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Original Article

Effect of Sodium Valproate on the Toxicity of Cyclophosphamide in the Testes of Mice: Influence of Pre- and Post-Treatment Schedule

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ABSTRACT

Recently, sodium valproate (VPA) has been proven as histone deacetylase (HDAC) inhibitor and potentiates the cytotoxicity of anticancer drugs, and also exhibit promising anti-cancer activity. Present study aimed to investigate the influence of pre- and post-treatment of VPA on cyclophosphamide (CP) induced genotoxicity and germ cell toxicity in mice. All the animals were treated with VPA at the dose of 500 mg/kg/day on alternate day thrice/week for a period of two weeks, CP at the dose of 200 mg/kg on 7th and 15th day and sacrificed 24 h after administration (*i.p.*) of the last dose. End point of evaluation includes sperm count, sperm head morphology, sperm comet assay and histology. VPA treatment significantly decreases CP induced sperm count, testes and epididymis weight; increased sperm head abnormality and sperm DNA damage. Both VPA pre- and post-treatment augmented CP induced DNA damage and the germ cell toxicity; however, pre-treatment induced more cytotoxicity and genotoxicity as compared to post-treatment.

Key words: Cyclophosphamide, DNA damage, histone deacetylase inhibitor, mice, sodium valproate

INTRODUCTION

Recently, VPA has been shown to have histone deacetylase (HDAC) inhibition activity and potentiate the cytotoxicity of commonly used anti-cancer drugs through epigenetic mechanisms that shown promising effects in clinical development for cancer treatment.[1-3] Several clinical trials are in progress for the evaluation of anti-cancer efficacy of the combination treatment of HDAC inhibitors with the conventional anti-cancer agents in various types of cancers.[2,4] However, many questions have been raised for this relatively non-specific therapy such as precise mechanisms of *in vivo* activity or resistance, mechanisms of toxicity, minimally effective dose and predictive markers for response.[5,6] Cyclophosphamide (CP) is a cytotoxic bifunctional alkylating agent belongs to the nitrogen mustard class and extensively used for the treatment of various cancers, immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and other benign diseases also.[7,8]

VPA has been reported to function as a histone deacetylase inhibitor, leading to the increased acetylation of the histone tails. Increased histone acetylation results in chromatin decondensation owing to the attenuation of the electrostatic charge interactions between histones and DNA.[9,10] VPA induced chromatin decondensation may lead to enhanced sensitivity of DNA to nucleases and increased DNA binding by intercalating agents, thereby increasing the access of macromolecules and xenobiotics to DNA.[11] Moreover, it has been reported that the VPA-induced modulation of the chromatin structural proteins to be a dose- and

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time-dependent phenomenon.[12] The HDAC inhibitors induces a diverse array of biological effects on cancerous, as well as normal cells, it supported the cell-cycle arrest and induced apoptosis.[13] Further, HDAC inhibitors also induces apoptosis *via* both the extrinsic (death receptor) and intrinsic (mitochondrial) pathways.[3] HDAC inhibitors including VPA could affect the epigenome and alter multiple cellular signalling pathways, which suggests that it potentially affect the fate of xenobiotics not only during critical period of development, but also in later life.[14] This has implications on drug action and toxic effects of xenobiotics and/or drugs, since cell-specific epigenetic variation could result in differential pharmacodynamics, pharmacokinetics and toxic response in different tissues.[15] Recent findings suggested that a list of epigenetic modifications in the mature sperm may have a potential role in the development of the embryo and alterations in the epigenetic profile have been associated with infertility in humans.[16,17]

Keeping this in view, we hypothesized that when both these drugs are used in combination, this may alter the toxicity profile of CP by perturbing DNA integrity and chromatin packaging (epigenetic mechanisms). Therefore, when HDAC inhibitors will be used in combination with cytotoxic drugs, the toxic effects may be exaggerated in the tissue and cell specific manner. Thus, an attempt has been made to evaluate toxicity of CP under the influence of VPA (HDAC inhibitor). Hence, the present study was undertaken to explore the possible influence of VPA pre- and post-treatment on the CP induce germ cell toxicity and DNA damage.

MATERIALS AND METHODS

Animals

All the animal experiment protocols were approved by the Institutional Animal Ethics Committee (IAEC) and the experiments on animals were performed in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experimentation on Animals) guidelines. Experiments were performed on male Swiss mice (25 ± 5 g) procured from the Central Animal Facility (CAF) of the institute. All the animals were kept under controlled environmental conditions at room temperature (22 ± 2°C) with 50 ± 10% humidity and an automatically controlled cycle of 12 h light and 12 h dark. Standard laboratory animal feed (purchased from commercial supplier) and water were provided *ad libitum*. Animals were acclimatized to the experimental conditions for a period of one week prior to the commencement of the experiment.

Chemicals

Sodium valproate (CAS no. 1069-66-5), cyclophosphamide (CAS no. 6055-19-2), bovine serum albumin (CAS no. 9048-46-8), hematoxylin and eosin (H and E), Trizma (CAS no. 77-86-1), dithiothreitol (CAS no. 3483-12-3), Proteinase-K (CAS no. 39450-01-6) and SYBR Green I (CAS no. 163795-75-3) were purchased from Sigma-Aldrich Chemicals, Saint Louis, MO, USA. Dimethyl sulphoxide (DMSO), normal melting point agarose (NMPA), low melting point agarose (LMPA), Triton X-100, ethylenediamine-tetraacetic acid (EDTA) and Hank's balanced salt solution (HBSS) were obtained from HiMedia Laboratories Ltd., Mumbai.

