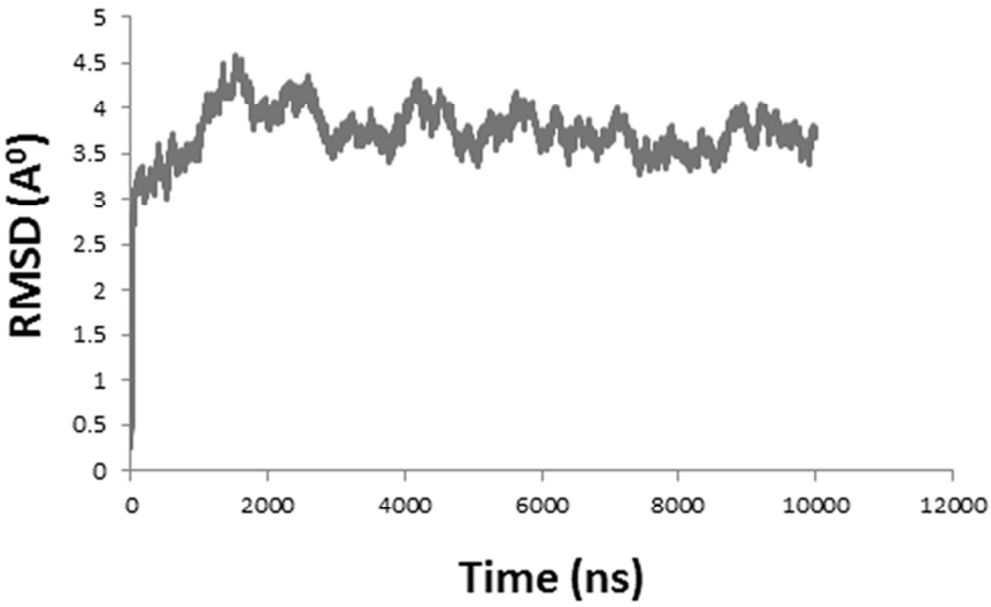
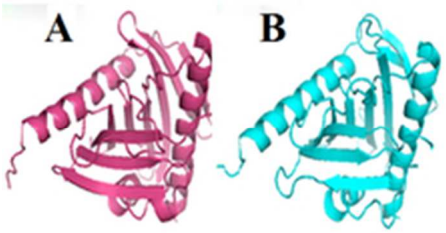
C. Uterus of 6th day pregnant rat mated with male rat exposed to 10µg BaP/kg bw during embryonic development (number of implantations 8.67 ± 0.58).
D. Uterus of 6th day pregnant rat mated with male rat exposed to 100µg BaP/kg bw during embryonic development (number of implantations 6.5 ± 0.71).

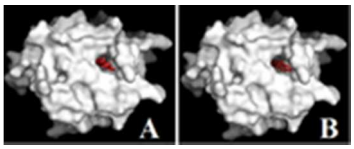
6x2mm (600 x 600 DPI)



Calculated RMSD graph of molecular dynamics simulations of StAR protein using NAMD software.
29x17mm (600 x 600 DPI)



Crystal (PDB: 3POL) (A) and simulated structure StAR protein (B). The simulated structure is received by energy minimization and equilibrium over the last 500,000 runs with 10 ns of molecular dynamic simulation.
9x5mm (600 x 600 DPI)



Binding site of StAR protein with cholesterol (A) and BaP (B).
7x2mm (600 x 600 DPI)