Original Article

Metanil Yellow (MY) Impairs Heart Ventricular Function by Promoting Cholinergic and Nitrergic Signaling Pathways

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ABSTRACT

Metanil yellow (MY), being a synthetic commercial azo dye, is not approved to be used as a food colour. Despite being non-permitted, it is still used as a food adulterant in unorganized food manufacturing and processing sectors. As a consequence, human exposure to this hazardous chemical occurs through the consumption of MY-contaminated food items resulting in the entry of MY into blood and finally to the heart via systemic circulation. Though the chemical is known for its potentiality to induce toxicity in several organ systems in animal models, its impact on mammalian heart has not been thoroughly reported till date. Therefore, this study has been designed to evaluate the effects of MY on structural and functional aspects of heart ventricular muscle in rat model in vivo. Exposure to MY has been found to significantly ($p<0.001$) decrease acetylcholinesterase (AChE) activity; significantly ($p<0.001$) increase nitric oxide synthase (NOS) activity; and concentrations of cytosolic and mitochondrial nitrite in heart ventricular muscle. Moreover, MY exposure has also been found to induce morphological alterations in heart ventricular muscle. So, from the results, it can be postulated that MY suppresses heart ventricular function in rats by compromising the structural integrity and organization of ventricular muscle. These detrimental effects seem to be mediated by changes in the activities of AChE and NOS enzymes, as well as alterations in nitrite concentration within the ventricular muscle and mitochondria.

Keywords: Metanil yellow, heart ventricular muscle, mitochondria, acetylcholinesterase, nitric oxide synthase.

INTRODUCTION

Adulteration of food by using various synthetic colouring agents, particularly those containing one or more azo groups, is a cost-effective but entirely non-permitted way to increase the attractiveness of food products supplied through the food service markets, ultimately destined for consumption by human beings. Metanil yellow (MY), also known as acid yellow 36, which is water soluble monosodium salt of 3[[4-(phenylamino phenyl)]azo]benzenesulfonic acid is one of the food adulterants used extensively for its ability to provide vivid yellow to orange spectrum of colour into the food products and beverages[1,2]. However, according to the Prevention of Food Adulteration Act of India, 2008, MY is enlisted as a non-permitted synthetic food colour and thereby should strictly not be used in food products[3,4].

Despite this legislation, it is being widely used as an adulterant in various food ingredients and food products such as spices and condiments including turmeric powder, substitute of kesar; pulses and its products including pigeon pea (arhar dal), chickpea flour (besan) and items produced from it; sweets and confectionaries including coloured sweets, jalebi, laddoo, ice creams, candy, lozenges, cake, pastry; prepared food items including biryani, snacks, bakery goods, cereals, gelatin; and also in a number of coloured beverages mostly in various unorganized food manufacturing and processing sectors of West Bengal, India as well as in other regions of the country[3,5,6,7,8]. Therefore, in most of the cases, unaware oral consumption of MY- contaminated food products by humankind becomes the sole source of exposure to MY by human beings.

However, scientific studies have shown that MY generates a number of toxic effects on physiological systems including its reported nature of being a carcinogenic agent[9]. Besides, it is reported to cause functional alterations in the hepatic, gastrointestinal and renal systems via histopathological and ultrastructural deteriorations in the liver, ileum, rectum and kidney in rats[10,11,12]. It has also been reported that oral administration of MY significantly altered the absolute and relative weights of testes in male rats through degenerative changes of spermatocytes and seminiferous tubules, leading to remarkable alterations in the secretion of testicular enzymes in pigs, mice and rats[13,14,15]. It can also cause degradation of seminiferous tubule and vacuolization in the Sertoli cell of rats[13,16]. Alongside, functional alterations of the stomach, ileum, spleen and ovary were reported to be observed in female mice during chronic exposure to MY[12]. Oxidative stress-mediated damages have also been reported to be found in ovary and uterus of female rats when subjected to MY[17].