Dose selection and chemical preparation

The dose of CP (200 mg/kg) was selected on the basis of studies conducted by[7,18,19] in mice to assess testicular toxicity after acute and chronic exposure; while the dose of VPA pre- and post-treatment (500 mg/kg) and duration of exposure were selected from the studies conducted by[11,20] to evaluated anticancer efficacy of VPA as HDAC inhibitor in mice. Both VPA and CP were dissolved in distilled water and were administered through *i.p.* route according to body weight.

Experimental design and animal treatment

The animals were randomized into 4 groups consisting of 8 animals in each group for the evaluating the influence of pre- and post-treatment of VPA on the germ cell toxicity and DNA damage of CP. All the four group label as, group-1; control receiving normal saline, group-2; CP alone (CP) receiving CP alone, group-3; pre-treatment (VPA: CP) receiving VPA prior to CP and group-4; post-treatment (CP: VPA) receiving CP prior to VPA. The drug treatment, VPA was given at the dose of 500 mg/kg/day at alternate day thrice/week for two weeks (six doses) and CP at the dose of 200 mg/kg/day on 7th and 15th day and all the animals were sacrificed 24 h after administration of the last dose [supplementary Figure 1]. Both VPA and CP were administered through *i.p.* route. For the administration of CP special consideration was taken, CP was injected at same day in the CP control, VPA pre- as well as post-treatment groups from a single solution to eliminated environmental, dosing variations and CP exposure time.

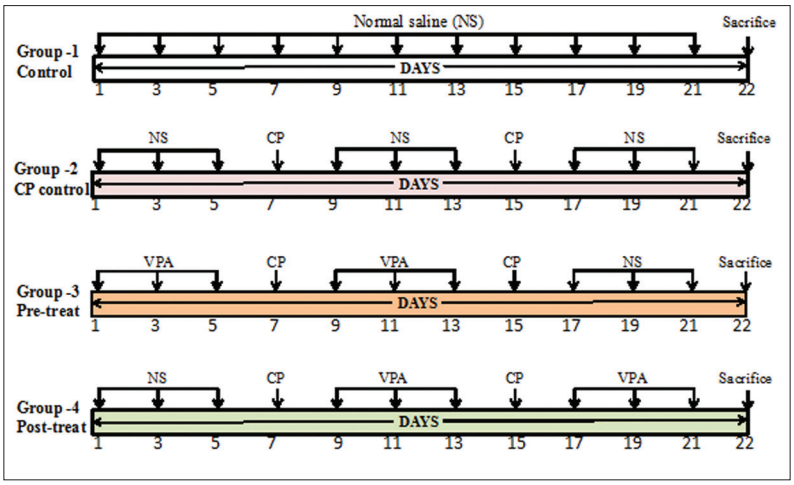
Animal observation, body and organ weight

All the animals were regularly observe for the signs of toxicity and survival rate was recorded during the entire study. The body weight of each animal was recorded at initiation of study and once in a week during study, as well as prior to sacrifice. The testes and epididymis were dissected immediately after sacrifice and wash with chilled normal saline then saline was soaked on blotting paper and organs weight were recorded for individual animal.

Sperm count and sperm head morphology

After the animal sacrifice, epididymis was removed and placed in a Petri-plate containing 2 ml of HBSS medium

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Supplementary Figure 1: Schematic diagram illustrated the experimental design. Group 1, received normal saline once alternative day for a period of 21 days and served as vehicle control. Group 2, received normal saline once alternative day and CP 200 mg/kg on the 7th and 15th day and served as CP control. Groups 3, received VPA 500 mg/kg prior to CP 200 mg/kg and serve as pre-treatment while group 4, received same dose CP prior to VPA once alternate day as shown above and served as post-treatment. All the animals were sacrificed 24 hour after receiving the last injection.

at room temperature. The epididymis was cut into small portions to allow the sperm to swim out. The solution containing the sperm was centrifuged at 1000 rpm for 3 min. After centrifugation, 1 ml of supernatant was taken and used for sperm counting and sperm head morphology. The epididymal sperm count was determined using Neubauer's hemocytometer. The sperm count was expressed as number of sperm per ml of solution containing sperm. For sperm head morphology, 0.5 ml of above solution containing the sperm and 0.5 ml of 2% eosin solution were mixed and kept for 1 h to stain the sperm. Smear was prepared using 2-3 drops of the above solution then air dried and fixed with absolute methanol for 3 min. Two hundred sperm per animal was examined to determine the morphological abnormalities under oil immersion. Sperm head morphology was categorised as normal, quasinormal and grossly abnormal as described by Burruel *et al.*[21] Sperm missing the rostral part of the acrosome and or the posterolateral region of the acrosome were classified as quasinormal sperms and those with collapsed, triangular and amorphous heads with highly deformed acrosomal caps and nuclei were classified as grossly abnormal sperm. Data was shown in terms of % of abnormal sperm.[22]

