Original Article

VITAMIN C REDUCES THE METANIL YELLOW INDUCED OXIDATIVE STRESS IN OVARIAN TISSUES OF RAT

Partha Pratim Nath[1], Kaushik Sarkar[2], Kamalesh Das[3], Goutam Paul[4]

[1]Assistant Professor, Department of Physiology, Government General Degree College at Kaliganj, Nadia, West Bengal-741137.
Email- nath.partha3@gmail.com
[2]Assistant Professor, Department of Physiology, Krishnagar Government College, Nadia, West Bengal-741101.
[3]Assistant Professor, Department of Physiology, Uluberia College, Uluberia, Howrah, West Bengal- 711315.
[4]Professor, Department of Physiology and Pro-Vice-Chancellor, University of Kalyani, Kalyani, West Bengal-741235.
Email: goutampaul.ku@gmail.com

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Abstract

Metanil yellow (MY) is a banned synthetic azo dye that induces oxidative stress in rat ovarian tissue. To investigate the mitigating role of vitamin C in MY-induced oxidative stress in ovaries, the activity of antioxidant enzymes in ovarian tissue homogenates and cytoarchitectural changes in ovarian tissue sections of MY with vitamin C-treated rats were investigated. Female Charles Foster rats (age 90-120 days, weight 110-120 grams) bred in the laboratory were used in the study. The study found no significant changes in the production of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and malondialdehyde (MDA) in groups of rats that received MY with vitamin C compared to control rats. In addition, we found no significant structural changes in ovarian tissue in rats that received MY with vitamin C compared to control rats. The results suggest that vitamin C exerts a protective effect against MY-induced oxidative stress in ovarian tissues, probably by antagonizing MY-induced reduction of antioxidant enzyme activity, MDA production through lipid peroxidation, and stress-induced damage of ovarian tissues.

Keywords: Metanil yellow, oxidative stress, lipid peroxidation, vitamin C.

Introduction

Food manufacturers often use the synthetic food color metanil yellow (MY) in many yellow or orange colored foods. It is mainly used as a dye in shops, paper, cotton, leather, dye, textile, and alcoholic beverage industries [1]. MY is a banned synthetic azo dye that is widely used to color various foods in many developing countries, including India. It is found in various yellow or orange colored foods like ladoo, papadum and spices like turmeric powder, sweets, ice cream etc. It is also found in prepared foods like biryani. It is chemically known as the sodium salt of 3-(-anilinophenylazo)benzenesulfonic acid and has the chemical formula $C_{18}H_1N_3NaO_3S$. MY is a banned colorant in India under the Food Adulteration Act (FDA) 1954, but despite this, small rural belt producers continue to use MY in various foods because they are not under the direct control of government vigilance [2].

It has been shown that MY promotes the structural degeneration of the ovary and generates oxidative stress in the ovaries, most likely via producing oxidative stress in the tissues [3]. Vitamin C (ascorbic acid) is a very pronounced antioxidant that reduces the deleterious effect of oxidative damage by scavenging the free radicals. Vitamin C can donate hydrogen to free radical molecules and neutralize them. By contributing a hydrogen molecule, vitamin C not only neutralizes hydroxyl (O), alkoxyl (OL), and peroxyl (LOO) radicals but also the radical forms of other

antioxidants like glutathione and vitamin E. In the past few decades, there have been some studies about the use of vitamin C as an antioxidant to lower the risk of chemically induced toxicity [4-9]. It was also reported that vitamin C is capable of protecting the ovary from chemically induced oxidative stress [10-14]. Previous research has suggested that taking vitamin C supplements reduces chemically induced oxidative stress in body cells.

Therefore, to investigate the mitigating role of vitamin C in MY-induced oxidative stress in ovaries, the activity of antioxidant enzymes was determined and the amount of MDA was measured biochemically in the ovarian tissue homogenate of rats treated with MY with vitamin C. In addition, the histological structure of ovaries was examined in rats treated with MY in combination with vitamin C to investigate the protective effect of vitamin C against MY accompanied by structural degenerations due to oxidative stress.

