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RESEARCH

Attenuation of sodium arsenite mediated ovarian DNA damage, follicular atresia, and oxidative injury by combined application of vitamin E and C in post pubertal Wistar rats

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Abstract

Arsenic being a toxic metalloid ubiquitously persists in environment and causes several health complications including female reproductive anomalies. Epidemiological studies documented birth anomalies due to arsenic exposure. Augmented reactive oxygen species (ROS) generation and quenched antioxidant pool are foremost consequences of arsenic threat. On the contrary, Vitamin E (VE) and C (VC) are persuasive antioxidants and conventionally used in toxicity management. Present study was designed to explore the extent of efficacy of combined VE and VC (VEC) against Sodium arsenite ($NaAsO_2$) mediated ovarian damage. Thirty-six female Wistar rats were randomly divided into three groups (Grs) and treated for consecutive 30 days; Gr I (control) was vehicle fed, Gr II (treated) was gavaged with $NaAsO_2$ (3 mg/kg/day), Gr III (supplement) was provided with VE (400 mg/kg/day) & VC (200 mg/kg/day) along with $NaAsO_2$. Marked histological alterations were evidenced by disorganization in oocyte, granulosa cells and zona pellucida layers in treated group. Considerable reduction of different growing follicles along with increased atretic follicles was noted in treated group. Altered activities ofΔ^5 3β-Hydroxysteroid dehydrogenase and 17β-Hydroxysteroid dehydrogenase accompanied by reduced luteinizing hormone, follicle-stimulating hormone and estradiol levels were observed in treated animals. Irregular estrous cyclicity pattern was also observed due to $NaAsO_2$ threat. Surplus ROS production affected ovarian antioxidant strata as evidenced by altered oxidative stress markers. Provoked oxidative strain further affects DNA status of ovary. However, supplementation with VEC caused notable restoration from such disparaging effects of $NaAsO_2$ toxicities. Antioxidant and antiapoptotic attributes of those vitamins might be liable for such restoration.

Keywords Sodium arsenite · DNA damage · Granulosa cell · Vitamin E · Vitamin C

Introduction

Various natural and anthropogenic substances on earth crust affect reproductive health of both animals and human by altering the structure and functions of reproductive organs even at low dose (Goralczyk 2021). Arsenic (As) is such a contaminant, widely exists in nature and humans are exposed to such pollutant through contaminated drinking water and food (Biswas et al. 2021). Presence of arsenic in food and water above the permissible limit (Rice: 200 µg/Kg for polished rice, 250 µg/Kg for parboiled; Water: 10 µg/L) set by regulatory authorities like FAO (FAO 2017) and WHO (WHO 2011) can cause in several physiological consequences (Martínez-Castillo et al. 2021). A research report illuminated multiple systematic disorders like melanosis, gastro enteritis, chronic liver disease, chronic kidney disease along with peripheral vascular disease (Jain and Chandramani 2018) caused by arsenic poisoning. Reproductive health outcomes due to arsenic ingestion drawn the attention of scientific communities in past decades

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(Hood et al. 1972). Embryotoxicity, prenatal toxicities and foetal development anomalies due to arsenic exposure was the most studied topics during 1970's, 80's and 90's (Hood et al. 1972; Baxley et al. 1981; Chaineau et al. 1990). Later, epidemiological studies from different provinces across the globe have documented an array of reproductive disorders, of which preterm birth, low birth weight, foetal loss and impaired fecundity in females are very constant (Vahter and Concha 2008; Shih et al. 2017; Susko et al. 2017).

Studies on mammalian model have emphasised arsenic mediated follicular atresia, luteal dysfunction and suppression of ovarian steroidogenesis (Chattopadhyay et al. 1999; Mondal et al. 2013; Wang et al. 2021). Exposure of As during prenatal and neonatal development has been linked to wide variety of effects, including alterations in brain sexual differentiation, male and female reproductive tract defects, pregnancy complications, and meiotic abnormalities in foetal oocytes (Navarro et al. 2004; Allan et al. 2015; Ishfaq Ahmad et al. 2021; Pinchoff et al. 2022). One recent report has demonstrated that application of sodium arsenite ($NaAsO_2$) at 1, 3 and 5 mg/kg body weight could initiate ovarian and uterine impairments and further demonstrated that 3 mg/kg body weight was the minimum dose with maximum clinical outcomes like impaired follicular growth pattern, increased total ROS productions and deleterious effects on biochemical as well as histological parameters (Mondal et al. 2022). Erstwhile studies have also reported evidence for the association between ovarian toxicity and arsenic induced redox imbalance (Biswas et al. 2019; Ommati et al. 2020). In this perspective, supplementation of some exogenous antioxidant agents, like betaine (Tazari et al. 2018), arjunolic acid (Sinha et al. 2008), taurine (Das et al. 2010) and N-acetyl cysteine (Hemalatha et al. 2013) had been reported to reduce arsenic induced ovarian toxicities. Vitamins as antioxidant got championed among the synthetic antioxidants and chelating agents due to their excellent bioavailability and less side effects (Flora and Pachauri 2010). There is evidence regarding the protection of arsenic mediated reproductive toxicity by using vitamin A as supplement (Chatterjee and Chatterji 2010). Ramanathan et al. (2002) has been documented the enhanced efficacy of combined application of vitamins E and C over the individual application in protecting arsenic induced oxidative stress. Another similar supplementation study reported that vitamin E and vitamin C could effectively restore zinc content and ameliorated lead mediated reproductive toxicity in adult rats (Ayinde et al. 2012).

Vitamin C being water soluble molecule is usually used in toxicity management due to its antioxidant potential (Pacier and Martirosyan 2015). Vitamin E can also alleviate toxicant induced oxidative damages in different systems by quenching free radicals and also by modification of specific transcription factors and enzymes (Gaeini et al. 2006; Gao et al. 2010). Available literature speculates antioxidant nature of vitamin E and C separately in mitigating arsenic induced system disorders. On the other hand, the protective effect of combined application of vitamin C and E against reproductive disorders had also been documented but there is insufficient information regarding the usefulness of combined use of vitamin E (VE) and vitamin C (VC) in management of $NaAsO_2$ induced ovarian anomalies. Therefore, the blue print of the study has been designed to discern the efficacy of vitamin E and C (VEC) in mitigating the deleterious effects of arsenic on ovarian structure–function. As a consequence, the objectives of this study were designed in two-tier: (1) determine the effect of $NaAsO_2$ on granulosa cell ultrastructure and nuclear damage (2) determine the efficacy of VEC in protecting $NaAsO_2$ induced structural and functional alterations along with the granulosa cell nuclear degeneration as well as ovarian DNA damage.

Materials and methods

Chemicals

Sodium arsenite ($NaAsO_2$) and collagenase type I and IV were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hyaluronidase, alpha-tocopherol, phenol–chloroform-isoamyl-alcohol mixture, chloroform-isoamyl alcohol mixture and the salts were purchased from Hi-Media, India. Haematoxylin, Eosin, Orange G, Leishman's stain, glutaraldehyde and ascorbic acid were procured from Merck, India. Hoechst 33342 dye and 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Invitrogen, USA and Thermo Fischer Scientific, USA respectively. All the primary culture media McCoy's 5a and M199 along with Penicillin Streptomycin, trypsin and foetal bovine serum were purchased from Gibco, Thermo Fischer, India. Enzyme-linked fluorescence assay (ELFA) kit was procured from Biomerieux, Etiole, France. LH and FSH Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Elabscience, Houston, Texas. Rest of the chemicals were purchased from SRL, India; Lobacheme, India and Ranbaxy Pvt. Ltd, India. Reagents used for biochemical assay and DNA extraction were of high analytical grade and molecular biology grade respectively.

Animals and ethics statement

Thirty-six female nulliparous Wistar rats (b.wt. 120 ± 10 gm) of 3 months old were procured from the registered breeder (Saha enterprise, Kolkata). All the rats were acclimatized for 10 days in polypropylene cages under controlled conditions of 14-h light/10-h dark cycle and at a constant temperature of ~ 22 °C. Animals were fed with standard laboratory diet composed of 71% carbohydrate, 18% protein

(consist of 6.32% wheat flour, 4.38% barley meal and 10% casein), 7% fat, 4% salt mixture and vitamins (Biswas and Kumar Mukhopadhyay 2020). In a regular basis during the experimental tenure (quarantine along with treatment period) cyclicity was checked by drawing vaginal lavage and also the body weight were recorded. All animal experiments were undertaken following the ethical guidelines of the Animal Ethics Committee of our University (Sanction No. PU/IAEC/PM/12).

Experimental design

All the rats were randomly divided into three different groups consisting twelve rats in each group. Treatment schedule was continued for 30 consecutive days. Dose selection and the route administration of chemicals were based on previously published works for $NaAsO_2$, α-tocopherol and ascorbic acid (Mondal et al. 2016; Ojo et al. 2018). In brief, the selected dose of $NaAsO_2$ was one tenth of its LD_{50} and for α-tocopherol and ascorbic acid it was 11,900 mg/kg and > 7000 mg/kg respectively.

Group I rats were received vehicle (distilled water) only. Group II rats were treated with aqueous solution (0.025% w/v) of $NaAsO_2$ at a dose of 3 mg/Kg/day. Group III rats were given α-tocopherol (VE) (400 mg/kg/day) and ascorbic acid (VC) (200 mg/kg/day) along with $NaAsO_2$ at above mentioned dose. Distilled water, $NaAsO_2$ solution, VE and VC vitamin solutions were orally administered by 2 ml gavaging syringe during the entire experiment period. Body weight and feeding behaviour were also monitored. The final body weight was measured prior to sacrifice. To avoid phase specific difference in ovary all animals were selected for euthanasia when the rats were at diestrus phase by following the CPCSEA guideline.