Moreover, our earlier study also established that MY is responsible for the oxidative stress-mediated alterations in cytosolic and mitochondrial metabolic activities of heart ventricular muscle in male albino rats[4]. However, the probable key mechanism(s) behind the cardiotoxicity induced by MY is not yet fully understood and needs to be explored further. Therefore, the present study was

designed to evaluate the probable involvement of cholinergic and nitrergic signalling pathways in MY-induced impairment of the function of heart ventricle.

MATERIALS AND METHODS

Chemicals and Reagents:

All the chemicals and reagents used to perform this study were of analytical grade. Study chemical metanil yellow (MY) having a purity of 98% was obtained from Sigma Aldrich Chemical Company, USA. Other chemicals and reagents were purchased from Sisco Research Laboratory (SRL) Private Limited, Mumbai, India and Merck Life Science Private Limited, Mumbai, India

Selection and Maintenance of Experimental Animals:

Swiss male albino rats (*Rattus norvegicus*), weighing 120-130 grams and aged about 15- 16 weeks were selected as the animal model for this experimental study. All the animals were housed in the departmental animal care house with a 12:12 light and dark cycle at an ambient room temperature of 25±2ºC. Throughout the study, the animals were fed a standard diet and continuously provided fresh tap water as per the recommendation of the Institutional Animal Ethical Committee of Kalyani University.

Experimental Design for Dose and Duration-Response Study to MY Exposure:

Group Division:

After acclimatization, the male rats were randomly divided into eight groups, each containing six animals. The following table shows the doses and exposure duration of MY for this study (Table 1).

Table 1: Group division of animals for the exposure to MY in dose and duration-response manner.

Duration of Exposure	Specification of the Groups	Name of the Groups	Description of the Groups
15 consecutive day	**Control group**	**Control**	**Not exposed to MY**
	MY exposed groups	Exposed group-I	**Orally administered with** 250 mg/kg BW/day dose of MY (i.e., 5% LD_{50})
		Exposed group-II	**Orally administered with** 500 mg/kg BW/day dose of MY (i.e., 10% LD_{50})
		Exposed group-III	**Orally administered with 7**50 mg/kg BW/day dose of MY (i.e., 15% LD_{50})
30 consecutive day	**Control group**	**Control**	**Not exposed to MY**
	MY exposed groups	Exposed group-I	**Orally administered with** 250 mg/kg BW/day dose of MY (i.e., 5% LD_{50})
		Exposed group-II	**Orally administered with** 500 mg/kg BW/day dose of MY (i.e., 10% LD_{50})
		Exposed group-III	**Orally administered with 7**50 mg/kg BW/day dose of MY (i.e., 15% LD_{50})

Animal Sacrifice and Sample Collection:

At the end of the said exposure durations, the animals were sacrificed by cervical dislocation. Then the heart was collected by opening the thoracic cage of the rat followed by washing with cold saline (pH 7.4). For biochemical experiments and isolation of mitochondria, the samples were either instantly homogenized or stored at -20º C. For histological and histochemical experiments, a portion of ventricular tissue was fixed in neutral buffer formalin (NBF) fixative for the preparation of tissue block. Another part of ventricular tissue was fixed in 3% glutaraldehyde fixative solution for scanning electron microscopic (SEM) experiments.

Preparation of Tissue Homogenate:

2% w/v tissue homogenate was prepared in 50mM ice-cold phosphate buffer (pH 7.4) to estimate the activity of acetylcholinesterase enzyme. Additionally, a 2% w/v tissue homogenate was prepared in 50mM ice-cold phosphate buffer (pH 7.4) with EDTA to measure the nitrite concentration[18].

Isolation of Heart Ventricular Mitochondria:

Heart ventricular mitochondrial suspension was prepared by following the method of Dutta and Paul, (2018) with slight modifications[19]. At first 5% heart ventricular tissue homogenate was prepared in mitochondrial isolation buffer (pH-7.8) at a cold temperature using a homogenizer apparatus (Remi Elektrotechnik Limited, Vasai, India (RQ-127A)). The freshly prepared homogenate mixture was centrifuged at 4000 rpm speed at 4ºC temperature for 10 minutes, the obtained supernatant was collected and again centrifuged at 10500 rpm speed at 4ºC temperature for 40 minutes. The pellet containing the mitochondrial portion was re-suspended in mitochondrial isolation

buffer to obtain mitochondrial suspension used in the experiments concerning mitochondria.