Materials and methods

Reagents and chemicals

All chemicals used in this study were of analytical grade. MY was purchased from Sigma-Aldrich, USA and 5,5'-dithiobis-2-nitrobenzene (DTNB), oxidized and reduced glutathione, NADPH.Na_4 was obtained from Sisco Research Laboratories Pvt. Ltd., India. Trichloroacetic acid (TCA), tris, BSA, NaCl, glacial acetic acid, K_2HPO_4, NaOH, Triton-X-100, EDTA, $CuSO_4$, Na-K tartrate, pyrogallol, 2-thiobarbituric acid (TBA), HCl, $K_2Cr_2O_7$, CDNB , KH_2PO_4, sodium azide, Na_2HPO_4, NaH_2PO_4, etc were obtained from E-Merck, India.

Animal selection and care

Healthy adult female Charles Foster rats aged 90-120 days, weighing 110-120 gm, bred in the laboratory were selected for the experiments. All experimental procedures were performed under the recommendation of the Kalyani University Animal Ethics Committee in accordance with national guidelines.

Experimental design

Before the experiments, the test animals were randomly chosen, marked, and housed in different cages. After acclimatization to the laboratory environment, the experimental animals were divided into seven groups (8 rats per group) according to the similar average body weight between the groups.

Table 1: Experimental design to investigate the effects of different doses of MY, and MY in combination with vitamin C on ovarian tissue with special influence on oxidative stress.

Groups	Exposures
I	Received distilled water for 20 and 30 days (control).
II	Received 250 mg/kgBW/day (i.e., 5% of LD_{50}) of MY for 20 and 30 days.
III	Received 500 mg/kgBW/day (i.e., 10% of LD_{50}) of MY for 20 and 30 days.
VI	Received 750 mg/kgBW/day (i.e., 15% of LD_{50}) of MY for 20 and 30 days.
V	Received 250 mg/kgBW/day (i.e., 5% of LD_{50}) of MY and 50 mg/kgBW/day Vitamin C for 20 and 30 days.

VI	Received 500 mg/kgBW/day (i.e., 10% of LD_{50}) of MY and 50 mg/kgBW/day Vitamin C for 20 and 30 days.
VII	Received 750 mg/kgBW/day (i.e., 15% of LD_{50}) of MY and 50 mg/kgBW/day Vitamin C for 20 and 30 days.

The drug was administered to rats by oral gavage for 20 and 30 consecutive days. A single oral dose was administered below the acute LD_{50} level of toxicity.

Preparation of ovarian tissue homogenate

Rats were sacrificed by cervical dislocation, and the ovaries were quickly removed, washed with ice-cold 0.9% NaCl solution, dried, and immediately weighed. A 2% ovarian tissue homogenate was prepared in phosphate buffer using a tissue homogenizer. The ovarian tissue homogenate was then centrifuged and the clear supernatant was stored in aliquots at −20°C for further biochemical analysis.

Biochemical estimations of oxidative stress related variables in ovarian tissue homogenate of rats

Superoxide dismutase (SOD) activity was measured by the method of Marklund and Marklund, 1974 [15]. Measurement of catalase (CAT) activity in tissue homogenates was performed according to the protocol of Sinha et al., 1972 with minor modifications [16]. Glutathione peroxidase (GPx) activity was measured according to the method of Rotruck et al., 1973 [17]. The activity of glutathione reductase (GR) was assayed according to the method of Staal et al., 1969 [18]. Glutathione-s-transferase (GST) activity was estimated according to the method of Habig et al., 1974 [19]. The degree of lipid peroxidation was measured according to the method of Devasagayan and Tarachand, 1987 [20]. Protein was determined according to the method of Lowry et al., 1951 by using bovine serum albumin as standard [21].