Sample collection

Blood was collected from dorsal aorta. Serum was separated by centrifugation for10 min at 1000 g and kept at – 80 °C for hormonal analysis. The ovaries were dissected out immediately and cleansed off adhering tissues. For biochemical studies and DNA isolation tissues were stored at – 80 °C until analysis. Cell isolation was performed just after the removal of tissues and was cultured in eukaryotic cell culture facility for Fluorescence activated cell sort (FACS) analysis. Ovarian tissues were immersed in Bouin's solution for histopathological studies.

Body weight and organ coefficient

Percentage of body weight (b.wt.) change and organ coefficient was calculated using the following formula:

$$\text{Total body weight change (\%)} = [(\text{Final b.wt.} - \text{Initial b.wt.}) / \text{Initial b.wt.}] \times 100$$

$$\text{Organ coefficient (\%)} = (\text{Ovary wt.} / \text{Final body wt.}) \times 100$$

Histopathology

All fixed tissues were dehydrated, processed and embedded in paraffin. Serial sections of ovarian tissues of 5 µm were cut by rotary microtome (OPTEX Brand, Pinkpearl Corporotaion, Osaka, Japan). The ovarian sections were stained with haematoxylin and eosin (H&E) and developmentally staged according to their morphology as: primordial, primary, preantral, antral or atretic follicles for folliculokinetics study along with morphological alterations. Repetitions of counting same follicle were avoided by choosing the alternative fifth and sixth serial sections. The slides were examined by using a microscope (Nikon Eclipse Ci microscope; Y-PB 55, Japan) at 400 × and 1000 × magnification. According to the severity of histopathological findings in the sections, they were evaluated as negative (−), mild (+), moderate (+ +), and severe (+ + +).

Follicular kinetic study

Follicles present in ovarian section were quantified by using the formula described by Abercrombie (1946).The total number of follicles in each section was quantified using the following formula.

$$N = P \times n[P = \frac{A \times M}{L + M}(A = \textit{crude number of follicles seen in the section},\ M = \textit{thickness of the section in } \mu m,\ L = \textit{average diameter of the follicle in } \mu m.); n = \textit{total number of observed sections}]$$

For oocyte diameter measurement the inbuilt software of Axiscope plus 2, Zeiss, Germany, microscopy was used and the scale bar unit was set in µm.

Steroidogenic enzyme activity

Ovary from each rat of all groups was homogenized separately in spectroscopic grade glycerol containing 5.0 mM

potassium phosphate and 1.0 mM ethylenediamine tetra acetic acid (EDTA) at 15 mg/mL tissue concentration. The homogenate was centrifuged at 10,000 g at 4 °C, and the supernatant was collected to assess ovarian Δ^5 3β-hydroxysteroid dehydrogenase (Δ^5, 3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β -HSD) activities. The Δ^5 3β-HSD activity was measured upon addition of 0.5 mM of nicotinamide adenosine dinucleotide (NAD) and 30 mg of dehydroepiandrosterone to the tissue supernatant using a spectrophotometer (JASCO v-530, Easton, MD) at 340 nm against reagent blank containing no NAD. The residual supernatant was used to measure the 17β –HSD activity. The supernatant was mixed with of crystalline bovine serum albumin, 0.3 mM testosterone and 1.1 mM of nicotinamide adenosine dinucleotide phosphate (NADP). The activity was measured at 340 nm wavelength against a blank (Jarabak et al. 1962). One unit of enzyme activity is correspondent to change in absorbance of 0.001/min at 340 nm.

Serum LH, FSH, and estradiol estimation

According to the protocol provided by manufacturer (Rat LH and FSH ELISA kit Elabscience, Houston, Texas; ELFA Kit: VIDAS Estradiol II, Biomerieux, Etiole, France) serum levels of LH, FSH and estradiol (E_2) were estimated following the principle of ELISA and ELFA respectively. The analytical detection limit of LH, FSH and E_2 were set at 1.56–100mIU/mL, 3.13–200 ng/mL and 9 pg/mL serum respectively.

Estrous cyclicity

The different stages of the estrous cycle were studied in all rats following the collection of vaginal smears. Vaginal fluid was collected using blunt edged glass pipette filled with 50–100 µL of 0.9% physiological saline. The collected vaginal fluid was uniformly spread on a glass slide. The glass slides were allowed to dry for a few min and finally were stained by Papanicolaou stain (Srinivasan et al. 2017) and the different phase of the estrous cycle was identified using a microscope (Di winter; 100 × magnification). Different types of epithelial cells were found along with leucocytes Epithelial cells were classified as small or large basophilic cells (SBCs, LBCs; bluish in colour), nucleated or enucleated acidophilic cells (NACs, EACs; orange colour) or preacidophilic cells (PACs, cells exhibiting a mix of blue and orange staining). Thus, each epithelial cell and number of leucocytes were scored as follows: number of cells on a scale of 1 + to 4 + as described: + (up to 10/field); + + (11 to 25/field); + + + (26 to 50/field); + + + + (more than 50/field); Cycles with mean duration of 4–5 days were considered regular, and continuation of the diestrus phase or any other stage for more than 3 days was considered as irregular cycle. The cycle count was used in this study for the determination of estrous cycle pattern.

Oxidative stress assessment

The super oxide dismutase (SOD) level was assessed by measuring the auto oxidation of haematoxylin as described by Martin et al.(1987). Activity was expressed as units per mg tissue after taking spectrophotometric readings at 560 nm (SYSTRONICS 2202 Double Beam, India).

The catalase (CAT) activity was measured biochemically following Beers & Sizer (1952). The enzymatic activity was expressed in mM of H_2O_2 consumed per mg tissue per min.

Ovarian GST activity was measured spectrophotometrically following the protocol of Boyland and Chasseaud (1969). The activity was expressed in µM NADPH consumed/ minute/ mg protein.

The activity of ovarian glutathione peroxidase (GP_X) was measured spectrophotometrically following Paglia and Valentine (1967). GPx activity was expressed as nM NADPH oxidized/ mg tissue/ minute using a molar extinction coefficient 6.22×10^3.

Reduced glutathione (GSH) content of the ovarian tissue was measured by assessing the reduction of 5,5-dithiobis-2-nitrobenzoate to 2-nitro-5- thiobenzoate. The changes were monitored by taking the absorbance at 412 nm in a spectrophotometer (JASCO v-530, Tokyo, Japan), which reflected the rate of reduction of 5,5-dithiobis-2-nitrobenzoate to 2-nitro-5-thiobenzoate. A standard curve was obtained with standard GSH and the level of GSH was expressed as µm (Rahman et al. 2007).

Malondialdehyde (MDA) level in tissues were measured spectrophotometrically by the method of Ohkawa et al. (1979).The absorbance was recorded at 535 nm and level was expressed in nM per mg tissue.

Protein oxidation was quantified by carbonyl content formed after reaction with 2, 4-Dinitrophenyl hydrazine (DNPH) as per Levine et al.(1994) at 370 nm. Protein carbonyl (PC) level was expressed as µmol/mg protein.

Reactive oxygen species (ROS) estimation

Fresh ovary was decapsulated and washed using Hank's balanced salt solution (HBSS), pH 7.4. Ovaries were punctured in a petriplate containing Mc. Coy's 5amedium with the help of 27-gauge hypodermic needles under stereomicroscope for isolation of granulosa cells. Rest of the ovarian tissues were digested with collagenase and hyaluronidase and were kept at 37 °C in a shaker incubator for 30 min for thecal cell

isolation. Centrifugation was performed at 600 g in cooling centrifuge for removal of debris and a further centrifugation was performed at 9,500 g for 10–15 min. Supernatant was discarded and pellet was suspended using McCoy's 5a medium (Tian et al. 2015). Both the granulosa and thecal cells were counted and cell viability was checked using Neubar's chamber.

Reactive oxygen species activity within a cell was quantified by the cell-permeable fluorogenic probe 2', 7'- Dichlorodihydrofluoresceindiacetate (DCF-DA) (Eruslanov & Kusmartsev 2010). The DCF fluorescence intensity is proportional to the ROS levels within the cell. In this experiment DCFDA at 100 µM was added to the cell suspension followed by 30 min incubation at 37 °C, and ROS was measured with BD accuri C6 (Becton, Dickinson and Company, Franklin Lakes, U.S) cell analyzer.

DNA fragmentation assay

Ovary was minced in phosphate-buffered saline (PBS) for 15 min. Lysis buffer (10 mM Tris, 400 mM NaCl, 1 mM EDTA and 1% Triton X-100) was directly added to minced tissues at 30 mg/mL tissue concentration and homogenized. Homogenate was centrifuged (1,500 g) at 4 °C for 10 min and supernatant was treated with 10% SDS (0.5% w/v). Next the supernatant was incubated with RNase (0.2 mg/mL) at 56 °C for 2 h and proteinase K (0.1 mg/mL) for overnight at 37 °C. DNA was extracted using the conventional phenol/chloroform/isoamyl alcohol method and 3 M sodium acetate was used as chelating agent. DNA pellet was obtained by centrifugation (16,000 g) at 4 °C for 20 min (Wyllie et al. 1980). The isolated DNA separated in a 0.8% agarose gel containing ethidium bromide and visualized under a UV transilluminator Bio Rad Gel Doc System.

