The present study was designed to evaluate the dose and exposure duration dependent effects of malathion on testicular tissue of mature goat (Capra hircus), with or without supplementation of vitamin C and vitamin E for 4 and 8 hours of exposure in vitro. In experimental group, the testicular tissues were treated with malathion (in vitro) at lower concentrations (10^{-3} nM, 1nM) to higher concentration (100 nM), each for 4h and 8h exposure duration. These doses were further supplemented with vitamin C (0.1µM) and vitamin E (0.1µM) individually to evaluate their ameliorative activity. In each case, the testicular cells/tissues were cultured in medium 199 fortified with antibiotics (gentamycin and streptomycin) with malathion at different doses with or without vitamin C and vitamin E supplementation in CO₂ incubator at 39°C, 5% CO₂ and 95% humidity according to exposure duration. After in vitro culturing, the testicular cells/tissues were subjected to further analysis. The cellular parameters were analyzed using trypan blue staining, acridine orange staining, and flow cytometry. The enzymatic changes were evaluated by studying the level and activity of ferric reducing antioxidant power, lipid peroxidation, catalase, superoxide dismutase, glutathione peroxidase and 3β hydroxysteroid dehydrogenase (3β HSD). The molecular analysis was done using TUNEL assay, comet assay, DNA Ladder assay and Real Time PCR to ascertain the extent of damage caused by malathion and its amelioration by vitamin C and vitamin E.

Trypan blue staining was used to differentiate between live and degenerated/dead cells of testicular tissue and revealed a significant increase (P<0.05) in the number of dead cells with increasing concentration of malathion (10^{-3} nM, 1 nM, and 100 nM) however, supplementation of vitamin C and vitamin E (0.1µM) decreased significantly (P<0.05) the percentage of dead cells after 4h and 8h of exposure.

Similarly, supra-vital acridine orange staining revealed a facilitated visualization of apoptotic germ cells under fluorescent microscope. The acridine orange stained preparations revealed chromatin condensation of apoptotic cells, probably in an early phase of death as punctuating bright green fluorescence while
normal cells appeared red in colour. The percentage apoptosis of testicular cells per 10,000 cells were counted by flow cytometry (using Annexin V/PI double staining) assisted in exact quantification of apoptotic potential of malathion. With increasing concentration of malathion a significant rise (P< 0.05) in the number of apoptotic cells were observed whereas on supplementation of vitamin C and vitamin E with different concentration of malathion, the percent apoptotic cells declined significantly (P<0.05). The percentage of total apoptosis increased to 39.0 % after treatment with malathion at 100 nM concentration as compared to control 3.8 %. The malathion treated tissues, supplemented with vitamin C, showed decrease in late apoptotic cells percent i.e. 3.4 % as compared to treated cells.

To evaluate the oxidative stress generated by malathion, ferric reducing antioxidant power (FRAP) and lipid peroxidation level were assessed. It was found that with the increasing malathion concentration (10⁻³ nM, 1nM, 100nM) at different exposure duration (4h and 8h) ferric reducing antioxidant power (FRAP) decreased and that of lipid peroxidation level significantly (P<0.05) increased. This trend was mitigated by vitamin C and vitamin E (0.1µM) supplementation. Lipid peroxidation level was found to increase to 2.9 ± 0.07 (10⁻³ nM), 3.4 ± 0.08 (1 nM), 3.9 ± 0.13 (100 nM) after 4h exposure duration as compared to control (1.4 ± 0.03). Similar trend in lipid peroxidation was recorded after 8h exposure duration. Furthermore, supplementation of vitamin C and E diminished the activity of lipid peroxidation significantly (P<0.05).

Antioxidants inhibit oxidation of free radical thus decreasing the reactive oxygen species level. Malathion decreased the activity of antioxidant enzymes facilitating the formation of reactive oxygen species leading to oxidative stress. Enzymatic analysis revealed a significant (P<0.05) decline in the activity of three antioxidant enzymes viz. catalase, superoxide dismutase and glutathione peroxidase, after malathion exposure at different dose level and exposure duration that was ameliorated with vitamin C and vitamin E supplementation. Malathion exposure resulted in reduced activity of catalase from 3.10 ± 0.02 (in control) to 2.84 ± 0.04 (10⁻³nM), 2.67 ± 0.09 (1 nM), 2.25 ± 0.02 (100 nM) after 4h exposure duration. At the lowest selected dose of malathion (10⁻³nM), the activity of catalase increased significantly (P<0.05) from 2.84 ± 0.04 in 10⁻³nM to 2.92 ± 0.04 after vitamin C
Superoxide dismutase showed a similar pattern of decrease in malathion treated testicular tissues. Another antioxidants enzyme analysis revealed a significant reduction (P<0.05) in the activity of superoxide dismutase to 6.15 ± 0.11, 4.25 ± 0.17 and 3.36 ± 0.10 with rise in malathion concentration, from 10^{-3} nM, 1nM, to 100nM, respectively in comparison to control 6.62 ± 0.26 at 4h exposure duration. Similar declining trend was recorded after malathion exposure for 8h duration. Vitamin C and vitamin E (0.1µM) significantly mitigated the effects of malathion on testicular tissue by significantly enhancing the activity of superoxide dismutase to 6.30 ± 0.20 (10^{-3} nM + vitamin C) and 6.21 ± 0.44 (10^{-3} nM + vitamin E) in comparison to 6.15 ± 0.11 (10^{-3} nM) after 4h malathion exposure. Same pattern of changes in activity of superoxide dismutase was observed after treating the testicular cells at all selected doses and exposure duration.