Measurement of Acetylcholinesterase (AChE) Activity:

The AChE activity was measured according to the method of Ellman et. al., (1961) with some modifications[20]. The assay mixture had a total volume of 3.12ml, containing 2.6ml of 0.1M phosphate buffer, 0.1ml of 0.01M of DTNB, 0.02ml of 0.075M aqueous acetylthiocholine iodide solution and 0.4ml of 2% w/v tissue homogenate. The colour intensity of the mixture was measured spectrophotometrically at 412nm by using a UV-VIS spectrophotometer (Thermo Fisher Scientific, USA (Genesys 10S UV-VIS) and the enzyme activity was expressed as µmoles substrate hydrolysed/min/mg of protein.

Estimation of Nitrite Concentration:

Cytosolic and mitochondrial nitrite concentrations were measured according to the method of Griess et. al., (1879) adapted by Sun et. al., (2003) with slight modifications[21,22]. The assay mixture consisted of 100µl Griess reagent, 300µl of 2% w/v tissue homogenate or mitochondrial fraction and 1.6ml distilled water. The Griess reagent is the mixture of reagent A (1% sulphonyl amide dissolved in 5% phosphoric acid) and reagent B (0.1% NEDA) at the ratio of 1:1. The absorbance of this assay mixture was measured spectrophotometrically at 548nm and the value was expressed as µM nitrite/mg of protein.

Histological, Morphological and Histochemical Observations:

Hematoxylin and Eosin (H & E) Staining:

The ventricular tissue of both control and MY-exposed rats was subjected to several steps for the preparation of tissue blocks according to the method of Bancroft and Gamble, (2008)[23]. After that 5 µm thick sections of ventricular muscle were done with the help of a rotary microtome machine (MT-1090 A, Weswox Optik, India) from the prepared tissue block and stained with hematoxylin and eosin Y stain. The stained ventricular tissue section was then visualized under a phase contrast light microscope and the photomicrographs were captured by a digital SLR Olympus camera (E-620) attached to a light microscope (CH20i).

Histochemical Technique for Examination of Acetylcholinesterase Enzyme Expression in Heart Ventricular Tissue:

The expression of acetylcholinesterase A(ChE) in heart ventricular tissue from both control and MY-exposed rats was assessed using a modified histochemical staining technique based on Karnovosky and Roots (1964)[24]. A neutral buffer formalin-fixed tissue block was prepared by embedding dehydrated samples in paraffin, and 7-10 micrometer sections were cut using a rotary microtome. After deparaffinization with xylene and rehydration with graded alcohol, the sections were washed with phosphate buffer saline (PBS) and incubated in a solution containing acetylthiocholine iodide and various buffers for 10-12 hours. Following incubation, the sections were rinsed, counterstained with hematoxylin, dehydrated with ethanol, cleared with xylene, and mounted with DXP for microscopic evaluation of AChE expression.

Histochemical Observation of Nitric Oxide Synthase (NOS) Expression in Heart Ventricular Muscle:

For the histochemical observation of NOS expression in heart ventricular muscle, the NADPH- diaphorase staining technique, based on Sandell's method from 1986 with minor modifications, was employed[25]. A 7-10µm tissue section was obtained by using a rotary microtome machine from a prepared tissue block. The tissue section was then incubated for 12-18 hours in an incubation buffer containing 0.1M Tris buffer with 0.5% Triton-X-100, 1mg/ml β-NADPH, and 0.25mg/ml nitroblue tetrazolium. Following the incubation, the tissue section was washed with Tris buffer and dehydrated with graded alcohol before being cleared with xylene and mounted with DPX for further microscopic observation.

