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Metanil Yellow Induces Oxidative Stress in Duodenal Visceral Smooth Muscle of Rat

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ABSTRACT

Metanil Yellow, a synthetic dye has huge application as a dye in the paint, textile, porcelain, and leather industries. Besides, it is widely used as a food colorant in the food industry because of its persistent yellow color despite of being categorised as non-permitted food color to attract customers. Consequently, eating food tainted with MY exposes humans to MY on a regular basis. Thus, the study was to examine any induction of oxidative stress on acute exposure of MY in the smooth muscles located at the wall structure of the duodenum, that with its contractile activity provides motility to it that performs the digestive and absorptive functions of the small intestine. From the results, we have found significant decrease in the activity of antioxidant enzymes- catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR); and increase in the levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH) activity, biomarkers of oxidative stress in the duodenal visceral smooth muscles in a significant manner. In conclusion, MY significantly induces oxidative stress in smooth muscles found in the muscularis externa of the duodenal wall that provides the motility of the duodenum which is prerequisite for digestion and absorption of the food stuffs. The induction of oxidative stress in the dVSM might cause degenerative alterations that results in the impairment of its digestive and absorptive functions.

Keywords: Metanil yellow, duodenal visceral smooth muscle, oxidative stress, antioxidant enzymes, biomarkers of oxidative stress

INTRODUCTION

The most essential factor for good health and nutrition is a proper healthy diet. It also provides protection against many diseases and helps to maintain fitness and a healthy lifestyle. But, with the increase in population and food demand, certain substances are added to the food products available in the market with the intention of making it more attractive and to increase the quantity of food production for making maximum profit. This practice is known as food adulteration and the added substances are called food adulterants, which are poor quality materials and reduce the value of nutrients in food. These substances can be pigments, dyes or any other compounds which impart color to the materials in which they are added (Yadav et al., 2016).

Metanil Yellow is a water soluble food colourant which is used extensively in different food products like turmeric powder, biryani, sweets, ice-creams, cheese, beverages, ladoo and many more (Sarkar and Ghosh, 2012; Ghosh et al.,2017). It is a yellow azo dye which is made from diphenylamine and diazotized metanilic acid (Khanna and Das, 1991) and is generally allowed to be used in industries for colouring wool, nylon, silk, paper, ink, aluminium, detergent, etc. (Ghosh et al.,

2017). Metanil yellow is a banned dye as per PFA Act (1954), by the Government of India (Nath et al., 2016), because of its mutagenic (Das and Mukherjee, 2004) and carcinogenic (Gupta et al., 2003) nature. But it has been observed that MY is being added to various food items due to its availability and cheap price (Nath et al., 2016).

Regular intake of MY may lead to several toxic effects on the various physiological systems of the human body (Nagaraja et al.,1993; Ramchandani et al., 1997). MY is also responsible for generating oxidative stress in various vital organs such as heart, liver, kidneys (Dome et al., 2017; Hazra etal., 2016), nervous tissue, intestines, gastric tissue etc. ultimately leading to damage. MY gets absorbed from the intestine when consumed with food and enters the bloodstream. The toxic chemical travels in blood and reaches various organs and affects various cellular metabolic processes (Ghosh et al., 2017).

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with oxidative damage to proteins, lipids, and nucleic acids. MY causes degenerative changes in the lining of the stomach, kidneys, and liver (Sarkar and Ghosh, 2010) probably by the induction of oxidative stress. It adversely affects the ovaries and testes, leading to damage in the reproductive organs (Sarkar and Ghosh, 2012). It also causes insufficient oxygen supply to skin and mucous membranes resulting in cyanosis (Chandro et al., 1987). It is also responsible for causing toxic methemoglobinemia (Reyes et al., 1996).

Despite extensive research on the physiological effects of MY, there remains a significant gap in our understanding of the effect of MY on the oxidative stress in the duodenal visceral smooth muscle. So, the purpose of this study is to examine the effect of MY on the antioxidant enzymes, and stress markers in the duodenal visceral smooth muscle of rats to know about the damage caused by this chemical on the gastrointestinal system.

