Metanil yellow suppresses contraction mediated ejection functions of heart ventricular muscle by inducing fibrillar and mitochondrial oxidative stress

Anamika Biswas, Debarati Roy, Mousumi Dutta and Goutam Paul

Molecular Neurotoxicology Laboratory, Department of Physiology, University of Kalyani, Kalyani, Nadia-741235, West Bengal, India.

Corresponding author: goutampaul.ku@gmail.com

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Abstract

The presence of metanil yellow (MY), a synthetic azo-dye, has been scientifically proved in different food products. It is mostly used in unorganized food industries as a food colorant due to its cost-effectiveness. Humans are often exposed to MY through ingested food products, colored with MY. The toxic effects of MY have already been reported in animal models. Till date, the effects of MY on heart ventricular functions have not been reported. So, our study was aimed to evaluate the effects of MY on the functions of heart ventricular muscle in rat model in vivo. We have observed significant (P<0.05) increase in the level of malondialdehyde and content of reduced glutathione in heart ventricular muscle of MY exposed groups of rats in comparison with control rats. The enzymatic activities of cytosolic copper-zinc-superoxide dismutase, glutathione peroxidase, glutathione reductase; and mitochondrial manganese superoxide dismutase and ATPase were significantly (P<0.05) increased in exposed groups. Whereas the activities of cytosolic catalase and acetylcholinesterase; mitochondrial catalase, Krebs cycle and electron transport chain enzymes were decreased significantly (P<0.05). Furthermore, myodegeneration of heart ventricular muscle and myofibrillar mitochondria of exposed rats have been identified through prominent signs of lesions and disintegrations by scanning electron microscopic and histological studies. So, from our observations we can conclude that MY depresses the contraction mediated ejection functions of heart, probably by inducing fibrillar and mitochondrial oxidative stress in heart ventricular muscle. The results obtained through this study in rat model could be extrapolated in humans.

Keywords Metanil yellow, Heart ventricular muscle, Oxidative stress, Myofibrillar mitochondria, AChE activity

Introduction

Colouration is an important aspect of food industries as colouration of foods makes them visually attractive as well as aids in their recognition while choosing the food products. Use of various synthetic dyes as food colorants is very ancient in its mode of practice. Even nowadays, synthetic dyes are favoured over their naturally existing colouring counterparts due to their comparative inexpensiveness and longer colour persistency (Gupta et al., 2003; El-Wahab &Moram, 2013). Although, scientific research has already shown that a number of synthetic dyes are potent carcinogens which exert deleterious effects on human health. For this reason, the Food Safety and Standard Authority of India permits the usage of only eight synthetic agents as food colours, though confining their permissible limit not exceeding the value of 100 mg/kg of food (Ashida et al., 2000; Elwan, 2018; Dixit et al., 2011). Most of the synthetic chemicals contain one or more azo groups (-N=N-), predominantly responsible for their inherently existing colouring property. Metanil yellow (MY) belongs to one of those renowned azo-dyes (Jain et al., 2009; Nath et al., 2015). It is a highly water-soluble dye profoundly used in leather, paper and textile industries (Khanna et al., 1978; Prasad & Rastogi, 1983). Considering its probable toxicity to human beings, it has been registered as a non-permitted food colour according to the Prevention of Food Adulteration Act of India. However, for its cost-effectiveness, unorganized food industries in West Bengal and certain other states in India still use MY as a food colouring agent to prepare different food items, namely turmeric powder, biryani, sweets, ice-creams, cheese, beverages, ladoo and many more (Sarkar and Ghosh, 2012; Ghosh et al., 2017). Several scientific studies have revealed the toxic effects of MY in different animal models. It has been reported to cause histopathological and ultrastructural alterations in stomach, intestine, liver and kidney in rats; as well as showed...
degenerative changes and metastasis in kidney, ilium, rectum and ovary in female mice (Mehrotra and Singh, 1974; Sarkar and Ghosh, 2010; Prasad & Rastogi, 1982). It has also been claimed to significantly alter the activities of testicular enzymes and produce remarkable degradation of spermatocytes and seminiferous tubules in guinea pigs, mice and rats (Singh, 1998; Khanna and Singh, 1973; Prasad & Rastogi, 1982). Further, MY, when combined with sunset yellow and tartrazine, has been reported to affect the activities of several brain regions, as well as altered the serotological content in Swiss albino rats (Nagaraja&Desiraju, 1993; Sundarrajan et al., 2000; Saxena & Sharma, 2014). Moreover, our laboratory has previously established that long-term exposure to MY significantly reduced the secretion of reproductive hormones, involving the induction of oxidative stress in female rats in vivo (Nath et al., 2016). Although previous studies have elaborated about the MY induced toxicity on the functions of several physiological systems, the information regarding the toxicity of MY on the functions of heart ventricular muscle is still limited in literature. Heart is one of the basic life support organs in human body, as its rhythmic movement sustains the circulation of blood through blood vasculature. The contraction of the heart ventricular muscle during systole forces the blood to flow into the pulmonary and systemic arteries from right and left ventricle; and the relaxation of the ventricular muscle during diastole allows the right and left ventricles to fill with blood that moves from the right and left atrium respectively. Thus, the contraction and relaxation of ventricular muscle play the pivotal role in controlling the ejection functions of the heart, so that the heart centric blood circulation in human body is maintained steadily for cardiovascular homeostasis. There remains a possibility of intoxication of heart ventricular muscle through consumption of MY tainted food products that might ultimately lead to structural and functional impairments of heart. So, the present study was aimed to evaluate the probable toxic effects of MY on contractile functions of heart ventricular muscle of rat in vivo.