Histological studies of the ovary tissue

Ovaries of each group were collected immediately after rat sacrifice by cervical dislocation and immediately fixed in 10% neutral buffer saline. The fixed samples were then treated with a series of graded alcohol solutions and embedded in paraffin wax to generate paraffin blocks. Paraffin blocks were cut into 5–7 µm thick ribbon, deparaffinized, rehydrated, and stained with hematoxylin and eosin [22] for morphological evaluation with an Olympus light microscope (CH20i).

Statistical analysis

Data obtained from the results were expressed as mean ± SEM. Statistical comparisons between values obtained in control and treated rats were performed by using ANOVA followed by Student's t-test and Mann-Whitney U-test for non-parametric data where appropriate. $P \leq 0.05$ was considered as significant.

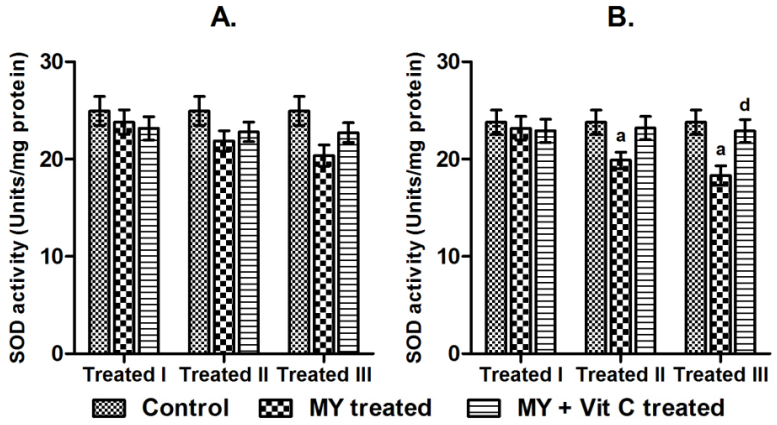
Results

Effect of vitamin C on the SOD activity of ovarian tissue homogenate of MY exposed rats

Figure 1 shows the protective effect of vitamin C on ovarian SOD activity in MY-exposed rats. From the results, it was observed that MY significantly reduced SOD activity in ovarian tissue homogenates of MY-exposed rats over a 30-day exposure period compared to control rats. However, co-exposure of vitamin C and MY showed a

significant counteraction in SOD activity in rat ovarian tissue homogenate compared to that in MY-treated rats. It was also observed that vitamin C exhibited a non significant counteraction in the activity of SOD during the 20-day exposure period in ovaries compared to SOD activity in ovaries of MY-exposed rats.

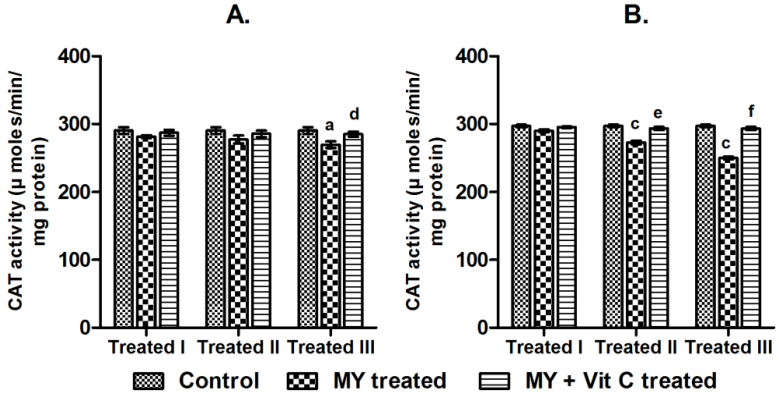
Figure 1: Graphical representation of the effect of vitamin C on ovarian SOD activity in MY exposed rats. Data are expressed as mean ± SEM (n=8). [a]p<0.05 vs. control, [d] p<0.05 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration.