Scanning Electron Microscopic (SEM) Study:

Initially, ventricular tissue sections were immersed in a 3% aqueous glutaraldehyde solution for fixation. After allowing the samples to be fixed for 72 hours, tissue sections were meticulously air-dried to prepare them for imaging. This preparation aimed to capture the intricate topology of the ventricular heart tissue. To achieve this, scanning electron microscopy (SEM) was employed, utilizing a Zeiss Evo 18 model system EDS 8100. This advanced imaging technique allowed for a detailed evaluation of the morphological structure of the heart ventricular tissue, revealing insights into its cellular organization and surface characteristics at a microscopic level.

Statistical Analysis:

The data obtained in this study were analysed through 'one-way' analysis of variance (ANOVA) followed by Tukey's multiple comparison test at the significance level of 0.001 by using 'Microcal Origin' software (version 7.0) and represented as mean±SEM. Graphical representations of the data in this study were accomplished by 'GraphPad Prism' statistical software (version 5.03, GraphPad Software, Inc).

RESULTS AND DISCUSSION

The pacemaker cells that generate electrical impulses responsible for the auto-contractility and rhythmicity of the heart are primarily located within the specialized structure of the cardiac conduction system[26]. These cells are innervated by the cholinergic, adrenergic and nitrergic efferents[27]. Both the cholinergic and nitrergic efferents play critical roles in reducing cardiac activity. Specifically, stimulation of these pathways leads to the release of acetylcholine (ACh) and nitric oxide (NO) from nerve endings, which act as major inhibitory neurotransmitters.

Upon release, ACh binds to muscarinic receptors on cardiac tissues, resulting in decreased heart rate and reduced contractility. At the same time, nitric oxide provides a secondary mechanism for vasodilation and further modulation of cardiac function[28,29]. Conversely, the activation of adrenergic efferents results in the release of adrenaline (epinephrine) from nerve terminals, which enhances the contractile activity of heart. This adrenergic signalling increases heart rate and contractility through interacting with beta-adrenergic receptors, thereby facilitating the heart's capacity to respond to physiological stressors[30,31]. Given the intricate interplay of these signalling pathways, we hypothesized that exposure to MY could disrupt these mechanisms. Therefore, in this section, we have evaluated the potential signalling pathways that may be affected by MY-induced alterations, focusing on their impact on ventricular function and overall cardiac performance.

Effects of MY on Acetylcholinesterase (AChE) Anzyme:

Acetylcholine (ACh), an endogenous cholinergic neurotransmitter acts as one of the predominant modulators of cardiac functions upon release in the neuromuscular junction (NMJ) from nerve terminals and is responsible for decreased impulse generation in pacemaker cells via stimulation of vagus nerve. Along with ACh, the enzyme acetylcholinesterase (AChE), profoundly located in the vicinity of NMJ of all voluntary as well as involuntary contractile muscle, subordinately acts as the regulatory component to maintain the rhythmic contraction and relaxation of cardiac muscle by being the entity responsible for the catalysis of hydrolysis of the neurotransmitter ACh to its precursor molecules[32]. Therefore, the activity level of AChE provides fundamental control to stabilize the homeostatic balance of ACh signalling on cardio-myocytes that further capacitates the cardiac muscle to achieve the desired function[33].