Sperm comet assay

The sperm comet assay was performed as described by Haines *et al*. with some modification.[18,23] Sperm sample (5 µl) containing 1-3 × 10^4 sperms per ml were suspended in 95 µl of 0.5% (w/v) LMPA. From this suspension, 80 µl was applied to the surface of a microscope slide (pre-coated with 1% NMPA) to form a microgel and allowed to set at 4°C for 5 min. A second layer of 1% LMPA was added and allowed to set at 4°C for 5-10 min. Slides were dipped in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl, pH 10.0 containing 1% Triton X-100 and 40 mM Dithiothreitol) for 24 h at room temperature and protected from light. Following the initial lysis, proteinase K was added to the lysis solution (0.5 mg/ml) and additional lysis was performed at 37°C for 24 h. Following cell lysis, all slides were washed three times with deionized water at 10 min intervals to remove salt and detergent from the microgels. Slides were then coded and placed in a specifically designed horizontal electrophoresis tank (Model, CSLCOM20, Cleaver Scientific Ltd., UK) and DNA was allowed to unwind for 20 min in an alkaline solution containing (500 mM NaCl, 100 mM Tris-base and 1 mM EDTA, pH 9). Electrophoresis was conducted at 28 V, 250 mA for 30 min. After electrophoresis, slides were neutralized and the DNA fluorochrome SYBR Green I (1:10,000 dilution) was applied for 1 h. Slides were rinsed briefly with double-distilled water and cover slips were placed before image analysis. The fluorescent labelled DNA was visualized (200x) using an AXIO Imager M1 fluorescence microscope (Carl Zeiss, Germany) and the resulting images were captured on a computer and processed with image analysis software (Comet Imager V.2.0.0). Duplicate slides were prepared for each treatment and were independently coded and scored without the knowledge of the codes. The edges of the slides, any other damaged part of the gel, debris and superimposed comets without distinct head ('hedgehogs' or 'ghost' or 'clouds') were not taken in to account for scoring and analysis. The parameters for the DNA damage analysis include: tail length (TL, in µM), tail moment (TM), tail moment olive (OTM) and % tail DNA (% DNA). TL is the maximum distance that the damaged DNA migrates from the centre of the cell nucleus. % DNA is the total DNA that migrates from the nucleus into the comet tail. TM is the product of the TL and % DNA, which gives a more integrated measurement of overall DNA damage in the cell. OTM is the product of % DNA and median migration distance.

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Histological evaluation and quantification

Histological slides were prepared as previously standardized in our laboratory and described by khan *et al.*[24] The testes were fixed in 10% formalin, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Tissue sections (5 µm) were mounted on glass slides coated with Mayer's albumin and dried overnight. The sections were then deparaffinized with xylene, rehydrated with alcohol and water. The rehydrated sections were stained using H and E, mounted with DPX mounting media and examined under the microscope (Olympus B×51 microscope, Tokyo, Japan). The sections from each animal were evaluated for structural changes.

Histological quantification was performed in the testicular sections by allotting a Johnsen's score following the criteria of scoring as described.[25] Johnsen's score 10 indicates full spermatogenesis, 9-disorganized tubular epithelium, 8-few late spermatids, 7-no late spermatids, 6-few early spermatids, 5-no spermatids, 4-few spermatocytes, 3-spermatogonia only, 2-no germ cells, Sertoli cells only and 1-no seminiferous epithelial cells. Thirty seminiferous tubules from each animal were randomly examined and Johnsen's score was given based on the type of the cells damaged in the seminiferous tubule. Johnsen's score was calculated by dividing the sum of all the scores with the total no. of seminiferous tubules examined. Further number of seminiferous tubule per unit area of testes section at 40x were also counted as described.[26] Twenty focuses from each animal were random examined. Number of seminiferous tubule/unit area was calculated by dividing the sum of number of tubule in each focus with total number of focus examined.

Statistical analysis

Results were shown as mean ± standard error of mean (SEM) for each group. Statistical analysis was performed using Jandel Sigma Stat (version 3.5) statistical software. Significance of difference between multiple groups, one-way analysis of variance (ANOVA) was used. In case ANOVA showed significant differences, post-hoc analysis was performed with Tukey's test. $P < 0.05$ was considered to be statistically significant. For the survival analysis LogRank tests was used. In case LogRank showed significant differences, multiple comparisons between the experimental groups was performed with Holm-Sidak tests. Linear regression analysis was performed in order to observe the correlation between the OTM, as well as % DNA and % grossly abnormal sperm head, sperm count and Johnsen's score.

RESULTS

Body and organ weight

Both CP alone and VPA pre-treatment induced significant decrease in the body weight as compared to control and CP alone, respectively [Figure 1a]. In addition, the VPA pre- and post-treatment were significantly decreased the testes and epididymis weight as compared to the CP alone; while CP alone also significantly decreased the testes weight [Figure 1b]. Further, CP alone and VPA pre-treatment, as well as VPA post-treatment were significantly decreased the relative testes weight while no effect on the relative epididymis weight as compared to respective control groups [Figure 1c]. The VPA pre-treatment was shown to more decrease in the body and organ weight as compared to the VPA post-treatment.