### Materials and Methods

#### Chemicals and reagents

All the chemicals and reagents used to conduct this study were of analytical grade. 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, thiocelone iodide, copper sulphate, trichloroacetic acid, pyrogallol, follin reagent, sodium chloride, sodium bicarbonate, glucose, disodium hydrogen phosphate, potassium dihydrogen phosphate, and ethanol were purchased from EMark, Mumbai, India. 5-5’dithiobis 2-nitrobenzene, potassium ferricyanide and sodium pyruvate were obtained from Sisco Research Laboratory (SRL), India.

#### Experimental animal and their maintenance

Adult male albino rats of Charles Foster strain weighing around 100-120 grams and ageing about 15-16 weeks were selected as the experimental model. Animals were kept at departmental animal care house with 24 hours light-dark cycle at the room temperature of 25-27°C and were fed with standard diet and water ad libitum as per the guidelines of Institutional Animal Ethics Committee.

#### Design of the experiments

After acclimatization with laboratory environment, animals were at first divided into two categories namely control group and MY exposure group. Animals in control group were devoid of exposure to MY. Whereas MY exposure group was further sub-divided into three groups according to exposure to three graded doses of MY through oral gavage and were named as exposed group I, exposed group II and exposed group III. All of these four groups contained six animals in each. The above-described group division pattern was followed for two separate exposure durations i.e. 15 and 30 consecutive days. Doses of MY were selected based on LD₅₀ value of the compound and are summarized with their particular specifications in Table 1.

#### Animal sacrifice and collection of samples

At the end of the said exposure durations, animals were sacrificed by performing cervical dislocation. Then the heart was collected, washed by using 0.9% NaCl solution and preserved at -20°C for performing biochemical experimentalations. For histological experiments, a portion of ventricular tissue of heart was fixed in neutral buffer formalin fixative and dehydrated using graded concentration of ethanol. The dehydrated tissues were then embedded in liquid paraffin for preparation of tissue blocks which were used to conduct the histological study by Hematoxyline and Eosin staining. Another set of heart ventricular tissues was prepared by fixing in 3% glutaraldehyde solution for scanning electron microscopy.

| Group name      | Doses of metanil yellow (MY)                                                                 |
|-----------------|---------------------------------------------------------------------------------------------|
| Exposed group-I | Administered with 250 mg/kg BW/day of MY (5% of LD₅₀ of MY) for 15 and 30 days durations.   |
| Exposed group-II| Administered with 500 mg/kg BW/day of MY (10% of LD₅₀ of MY) for 15 and 30 days durations.  |
| Exposed group-III| Administered with 750 mg/kg BW/day of MY (15% of LD₅₀ of MY) for 15 and 30 days durations. |
Preparation of tissue homogenate

5% (w/v) tissue homogenate of heart ventricle was prepared in ice-cold phosphate buffer (pH 7.4) solution except for lipid peroxidation assay where 0.9% sodium chloride solution was utilized. 2% (w/v) tissue homogenate was prepared for acetylcholinesterase (AChE) enzyme assay. Homogenization was performed by using tissue homogenizer (RQ-127A, REMI, India) (Roy et al., 2021).

Isolation of heart ventricular mitochondria

Heart ventricular mitochondria were isolated by following the method of Dutta and Paul, (2018). 0.15 gm tissue was mixed with 3 ml of sucrose buffer (pH 7.8) consisting of sucrose, EDTA and Tris-HCl and homogenized at 4°C by using a homogenizer machine. Then the tissue homogenate was centrifuged by using a cooling centrifuge apparatus (C24BL, REMI, India). Pellet portion containing mitochondrial fraction, obtained after differential centrifugation, was resuspended using the same sucrose buffer and was used for the mitochondrial experiments.

Measurement of cellular oxidative stress indicators

Measurement of cellular lipid peroxidation (LPO)

The lipid peroxidation level was measured by estimating the level of thiobarbituric acid reactive substances (TBARS), i.e. malondialdehyde (MDA) according to the method of Buege and Aust, (1978) with slight modifications (Dutta et al., 2014d). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were dissolved into diluted hydrochloric acid. Then 5% heart ventricular tissue homogenate was added into TBA-TCA-HCL reagent mixture before heating for 20 minutes at 80°C by using a water bath. After cooling down the solution under running tap water, it was centrifuged at 5000rpm. The supernatant was collected, and the absorbance was measured by using a spectrophotometer(Genesys 10S UV-VIS, Thermofisher, Scientific, India) at 532nm wavelength.

Measurement of reduced glutathione (GSH) content

Reduced glutathione (GSH) content was measured by following the method of Sedlak and Lindsay, (1968). 10% TCA was added into 5% tissue homogenate and centrifuged at cold condition. Then the supernatant was collected and 0.8M Tris-HCl and 0.01 (M) DTNB were sequentially added with the supernatant. Colour intensity of the mixture was measured spectrophotometrically at 412nm.

Measurement of the activities of cytosolic and mitochondrial antioxidant enzymes

Measurement of cytosolic copper-zinc superoxide dismutase (Cu-Zn-SOD) and mitochondrial manganese superoxide dismutase (Mn-SOD) activities

The activity of Cu-Zn-SOD and Mn-SOD were measured by the autoxidation of pyrogallol according to the method of Marklund and Marklund, (1974) with some modifications (Dutta et al., 2014a). Tris-HCl buffer (pH 8.2) and pyrogallol were added either into 5% tissue homogenate samples or isolated mitochondrial preparations respectively for estimating Cu-Zn SOD or Mn SOD activity. The increasing absorbance was recorded spectrophotometrically for 5 minutes at 420 nm.