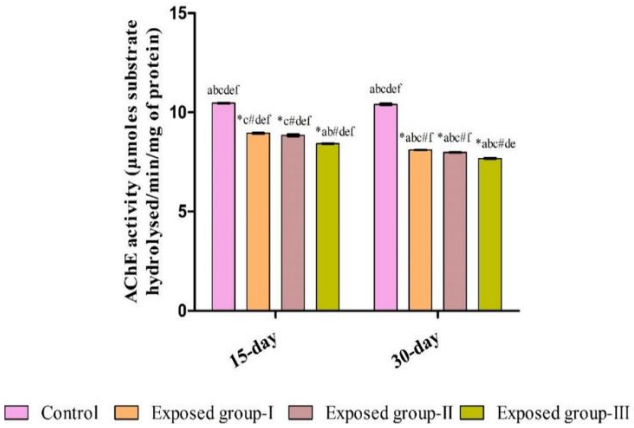

Figure 1: Bar diagram showing the activity of acetylcholinesterase (AChE) in heart ventricular muscle homogenate of control and metanil yellow (MY) exposed groups of rats for 15-day and 30-day exposure durations. The values are represented here as mean±SEM of n=6 rats. Statistical significance denoted as *p<0.001 vs. control of 15-day exposure duration; [a]p<0.001 vs. exposed group-I of 15-day exposure duration; [b]p<0.001 vs. exposed group-II of 15-day exposure duration; [c]p<0.001 vs. exposed group-III of 15- day exposure duration and #p<0.001 vs. control of 30-day exposure duration; [d]p<0.001 vs. exposed group-I of 30-day exposure duration; [e]p<0.001 vs. exposed group-II of 30-day exposure duration; [f]p<0.001 vs. exposed group-III of 30-day exposure duration.

In the present study, the evaluation of the effects of MY on the levels of enzyme activity and enzyme expression of AChE within NMJ of heart ventricular muscle has been performed by biochemical and histochemical experimentations respectively. The result of biochemical experiment shown in Figure 1 indicates significant (p<0.001) decrease in the activity of AChE in the groups of rats exposed to MY in a dose dependent manner in comparison to the control group of rats in case of both the 15-day and 30-day exposure durations. Moreover, besides following the dose dependent pattern, the MY-induced decrease in AChE activity also shows significant (p<0.001) duration dependent pattern. This observation indicates that the MY- induced decrease in AChE activity generated by the 30-day exposure duration is significantly more pronounced than the effect generated by 15-day exposure duration.

The observation of biochemical result is further validated by the observation obtained from the histochemical experiment conducted upon heart ventricular muscle shown in Figure 2 where a similar pattern of MY-induced dose and duration dependent decrease of AChE expression in the form of presence of lesser numbers of brown to orange precipitated aggregates in MY- exposed groups of rats in comparison to the control group of rats has been visualized microscopically in ventricular tissue sections. In order to obtain the enzyme expression for AChE, acetylthiocholineiodide is used as the main substrate for the enzyme AChE scattered throughout the ventricular NMJ area where it catalyzes the hydrolysis of exogenously added acetylthiocholineiodide and thereby produces acetic acid and thiocholineiodide conjugates. The more prominent aggregation of brown to orange precipitation of thiocholineiodide conjugates indicates increased expression of AChE whereas a decreased AChE expression leads to precipitation of lesser aggregation in MY-exposed ventricular tissue sections. Therefore, a gradual decrease in the deposition of thiocholineiodide conjugates present in MY- exposed ventricular tissue sections compared to the control tissue sections provides strong evidence of decreased AChE enzyme expression within cardiac myocytes due to MY exposure.

These results clearly indicate that MY exposure suppresses the function of AChE enzyme in heart ventricular muscle. Moreover, the breakdown product of MY named *p*- aminodiphenylamine has already been linked with the alteration of AChE activity. The compound MY is capable of crossing the lipid-containing cell membrane owing to its lipophilic nature and relatively higher solubility in non-polar solvents. Due to its penetration efficacy, MY probably enters the NMJ space and might get access to AChE, ultimately causing functional alteration of the enzyme[34]. Therefore, this notion can be derived that MY decelerated the hydrolysis of ACh molecule by diminishing the activity of AChE

enzyme in the heart ventricular NMJ. As a consequence, the decreased AChE activity leads to an elevated performance of the neurotransmitter ACh, which generates an inhibitory cholinergic signalling response towards cardiac muscle. The activity of AChE has previously been reported to be associated with alteration of lipid rafts, which are specialized membrane bound components made up of cholesterol that helps in maintenance of muscle integrity through controlling several cellular signalling processes. Alteration in the activity of AChE can affect muscle integrity by modulating lipid rafts and cellular cholesterol levels, which induces cellular oxidative stress[35]. Additionally, the enhancement of peroxidation of lipids has been reported to have a correlation with decreased AChE activity[36]. This report supports the observation of our previous study where a significant increase in the level of malonedialdehyde (MDA), a lipid peroxidation product, has been found to occur following exposure to MY that might play a role to decrease AChE activity as well. Based on this notion, it can be speculated that MY may induce the functional alterations of cardiac muscle probably by inducing oxidative stress, which in turn might decrease the activity of AChE that modifies the lipid rafts of cellular membrane, ultimately causing a degenerative effect on myocytes.