MATERIALS & METHODS

Chemicals and reagents

All the reagents and chemicals that were used to conduct this study were of analytical grade. The chemical used for the present study was Metanil Yellow; 3-[[4-(Phenylamino) phenyl]azo]benzenesulfonic acid monosodium salt. Metanil yellow (CAS no. 587-98-4, Dye content 70%) was purchased from Sigma Aldrich Chemical, USA. Ethylene di-amine tetra acetic acid, sodium-potassium tartrate, hydrochloric acid, thiocholine iodide, copper sulphate, trichloroacetic acid, pyrogallol, folin reagent, sodium chloride, sodium bicarbonate, glucose, disodium hydrogen phosphate, potassium dihydrogen phosphate, and ethanol were purchased from EMerck, Mumbai, India. 5-5´dithiobis 2-nitrobenzene, potassium ferricyanide and sodium pyruvate were obtained from Sisco Research Laboratory (SRL), India.

Experimental Animals and Care

As the experimental model, adult male albino rats of Sprague-Dawley strain with body weight ranging around 130-150 g and age around 2-3 months were selected. They were kept in the room temperature of 25-27°C at the departmental animal care room with 24 hours light-dark cycle and were fed with standard diet and sufficient water. All the experiments were performed following the recommended guidelines of the Kalyani University Animal Ethics Committee. Animals were kept in fasting condition overnight before the experiments.

Experimental design

The experimental animals were divided into four groups respectively viz., one control group and three Metanil Yellow (MY) exposed groups. Animals in the control group were exposed to distilled water only. Moreover, the exposure groups were further subdivided into three groups according to the exposure of three graded doses of MY through oral administration and were categorised as Treated group I, Treated group II and Treated group III. All of the

four groups contained six animals in each. The doses of MY were selected based on the LD_{50} value of the compound and are summarised in the following table 1.

Table 1. Tabular representation of the group division of experimental animals according to dose selection

GROUP NAME	DOSES OF METANIL YELLOW (MY)
Control Group	Received distilled water only
Treated group I	Received 200 mg/kg BW/day of MY (approximately 4% of LD_{50} of MY)
Treated group II	Received 400 mg/kg BW/day of MY (approximately 8% of LD_{50} of MY)
Treated group III	Received 600 mg/kg BW/day of MY (approximately 12% of LD_{50} of MY)

Tissue Collection

The animals were sacrificed by performing cervical dislocation and the abdominal cavity is opened up. The small intestine was exposed and the duodenum, which is the initial part of the small intestine, was identified. After that, the muscularis externa layer which is made up of smooth muscle was separated to obtain the visceral smooth muscle (VSM) tissue that was further used to prepare the homogenate for performing the experiments of this study.

Preparation of Tissue Homogenate

The duodenal tissue was weighed and 10% w/v homogenate was prepared by the utilization of a homogenizer (RQ-127A, REMI, India) in cold condition. As the homogenization medium, 50mM ice-cold phosphate buffer (pH 7.4) was used and for lipid peroxidation assay, 0.9% sodium chloride solution was used instead of phosphate buffer.

Measurement of the Activities of Antioxidant Stress Related Enzymes

Superoxide dismutase (SOD) activity was measured by the inhibition of pyrogallol (2mM) autoxidation at 420 nm for 3 min at an interval of 1 min according to the method of Marklund & Marklund (Marklund and Marklund, 1974). The enzyme activity was expressed as U/mg protein, where 1 U is the amount of enzyme required to bring about 50% inhibition of the autoxidation of pyrogallol. Catalase (CAT) activity was measured according to the method of Aebi (Aebi, 1974) by following the decrease in absorbance of H_2O_2 at 240 nm for 1 min. The enzyme activity was expressed as µ moles H_2O_2 decomposed/min/ mg protein. Glutathione peroxidise (GPx) activity was assayed by the method of Rotruck et al., 1973(Rotruck et al., 1973) using H_2O_2 as the substrate. Enzyme activity was expressed as µ moles glutathione oxidized/min/mg protein. The activity of GR was measured spectrophotometrically according to the method of Staal et al., 1969 (Staal et al., 1969) with some modifications. The activity of GR was expressed as µ moles of NADPH oxidized/min/mg protein.

Measurement of the Oxidative Stress Biomarkers

The degree of lipid peroxidation was examined by measuring the level of malondialdehyde (MDA) produced in the dVSM as a consequence of oxidative stress with the help of the method of Devasagayam and Tarachand, 1987 with slight modifications. The amount of MDA produced was estimated using the molar co-efficient of $1.56*10^5$ $mol^{-1}cm^{-1}$ and expressed as n moles MDA/mg protein. The LDH activity was measured following the method of Wróblewski & Ladue (1955) and expressed as Unit/ml/min.