Effect of vitamin C on the CAT activity of ovarian tissue homogenate of MY exposed rats

Ovarian CAT activity was significantly decreased in the MY-exposed group of rats in a dose-dependent manner on both 20 and 30 days of treatment durations compared to the control group of rats. But, co-administration of vitamin C and MY showed a significant counteraction in CAT activity in rat ovarian tissue homogenates compared with that in MY-treated rats. Figure 2 shows the effect of vitamin C on ovarian CAT activity in MY-exposed rats.

Figure 2: Graphical representation of the effect of vitamin C on ovarian CAT activity in MY exposed rats. Data are expressed as mean ± SEM (n=8). [a, c] p<0.05, 0.001 vs. control, [d, e, f] p<0.05, 0.01, 0.001 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration.

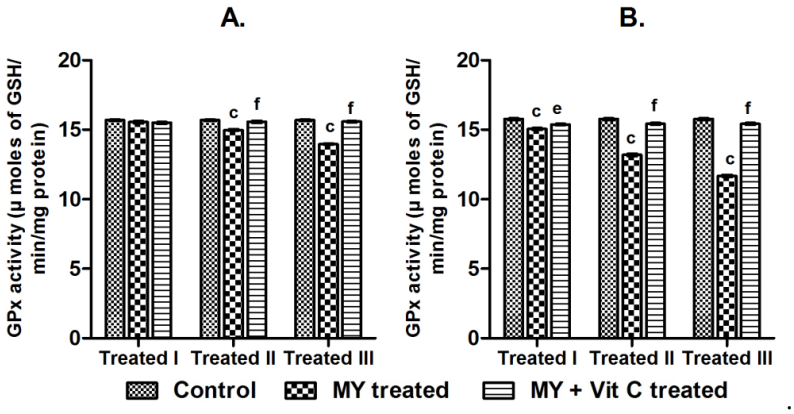


Effect of vitamin C on the GPx activity of ovarian tissue homogenate of MY exposed rats

We found a significant decrease in GPx activity in the ovarian tissue homogenate of MY-exposed rats in a dose-dependent manner compared to control rats. In addition, we did not observe a significant decrease in GPx activity

in ovarian tissue homogenate from rats treated with vitamin C in combination with MY during both treatment periods compared to GPx activity in control rats (Figure 3).

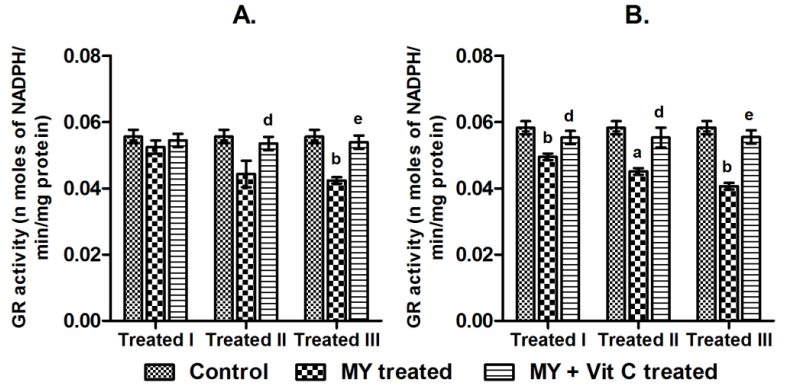
Figure 3: Graphical representation of the effect of vitamin C on ovarian GPx activity in MY exposed rats. Data are expressed as mean ± SEM (n=8). [c]p<0.05 vs. control, [e, f] p< 0.01, 0.001 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration



Effect of vitamin C on the GR activity of ovarian tissue homogenate of MY exposed rats

Figure 4 shows glutathione reductase activity in rat ovarian tissue homogenate after co-administration of vitamin C with MY. Treatment with MY at various concentrations caused a significant dose-dependent decrease in GR activity in ovarian tissue homogenate in both treatment periods of rats compared to control rats. Ovarian GR activity was significantly restored to normal levels when vitamin C was co-administered with MY for a 20 and 30-day treatment duration in rats.