Comet assay

The isolated ovarian cells (described in "ROS estimation" section) were mixed with 1% low melting point agarose and embedded in a thin 1% normal melting point agarose on a glass slide. Cells were lysed in 2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Trizma base, 1% Triton X and 10% DMSO and incubated overnight in 4 °C freezer. DNA was allowed to unwind under alkaline condition using electrophoresis buffer (300 mM NaOH and 1 Mm EDTA, pH 13) at 280 mA and 24 V (0.74 V/cm) for 20 min. DNA fragments or damaged DNA undergoing electrophoresis migrate away from the nucleus. In this process, the length of migration is inversely proportional to the size of fragment. The slides were then neutralized using neutralizing buffer (0.4 M Tris, pH 7.5) followed by staining with a fluorescent dye (Ethidium bromide 2 mg/mL), and DNA 'comet' was visualized by fluorescent microscope (Axiscope plus 2, Zeiss, Germany) (Singh et al. 1998). The tail length (in µm), tail moment (TM) and olive tail moment (OTM) were analysed using CASP version1.2.3 beta2 (Figure).

Scanning electron microscopy

Ovary of euthanized rat was collected and was fixed in 2.5% buffered glutaraldehyde for 48 h followed by graded ethanolic dehydration (50–100%). The tissues were kept in absolute alcohol and 100% acetone solution (1:1, v/v) followed by the immersion in 100% acetone. Cubes of 1 mm × 1 mm tissues were incised from each section with a sterile new blade to avoid distortion. The samples were dried in a vacuum chamber. Aluminium stubs were used for fixation and coating was performed with 5 ng platinum using an agar sputter coater (Q150T; Quorum technologies) (Mahapatra and Chandra 2017). At the end the sections were examined in a scanning electron microscopy (SEM) (ZEISS EVO-MA 10).

Hoechst staining

Fixed ovarian tissues were stained with Hoechst 33342 stains (1 µg/mL) to determine the chromatin condensation and DNA nick formation. The ovarian sections were covered with PBS dissolved Hoechst 33342 and kept in dark followed by 15 min incubation at room temperature. Finally excess stain was removed by PBS washing. This stain has the capacity to bind with the DNA nicks and fluoresce. Finally the cells were stained and observed under a fluorescence microscope at 100 × magnification (Axiscope plus 2, Zeiss, Germany) (Lima et al. 2017). The representative photographs were taken and the fluorescence intensity of the captured image area was quantified by Image J (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation) tool.

Statistical evaluation

SPSS 20.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Significance levels and statistical differences were determined by using the "one-way analysis of variance (ANOVA)" test, and Tukey's test was used to determine differences among the groups. All the values were expressed as mean ± standard error (± SEM), while the results at $p < 0.05$, 0.01, 0.001 were considered significant. The Kruskal–Wallis test, one of the nonparametric tests, was used for the analysis of the data obtained semi-quantitatively in histopathological examinations in terms of the differences between groups and $p < 0.05$ was considered as significant.

Table 1 Role of VEC supplementation on the change of body weight and organ coefficient of ovary in $NaAsO_2$ treated rats

Animals	Body weight changes (%)	Organ coefficient (mg/100 g) Ovary
Gr I	11.241 ± 4.617	0.015 ± 0.001
Gr II	-13.504 ± 3.828 ^{a***}	0.007 ± 0.005 ^{a*}
Gr III	17.852 ± 2.900 ^{b***}	0.018 ± 0.001^{ab**}

The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The different letters (a & b) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$

Table 2 Effect of VEC on the restoration of histopathological alterations of ovary after $NaAsO_2$ treatment

Histopathological features	Gr I	Gr II	Gr III
Oocyte nest degeneration	+	+++	+
Pycnotic granulosa cells	+	+++	+
Thecal layer degeneration	+	++	+
Zona pellucida disorganization	−	+++	+

No changes (−), mild (+), moderate (++), severe (+++)

Results indicate the Kruskal–Wallis H test for comparison among Gr I, II, & III for histopathological scorings of ovarian tissues. $p < 0.05$ was considered as significant level

Results

Effect of VEC on change of body weight and ovarian coefficient in $NaAsO_2$-exposed rats

Experimental rats were monitored daily during the treatment period for any behavioral changes. Symptoms like hair loss, aggression, and reduction of food intake were seen to appear in $NaAsO_2$ treated rats. Body weight along with the ovarian coefficient were reduced significantly in treated groups as compared to control ($p < 0.001$ & $p < 0.05$ respectively). VEC supplementation maintained the body weight and ovarian coefficient at the control levels ($p < 0.001$ & $p < 0.01$ respectively) in spite of $NaAsO_2$ treatment (Table 1).

Effect of VEC on ovarian histology in $NaAsO_2$-treated rats

The ovarian histology was assessed and representative photomicrographs were presented in Fig. 1 and the findings were summarized in Table 2. Usual arrangement of Graafian and different growing follicles were observed in the control ovary (Fig. 1a). Regularly arranged zona pellucida layer with proliferating granulosa cells were also seen under higher magnification (Fig. 1d). In contrast to the control group, increased presence of atretic follicles was observed in $NaAsO_2$ treated group (Fig. 1b). Severe disorganization in zona pellucida, shrunken oocyte, degenerated ooplasm, and oocyte nest were also noted in treated ovary (Fig. 1e; Table 2). Observation further revealed severe degeneration and depletion of granulosa cells along with pycnotic nuclei and moderate level degeneration of thecal cells due to

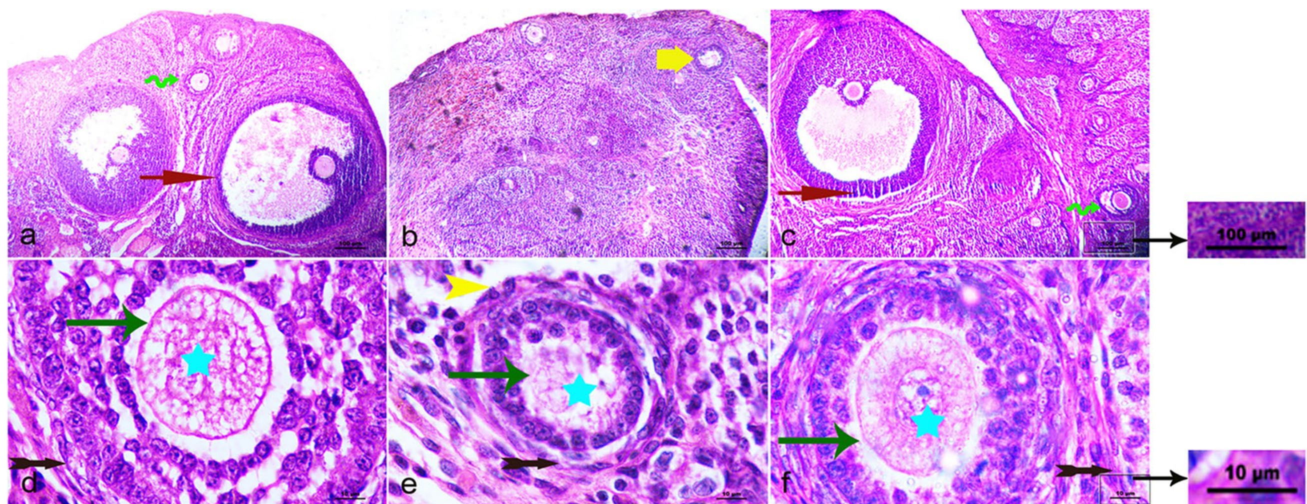

Fig. 1 Representative photomicrographs of haematoxylin and eosin stained histoarchitecture of ovary (**a–f**), where a, b and c are at 100× (scale: 100 μm) magnification and d, e, and f are at 1000× (scale: 10 μm) magnification. Group I (**a** & **d**) sections depicts normal histological features. In contrast, Group II (**b** & **e**) reveals noticeable pathological changes. Group III (**c** & **f**) denotes rescue effect of VEC against $NaAsO_2$-induced ovarian alterations. Healthy growing follicle (light green curved arrow), pycnotic granulosa cells (yellow arrow head), atretic secondary follicle (yellow thick arrow), zona pellucida layer (dark green thin arrow), Graafian follicle (brown short arrow), thecal layer (black tailed arrow), ooplasm (sky blue asterisk)

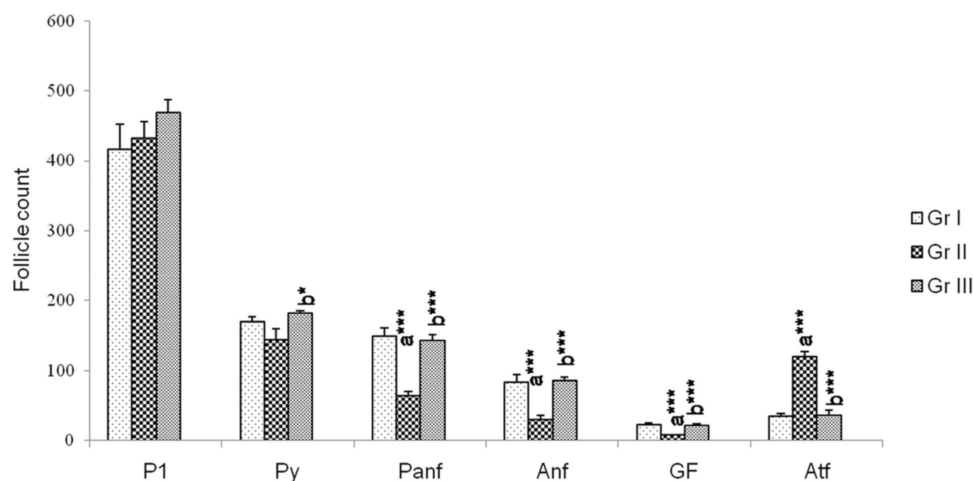

Fig. 2 Role of VEC on ovarian follicular growth in $NaAsO_2$ treated rats. The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The bar diagrams depict insignificant changes in primordial follicles (P1) in all the groups. The number of primary follicles (P_y), preantral follicles (P_{anf}), antral follicles (Anf), Graafian follicles (Gf) and atretic follicle (Atf) varies in Gr II. VEC supplementation prevented the attenuating effect of $NaAsO_2$ on the follicles. The different letters (a & b) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; $*p < 0.05$, $***\ p < 0.001$

$NaAsO_2$ toxicity (Fig. 1b and e; Table 2). VEC supplementation restored the structure of ovarian follicles showing the presence of Graafian and healthy antrum follicles, regular arrangements of oocytes with distinct zona pellucida layer (Fig. 1c and f; Table 2). Similar to the control, the VEC supplementation had also shown intact arrangement of oocyte nest, granulosa cells and thecal layers (Fig. 1f; Table 2).