Statistical Analysis

The values were represented as the mean ± SEM. Difference among the mean values in groups of each drug treatment and the control was inspected by one way ANOVA in GraphPad Prism 8 software. $P < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Oxidative stress is a cytological outcome brought on by an imbalance between the generation of free radicals and antioxidants' capacity to scavenge them. Owing to this unbalance, oxidatively changed molecules that might lead to oxidative stress. Oxidative stress caused by any drug has been linked to toxicity in a variety of organ systems and tissues, including the kidney, liver, intestines, heart, and neurological systems. Drug metabolism can produce reactive oxygen species (ROS), disrupt oxidant equilibrium, and cause mitochondrial dysfunction, all of which can lead to side effects that are clinically significant. Oxidative stress plays a role in the pathophysiology of several gastrointestinal disorders, such as peptic ulcers, gastrointestinal malignancies, and inflammatory bowel disease. In order to examine the effect of acute exposure MY on oxidative stress related parameters, the activity of antioxidant enzymes viz., SOD, CAT, GPx and GR and also two oxidative stress biomarkers – level of MDA and activity of LDH have been examined in the MY exposed dVSM homogenates and control dVSM homogenates.

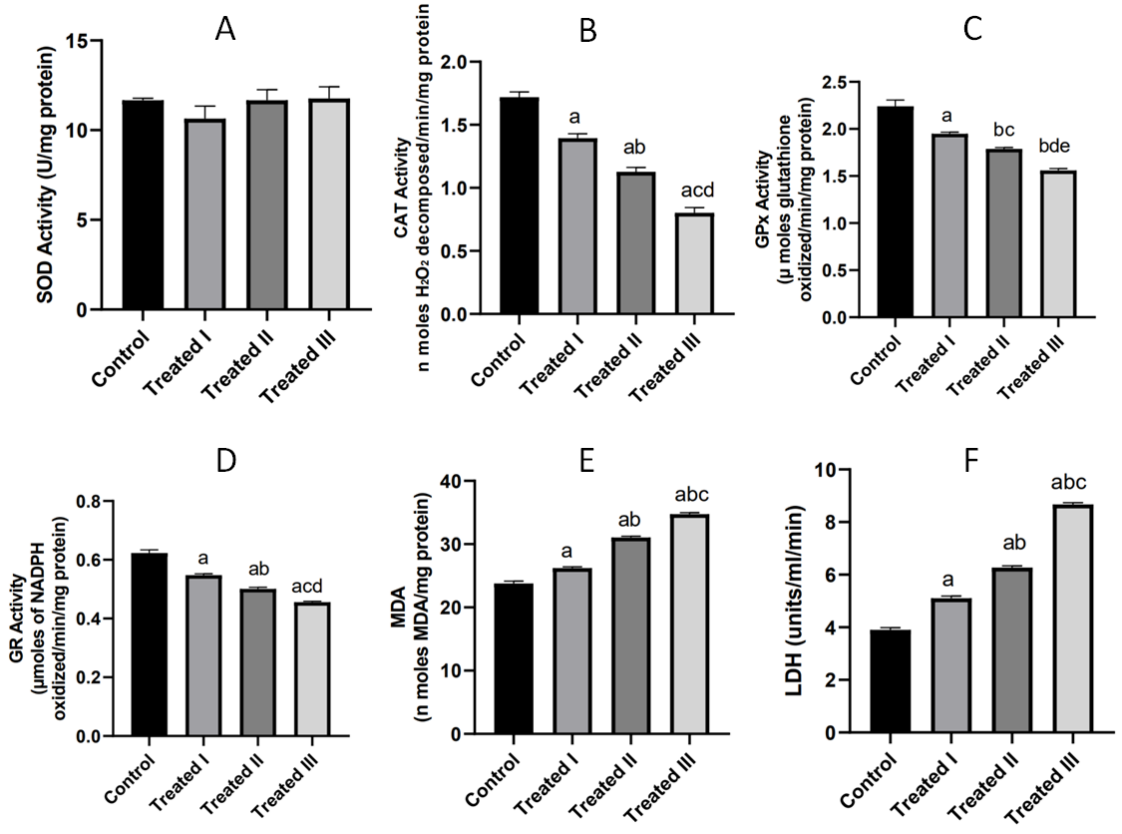

Figure1. Bar diagram showing the activities of antioxidant enzymes (SOD,CAT, GPx and GR) and level of stress biomarkers (MDA and LDH) in MY exposed and control rats. ^{a}P<0.0001 Vs Control, $^{c,b}P$<0.0001, 0.001 Vs Treated I, ^{d}P<0.0001 Vs Treated II (B). $^{b,a}P$<0.0001, 0.001 Vs Control, $^{d,c}P$<0.0001, 0.05 Vs Treated I, ^{e}P<0.01 Vs Treated II (C). ^{a}P<0.0001 Vs Control, $^{c,b}P$<0.0001, 0.001 Vs Treated I, ^{d}P<0.001 Vs Treated II (D). ^{a}P<0.0001 Vs Control, ^{b}P<0.0001 Vs Treated I, ^{c}P<0.0001 Vs Treated II (E). ^{a}P<0.0001 Vs Control, ^{b}P<0.0001Vs Treated I, ^{c}P<0.0001 Vs Treated II (F).

From the results, we have found that though MY did not produce significant alterations in the SOD activity but the activity of CAT, GPx and GR have been decreased significantly in a dose response manner in MY exposed rats compared to control rats (Figure 1 and Table 2). Further, we have also found significant increase in the level of MDA production and increased activity of LDH in MY exposed dVSM homogenates compared to control homogenates. It is evident that MY on

exposure induces generation of ROS in the dVSM and suppressed the activity of antioxidant enzymes that ultimately failed to neutralize the MY induced ROS generation leading to induction of oxidative stress in the dVSM.

Table 2. The oxidative stress related variables in MY exposed and Control rats.