Figure 4: Graphical representation of the effect of vitamin C on ovarian GR activity in MY exposed rats. Data are expressed as mean ± SEM (n=8). [a, b] p<0.05, 0.01 vs. control, [d, e] p<0.05, 0.01 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration.

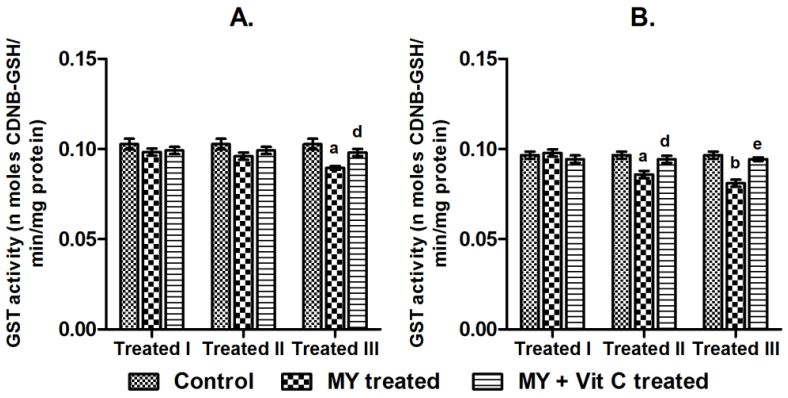


Effect of vitamin C on the GST activity of ovarian tissue homogenate of MY exposed rats

The effects of MY; and co-administration of MY with vitamin C on GST activities of ovarian tissue homogenate are shown in Figure 5. From the results it was observed that, in case of 20 days treatment duration the GST activity has been significantly decreased only in last dosage of MY whereas, in case of 30 days treatment duration the GST activity was significantly decreased in last two dosages of MY respectively. But, when the doses of MY was

co-administered with Vitamin C, it was observed that vitamin C significantly counteract the effect of MY on activity of GST in ovarian tissue homogenate of rats when compared to MY treated rats.

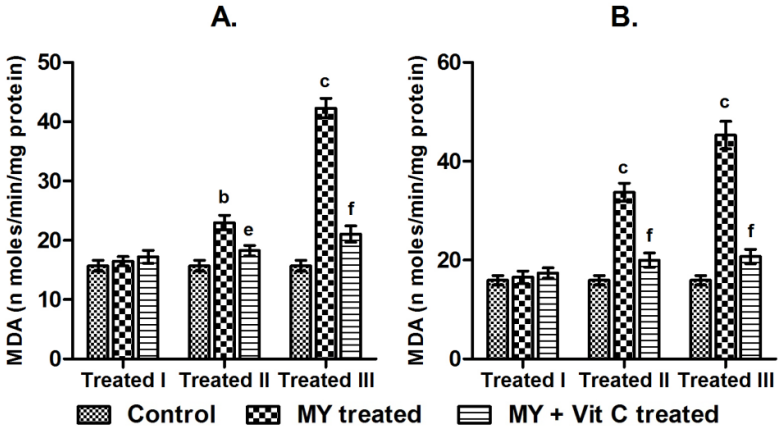
Figure 5: Graphical representation of the effect of vitamin C on ovarian GST activity in MY exposed rats. Data are expressed as mean ± SEM (n=8). [a, b] $p<0.05$, 0.01 vs. control, [d, e] $p<0.05$, 0.01 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration.



Effect of vitamin C on ovarian MDA production in MY exposed rats

Figure 6 shows the effect of vitamin C on ovarian MDA production (a marker of lipid peroxidation) in MY exposed rats. It was observed that MY significantly increase the amount of MDA production in ovarian tissue homogenate of rats in both treatment durations compared to the control groups of rats. But, co-administration of MY with vitamin C showed significant reduction in the production of MDA in ovarian tissue homogenate for 20 and 30 days treatment durations compared to the production of MDA in ovarian tissues of MY exposed rats.