Effect of VEC on ovarian follicular growth in $NaAsO_2$-treated rats

Results depicted in Fig. 2 revealed the protective ability of VEC supplementation against altered developmental pattern of ovarian follicles due to $NaAsO_2$ treatment. $NaAsO_2$ treatment caused substantial reduction of the number of different follicle populations (preantral, antral and Graafian) along with the increase of atretic follicle number as compared to those of control group ($p < 0.001$ in all the cases). However, the numbers of primordial and primary follicles remain unaltered. Supplementation of VEC offered a significant restoration of the number of affected follicle populations and the concomitant decrease of atretic follicles number ($p < 0.001$ in all the cases). Supplementation also caused the significant increase of primary follicle number as compared to that of treated animals ($p < 0.05$).

Effect of VEC on oocyte nucleus diameter in $NaAsO_2$-exposed rats

There were substantial decreases in the diameter of oocyte nucleus in case of Py, Panf, Anf follicles in Gr II ovaries (Fig. 3 and Table 3). In Gr II the nuclei diameter of Py (a), Panf (b) and Anf (c) follicles reduced significantly compared to that of Gr I ($p < 0.001$ in all cases). Supplementation of VEC restored the nuclei diameter successfully in Py, Panf and Anf follicles ($p < 0.001$ in Py & Anf follicles; $p < 0.01$ in Panf follicles). However, the nuclei diameter of P1 follicles showed insignificant differences among the groups.

Effect of VEC on the activities of Δ5 3β HSD and 17β HSD in $NaAsO_2$-exposed rats

Within the scope of the study, the activity of ovarian key steroidogenic regulatory enzymes, i.e., Δ^5 3β HSD and 17β HSD were assessed in order to evaluate the protective effect of VEC on $NaAsO_2$-induced steroidogenic imbalance (Table 4). It was observed that the Δ^5 3β HSD activity in treated group was significantly decreased than that of control ($p < 0.001$). The activity of 17β HSD was also seen to be decreased considerably ($p < 0.001$). Noticeable recovery was observed in the activity of these enzymes due to VEC supplementation ($p < 0.001$ in both the cases). Though, significant differences were also seen to exist between the control and supplemented rats for both the enzyme activities ($p < 0.001$ in both the cases).

Effect of VEC on LH, FSH, and E_2 levels under $NaAsO_2$-exposed state

The protective ability of VEC on serum LH, FSH and E_2 levels in $NaAsO_2$ exposed rats is presented in Fig. 4. The levels of LH(a), FSH(b) and E_2(c) were found to significantly reduced in treated group as compared to that of control ($p < 0.001$ in all the cases). Notable restoration was seen

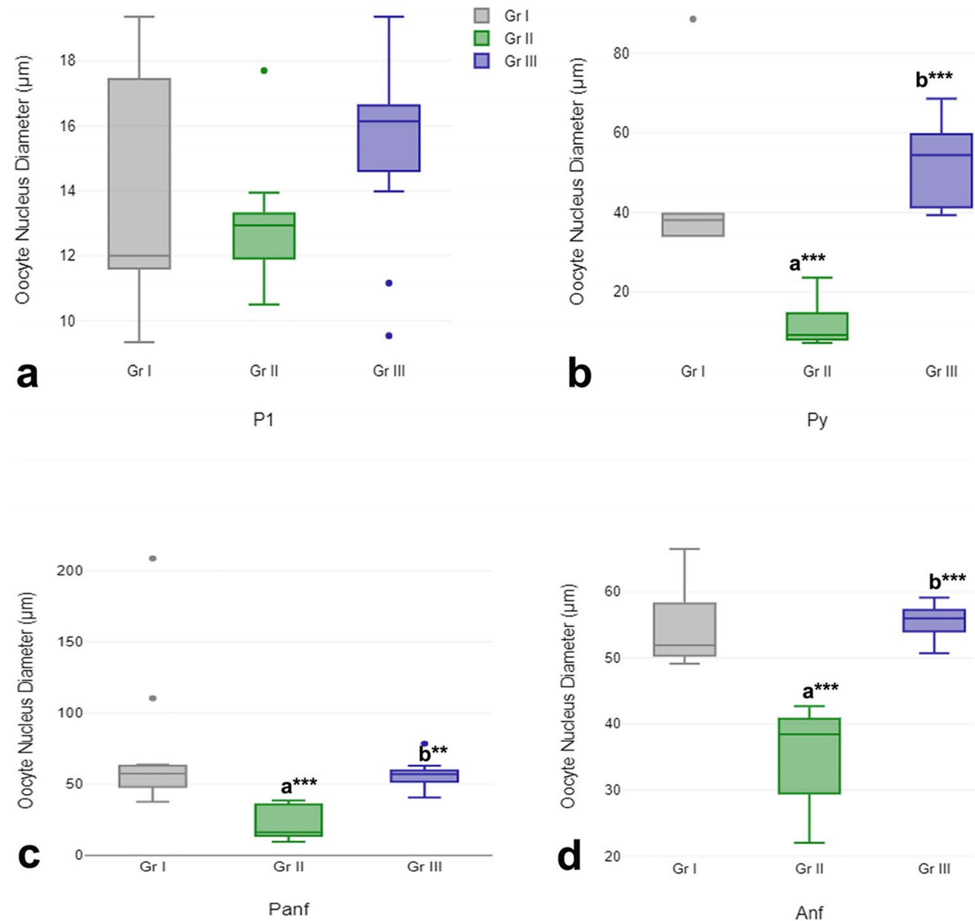

Fig. 3 Box plot showing the distribution and the variability of oocyte nucleus diameters of ovarian (**a**) Primordial follicle (P1); (**b**) Primary follicle (P_y); (**c**) Preantral follicle (P_{anf}) & (**d**) Antral follicle (Anf) in VEC and $NaAsO_2$ co exposed rats. The graph illustrates the median values (inner line inside the box), the upper and lower quartiles (the box) & the outliers (dot within the plot area). Significant differences were obtained from one-way ANOVA followed by Tukey's post hoc test. a = Gr I vs Gr II & III; b = Gr II vs Gr III; ** $p < 0.01$, *** $p < 0.001$

Table 3 Effect of VEC on the restoration of oocyte nucleus diameter after $NaAsO_2$ exposure

Animals	Oocyte nucleus diameter (µm)			
	P1	Py	Panf	Anf
Gr I	13.80 ± 0.93	45.39 ± 8.68	71.87 ± 14.90	54.52 ± 2.60
Gr II	12.90 ± 0.48	11.93 ± 2.57	19.16 ± 2.90	34.56 ± 3.07
Gr III	15.38 ± 0.71	55.81 ± 3.92	56.84 ± 2.36	55.70 ± 1.13

The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test

Table 4 Role of VEC supplementation on the activities of Δ^5 3β HSD and Δ17 β HSD in $NaAsO_2$-treated rats

Animals	Parameters	
	Δ^5 3β HSD (unit/mg tissue/hr)	17β HSD(unit/mg tissue/hr)
Gr I	4.53 ± 0.367	1.67 ± 0.067
Gr II	$1.27 \pm 0.122^{a***}$	$0.57 \pm 0.095^{a***}$
Gr III	$2.55 \pm 0.09^{ab***}$	$1.14 \pm 0.067^{ab***}$

The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The different letters (a & b) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; *** $p < 0.001$

when VEC supplementation was given ($p < 0.001$ in case of LH and FSH; $p < 0.05$ in case of E_2) though the E_2 level did not attain the normal level ($p < 0.001$).

Role of VEC on estrus cyclicity in $NaAsO_2$-treated rats

Supplementation of VEC exhibited potent effect in sustaining estrus cyclicity under $NaAsO_2$ exposed state (Table 5). Scores of epithelial cell populations and leukocytes in each stage of the estrous cycle are shown in Table 5. The number of epithelial cells, i.e., EACs, NACs, LBCs, PACs, SBCs and leucocytes appeared in normal fashion in all three groups during 19 ± 5 days of treatment (Fig. 5a–c). The number of EACs and NACs declined significantly after 19 ± 5 days of treatment in treated groups ($p < 0.05$; Fig. 5e). However, in case of control and supplement group, number of these two cells were more abundant during the transition from proestrus to estrus ($p < 0.05$; Fig. 5d and f). The number of LBCs, PACs and SBCs were declined considerably in treated group before the onset of continuous