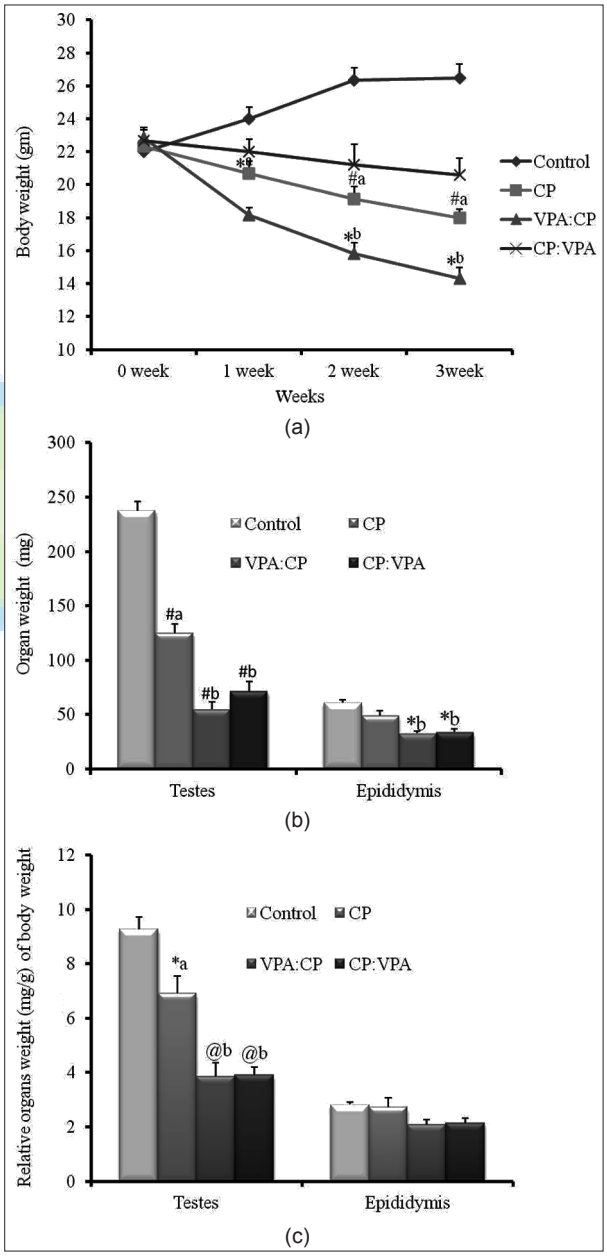


Figure 1: Effect of CP alone and pre- as well as post-treatment of VPA with CP on (a) body weight, (b) organs weight and (c) relative organs (testes and epididymis) of mice, respectively. All the values were expressed as mean ± SEM, (n = 4-8), #$P < 0.001$, @$P < 0.01$ and *$P < 0.05$, 'a' *vs.* control and b *vs.* CP control

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Survival and mortality analysis

The VPA pre-treatment significantly affected the survival/mortality rate of the animal as compared to control and CP alone. Further, both VPA pre- and post-treatment were shown 50% and 12.5% mortality (50% and 87.5% survival) rate as compared to CP alone respectively, whereas CP alone did not affect the survival/mortality rate of the animal as compare to control [Figure 2]. The VPA pre-treatment showed mortality 25% on 10th, 12.5% on 13th and 16th day of experiment, while in the post-treatment

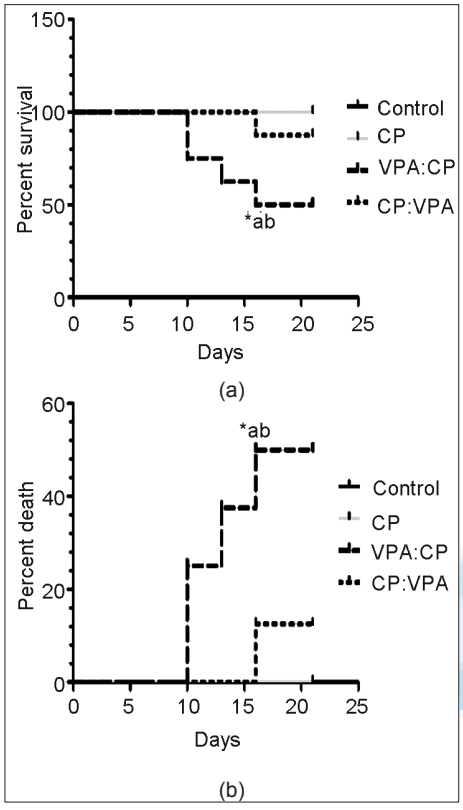


Figure 2: Effect of CP alone and pre- as well as post-treatment of VPA with CP on (a) survival and (b) mortality rate of the animals. The VPA pre-treatment affect survival/mortality rate of the animals on 10th, 13th and 16th day of experiment. All the values were expressed as percent, (n = 4-8), *P < 0.05, 'a' *vs.* control and b *vs.* CP control