Glutathione peroxidase, an antioxidant enzyme that reduces hydrogen peroxides, was also found to be reduced in malathion treated testicular tissue with increase in the concentration of malathion. In the experimental group, the increase in malathion dose resulted in decreased activity of glutathione peroxidase from 1.53 ± 0.01 (control) to 1.48 ± 0.02 (10^{-3} nM), 1.44 ± 0.05 (1nM), and 1.21 ± 0.08 (100 nM) after 4h exposure duration. Vitamin C and vitamin E (0.1µM) supplementation individually, along with malathion, significantly (P<0.05) improved this decline in glutathione peroxidase activity. With vitamin C and E supplementation, glutathione peroxidase activity was found to be increased to 1.48 ± 0.01 (10^{-3} nM + vitamin C) and 1.47 ± 0.02 (10^{-3} nM + vitamin E) as compared to 1.42 ± 0.01 (10^{-3} nM) after malathion exposure for 4h. Similar trend was observed at all malathion doses for 4h and 8h exposure durations.

Steroidogenic enzyme, 3βHSD catalyzes the synthesis of progesterone from pregnenolone and the present study revealed the role of malathion in altering the activity of 3βHSD. The activity of 3βHSD decreased from 5.10 ± 0.08 in control to 3.83 ± 0.04, 3.52 ± 0.09, 3.26 ± 0.06 in malathion treated testicular tissue with increasing concentration of 10^{-3} nM, 1 nM and 100 nM respectively after 4h exposure duration.
duration. Vitamin C and vitamin E (0.1µM) significantly (P<0.05) mitigated the effect of malathion (10^{-3} nM) by increasing the activity of 3β HSD to 4.41 ± 0.04 and 4.28 ± 0.09 respectively in comparison to control (3.83 ± 0.04) after 4h exposure. Similar trend in activity of 3βHSD was recorded at all doses and exposure duration.

Besides triggering apoptosis and altering enzyme levels, malathion was observed to induce DNA damage in treated testicular cells that showed significant amelioration after supplementation of vitamin C and E. Comet assay, also known as single cell gel electrophoresis (SCGE) technique, was adopted as a useful tool in short term genotoxicity for detecting DNA single and double strands breaks, alkali labile sites, incomplete excision repair sites and genomic structural discontinuities. The extent of DNA damage was measured quantitatively as tail moment (TM) value using comet assay IV software. Malathion increased the DNA damage in testicular cells, as measured by tail moment, with increasing concentration of malathion (10^{-3} nM, 1 nM and 100 nM) and exposure duration (4h and 8h) that was decreased by vitamin C and E supplementation. The testicular cells, treated with malathion at 100 nM concentration, showed higher amount of DNA damage with TM value 5.58±0.19 at 4h and 7.43±0.20 at 8h time duration. Whereas on supplementing the testicular cells with vitamin C and E for 4h exposure duration, the TM value decreased to 5.32±0.09 and 5.47±0.11 respectively after 8h of 100 nM malathion exposure, the TM value were 7.32±0.09 and 7.37±0.21 respectively. Significant (P<0.05) amount of DNA damage was found at lower malathion doses revealing a similar pattern of DNA damage and amelioration by vitamin C and E.