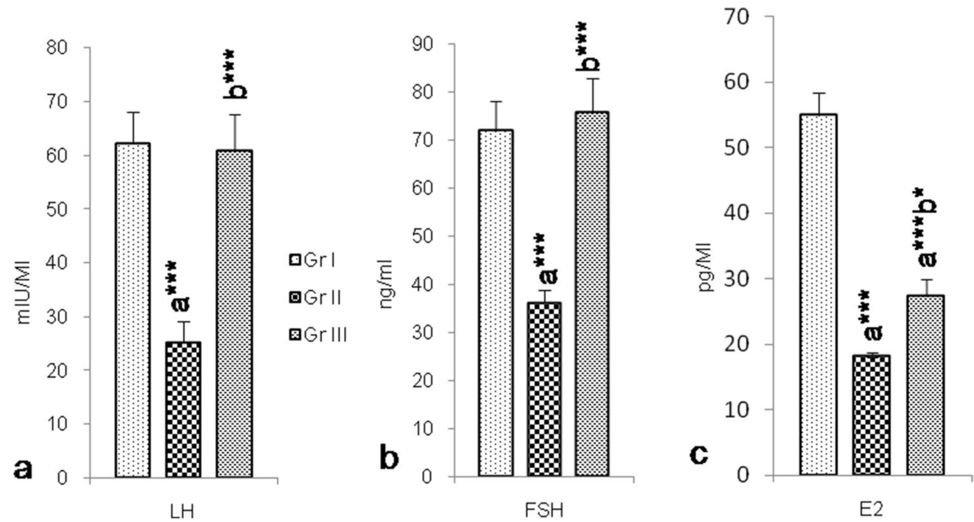

Fig. 4 Bar diagrams represent attenuation of (**a**) LH, (**b**) FSH & (**c**) E_2 in Gr II rats. Supplementation of VEC restores the levels of hormone in Gr III. The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The different letters represent the statistically significant differences between control and treatment groups and the sign is changed gradually when the significant level differs. a = Gr I vs Gr II & III; b = Gr II vs Gr III; *$p < 0.05$, *** $p < 0.001$

Table 5 Effect of VEC on scores of vaginal epithelial cells and total number of complete estrous cycles after $NaAsO_2$ treatment

Epithelial cells	Gr I	Gr II	Gr III	Gr I	Gr II	Gr III
	Day 1-Day 19 ± 5			Day 19 ± 5-Day 30		
Leucocytes	+++	+++	+++	+++	+++	+++
EAC	++++	++++	++++	++++	-	+++
NAC	+++	+++	+++	++++	-	+++
LBC	++	++	++	++	-	+
SBC	++	++	++	++	+	+
PAC	++	+	++	++	-	++
Animals	Number of cycles					
Gr I	5.54 ± 0.207					
Gr II	4.01 ± 0.500^{a***}					
Gr III	5.38 ± 0.263^{b***}					

The data represent the means ± SEM. Results indicate the Kruskal–Wallis H test for comparison among Gr I, II, & III for vaginal smear scorings. $p < 0.05$ was considered as significant level. Leukocytes (Lkc), enucleated acidophilic cells (EAC), nucleated acidophilic cells (NAC), large basophilic cells (LBC), small basophilic cells (SBC), pre-acidophilic cells (PAC). Absent (-), low (+), moderate (++), high (+++), very high (++ ++)

Rest of the statistical data were derived from ANOVA followed by Tukey's post hoc test. The different letters represent the statistically significant differences between control and treatment groups and the sign is changed gradually when the significant level differs. a = Gr I vs Gr II & III; b = Gr II vs Gr III; *** $p < 0.001$

diestrus phase ($p < 0.05$) while notable restoration of such number was observed in supplement group ($p < 0.05$). The number of complete estrus cycles was decreased significantly ($p < 0.001$) in treated group due to prolonged diestrus phase. The estrus cyclicity was seen to be restored in supplemented group ($p < 0.001$).

Effect of VEC on redox balance in $NaAsO_2$-exposed ovarian tissues

Within the scope of the study, the activities of SOD, CAT, GST and GP_x along with the levels of GSH, MDA and PC were determined in order to evaluate the effect of VEC on $NaAsO_2$ induced oxidative damages in ovary (Fig. 6). It was noted that SOD and CAT activities were decreased significantly in the treated group compared to that of control (Fig. 6a and b; $p < 0.001$ in both the cases). Moreover, the activities of GST, GPx, and also the level of GSH were seem to be reduced significantly in treated ovaries due to $NaAsO_2$ intoxication (Fig. 6c, d and e; $p < 0.001$, 0.01 & 0.05 respectively). The supplementation of VEC resulted noticeable increase in all the enzymatic (Fig. 6a, b and c; $p < 0.001$; Fig. 6d; $p < 0.01$) and non-enzymatic parameters (Fig. 6e; $p < 0.05$) but striking differences still existed with that of control group (Fig. 6a, b and c; $p < 0.001$; Fig. 6d; $p < 0.05$ respectively). However, the GSH level obtained in

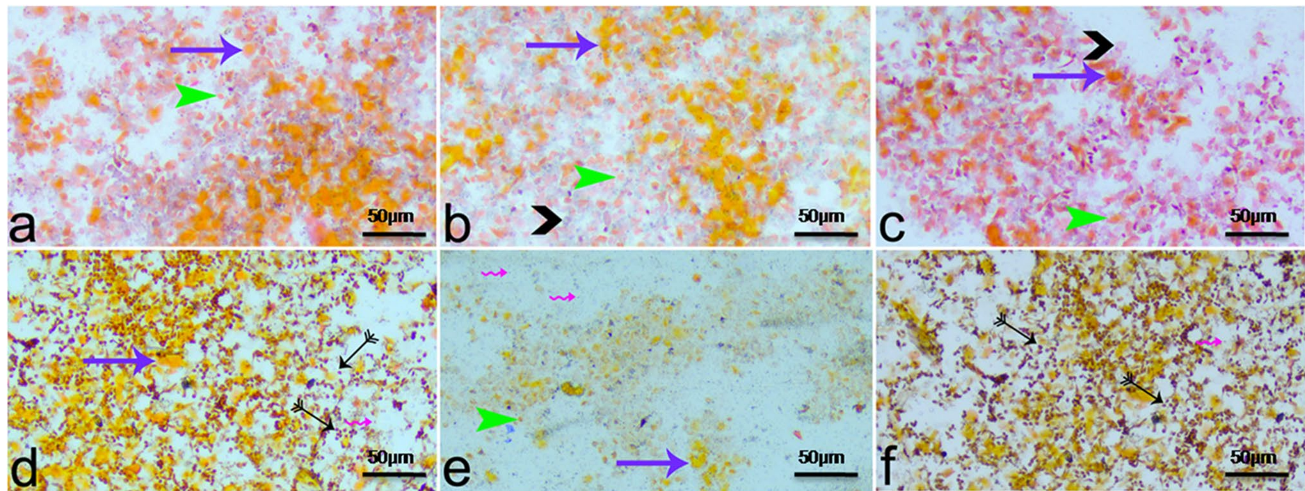

Fig. 5 Papanicolaou staining of vaginal cytology of Gr I (**a** & **d**- 400X), Gr II (**b** & **e**- 400X) and Gr III (**c** & **f**- 400×) (scale: 50 µm) rats. Light microscopic images showing estrus phases (upper panel; **a**-**c**) during 19 ± 5 days and proestrus to estrus transition period (lower panel; **d**-**f**) after 19 ± 5 days in all the groups. Equal distribution of pre-acidophilic cells (PACs; green arrow head) & enucleated acidophilic cells (EACs; blue thin arrow) present in all the groups. After 19 ± 5 days number of small basophilic cells (SBCs; black tailed arrow), large basophilic cells (LBCs; black short arrow head), PACs & EACs drop in Gr II. VEC supplementation restores the vaginal cytology. Number of leucocytes (pink curved arrow) remains unaltered among all the groups

Gr III was insignificant to Gr I. The MDA and PC contents were also seen to be increased significantly in treated group compared to that of control (Fig. 6f and g; $p < 0.001$ in both the cases) while in VEC supplemented group the values were evidently decreased to near normal level (Fig. 6f and g; $p < 0.001$ in both the cases).

Effect of VEC supplementation on ovarian total ROS generation in $NaAsO_2$-exposed state

The results demonstrated that DCF fluorescence, which is a standard indicator of cellular ROS generation, was significantly increased following $NaAsO_2$ treatment in isolated ovarian cells ($p < 0.001$; Fig. 7; Table 6) compared with those in the untreated cells and DCFDA treated control cells also. Interestingly, study revealed tremendous reduction of ROS level in VEC co-administered groups than treated group ($p < 0.001$).

Effects of VEC on $NaAsO_2$ induced ovarian DNA integrity

In order to measure the DNA integrity in isolated ovarian single cell, we measured the tail length (in µm), tail moment, and olive tail moment (Fig. 8a and Table 7). The parameters were quantified and evaluated with CASP 1.2.3 beta 2 (Fig. 8a and Table 7). Likewise, DNA fragmentation results, in case of Gr II cells showed longer tail than Gr I ($p < 0.001$) along with an increase in tail moment and olive tail moment ($p < 0.001$). As predicted, isolated ovarian cells from Gr III rats displayed significant restoration of DNA integrity. The significant recovery was noticed in tail length, moment and olive tail moment in Gr III rats than that of Gr II rats ($p < 0.001$). However, significant difference was present in tail length and olive moment between Gr I and III ($p < 0.01$ & $p < 0.05$ respectively).

Effects of VEC on ovarian DNA fragmentation in $NaAsO_2$-exposed state

The adverse impacts of $NaAsO_2$ intoxication and the potential ameliorative role of VEC on DNA fragmentation was reflected in Fig. 8(b). Data revealed from this assay showed increase of smear in Gr II rats which was a direct evidence of arsenic mediated DNA damage. The supplementation of VEC showed significant reduction of DNA smear.

Role of VEC on ultra-structure of granulosa cells in $NaAsO_2$-exposed state

The adverse impacts of $NaAsO_2$ intoxication and the potential ameliorative role of VEC on the ultra-structure of ovarian granulosa cells (GCs) were reflected in Fig. 9. The GCs of control rats appeared more or less spherical in shape and were seen to be connected through cytoplasmic extensions and matrix (Fig. 9a). In $NaAsO_2$-treated rats, the sign of severe degenerations was noted as the outline of the GCs was irregular and short stumpy projections on their surface were observed. Moreover, the connections between neighbouring GCs were lost due to condensation of cytosol.