Measurement of catalase activity

Catalase activity was measured according to the method of Sinha, (1972) with some modifications. Phosphate buffer (pH 7.0) and hydrogen peroxide (H2O2) solution were added either into 5% tissue homogenate or isolated mitochondrial preparation for estimating cytosolic or mitochondrial catalase activity respectively. Then 5% aqueous dichromate solution was added to it and heated for 10 minutes. After cooling down the solution, the activities of catalases were measured spectrophotometrically at 570 nm.

Measurement of glutathione peroxidase (GPxs) activity

The activity of GPxs was measured by following the method of Paglia and Valentine, (1967) with a slight modification (Dutta et al., 2014c). The increasing absorbance of assay mixture containing tissue homogenate, phosphate buffer (pH 7.6), H2O2, NADPH, sodium azide, and GSH, was measured spectrophotometrically at 340nm.

Measurement of glutathione reductase (GR) activity

The activity of GR was measured spectrophotometrically according to the method of Stall et al., (1969) with some modifications (Dutta et al., 2014b). 5% tissue homogenate was mixed with phosphate buffer (pH 7.4), NADPH, oxidized glutathione (GSSG) and BSA to obtain a final assay mixture volume of 1ml. Then the absorbance was measured at 340nm.

Measurement of the activities of Krebs cycle enzymes

Measurement of pyruvate dehydrogenase (PDH) activity

Activity of PDH was measured by following the method of Chrétine et al., (1995) with slight modifications. A final volume of 1ml assay mixture contained phosphate buffer (pH 7.6), NAD+, sodium pyruvate and mitochondrial suspension. The absorbance was estimated spectrophotometrically at 340nm.

Measurement of isocitrate dehydrogenase (ICDH) activity

The activity of ICDH was measured by following the method of Duncan and Fraenkel, (1979). A final volume of 1ml assay mixture contained NAD+, MnSO4 and phosphate buffer (pH 7.6) with mitochondrial suspension considered as an assay mixture. The absorbance was measured spectrophotometrically at 340nm.

Measurement of alpha-ketoglutarate dehydrogenase (α-KGDH) activity

The activity of α-KGDH was measured by following the method of Duncan and Fraenkel, (1979). A final volume of 1ml assay mixture contained α-ketoglutarate, phosphate buffer (pH 7.6), H2O2, acetyl-CoA, α-ketoglutarate dehydrogenase (α-KGDH), and Tris-HCL buffer (pH 7.6).
NAD⁺ and mitochondrial suspension. The changes in absorbance were measured spectrophotometrically at 340nm.

**Measurement of succinate dehydrogenase (SDH) activity**

The activity of SDH was measured according to the method of Veeger et al., (1969). A final volume of assay mixture that contained potassium ferricyanide K₃[Fe(CN)₆], succinate, BSA, phosphate buffer (pH 7.6) and mitochondrial suspension. The enzyme activity was measured at 420nm.

**Measurement of the activities of respiratory chain enzymes**

**Measurement of NADH cytochrome c oxidoreductase activity**

The activity of cytochrome c oxidoreductase of heart ventricular tissue was measured spectrophotometrically according to the method of Goyal and Srivastava, (1995) with slight modifications. A final volume of 1ml assay mixture contained oxidized cytochrome c, BSA, NADH, phosphate buffer (pH 7.6) and a suitable amount of mitochondrial suspension. Changes in absorbance were determined at 565nm wavelength.

**Measurement of cytochrome c oxidase activity**

The activity of cytochrome-c oxidase was measured according to the method of Goyal and Srivastava, (1995) with some modifications. A final volume of 1ml assay mixture contained reduced cytochrome c, phosphate buffer (pH 7.6) and mitochondrial suspension. The activity of enzyme was measured spectrophotometrically at 550nm.

**Measurement of ATPase activity**

ATPase activity of heart ventricular mitochondrial suspension was measured according to the method described by Yang et al., (2004) by accounting the release of inorganic phosphate according to the method of Penney and Bolger, (1978).

**Measurement of acetylcholinesterase (AChE) activity**

The activity of AChE was measured according to the method of Ellman et al., (1961) with a minute modification. 2.6ml phosphate buffer (pH 8.0) was added with 0.1ml of DTNB, 0.01ml of acetylthiocholine iodide and 0.4ml of tissue homogenate to having a final volume of 3.12ml assay mixture. The absorbance was measured spectrophotometrically at 412nm.

**Histological and morphological studies**

**Hematoxylin and eosin staining method**

5 µm thick sections of heart ventricular muscle were obtained by using rotary microtome apparatus (MT-1090A, Weswox Optik, India). Hematoxylin and eosin staining procedure by Bancroft and Gamble, (2008) was adopted to stain the paraffin-embedded heart ventricular tissue sections. The stained tissue sections were observed under a light microscope at 400X magnification. Then the images were captured by a digital SLR Olympus camera (E-620) coupled with Olympus light microscope (CH20i).

**Scanning electron microscopic study of heart ventricular muscle**

Heart ventricular tissue segments were fixed by using aqueous glutaraldehyde solution (3%). After fixation, the samples were air dried and examined by scanning electron microscopy (SEM, Zeiss Evo 18 model EDS 8100) to evaluate the morphology of heart ventricular tissue. The images were captured at 5000X magnification (Mukherjee et al., 2015).