Figure 6: Graphical representation of the effect of vitamin C on ovarian MDA levels in MY exposed rats. Data are expressed as mean ± SEM (n=8).[b, c] $p<0.01$, 0.001 vs. control, [e, f] $p<0.01$, 0.001 vs. MY treated groups. A indicate the 20 days exposure duration and B indicate the 30 days exposure duration.

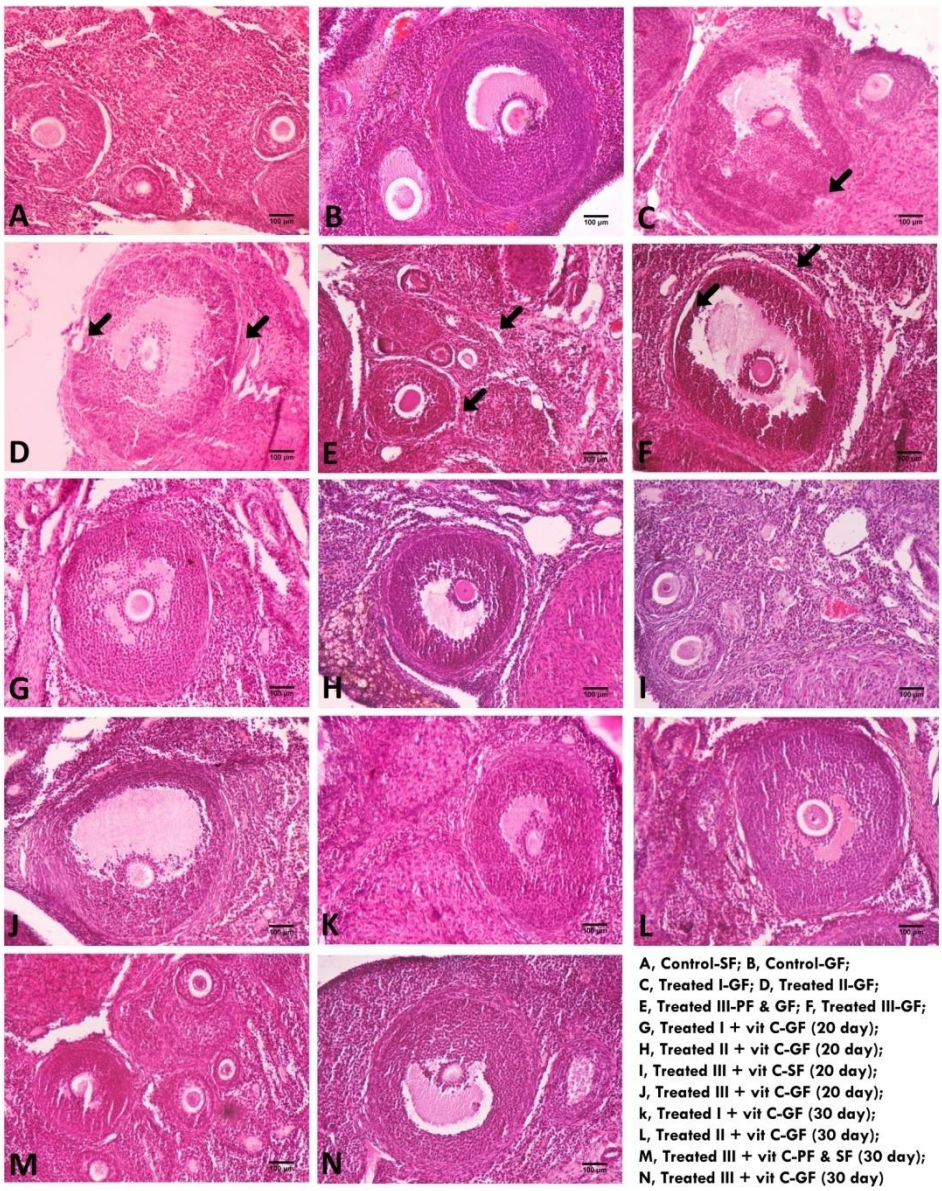


Effect of vitamin C on MY induced cytoarchitectural changes in ovary tissue of rats

We did not observe any significant changes in the ovarian structure of vitamin C-treated rats compared to control rats. The number of primordial follicles, primary follicles, graphite follicles, and atretic follicles was not significantly altered in the ovaries of MY and vitamin C-treated rats compared with control animals. The diameter

of the ovary and blood vessels did not show deviations from the control ovary. In addition, no degenerations of graafian follicles were observed (Figure 7).