Fig. 6 Role of VEC on ovarian redox balance in $NaAsO_2$ exposed rats. The bar diagrams show significant attenuation in (**a**) SOD, (**b**) CAT, (**c**) GST, (**d**) GPx & (**e**) GSH activities in Gr II. In Gr II (**f**) MDA & (**g**) PC levels shot over the control. VEC supplementation restores the parameters to near normal level. The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The different letters (**a** & **b**) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$

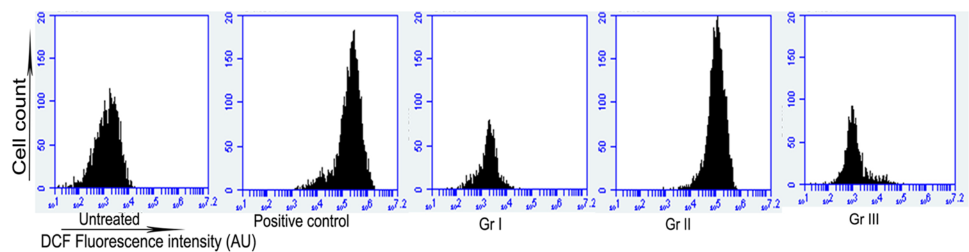

Fig. 7 Effect of VEC on total ROS generation in ovary after $NaAsO_2$ treatment. Representative flow cytometry histogram of DCFDA staining. Untreated and positive control histograms represent the unstained & H_2O_2 treated Gr I cells. AU = Arbitrary unit

Decrease in gap junction was also noted by the presence of numerous flattened and loosely arranged GCs (Fig. 9b). However, VEC supplementation resulted in restoration of GCs morphology (Fig. 9c). Cytoplasmic extensions were seen like that in normal rats and stumpy projections were also reduced in number.

Table 6 Effect of VEC supplementation on total ROS generation in ovary after $NaAsO_2$ treatment

	Untreated	Positive control	Gr I	Gr II	Gr III
Fluorescence Intensity (AU)	$435.07 \pm 53^{a***}$	160520 ± 134.23	$1807.06 \pm 125.43^{a***}$	$70593.42 \pm 147.41^{ab***}$	$2722.43 \pm 394.96^{abc***}$

Mean fluorescence intensity values of DCFDA. The data represent the means ± SEM. Results were derived from oneway-ANOVA followed by Tukey's post hoc test. The different letters represent the statistically significant differences between control and treatment groups and the sign is changed gradually when the significant level differs. a = + Ve control vs. unstained, Gr I, Gr II, Gr III; b = Gr I vs. II &III; c = Gr II vs Gr III. *** $p < 0.001$

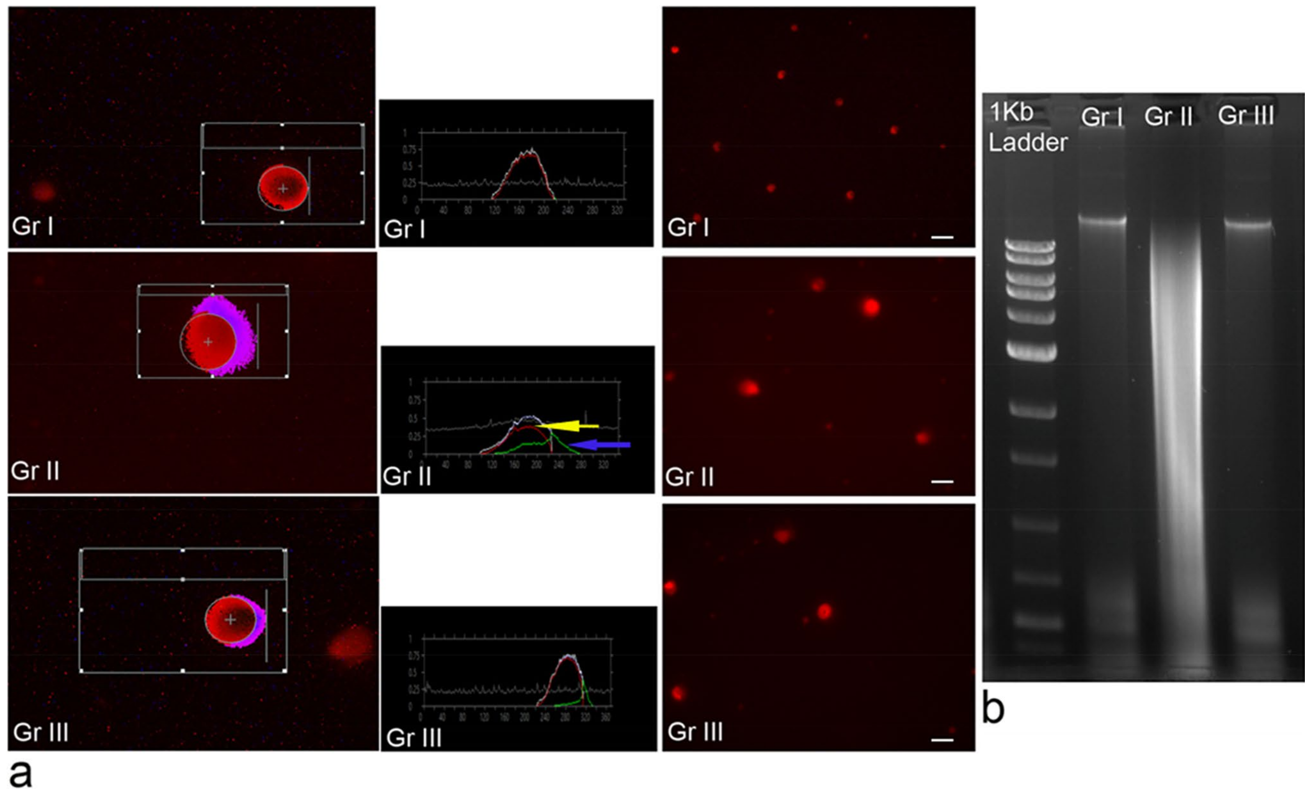

Fig. 8 Effect of VEC on DNA fragmentation & integrity after $NaAsO_2$ treatment. (**a**) Comet assay results of ovarian single cells (400×; scale: 50 µm) & (**b**) DNA fragmentation assay depict decrease of tail length & smear formation respectively after co exposure of $NaAsO_2$ & VEC supplement. (**a**) Red lines indicated by yellow arrow represents the head of comet, & green lines indicated by blue arrow represents the tail of comet. (8b) Lane 1 (1 kb ladder), Lane 2 (Gr I), Lane 3 (Gr II), Lane 4 (Gr III)

Table 7 Role of VEC supplementation on ovarian DNA integrity in $NaAsO_2$-treated rats

Animals	Parameters		
	Tail length (µm)	Tail moment (Arbitrary unit)	Olive tail moment (Arbitrary unit)
Gr I	6.92 ± 1.56	0.09 ± 0.05	0.28 ± 0.15
Gr II	$53 \pm 3.49^{a***}$	$21.09 \pm 3.8^{a***}$	$14.92 \pm 1.68^{a***}$
Gr III	$19.25 \pm 2.32^{a**b***}$	$2.13 \pm 0.73^{b***}$	$3.6 \pm 0.99^{a*b***}$

The data represent the means ± SEM. Results were derived from oneway ANOVA followed by Tukey's post hoc test. The different letters (a & b) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Effect of VEC on DNA damage of granulosa cells in $NaAsO_2$-exposed condition

Fluorescence microscopic study of granulosa cell Hoechst 33258 staining revealed that the fluorescent intensities of Hoechst 33258 were increased significantly ($p < 0.001$) with the application of $NaAsO_2$ treatment (Fig. 10). This observation clearly suggested the formation of DNA nick in treated group (Fig. 10b and d; $p < 0.001$). However, in VEC supplemented group the mean intensity dropped significantly near to control than $NaAsO_2$-treated group (Fig. 10a, c and d; $p < 0.001$).

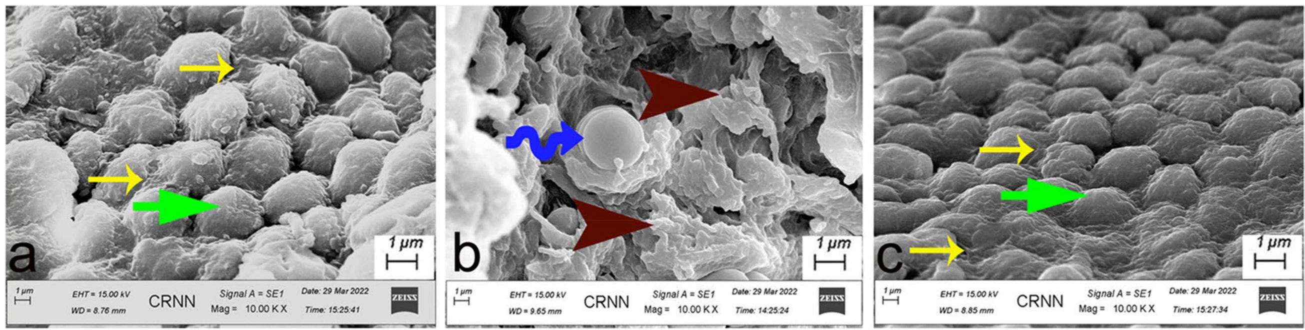

Fig. 9 Effect of VEC on ultra-structural analysis of granulosa cell of secondary follicles after $NaAsO_2$ exposed state. Secondary follicle of Gr I with organized granulosa cells (**a**); degenerated granulosa cells in Gr II ovary (**b**); near normal organization in Gr III (**c**). Smooth textured spherical granulosa cell (green short arrow); cytoplasmic connection (yellow thin arrow); stumpy projection of degenerated granulosa cell (brown arrow head); erythrocyte (blue curved arrow). Scale: 1 μm