**Method of staining of isolated mitochondria**

The mitochondrial suspension was spread and air dried on the surface of a clear glass slide before staining with aqueous Janus green B solution for 5 minutes. The excess stain was rinsed with distilled water before mounting with cover slip. Images of stained mitochondria were observed by using Olympus light microscope (CH20i) at 400X magnification and capture by a digital SLR Olympus camera (E-620) (Dutta and Paul, 2018).

**Measurement of protein**

Protein of heart ventricular tissue homogenate and mitochondrial suspension were estimated according to the method of Lowry et al., (1951).

**Statistical analysis**

All the data obtained from the studies were expressed as mean ± SEM and comparison between the values of control groups and exposed groups were done by student’s ‘t’-test with the help of graph pad prism 5.03 software (Graph Pad Prism Software, Inc). p<0.05 was considered as the significance level.

**Results and Discussions**

Reactive oxygen species (ROS), predominantly produced during cellular metabolic processes, are highly reactive toward cellular components due to the presence of one or more unpaired electron(s) in their orbital structure (Birben et al., 2012). Being present at low concentrations, they promote a number of physiological processes but at high concentrations, they can damage the cellular constituents as well (Birben et al., 2012; Patekar et al., 2013; D’Oria et al., 2020). MY has previously been indicated to induce oxidative damage in brain and ovary of female rats (Nath et al., 2016). So, to investigate any probable effects of MY on inducing oxidative imbalance in heart ventricular muscle, we have performed experiments on various indicators of oxidative stress by estimating the levels of oxidative stress biomarkers and measuring the activities of antioxidant enzymes in our experimental model.
Table 2. Showing the changes in the level of MDA and content of GSH in MY exposed heart ventricular tissue homogenate of rats

| Groups              | Level of MDA (nM MDA/mg of protein) | Content of GSH (µM GSH/mg of protein) |
|---------------------|------------------------------------|--------------------------------------|
|                     | 15 day exposure                    | 30 day exposure                      |
| Control             | 59.71±0.983                        | 42.72±2.881                          |
| Exposed Group-I     | 73.92±0.782c                       | 111.26±5.62c                         |
| Exposed Group-II    | 103.58±0.604c                      | 117.72±1.404c                        |
| Exposed Group-III   | 110.04±0.584c                      | 125.17±0.485c                        |

Values are represented as mean±SEM, (n=6) and 'a, b, c' indicate the level of significance at  p<0.05, p<0.01, p<0.001 respectively, in comparison with control group.

Table 3. Showing the changes in the activities of cytosolic antioxidant enzymes in MY exposed heart ventricular tissue homogenate of rats

| Groups                                      | Control                      | Exposed group-I | Exposed group-II | Exposed group-III |
|---------------------------------------------|------------------------------|-----------------|------------------|------------------|
| Copper-zinc superoxide dismutase activity   |                              |                 |                  |                  |
| (µM/min/mg of protein)                      | 15-day exposure              | 3.17±0.034c     | 3.385±0.003c     | 3.642±0.034c     |
|                                             | 30-day exposure              | 3.20±0.003c     | 3.71±0.012c      | 4.331±0.027c     |
| Catalase activity                           |                              |                 |                  |                  |
| (µM H₂O₂ consumed/min/mg of protein)        | 15-day exposure              | 21.26±0.778c    | 18.93±0.177c     | 16.06±0.062c     |
|                                             | 30-day exposure              | 16.82±0.241c    | 15.75±0.056c     | 14.76±0.118c     |
| Glutathione peroxidase activity             |                              |                 |                  |                  |
| (µM/min/mg of protein)                      | 15-day exposure              | 2.42±0.055b     | 2.64±0.089b      | 3.63±0.224b      |
|                                             | 30-day exposure              | 2.99±0.059b     | 3.17±0.038b      | 4.03±0.046b      |
| Glutathione reductase activity              |                              |                 |                  |                  |
| (µM/min/mg of protein)                      | 15-day exposure              | 1.21±0.007c     | 1.32±0.013c      | 1.49±0.045c      |
|                                             | 30-day exposure              | 1.51±0.023c     | 1.62±0.029c      | 1.67±0.030c      |

Values are represented as mean±SEM, (n=6) and ‘b, c’ indicate the level of significance at p<0.01, p<0.001 respectively, in comparison with control group.

Table 4. Showing the changes in the activities of mitochondrial antioxidant enzymes in MY exposed heart ventricular mitochondria of rats

| Groups                    | Mitochondrial manganese superoxide dismutase (Mn-SOD) activity (µM/min/mg of protein) | Mitochondrial catalase activity (µM H₂O₂ consumed/min/mg of protein) |
|---------------------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------|
|                           | 15-day exposure                      | 30-day exposure                      | 15-day exposure                      | 30-day exposure                      |
| Control                   | 2.767±0.084                          | 2.975±0.043                          | 64.23±0.220                          | 53.30±0.201                          |
| Exposed group-I           | 3.04±0.052a                         | 3.32±0.085b                         | 46.65±0.116c                         | 40.18±0.215c                         |
| Exposed group-II          | 3.98±0.068c                         | 4.00±0.052c                         | 41.90±0.256c                         | 32.07±0.285c                         |
| Exposed group-III         | 4.10±0.042c                         | 4.28±0.072c                         | 39.55±0.203c                         | 25.82±0.525c                         |