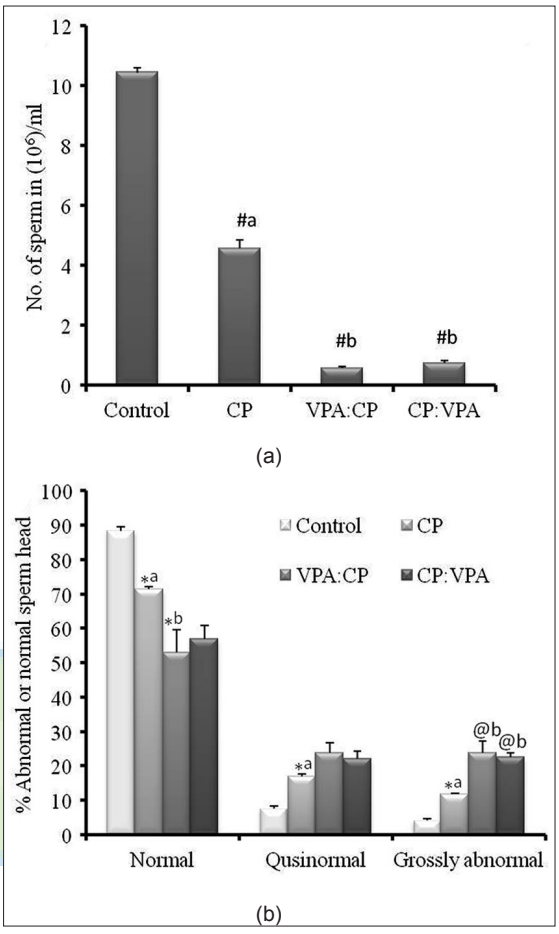


Figure 3: Effect of CP alone and pre- as well as post-treatment of VPA in combination on (a) the sperm count and (b) % abnormal or normal sperm head, respectively. All the values were expressed as mean ± SEM, (n = 4-5), #P < 0.001, @P < 0.01 and *P < 0.05, 'a' *vs.* control and 'b' *vs.* CP control

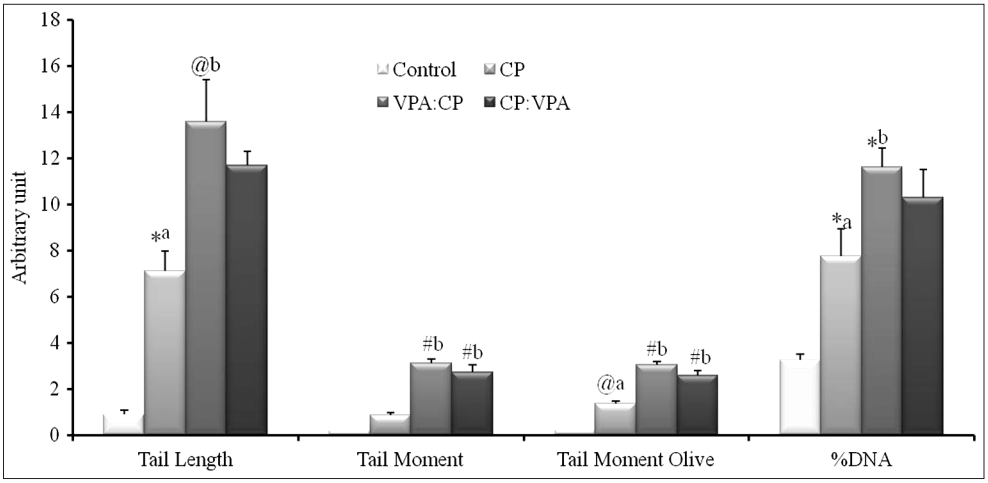


Figure 4: Effect of the CP alone and pre- as well as post-treatment of VPA in combination with CP on the sperm DNA damage. All the values were expressed as mean ± SEM, (n = 4-5), #P < 0.001, @P < 0.01 and *P < 0.05, 'a' *vs.* control and b *vs.* CP control

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only 12.5% mortality was observed on 16th day. The above result indicated VPA pre-treatment increased the accessibility DNA to CP and enhanced the cytotoxic effect of CP which favor to the hypothesis.

Sperm count and sperm head morphology

A significant decline in the sperm count was observed in the CP alone and VPA pre- as well as post-treatment as compared to the control and CP alone [Figure 3a]. However, the decline in the sperm count is more in VPA pre-treatment as compared to post-treatment. The CP alone and VPA pre- as well as post-treated were induces abnormality in the sperm head as compared to the control and CP control. Further, the % grossly abnormal sperm head was significantly increased after CP alone and VPA pre- as well as post-treatment while CP alone also significantly increased the % quasinormal sperm head as compared to respective control groups [Figure 3b].

Sperm DNA damage

The CP alone and VPA pre-treatment with CP induced DNA damage in sperm as observed from a significant increase in different comet parameters such as TL, TM, OTM and % DNA as compared to control and CP alone. However, VPA post-treatment also significantly induced the DNA damage in the sperm as observed in TM and OTM only as compared to CP control. Further, as expected the pre-treatment of VPA induced more DNA damage in the sperm as compared to post-treatment [Figure 4].