Stress Variables	Control	Treated I	Treated II	Treated III
SOD (U/mg protein)	11.67±0.1117	10.64±0.7087	11.66±0.5907	11.77±0.0.6551
CAT (µ moles H_2O_2 decomposed/min/mg protein)	1.720±0.0418	1.395±0.0351	1.127±0.0351	0.8023±0.0418
GPx (µ moles glutathione oxidized/min/mg protein)	2.240±0.0682	1.949±0.0175	1.788±0.0156	1.56±0.0209
GR (µ moles of NADPH oxidized/min/mg protein)	0.6228±0.0107	0.5479±0.0044	0.5012±0.0049	0.4557±0.0032
MDA (n moles MDA/mg protein)	23.78±0.3814	26.22±0.2007	31.05±0.1913	34.74±0.2317
LDH (Unit/ml/min)	3.90±0.0856	5.10±0.0856	6.267±0.0666	8.667±0.0666

Lipid peroxidation only becomes apparent when an increase in free radical production is the cause of the oxidative damage. Under normal circumstances, animals typically manage the production of reactive oxygen species (ROS) and their neutralization. However, the rate at which certain environmental toxicants, such as O_2^-, H_2O_2, OH^-, and ROO^-, are produced by organisms when exposed to them surpasses their capability for scavenging. Every living thing has a cellular antioxidant defense mechanism that is made up of both enzymatic and non-enzymatic elements. The components of the enzymatic antioxidant pathway are GPX, CAT, and SOD. Superoxide anion O_2^- is dismutated by SOD to H_2O_2, which is then reduced to water and molecular oxygen by CAT, or neutralized by GPX, which catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. Glutathione reductase (GR) regenerates GSH from oxidized glutathione (GSSG), which is a ROS scavenger and substrate for other enzymes.

Lipid hydroperoxide breakdown products such malondialdehyde and 4-hydroxynonenal may lead to chaotic cross-linkage with proteins and nucleic acids, which is a crucial step in the carcinogenesis process. Duodenal VSM lipid peroxidation (LPO) activities increased significantly in this study as a result of MY intoxication. Moreover, significant tissue damage in a free radical-mediated LPO leads to membrane damage, which lowers the fluid content of the membrane. Furthermore, lactate dehydrogenase (LDH) is an index of cellular damage, including organ toxicity and vascular endothelial breakdown. The significant increase in LDH activity observed may indicate the onset of cytolysis, which may indicate membrane damage. This disruption of the smooth muscular membrane, directly or indirectly, involves the generation of reactive oxygen species in dVSM cells. Free radicals attack unsaturated fatty acids in membranes, causing membrane lipid peroxidation, which reduces membrane fluidity, enzyme leakage and loss of receptor activity, and damages membrane proteins, leading to cell

inactivation (Airaodion et al., 2019). As lipid peroxidation gradually increases, the antioxidant defense system is correspondingly impaired, leading to oxidative stress (Tiwary and Rao, 2002).