Values are represented as mean±SEM, (n=6) and ‘a, b, c’ indicate the level of significance at p<0.05, p<0.01, p<0.001 respectively, in comparison with control group.
Effects of MY on oxidative stress biomarkers, activities of acetylcholinesterase (AChE), and cytosolic antioxidant enzymes

To examine the effects of MY on the oxidative stress variables in heart ventricular muscle, we have measured the level of malondialdehyde (MDA) and content of reduced glutathione (GSH) in both control and MY exposed groups of rats. We have found a significant (p<0.05) increase in MDA level and GSH content in MY exposed groups, in comparison to the control group, in a dose response manner for both 15 and 30 days exposure durations (Table 2). We speculate that increase in the level of these two oxidative stress biomarkers imply towards enhancement of ROS production within heart ventricular muscle. Increase in the level of MDA occurs due to elevation of peroxidation of cellular lipid molecules (Ayala et al., 2014; Ramachandani et al., 1992; Cheeseman, 1993; Tarafder et al., 2013). Enhancement of peroxidation of cellular lipids denotes a state of non-enzymatic oxidation of lipid molecules (Ramachandani et al., 1992). Occurrence of lipid peroxidation can be confirmed by quantitation of MDA level, as MDA is

Table 5. Showing the changes in the activities of pyruvate dehydrogenase and Krebs cycle enzymes in MY exposed heart ventricular mitochondria of rats

| Groups                                      | Control   | Exposed group-I | Exposed group-II | Exposed group-III |
|---------------------------------------------|-----------|-----------------|------------------|-------------------|
| Pyruvate dehydrogenase (PDH)               |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.024±0.001 | 0.016±0.001a    | 0.013±0.000b     | 0.012±0.002b      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.026±0.001 | 0.016±0.001b    | 0.013±0.001b     | 0.011±0.001c      |
| Isocitrate dehydrogenase (ICDH)            |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.028±0.000 | 0.026±0.000b    | 0.019±0.000c     | 0.017±0.001c      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.025±0.000 | 0.023±0.001b    | 0.015±0.003c     | 0.013±0.001c      |
| Alpha-ketoglutarate dehydrogenase (α-KGDH) |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.007±0.001 | 0.006±0.001     | 0.005±0.001a     | 0.003±0.000b      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.009±0.001 | 0.006±0.001     | 0.004±0.001a     | 0.003±0.010b      |
| Succinate dehydrogenase (SDH)              |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.020±0.001 | 0.016±0.001b    | 0.014±0.001c     | 0.011±0.001c      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.023±0.001 | 0.019±0.001b    | 0.017±0.001c     | 0.016±0.001c      |

Values are represented as mean±SEM, (n=6) and ‘a, b, c’ indicate the level of significance at p<0.05, p<0.01, p<0.001 respectively, in comparison with control group.

Table 6. Showing the changes in the activities of electron transport chain (ETC) enzymes and ATPase in MY exposed heart ventricular mitochondria of rats

| Groups                                      | Control   | Exposed group-I | Exposed group-II | Exposed group-III |
|---------------------------------------------|-----------|-----------------|------------------|-------------------|
| NADH Cytochrome c oxidoreductase            |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.104±0.002 | 0.089±0.003a    | 0.085±0.002b     | 0.083±0.002b      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.099±0.001 | 0.082±0.001c    | 0.073±0.002c     | 0.066±0.002c      |
| Cytochrome c oxidase                        |           |                 |                  |                   |
| (Units/min/mg of protein)                  | 15-day    |                 |                  |                   |
| exposure                                    | 0.053±0.002 | 0.047±0.002    | 0.043±0.002a     | 0.035±0.003b      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 0.060±0.001 | 0.054±0.002    | 0.043±0.002c     | 0.032±0.001c      |
| ATPase                                      |           |                 |                  |                   |
| (nM Pi released/min/µg of protein)          | 15-day    |                 |                  |                   |
| exposure                                    | 362.6±10.200 | 399.7±3.085b   | 412.8±2.648c     | 435.4±2.378c      |
|                                            | 30-day    |                 |                  |                   |
|                                            | 373.1±1.746 | 400.2±3.170c   | 422.7±9.492c     | 442.7±2.050c      |

Values are represented as mean±SEM, (n=6) and ‘a, b, c’ indicate the level of significance at p<0.05, p<0.01, p<0.001 respectively, in comparison with control group.
known to be a major by-product of lipid peroxidation (Zeb and Ullah, 2016; Cheeseman, 1993; Wills, 1971). Hence, increased MDA level might be considered to be a consequence of increased lipid peroxidation (Tsikas, 2017), as observed in our study, due to MY exposure. Increase in cardiac lipid peroxidation has previously been correlated with reduced acetylcholinesterase (AChE) activity. Alteration in AChE activity might affect the contraction mediated ejection functions of heart ventricular muscle. Hence, we have examined AChE activity in heart ventricular muscle and have noticed a significant (p<0.05) decrease in this variable in MY exposed groups, dose dependently, in comparison to the control group for both 15 and 30 days exposure durations (Fig. 1). Decreased activity of AChE might hinder the hydrolysis of acetylcholine (ACh) molecules by AChE, present in cholinergic neuron terminals and heart ventricular synaptic junctions (Mondal et al., 2017). We assume that MY exposure is involved in decreasing AChE activity probably by increasing peroxidation of lipids (Liu et al., 2017), as lipid peroxidation inducing agents have previously been reported to inhibit enzymatic activity of AChE (O’Malley et al., 1966; Tsakiris and Schulpis, 2000). Lipid peroxidation inducing agents primarily consists of peroxyl radical, hydrogen peroxide molecule (H₂O₂), and hydroxyl radical (HO•), involved in ROS mediated damages to unsaturated fatty acids present as constituents of cellular lipid molecules (Cheeseman, 1993; Wills, 1971; Mylonas and Kouretas, 1999). Therefore, we hypothesise that induction of ROS generation might as well impact cellular GSH pool, because GSH contribute to maintain the homeostasis of cellular redox status and oxidation-reduction reactions by supplying its reducing equivalents, thereby itself being converted into oxidised glutathione (GSSG) (Zitka et al., 2012; Nooris et al., 2016). Our observation, regarding MY induced increased cellular GSH content, complies with the notion that MY probably elevates ROS generation that triggered the simultaneous elevation of cellular GSH content in an attempt to counteract the above scenario.