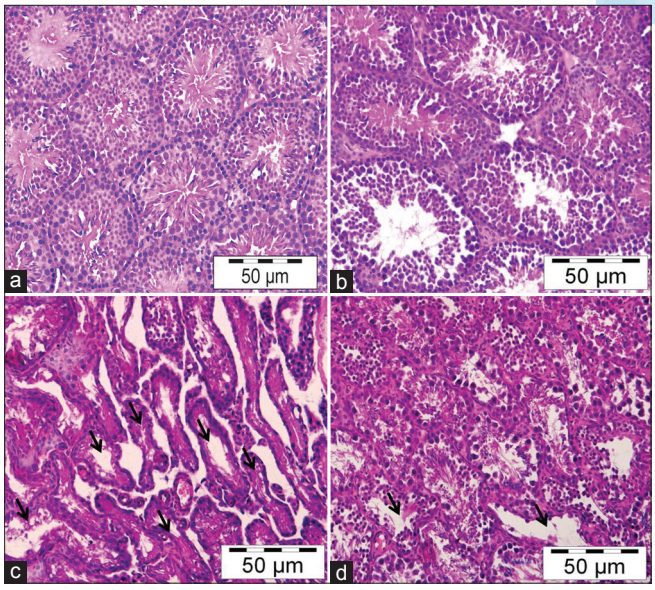


Figure 5: Representative photomicrographs of mice testes sections stained with hematoxylin and eosin (H and E). (a) Control, (b) CP control, (c) VPA pre-treatment and (d) VPA post-treatment at the dose of CP (200 mg/kg) alone and VPA (500 mg/kg) pre- as well as post-treatment with CP. The arrow indicated the severely damage and distorted seminiferous tubule of mice testes after VPA pre- and post-treatment

Histological observation and quantification

The morphological and structural alteration such as disorganization and distortion of the seminiferous tubules, vacuolization, as well as reduction in the size of the seminiferous tubules of testes and also degeneration in spermatogonia, spermatocytes and spermatids were induced by both VPA pre- as well as post-treatment but pre-treatment completely disrupted testicular structure as shown in testes histology [Figure 5]. However, CP alone was also induced degeneration of spermatocytes and spermatids in the testes. The quantitative assessment of the seminiferous tubules was done based on the type of cells damaged, as well as extends of damage, the Johnsen's score was allotted from 1 to 10 accordingly and no. of seminiferous tubules/unit area was also counted. A significant decreased in the Johnsen's score were observe in CP alone and VPA pre- as well as post-treatment as compared to control and CP alone [Figure 6a]. Further, the VPA pre-treatment significantly increases the no. of seminiferous tubule/unit area (size of tubule) as compared to CP alone while CP alone, as well as VPA post-treatment did not affect size of seminiferous tubules as compared to control and CP alone [Figure 6b].

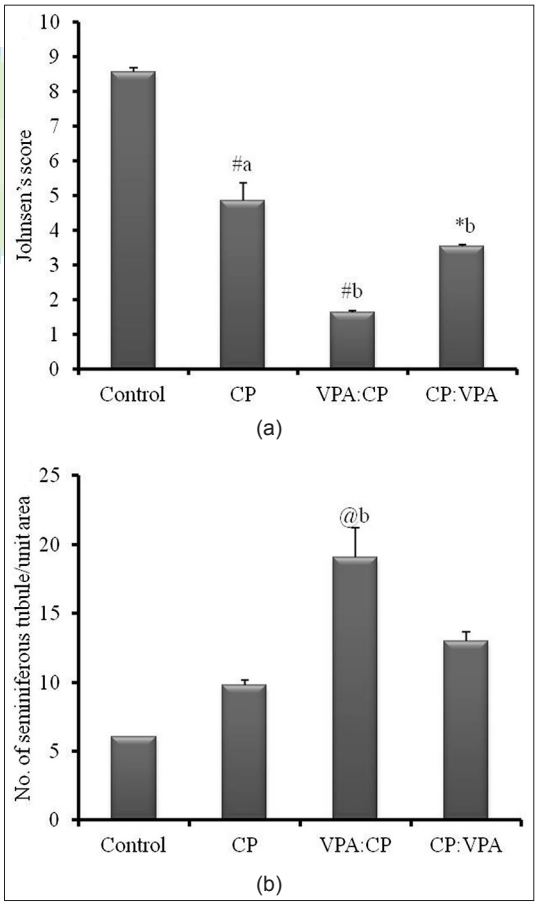


Figure 6: Effect of the CP alone and pre- as well as post-treatment of VPA on testicular structural alterations in the mice (a) the Johnsen's score and (b) the seminiferous tubule size. All the values were expressed as mean ± SEM, (n = 4-5), #P < 0.001, @P < 0.01 and *P < 0.05, 'a' *vs.* control and 'b' *vs.* CP control