To further evaluate the effects of malathion on the induction of apoptosis in the testicular tissue of goat in vitro, fragmentation of DNA (a hallmark of apoptosis) was investigated by DNA Ladder assay using agarose gel electrophoresis. The results showed DNA Ladder formation with intensive laddering and increased smearing of intact bands in malathion treated testicular tissue for 4h and 8h indicating random DNA cleavage, and necrotic cell death as compare to control which revealed intact bands in ladder assay. Supplementation of vitamin C showed less visible apoptotic laddering and DNA bands. Similarly, supplementation with vitamin E also resulted in less banding as compared with treated group.
To further access the role of malathion on the expression of pro apoptotic gene \( \textit{Bax} \) and anti-apoptotic \( \textit{Bcl-2} \), Real time PCR assay was optimized for valid amplification of these genes along with GAPDH as an endogenous control used for normalizing relative abundance values for respective transcripts. Malathion significantly increased the expression of \( \textit{Bax} \) gene from \( 2.0 \pm 0.22 \) (control) to \( 2.33 \pm 0.21, 2.68 \pm 0.04 \) and \( 3.60 \pm 0.15 \) with increasing concentration to \( 10^{-3} \) nM, 1nM, 100 nM respectively after 4h exposure duration. Whereas, anti-apoptotic, \( \textit{Bcl-2} \) gene expression decreased significantly (P<0.05) from \( 2.0 \pm 0.06 \) (control) to \( 1.89 \pm 0.08, 0.95 \pm 0.10, 0.10 \pm 0.11 \) after 4h exposure duration with increasing malathion concentration to \( 10^{-3} \) nM, 1nM, 100 nM respectively. Similar trends were recorded after 8h exposure duration and vitamin C and vitamin E were found to ameliorate malathion toxicity by significantly decreasing \( \textit{Bax} \) gene expression and increasing \( \textit{Bcl-2} \) gene expression both after 4h and 8h exposure duration.

From the present study it is evident that malathion induces changes in physiology of testicular tissue by production of oxidative stress as documented by FRAP, and lipid peroxidation suppressing the antioxidant enzymes SOD, GPx and Catalase reducing, 3β HSD activities, resulting in DNA damage as demonstrated by comet assay, ladder assay and expression of pro-apoptotic gene \( \textit{Bax} \) and suppression of anti-apoptotic gene \( \textit{Bcl-2} \). Vitamin C and vitamin E ameliorated malathion induced changes at cellular, enzymatic and molecular levels.

**Salient findings of the present study are:**

1. Malathion induced decline in number of live germ cells and increased percentage of germ cell apoptosis with elevating doses and exposure duration.
2. A decline in activity of ferric reducing antioxidant power (FRAP) and an increase lipid peroxidation was observed with increasing malathion concentration after 4h and 8h exposure duration.
3. The activities of various antioxidant enzymes namely catalase, superoxide dismutase and glutathione peroxidase, declined in testicular tissues after malathion treatment in dose and time dependent manner.
4. Malathion treated testicular tissues revealed a significant decrease in steroidogenic enzyme, 3βHSD activity.
5. Malathion induced genotoxicity in testicular cells by increasing the DNA damage and fragmentation as revealed by increased tail moment and ladder bands in testicular tissues.

6. Malathion exposure increased the expression of pro-apoptotic gene, Bax whereas it decreased the expression of anti-apoptotic, Bcl-2 gene in testicular tissues.

7. Vitamin C and vitamin E ameliorated the effects of malathion in testicular cells thereby increasing the percentage of live cells and decreasing the apoptotic germ cells percentage in malathion treated testicular tissues.

8. Vitamin C and vitamin E decreased malathion induced oxidative stress in testicular tissues hence, decreased ferric reducing antioxidant power (FRAP) and lipid peroxidation.

9. Vitamin C and vitamin E supplementation also ameliorated the malathion induced decrease in antioxidant level of catalase, super oxide dismutase and glutathione peroxidase enzymes.

10. Vitamin C and vitamin E decreased tail movement and ladder bands in testicular cells treated with malathion, suggesting reduction in DNA damage or fragmentation.

11. Vitamin C and vitamin E increased the activity of steroidogenic enzyme, 3βHSD along with decreased expression of pro-apoptotic gene, Bax and increased the expression of anti-apoptotic, Bcl-2 gene in comparison to malathion treated testicular tissues.

In conclusion, Malathion, an organophosphorous pesticide, was found to be a potent reproductive toxicant that induces genotoxic and oxidative damage within testicular tissue including spermatogenic cells. It reduced FRAP and enhanced lipid peroxidation, decreased the antioxidant and steroidogenic enzyme level in spermatogenic cells along with expression of apoptotic and anti-apoptotic gene, leading to apoptosis of germ cells. The loss of germ cells by activation of apoptotic gene expression resulted in decline in sperm production thereby, affecting the fertility of the organism. Furthermore, Vitamin C and vitamin E ameliorated the malathion induced oxidative stress, cytotoxicity, and DNA damage. It is therefore recommended that vitamin C and vitamin E has potential to combat malathion induced infertility in mammals and should be supplemented in diet for reproductive health.