Fig 1. Graphical representation shows the alteration of acetylcholinesterase (AChE) activity in heart ventricular muscle homogenate of metanil yellow exposed groups of rats for 15 and 30 days exposure durations. Values are represented here as mean±SEM (n=6), *p< 0.001 vs control.
Fig 2. Photomicrographic representations of hematoxylin and eosin-stained (H & E) longitudinal sections of heart ventricular muscle of control and MY exposed groups of rats for 15 and 30 days exposure durations. A₁ & A₂: heart ventricular sections of control rats for 15- and 30-days durations respectively; B₁, C₁, D₁: heart ventricular sections of MY exposed (250, 500, 750 mg kgBW⁻¹day⁻¹) rats for 15-day exposure durations. B₂, C₂, D₂: heart ventricular sections of MY exposed rats (250, 500, 750 mg kgBW⁻¹day⁻¹) for 30-day exposure durations. Here arrow heads indicate lesions of muscle layer and circles indicate disruptions and disintegrations of muscle fibres. Images were captured by a digital SLR Olympus camera (E-620) coupled with Olympus light microscope (CH20i) at 400X magnification.

Fig 3. Scanning Electron Microscopic images of heart ventricular muscle of control and metanil yellow exposed groups of rats for 15 and 30 days exposure durations. A₁ & A₂: heart ventricular sections of control rats for 15 and 30 days durations respectively; B₁, C₁, D₁: heart ventricular sections of MY exposed rats (250, 500, 750 mg kgBW⁻¹day⁻¹) for 15-day exposure durations. B₂, C₂, D₂: heart ventricular sections of MY exposed (250, 500, 750 mg kgBW⁻¹day⁻¹) rats for 30-day exposure durations. Here arrow heads indicate disruptions of muscle fibres and formation of blebs on the surface of heart ventricular muscle. Images were captured by scanning electron microscope at 5000X magnification.
Fig 4. Photomicrographic representations of Janus green B-stained heart ventricular mitochondria of control and metanil yellow exposed groups of rats after 15 and 30 days exposure durations. A₁ & A₂: heart ventricular mitochondria of control rat for 15 and 30 days durations respectively; B₁, C₁, D₁: heart ventricular mitochondria of MY exposed rats (250, 500, 750 mg kgBW⁻¹ day⁻¹) for 15-day exposure durations. B₂, C₂, D₂: heart ventricular mitochondria of MY exposed (250, 500, 750 mg kgBW⁻¹ day⁻¹) rats for 30-day exposure durations. Here arrow heads indicate formation of blebs and furrows on mitochondrial surface. Images were captured by a digital SLR Olympus camera (E-620) coupled with Olympus light microscope (CH20i) at 400X magnification.