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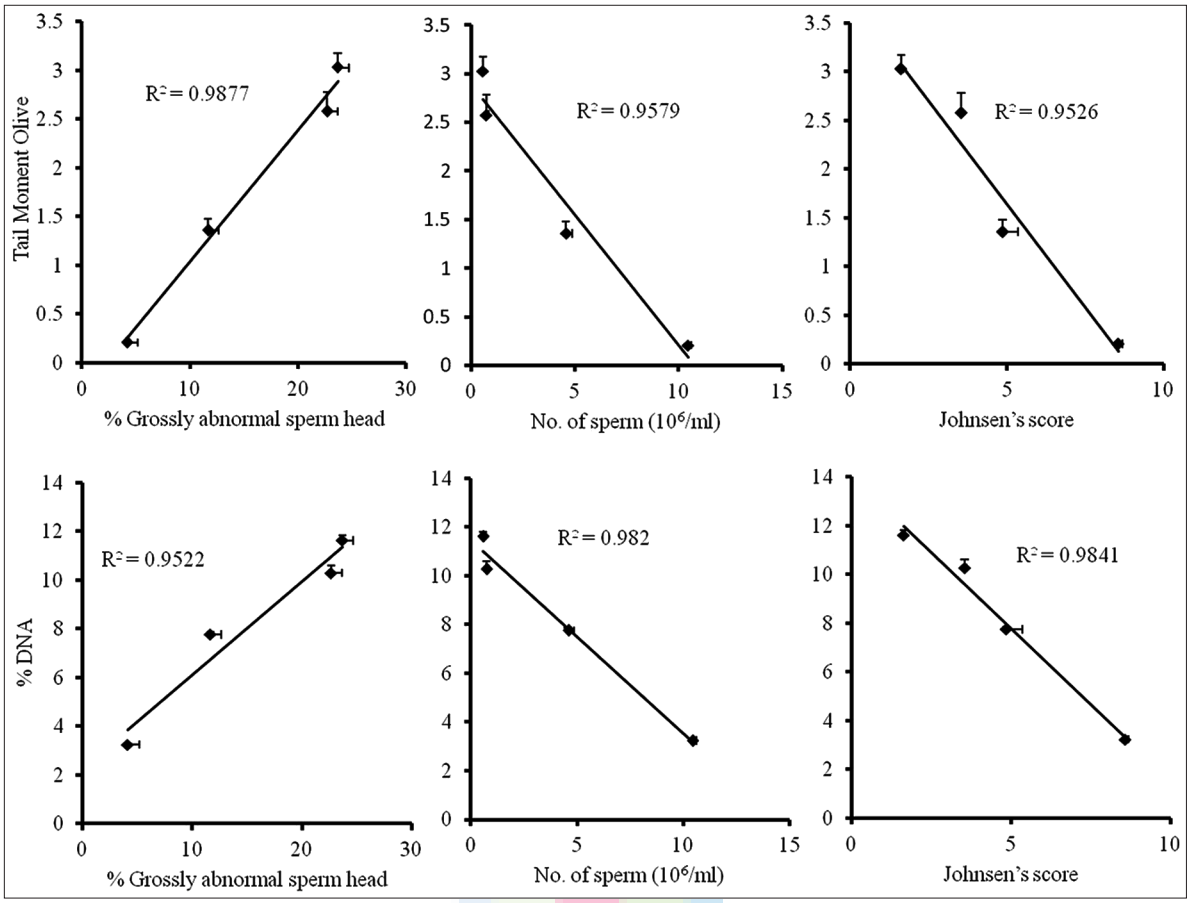


Figure 7: The linear regression analysis showing the correlation of the OTM, as well as % DNA (DNA damage) and sperm count, % grossly abnormal sperm head and Johnsen's score of the mice treated with CP alone and VPA pre- as well as post-treatment at the doses of 200 and 500 mg/kg, respectively. All the values are expressed as mean ± SEM, (n = 4-5)

Correlation between OTM, as well as % DNA and % grossly abnormal sperm head, sperm count and Johnsen's score

The simple linear regression analysis showed a strong positive correlation between the OTM, as well as % DNA and % grossly abnormal sperm head (R^2 = 0.987 and 0.952) respectively [Figure 7]. Further, the strong negative correlation were also be observed between the OTM, as well as % DNA and sperm count and Johnsen's score (R^2 = 0.957, 0.952, 0.982 and 0.984) respectively [Figure 7]. The R^2 value of all correlation analysis with OTM and % DNA (DNA damage) and other parameter were near to unit indicated DNA damage is one of the major factors responsible for the germ cell toxicity of CP alone as well as VPA pre- and post-treatment.

DISCUSSION

In the present study, CP alone and VPA pre-treatment resulted into a statistically significant reduction in the body weight at the doses of 200 mg/kg and 500 mg/kg as compared to control and CP alone. CP alone and VPA pre- as well as post-treatment significantly decreased the normal weight of the testes and epididymis, as well as the relative weight of testes only as compared to control and CP alone. The reduction in the body weight by CP treatment may be due to the direct cytotoxic effect of the drug, while further reduction in the body weight by VPA pre-treatment might be due to HDAC inhibition activity of the VPA and the subsequent effects on the chromatin integrity, which further enhanced the cytotoxic potential of CP as compared to CP alone and post-treatment. Further, the reduction in the weight of testes and epididymis by CP alone and VPA pre- as well as post-treatment may be due to degeneration and inhibition of spermatogenic cell proliferation, as well as direct cytotoxic effects of the drug on the highly proliferating germ cells as evident from the histological evaluation. In the testes histological sections increased disorganization, vacuolization, decreased spermatogonial, spermatocytes and spermatid counts were observed which indicated that VPA pre-treatment have more toxic effects as compared to post- and CP alone, which is clearly evident from the structural alterations in the histological sections and quantitative histological evaluations such as the Johnsen's score, as well as the number of seminiferous tubule/unit area. Almost all of the seminiferous tubules were found to be completely deprived of the spermatogenic cells

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and cellular structures in CP alone and VPA pre- as well as post-treatment group, whereas the extent of this effect very much prominent in VPA pre-treatment as compared to post-treatment and CP alone.