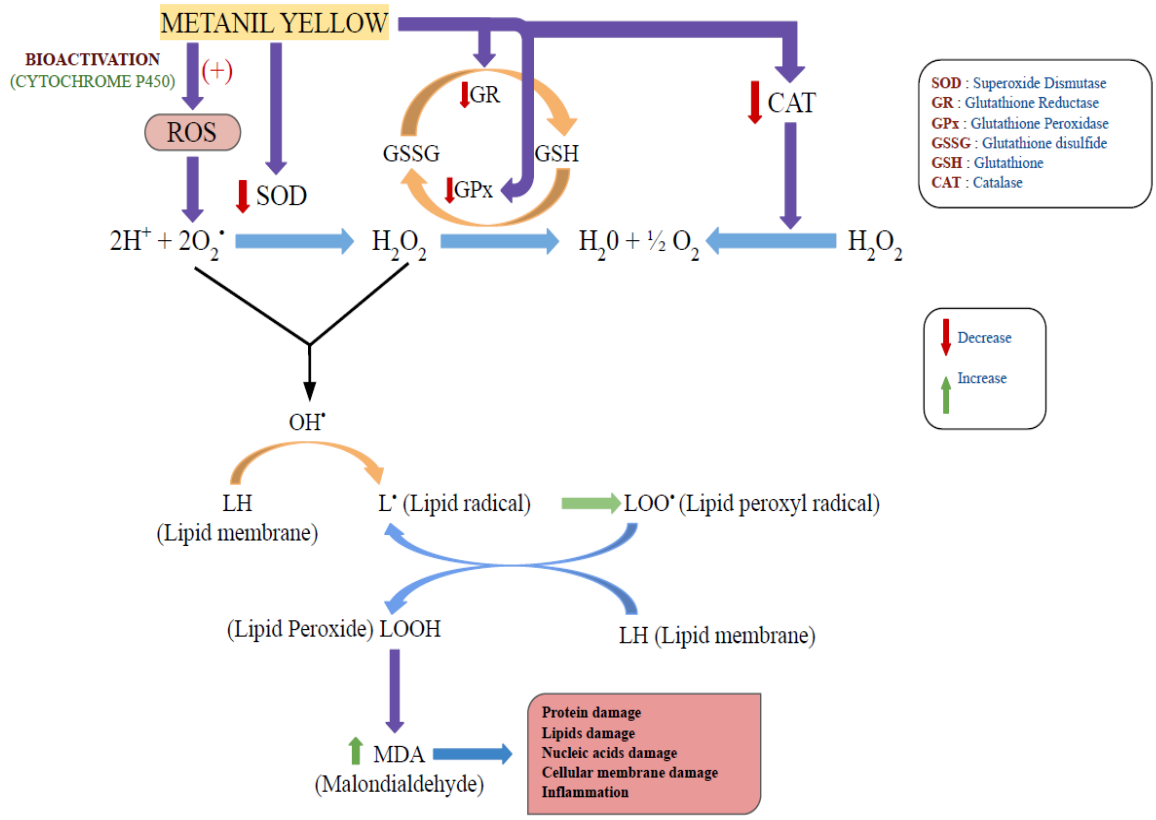

Figure 2. Schematic representation of the probable mechanisms involved in the MY induced oxidative stress in the dVSM. (+); indicates stimulation, (↑); increase in levels, (↓); indicates decrease in levels.

This suggests that MY on administration may have induces generation of ROS and depressed the activity of enzymes and thus results in oxidative stress induced cellular damage of the smooth muscle located at the wall structure of the duodenum. The results from the study could be extrapolated in humans, and it can be assumed that MY on exposure might impair the digestive and absorptive functions of the dVSM by impairing the contractile activity through MY induced oxidative degenerative alterations in the visceral smooth muscle located at the wall structure of the duodenum that provides motility to it.

CONCLUSION

In conclusion, MY significantly induces oxidative stress in smooth muscles found in the muscularis externa of the duodenal wall that provides the motility of the duodenum which is prerequisite for digestion and absorption of the food stuffs. From the results, it can be suggested that MY might impair the contractile function of the dVSM and thus, impair the digestive and absorptive functions of the duodenum probably by inducing oxidative stress.

Declaration by Authors

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