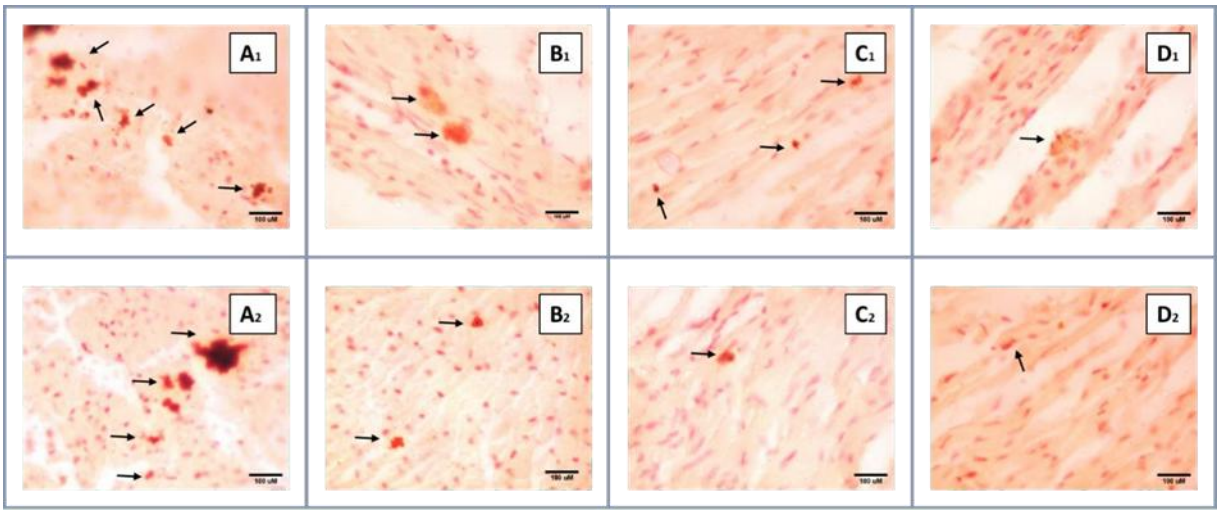

Figure 2: Representative photomicrographs showing histochemically stained heart ventricular tissue sections of both control and metanil yellow (MY)-exposed groups of rats for 15-day and 30-day exposure duration to indicate the depositions of acetylthiocholineiodide conjugates. A1 and A2 represent the heart ventricular tissue sections of control rats for 15-day and 30-day exposure duration respectively; B1, C1, and D1 represent the heart ventricular tissue sections of 15-day MY-exposed groups of rats at doses of 250, 500, 750 mg/kg BW/day respectively; B2, C2, and D2 represent the heart ventricular tissue sections of 30-day MY-exposed groups of rats at doses of 250, 500, and 750 mg/kg BW/day respectively. The arrowheads prominently indicate the site of depositions as the site of AChE expression in heart ventricular tissue. Images captured by using digital SLR Olympus camera (E-620) attached to Olympus bright field light microscope (CH20i) at the magnification of 400X.

Effects of MY on NOS Activity Expression and Nitrite Concentration

The diminished activity of the enzyme acetylcholinesterase (AChE) is closely associated with an increase in nitric oxide synthase (NOS) activity, which plays a crucial role in regulating cardiac function. AChE is responsible for breaking down the neurotransmitter acetylcholine, and when its activity decreases, acetylcholine levels may increase, which potentially affecting heart rate and contractility[37]. At the same time, the heightened activity of NOS results in increased production of nitric oxide, a signaling molecule that affects vascular tone, myocardial perfusion, and overall cardiac health. Consequently, understanding the interaction between AChE and NOS is crucial for comprehending cardiovascular physiology and the complex mechanisms that govern overall heart function[38]. Further, increased activity of NOS is reported to cause cellular damage through an overproduction of nitric oxide (NO), which then becomes oxidized to nitrite within the cell and thus elevates the level of nitrite in both the cytosol and the mitochondria, leading to induction of oxidative stress status responsible for decreasing the activity of AChE[39,40]. Therefore, the next section of the study has been directed towards the evaluation of the effects of MY on expression of NOS activity and nitrite concentration. The expression of NOS activity is determined through histochemical method whereas the nitrite concentration in cytosol and mitochondria of heart ventricular muscle is evaluated through biochemical experimentation. The histochemical observation performed to evaluate expression of NOS activity in heart ventricular muscle shown in Figure 3 reveals MY-induced dose and duration dependent increase in NOS activity in the form of presence of elevated portions of blue precipitation in MY-exposed groups of rats in comparison to the control group of rats as has been visualized microscopically in ventricular tissue sections. The intense blue precipitation is the result of the