Fig 5. Schematic diagram showing probable mechanisms of actions of metanil yellow in inducing oxidative stress mediated suppression of contraction mediated ejection functions of heart ventricular muscle. Here upward arrowheads (↑) indicate stimulation; downward arrow heads (↓) indicate inhibition. MY: Metanil yellow; ROS: Reactive oxygen species; GSH: Reduced glutathione; GSSG: Oxidized glutathione; GR: Glutathione reductase; GPx: Glutathione peroxidase; O₂⁻: Superoxide radical; Cu-Zn-SOD: Cupper-zinc superoxide dismutase; Mn-SOD: Manganese superoxide dismutase; CAT: Catalase; H₂O₂: Hydrogen peroxide; AChE: Acetylcholinesterase.
These results directed us to investigate the status of cellular antioxidant enzymes following MY exposure to heart ventricular muscle. As a consequence, we have examined the activities of four cytosolic antioxidant enzymes and have found significant (p<0.05) increase in the activities of Cu-Zn-SOD, GPx and GR, alongside a significant (p<0.05) decrease in the activity of catalase in MY exposed groups, compared to the control group, in a dose response manner for both 15 and 30 days exposure durations (Table 3). MY induced alterations in the activities of antioxidant enzymes further indicate imbalance of oxidation-reduction status that might directly expose heart ventricular muscle towards at the risk of ROS induced oxidative injuries. Generally, living organisms are highly prone to constant exposure to ROS molecules both extracellularly and intracellularly (Patekar et al., 2013). Therefore, intending to prevent their harmful effects, numerous antioxidant defence systems are also present in healthy cell and play a vital role in cell mediated detoxification of such ROS molecules by activating several antioxidant enzymes (Ighodaro and Akinloye, 2018). This in turn maintains the equilibrium between production and detoxification of ROS molecules. When the balance between ROS generation and ROS scavenging activity of antioxidant defence systems are shifted from its favourable ratio then this physiological condition is termed as “oxidative stress” (Pizzino et al., 2017; Pisoschi and Pop, 2015; Larosa and Remacle, 2018). SOD is an important ubiquitously distributed metalloenzyme antioxidant that performs catalysis of dismutation of superoxide free radicals (O$_2^-$) into relatively less harmful H$_2$O$_2$ and molecular oxygen (O$_2$). As Cu-Zn-SOD is considered as a first line defensive antioxidant, increase in its activity might be a consequence of higher cellular oxidative damage due to over-production of O$_2^-$ by MY. Previous studies have already shown that over expression of Cu-Zn-SOD activity is associated with chronic oxidative stress (Ighodaro and Akinloye, 2018; Larosa and Remacle, 2018; Marklund, 1984). So, above findings comply with our hypothesis that increase in the activity of Cu-Zn-SOD may occur probably to combat the harmful effects of overproduced free radicals due to MY exposure. Increased Cu-Zn-SOD activity probably causes overproduction of H$_2$O$_2$ within the cells (Maestro and McDonald, 1987; Xu et al., 2014). H$_2$O$_2$ at high concentrations acts as precursor for generation of harmful hydroxyl radicals (HO*) in presence of Cu$^{2+}$ or Fe$^{2+}$ through Fenton reaction. Harmful effects of HO* radicals might be accountable for peroxidation of membrane lipids, as indicated in our study through observed the elevated MDA level (Ighodaro and Akinloye, 2018). To limit the possibility of HO* formation from H$_2$O$_2$, catalase performs degradation of H$_2$O$_2$ molecules into molecular oxygen and water,thereby completing the detoxification reaction initiated by Cu-Zn-SOD (Ighodaro and Akinloye, 2018; Marklund, 1984; Chelikani et al., 2004; Maestro and McDonald, 1987). Hence, we speculate that MY might induce cellular oxidative stress by increasing the activity of Cu-Zn-SOD and decreasing the activity of catalase. In concordance with our result, previous studies have shown a significant correlation between decreased catalase activity and cellular oxidative stress (Escaribano et al., 2015; Das et al., 2013). Along with catalase, glutathione peroxidase (GPx), a selenium dependant antioxidant enzyme is crucial for cellular antioxidant defence system (Patekar et al., 2013; Cheng et al., 2017; Frampton et al., 1987). Activity of GPx is dependent on GSH content, which serves as a co-factor for GPx, by donating its reducing equivalents to H$_2$O$_2$ (Zitka et al., 2012). Further, GSH content depends on the activity of glutathione reductase, an enzyme belonging from glutathione family (Stall et al., 1969). GR replenishes GSH pool by catalysing the reduction of GSSG to GSH, thus maintaining the favourable ratio between GSH and GSSG (Cheng et al., 2017; Zitka et al., 2012; Noori, 2012). We speculate that, over-production of free radicals due to MY exposure may increase the activity of GR, thereby increasing the content of GSH, ultimately leading to increased GPx activity (Tarañder et al., 2013; Snezhkina et al., 2019; Cheng et al., 2017; Zitka et al., 2012; Noori, 2012). In accordance with our results, increase in the activities of GR and GPx, along with increased GSH content have previously been reported to be induced by ROS, and is considered a leading cause for induction of cellular oxidative stress (Vega et al., 2002; Melissinos et al., 1981). The production of ROS occurs predominantly in mitochondria and plasma membranes within a cell during cellular metabolism (Snezhkina et al., 2019). Being a source, as well as the nearest target of generated ROS molecules, there remains a possibility for mitochondria to be more susceptible towards MY induced alterations. Hence, we have investigated the status of antioxidant enzymes, metabolism and energy homeostasis of mitochondria in heart ventricular muscle following MY exposure for 15 and 30 days durations.

**Effects of MY on the activities of mitochondrial antioxidant enzymes, Pyruvate dehydrogenase (PDH), Krebs cycle enzymes, electron transport chain (ETC) enzymes and ATPase**