Figure 7: Photomicrographs of normal hematoxylin and eosin stained tissue section shows the morphological changes in ovary of rats exposed to MY, and MY in combination with vitamin C for 20 and 30 days treatment durations. Arrow heads indicate the sites of morphological changes. Images were taken by digital SLR Olympus Camera (E-620) fitted with Olympus light microscope (CH20i) (100X magnification). PF, primary follicles; SF, secondary follicles; GF, graafian follicle.



Discussion

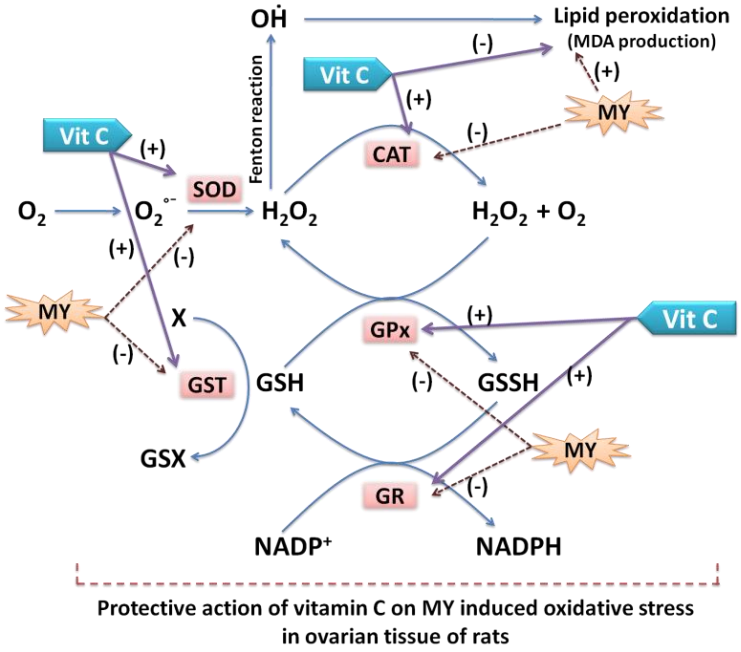
The aim of the study was to examine the role of vitamin C in the mitigation of MY induced oxidative stress in ovary tissues. In present study, we found no significant changes in the activities of SOD, CAT, GPx, GR, GST and MDA production in groups of rats received MY in combination with vitamin C compared with the control and MY treated rats. These results suggest that vitamin C might provides the protective role against the MY induced depression of the activities of antioxidant enzymes and damages of the structural membrane lipids by

means of MY induced peroxidation. In order to examine the protective role of vitamin C against the MY involved oxidative stress induced structural alterations of the ovary, the structure of the ovary of the rats received MY in combination with vitamin C was observed and compared with control and treated ovarian structures of the rats received MY. We did not find any significant structural alterations in the tissues of ovary in rats received MY in combination with vitamin C compared with the control groups of rats. The result suggests that vitamin C might provide mitigative role against the MY involved oxidative stress induced degeneration of the structure of the ovary.

Conclusion

From the results, it may be concluded that vitamin C provides protective functions against the MY induced oxidative stress in ovary.

Figure 8: The probable mechanism of action of vitamin C in the MY induced oxidative stress in ovarian tissue of rats. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-s-transferase; MDA, malondialdehyde; GSH, reduced glutathione; GSSH oxidized glutathione; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; MY, metanil yellow; O_2, oxygen; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical; $O_2^{\cdot -}$, superoxide anion.



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