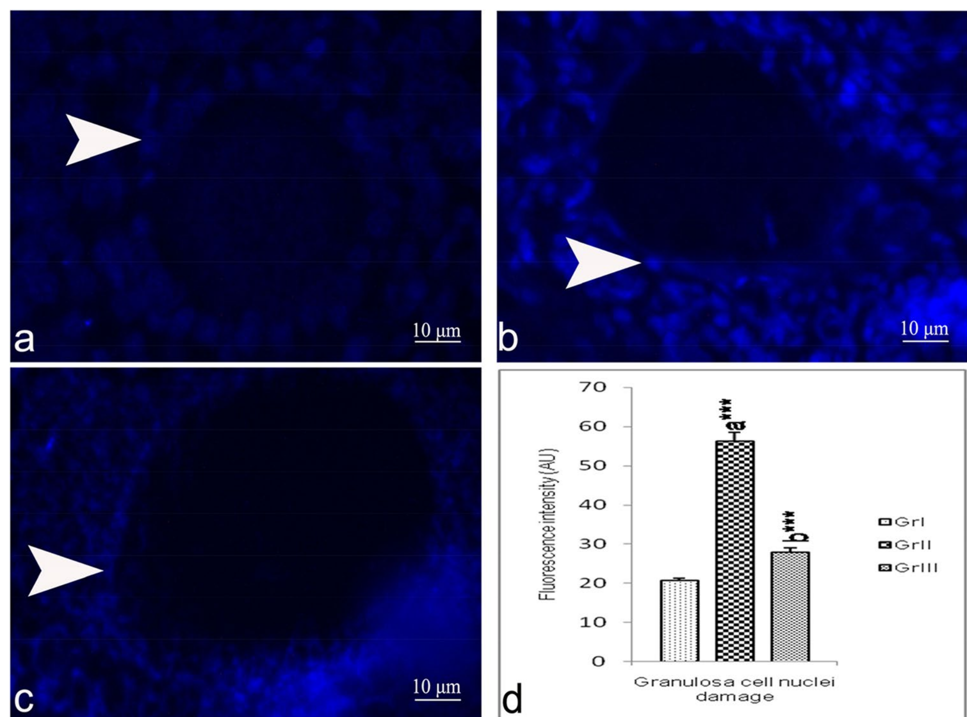

Fig. 10 Fluorescence microscopic images of GCs (arrow head; 1000×; scale: 10 μm) from Gr I, II and III rats after Hoechst staining (**a**-**c**). (**a**) Gr I & (**c**) III granulosa cells showing less fluorescence intensities than (**b**) Gr II. (**d**) Bar diagram represents the quantification of fluorescence intensities in all the groups. The data represent the means ± SEM. Results were derived from one-way ANOVA followed by Tukey's post hoc test. The different letters (**a** & **b**) represent the statistically significant differences between the groups. a = Gr I vs Gr II & III; b = Gr II vs Gr III; *** $p < 0.001$

Discussion

More than 230 million people worldwide are at risk caused by arsenic poisoning which is a significant public health concern (Shaji et al. 2021). The present data suggests that the ovarian coefficient along with percentage of body weight are diminished as a result of $NaAsO_2$ toxicity which are consistent with earlier reports (Barai et al. 2017; Dash et al. 2020). Body weight is positively correlated with metabolic activity, DNA synthesis, as well as total protein content and one earlier report suggested that inorganic arsenic effectively reduced all such parameters (Mitra et al. 1999). Moreover, literature also reported that, arsenic promotes thermogenesis by augmenting leptin level with a decline in adiponectin level which plays crucial role in weight loss (Handali and Rezaei 2021). These could be the possible explanation of body weight loss in $NaAsO_2$-treated groups. The restoration of body weight and organ coefficient is resulted due to VEC supplementation. Available data indicates that vitamin C improves growth rate, feed intake and feed efficiency (Sahin and Kucuk 2001) whereas vitamin E can restore the body weight in diabetic condition as observed in adult rats (Almeida et al. 2012). Therefore, application of VEC might act as protective weapon against ovarian degenerative changes.

Treatment with $NaAsO_2$ caused significant structural detoriation in the ovary including severe disorganization in zona pellucida layers and oocyte as well as appearance of huge number of pyknotic granulosa cells. Reduced number of preantral, antrum and Graafian follicles with a surge of atretic follicles was also observed in treated animals. Since 1996, it was assumed that preantral and pre-ovulatory follicles infrequently underwent atresia (Chun et al. 1996), recently it was known that granulosa cell death by apoptosis and/or autophagy were responsible for this phenomenon (Meng et al. 2018). Altered apoptosis and/or autophagy might be considered as underlying mechanism behind $NaAsO_2$ mediated such alterations. The restoration of ovarian histology and reduction in atretic follicle number were resulted upon VEC supplementation. The protective ability of vitamin C and E might be due to its anti-apoptotic and anti-autophagic actions (Rössig et al. 2001; Underwood et al. 2010). Oocyte diameter is positively correlated with follicular size and developmental competence (Raghu et al. 2002). Present study had shown $NaAsO_2$ intoxication resulted in decrease of oocyte diameter in preantral, antral and to some extent in primary follicles which was allied with the histological observation and follicular kinetic analysis. Literature study revealed that arsenic intoxication caused suppressed expansion of cumulus cells, expression of cumulus expansion-related genes (PTGS1, PTGS2, PTX3, TNFAIP6, and HAS2) in cumulus cells, and also the impaired extrusion of first polar body (Kang et al. 2022) and this might be considered as the probable underlying mechanism behind the reduction of oocyte diameter in different developing follicles.

The combined application of VEC successfully restored the oocyte diameter to near normal level. Asadi et al. (2012) reported that vitamin E can reduce the oocyte apoptosis in nicotine induced mice due to its direct antiapoptotic nature. Moreover, Zhang et al. (2020) stated that vitamin C could protect oocyte apoptosis induced by Microcystin-leucine arginine (MC-LR), a water body cyanotoxin. These antiapoptotic properties of both the vitamins might be the reason behind restoration of oocyte diameter and also these literatures could provide strength to the selection of combined application of VE and VC in mitigating the $NaAsO_2$ induced ovarian damages.

In addition, with the structural alterations, this study also demonstrates that $NaAsO_2$ inhibited ovarian steroidogenesis by reducing the activities of Δ^5, 3β-HSD and 17β-HSD. This finding is in agreement with previous report (Chattopadhyay et al. 2003). We also observed that supplementation of VEC offered restoration of the activities of these steroidogenic regulatory enzymes. This ameliorative property could be explained with the findings of Murugesan et al. (2007) where it was shown that vitamin C and E improved ovarian steroidogenesis by means of increased Δ^5, 3β-HSD and 17β-HSD mRNA expression in Aroclor 1254 treated rats.

Present study also depicts $NaAsO_2$ mediated disturbed ovarian endocrine milieu which was evidenced by impaired E_2, LH, and FSH levels, while VEC supplementation notably restored such levels. We can speculate that compromised steroidogenic activities are allied with reduced E_2, LH and FSH synthesis due to $NaAsO_2$ treatment. This observation is in accordance with recent report (Liu et al. 2021). As per literature, vitamin E is known to maintain the level of E_2 by acting as a key molecule for its synthesis and also by helping in the transportation of cholesterol in intestinal epithelial cells (Galmés et al. 2018), therefore promoting the synthesis of E_2. It also maintains the membrane integrity of granulosa cells, and thus regulates the development of follicle in addition to promoting the synthesis of steroid hormones (Sezer et al. 2020). On the other hand, vitamin C has role in the maintenance of adrenal and gonadal steroidogenesis along with the secretion of FSH and LH (Das et al. 1993; Karanth et al. 2001). Therefore, the use of VEC against $NaAsO_2$ mediated altered steroidogenesis offers better amelioration in restoring the steroidogenic and other endocrine activities.

Prolonged diestrus and reduction in estrus cycle number in $NaAsO_2$ exposed group were probably due to the low signalling of plasma E_2, and gonadotropins (Aritonang et al. 2017). Proestrus to estrus phase transition is highly monitored by LH surge (Fitzgerald 1987), and EACs are abundant in this phase whereas NACs are the main feature of proestrus. Preacidophilic cells started to increase at the end of diestrus along with LBCs, SBCs and leukocytes. High number of leucocytes are associated with increased progesterone level, which results an influx of neutrophils into the vagina (Paccola et al. 2013). From this study it is vibrant that in control and supplemented groups, the estrus phase showed abundant number of EACs but the treated group showed less number of EACs and PACs around day ~ 14–17. After 20 ± 2 days, the treated group showed continuous diestrus phase having a large number of leucocytes but the presence of PACs at the end of the diestrus was significantly lower than that of control and VEC supplemented group. This study clearly depicts that VEC supplementation protects the vaginal cytology and this was probably due to maintenance of LH, FSH and E_2 levels at near normal.

Endogenous antioxidants such as SOD, CAT and GP_X can be accounted as the primary line of antioxidant defence mechanism that makes a unanimously supportive system for maintaining intracellular redox equilibria (Aguilar et al. 2016). Increased production of free radicals due to arsenic exposure disrupts the redox system (Jomova et al. 2011). SOD being an antioxidant enzyme reduces the amount of superoxide anion (O_2^-) by its conversion to H_2O_2 and CAT decomposes H_2O_2 to oxygen and water (Usoh et al. 2005). We observed significant diminution of SOD, CAT and GPx

activities in ovary following $NaAsO_2$ exposure and this is in agreement with the earlier report (Dash et al. 2020). GST is one of the major enzymes involved in metabolism and transport of steroid hormones and this study reported significant decrease in GST and GSH levels in treated ovaries. The GST-alpha isoenzyme localized in steroid-producing cells and may be involved in controlling the steroid productions. On the other hand, the tripeptide glutathione (g–glutamylcysteinylglycine, GSH) acts as a character player in preventing electrophilic attack of toxicants (Deneke and Fanburg 1989). Ovarian GSH also acts as a shield for maturing follicles and protect from the byproduct like superoxide produced during steroid synthesis by the steroidogenic cytochrome P450 enzymes in follicle cells (Luderer et al. 2001).