Moreover, CP alone and VPA pre- as well as post-treatment induces significant degeneration in the germinal cells which lead to increased abnormality in the sperm head morphology and decline in sperm count. However, VPA pre-treatment induces prominent decline in the sperm count and increases the frequencies of abnormal sperm head as compared to VPA post-treatment and CP alone. This can be endorsed from the damage in spermatogonia and spermatocytes and hence impaired the spermatogenesis, which leads to significant reduction in the sperm count and an increase in sperm head abnormalities. Our earlier studies also suggested that VPA *per se* acts as a reproductive toxicant at high doses and in chronic treatment schedule. However, here this possibility can be ruled out due to limited treatment of VPA (only six dose at alternate days).[24] The sperm head morphology gives a rough assessment of the functional capability of the spermatozoa and reveal the quality of the sperm DNA.[27] However, sperm comet assay measure DNA damage in the sperm and hence can add further information on the quality of the sperm. It has also been reported that, there is a strong positive correlation between the sperm comet assay and the sperm head morphology.[28] In the present study, sperm comet assay revealed the DNA damaging potential of CP alone and VPA pre-treatment as evident from the significant increase in the TL, TM, OTM and % DNA, whereas VPA post-treatment significantly increased the TM and OTM as compared to respective control. The increase DNA damage in the sperm by VPA pre-treatment as compared to post-treatment and CP alone might be due to HDAC inhibition activity of VPA that can lead to histone hyperacetylation, which causes chromatin decondensation and increased accessibility of the DNA to CP and macromolecule that enhances the DNA damaging potential of CP. Recent report suggested that VPA alone or combination with cisplatin triggers the hyperacetylation of histone H3, production of reactive oxygen species, cytochrome c release from mitochondria are increases in the presence of valproate, indicated a mechanistic evidence of synergistic effects of the VPA on apoptosis and cell death.[29] Moreover, in the present study 50% and 12.5% mortality were observed in the VPA pre- and VPA post-treatment as compared to CP alone, respectively. The prominent effect on the mortality/survival of VPA pre-treatment might be due to the HDAC inhibition by the VPA leading to relaxation of chromatin, which further increases the interaction of DNA with xenobiotic or genotoxins and significantly potentiate the cytotoxic potential of CP, while the same effect is not as much in the VPA post-treatment due to limited alteration/accessibility of the chromatin (DNA) to CP as compared to CP alone. Additionally, correlation analysis between OTM and % DNA (DNA damage) and % grossly abnormal sperm head, Johnsen's score and sperm count have been well correlated. All of the parameters have been shown a considerable correlation ($R^2 \sim 1$) either positive or negative with both OTM, as well as % DNA (DNA damage), which further indicated that involvement of DNA damage is a most important factor responsible for the significant increase in the CP induced germ cell toxicity, as well as DNA damage in combination with VPA pre-treatment as compared to CP alone and post-treatment.

Recently, it has been reported that HDAC inhibitors induced apoptosis in normal, as well as tumor cells in a tissue and cell specific manner.[3,10,30] Further, during spermatogenesis and sperm maturation there are several epigenetic modifications such as methylation, acetylation and replacement of histone by protamine, which are essential for the functional competence of the sperm.[16,31,32] One of the plausible reasons for the further increases in the CP induced germ cell toxicity and DNA damage in combination with VPA pre- and post-treatment may be due to the HDAC inhibition by VPA lead to histone acetylation, that may alter the gene expression and chromatin decondensation, which facilitated DNA damage produced by CP.[11] From the above evidences, it can be envisaged that VPA might exert its enhanced germ cell toxicity and DNA damage induced by CP due to interfere in the acetylation of various protein involved in the replacement of histone by protamine which is essential for the functional competence of the sperm. The HDAC inhibitors constitute an attractive therapeutic intervention strategy in combination with other anticancer agents and presently undergoing an extensive pre-clinical and clinical testing.[4,9] Besides antiepileptic activity of VPA, it is currently under clinical development with different chemotherapeutic agents for the treatment of various types of cancers, so it is highly pertinent to assess its clinical efficacy, as well as toxicity of the combination therapy.

Overall, the present results clearly demonstrate that CP alone and VPA pre- as well as post-treatment induced germ cell toxicity and DNA damage as evident from the histological evaluation of testes, increased sperm head abnormalities, sperm DNA damage, and the decrease sperm count. Moreover, increase in the sperm DNA damage and % abnormality in sperm head indicated genotoxic potential of CP alone and in combination with VPA in germ cells. Further, both VPA pre- and post-treatment enhances the germ cell toxicity and DNA damage of CP, while the VPA pre-treatment have been shown to much prominent effect on the germ cell toxicity and DNA damage of CP. The present study provides plausible influence of pre- and post-treatment of VPA (HDAC inhibitor) on the germ cell toxicity and DNA damage of CP. Based on present results, it is essential to characterize the germ cell toxicity and DNA damaging potential of VPA, as well as different HDAC inhibitors with various cytotoxic drugs using other surrogate end points, as well as different study design in

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order to reduce the potential risk in the patient, who are on combination therapy particularly in the reproductive age.

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