ROS molecules are inevitably produced in mitochondria as by-products of oxygen metabolism during transfer of electrons through components of electron transport chain (ETC) (Noori, 2012; Larosa and Remacle, 2018; Raha and Robinson, 2000). Hence, our hypothesis of MY induced enhanced ROS formation, might as well affect the activities of antioxidant enzymes present in mitochondria. As a consequence, we have estimated the activities of Mn-SOD and catalase, two primary antioxidant enzymes found in mitochondria, and have found a significant (p<0.05) increase in Mn-SOD activity following a significant (p<0.05) decrease in catalase activity (Table 4). We assume, MY elevates Mn-SOD activity by enhancing the production of O$_2^-$ that may further increase the possibility of H$_2$O$_2$ production. Moreover, a simultaneous decrease in catalase activity might hinder with the neutralization of elevated H$_2$O$_2$ level, thereby increasing the possibility of HO* formation in heart ventricular mitochondria (Kitada et al., 2020; Zhou and Tian, 2018). Further, a number of Krebs cycle enzymes show susceptibility towards ROS molecules namely hydroxyl radical (HO*), superoxide anion (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$) (Lee et al., 2002; Martin et al., 2005; Tettet and Vizi, 2005). We have observed significant decrease in the activities of pyruvate dehydrogenase (PDH) and three Krebs cycle enzymes viz., isocitrate.
dehydrogenase (ICDH), alpha-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) following MY exposure for 15 and 30 days exposure durations (Table 5). PDH, ICDH and α-KGDH generate reducing equivalents in the form of NADH whereas, FADH₂ is generated from the activity of SDH (Zhou and Tian, 2018; Lee et al., 2002). We presume, MY induced ROS molecules decreased the activities of these four enzymes that might affect the generation of reducing equivalents (Singer et al., 1973; Anjali et al., 2021; Martin et al., 2005; Gibson et al., 1988; Gibson et al., 2000). These two reducing equivalents are directly fed into the ETC of mitochondria, for generation of chemical energy in the form of ATP through oxidative phosphorylation (Zhou and Tian, 2018; Lee et al., 2002; Mitchell, 1961). Therefore, we have examined the effects of MY exposure on the activities of two ETC enzymes of mitochondria, namely NADH cytochrome c oxidoreductase and cytochrome c oxidase, and have found a significant (p<0.05) decrease in their activities in a dose response manner for both 15 and 30 days durations (Table 6). MY induced decrease in the activities of these two ETC enzymes imply towards a hindrance of electron transfer, imposed by MY, either directly or by induction of ROS generation on ETC of heart ventricular mitochondria (Yang and Dettbarn, 1998; Dutta and Paul, 2019). A reduced performance of mitochondrial ETC might impact oxidative phosphorylation through alteration of the activity of mitochondrial ATPase. Consequently, we have observed significant (p<0.05) increase in ATPase activity in heart ventricular mitochondria in a dose response manner for both 15 and 30 days durations following MY exposure (Table 6). We speculate MY induced increased ATPase activity enhances the hydrolysis of ATP molecules, rendering lesser ATP molecules available for functioning of heart ventricular mitochondria. We apprehend, this might be the reason for altered metabolic status of heart ventricular mitochondria through decreasing Krebs cycle enzymes activities, as observed in our study due to MY exposure. Moreover, decreased ATP level has previously been linked with sub-optimal mitochondrial performance, rendering cardiomyocytes susceptible to hyper-contracture and rupture of cardiac sarcolemma (Lisa et al., 2001). Hence, we have examined the histo-anatomical features of heart ventricular mitochondria along with heart ventricular muscle to monitor the probable alterations induced by MY exposure for both 15 and 30 days exposure durations.

**Effects of metanil yellow on histo-anatomical structure of heart ventricular muscle and myofibrillar mitochondria**

In accordance with our study, oxidative stress induced alterations in cardiomyocytes have previously been reported to promote morphological and functional deteriorations and myocardial fibrosis of heart ventricular muscle (Pal et al., 2021; Cao et al., 2020). Besides, we have found altered architectural organizations in hematoxylin and eosin-stained tissue sections of MY exposed heart ventricular muscle under a light microscope in comparison with heart ventricular muscle of control group, where a normal structural arrangement and evenly distributed muscle fibres were observed in our study (Fig 2). Morphological examinations clearly indicate prominent signs of myodegeneration of heart ventricle, as characterized through presence of lesions, disruptions and loss of cardiac myofibers along with mononuclear cell infiltration of ventricular muscle fibres (Fig 2). The effects of MY induced myodegeneration occurred in a dose and duration dependant manner with maximum damages being observed at the dose of 750 mg/kg BW/day for 30-day exposure duration. To further validate the occurrence of MY induced myodegeneration, we have performed scanning electron microscopic (SEM) examinations to monitor surface topology of heart ventricular muscle of both control and MY exposed groups of rats for 15 and 30 days exposure durations. The images obtained through SEM show damages gradually, in the form of blebbings as early signs of alterations in MY exposed group-I and MY exposed group-II for 15-day exposure duration; blebbings along with disintegrations in MY exposed group-I for 30-day exposure duration; disintegrations with a number of cavities in MY exposed group-III for 15-day exposure duration; followed by disintegrations and severe disruptions in MY exposed group-II and MY exposed group-III for 30-day exposure duration in heart ventricular muscle of rats (Fig 3). Furthermore, from these results we hypothesise that MY induced heart ventricular myodegeneration might also affect heart ventricular myofibrillar mitochondria, which are responsible for attaining high energy demands for maintaining continuous contractile functions of heart ventricular muscle. Hence, we have examined mitochondrial morphology by Janus green B staining procedure in heart ventricular fibrillar mitochondria of both control and MY exposed groups of rats for 15 and 30 days exposure durations. We have observed structural alterations and signs of disintegrations following MY exposure indicating towards decrease in intactness of mitochondria of MY exposed groups, examined with bright field microscope (Fig 4). So, our present study evaluates for the first time, the effects of MY exposure on induction of oxidative stress mediated alterations in histological structure and contraction mediated ejection functions of heart ventricular muscle, and the probable mechanisms of actions involved in it are summarized here (Fig 5).

**Conclusion**

From our present study we can conclude that metanil yellow (MY) alters the redox status of heart ventricular muscle and induces cellular as well as mitochondrial oxidative stress through altering the activities of various antioxidant enzymes, Krebs cycle enzymes, ETC enzymes and ATPase enzyme and thus alters all ATP driven biochemical and metabolic processes of heart ventricular muscle. It also causes structural deformation and disintegration of heart ventricular muscle fibres and heart ventricular mitochondria through production and accumulation of excess free radicals within cellular content and thereby hampers the contraction mediated ejection functions of heart ventricular muscle by decreasing the activity of AChE enzyme.

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