MDA, one of the stable end products of lipid peroxidation is used to be produced during the decomposition of polyunsaturated fatty acids, can serve as a potent biomarker of oxidative stress (Abuja and Albertini 2001). According to our study, the tissue MDA level was ominously higher in the $NaAsO_2$ intoxicated group, which corroborated with one recent result (Nath Barbhuiya et al. 2021) and supported the involvement of oxidant–antioxidant instability in the pathogenesis of arsenic-provoked ovarian disorder. The observed increased PC content is due to the oxidation of protein in ovary which is at par with the earlier results, i.e., arsenic could cause oxidative damage to protein in the uterus, liver, kidney, and plasma (Kadirvel et al. 2007; Biswas et al. 2019). All these studied oxidative stress parameters in treated group denoted towards the inflation of ROS generation in ovary. Our study further reveals a significant increase of cellular ROS production in ovary and it was consistent with one recent result which showed that sodium arsenite at 5 mg/kg b.wt. for 28 days caused overproduction of ROS in testis (Seif et al. 2021).

We had shown that the VEC supplementation reduced the ROS accumulation followed by decrease of MDA and PC levels. Likewise, this study also demonstrates significant restoration of SOD, CAT, GST, and GP_x activities. Vitamin C has unique antioxidant capacity that can act to minimize the cytotoxic effects in reproductive tissues treated with various metals (Shen et al. 2021), by forming a poorly ionized but soluble complex (Flora et al. 2002). Similarly, it has also been shown that vitamin C and E were capable of reducing the augmented concentrations of lipid peroxides and recovered the concentration of antioxidant defence markers in the rat liver, kidney, and blood (Oliveira et al. 2003; Mondal et al. 2016; Jaturakan et al. 2017). These restoration of GSH could be explained by the regulatory role of gonadotropins in GSH synthesis by up regulating the transcription and/or the translation of the glutamate-cysteine ligase (GCL) which is rate-limiting enzyme for GSH synthesis (Luderer et al. 2001). The duo vitamin (VEC) could also up regulate the GSH-Px mRNA which could be plausible explanation of increased level of GSH in supplemented groups (Min et al. 2018). Though specific mechanism of VEC in mitigating the ROS level has not been detected till date, the chain breaking property of vitamin E can be taken into consideration. Vitamin E can attenuate ROS molecules by producing α-tocopheroxyl radical during fat oxidation and the propagation of free radical reactions (Valk and Hornstra 2000). It also acts as the first line of defence against lipid peroxidation, protecting the cell membranes from free radical attack as it is primarily located in the cell and organelle membranes and it can exert its maximum protective effect, even when its concentration ratio may be only one molecule for every 2,000 phospholipid molecules (Wang and Quinn 2000; Rizvi et al. 2014). Moreover, α-tocopheroxyl radical thus formed is converted back to α-tocopherol by cytosolic vitamin C (Wang and Quinn 2000; Traber 2013) and this could maintain the vitamin E pool in tissues. Thus, synergistic action of VE and VC could make appropriate rejoinder of the precise mechanism played by VEC in attenuating the ROS level.

Inorganic arsenic and its methylated metabolites can damage DNA indirectly through over production of free radicals which cause genomic instability and is often considered as the hallmark of cancer. In addition, the recent literature also states that the prolonged exposure to arsenic may generate hydroxyl radicals in ovarian DNA which react with nucleotide bases of DNA to yield DNA lesions by forming 8-oxo-7,8-dihydroguanine, 5-hydroxycytosine, and 5-hydroxyuracil (Tam et al. 2020). This study reveals that $NaAsO_2$ caused ovarian DNA damage as evidenced by increased comet tail length and presence of DNA ladder in treated rats. The supplementation of VEC evidently attenuated the DNA damage which is in agreement to previous findings that combined dose of vitamin C and E provide meaningful protection in fluoride induced DNA stand break in testis (Pal et al. 2022). A recent report further states that there is a positive effect of vitamin C on DNA repair by up regulation of a number of genes involved in DNA replication and repair (Tam et al. 2020). On the other hand, vitamin E can effectively reduce the generation of hydroxyl radicals which is potent DNA damaging agent (Royack et al. 2000). Hence, the supplementation of VE along with VC can act as an excellent protective agent against $NaAsO_2$ induced DNA damage and also it may play a crucial role in antagonizing genotoxicity followed by genomic instability in female reproductive environment.

During the development of the dominant ovarian follicle, the oocyte is regarded as a seed, and thecal cells along with GCs are regarded as the fecund soil, that directly influence the functions of an ovary (Holesh et al. 2022). Ovarian GCs nurse the oocyte and play a crucial physiological role in supporting of the development and selection of ovarian follicles by controlling oocyte maturation. These are also involved in the synthesis of the E_2 and progesterone that are important for the maintenance

of the ovarian folliculogenesis (Tian et al. 2015). Therefore, the study was aimed to reveal whether VEC could play a protective role in $NaAsO_2$ mediated change in DNA status and ultra-structure of the GCs. The damage in DNA of GCs was observed in treated group which was evidenced by increased Hoechst fluorescence intensity. In VEC supplemented group, significant reduction of fluorescence was observed which further attests the protective ability of VEC in $NaAsO_2$ mediated DNA damage in GCs. One recent study has shown that vitamin E can alleviate the mitochondrial DNA damage and can also reduce the number of autophagosome in granulosa cell in nicotine induced adult rats (Sezer et al. 2020). Another recent study has also reported that vitamin C and E supplementation can decrease the oxidative stress mediated granulosa cell apoptosis in glyphosate induced toxicity in caprine model (Bhardwaj et al. 2022). GSH depletion resulted in apoptosis both in vitro and in vivo studies (Lopez and Luderer 2004). This antiapoptotic activity of VE and VC followed by increased levels of E_2 and GSH which have anti apoptotic property itself (Lopez and Luderer 2004; Karvinen et al. 2021) may finally results in the restoration of DNA strand break in GCs.

The ultra-structural analysis of the granulosa cells exhibits atretic features like short stumpy projections on their surfaces, asymmetrical shrinkage, vacuolization of cytoplasm and increased number of gap junctions due to condensation of cytosol (Bhardwaj and Sharma 2011). The presence of cytoplasmic invaginations and spherical surfaces are positively related to the capacity of granulosa cells to bind LH (Channing and Kammerman 1973; Ryan and Lee 1976) and our study reports significant decrease in serum LH and FSH levels. Furthermore, gonadotropin receptors are located on the plasma membrane of granulosa cells (Han et al. 1974), and alterations of the surface ultra-structure by $NaAsO_2$ could change the ability to bind and respond to gonadotropins. Our results disclose that the VEC supplementation restored the GCs structures to near normal level in ovaries against the adverse effects of $NaAsO_2$. This might be explained by the fact that vitamin C plays a pivotal function to protect cellular aqueous compartments (Lin et al. 2003) and vitamin E protects the stabilization of bio membrane by virtue of lipid–lipid interactions between vitamins and unsaturated fatty acids (Chow et al. 1973).

Individually vitamin C and E exhibit antioxidant ability against xenobiotics and metal induced ovarian cytotoxicity (Traber 2004; Liu et al. 2020). The electrophilic nature of vitamin C and E could augment its protective nature by reducing the production of toxic metabolites (Traber and Stevens 2011). Vitamin E could also neutralize the lipid peroxidation products and promote its biliary excretion. These antioxidant vitamins could modulate the apoptotic events by reducing the expression of genes responsible for cellular death (He et al. 2017). The "vitamin E recycling" and regenerating capacity of vitamin C were the rationale behind selecting the combined vitamins as ameliorative agent. Vitamin C converts the oxidized alpha tocopheroxyl radical to active alpha tocopherol and thus stock up the pool of vitamin E in cellular atmosphere. From this study it is apparent that VEC is an efficient antioxidant combination against $NaAsO_2$ mediated ovarian disorders. Protective ability of these preferred vitamins may open up a new avenue towards managing the arsenic mediated reproductive toxicity in human population in near future. However, because of the limited numbers of animal approval and due to the small size of rat ovary, further exploration of the mechanism of these combined VEC in mitigating $NaAsO_2$ mediated ovarian damages become difficult. Therefore, further studies still require exploring the molecular mechanism of VEC in protecting the $NaAsO_2$ induced ovarian anomalies.

Conclusion

It can be concluded that VEC is an acceptable therapeutic combination against $NaAsO_2$ mediated ovarian disorders like follicular growth arrest, abnormal estrus cycle, impaired steroidogenesis, diminished gonadotropins and steroid hormone followed by oxidative stress induced DNA damage. The commendable success of VEC is due to their shielding nature against ROS molecules in cell membrane and their direct effect on preventing the cascade responsible for apoptosis initiation.

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Authors' contributions The authors declare that all data were generated in-house and that no paper mill was used. All authors contributed to the study design and experiments. Conceived and designed the experiments: RM, AM, and PKM. Performed the experiments: RM, PP and SB. Analysed the data: RM, PP, SB, AC, AB, AM, and PKM. Wrote the paper: RM. Overall manuscript checked: RM, PP, SB, AC, AB, AM, and PKM. Manuscript critically checked: RM, PP, PKM. Final approval was given by all the authors.

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Data availability The data sets generated and or analyzed during the study are included in the manuscript. The data that support this study will be shared upon reasonable request to the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval The experiments were performed following the national guidelines implemented by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India, with approval from the Animal Ethics Committee of Presidency University (Sanction number: PU/IAEC/PM/12).

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