expression of NOS activity as the enzyme catalyzes NADPH-dependent reduction of nitro-blue tetrazolium (NBT), which forms diformazan and appears as blue precipitation. Therefore, from this observation it can be speculated that MY might induce damage towards the heart ventricular muscle by elevating expression of NOS activity.

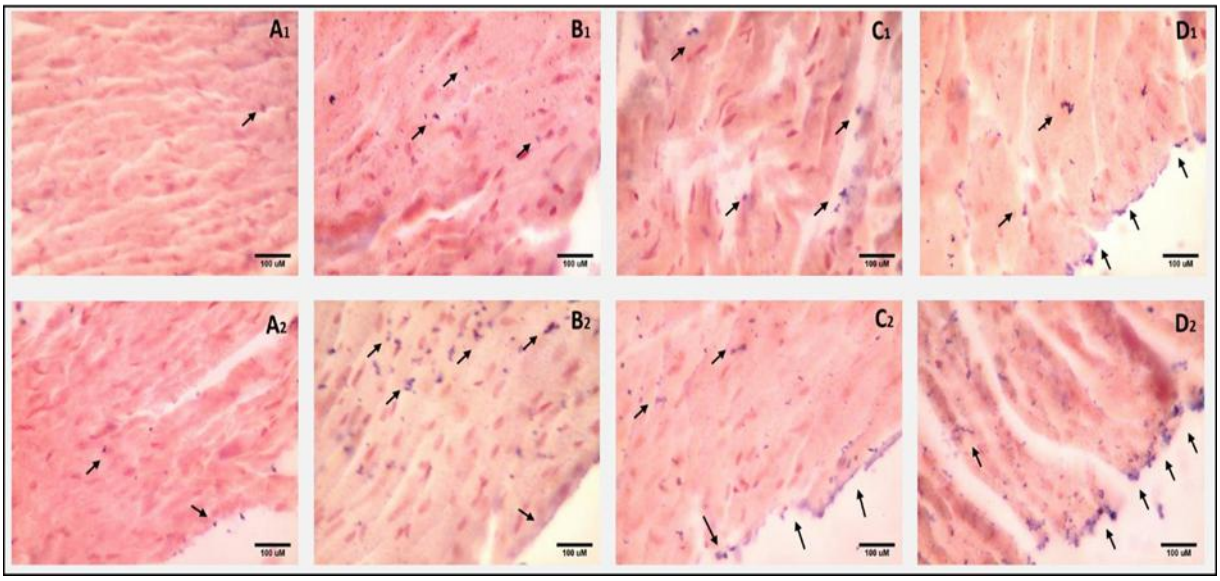

Figure 3: Representative photomicrographs showing NADPH diaphorase-stained heart ventricular tissue section of the control and MY exposed groups of rats subjected to MY exposure for 15-day and 30-day exposure durations. A1 and A2: represent heart ventricular tissue sections of control rats for 15-day and 30-day exposure duration respectively; B1, C1, D1: represent heart ventricular tissue sections of 15-day MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively); whereas, B2, C2, D2: represent heart ventricular tissue sections of 30-day MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively). Black arrowheads indicate the site of increased expression of NOS in a dose and duration-dependent manner within ventricular cardio- myocytes. Images captured using digital SLR Olympus camera (E-620) attached to Olympus bright-field light microscope (CH20i) at the magnification of 400X.

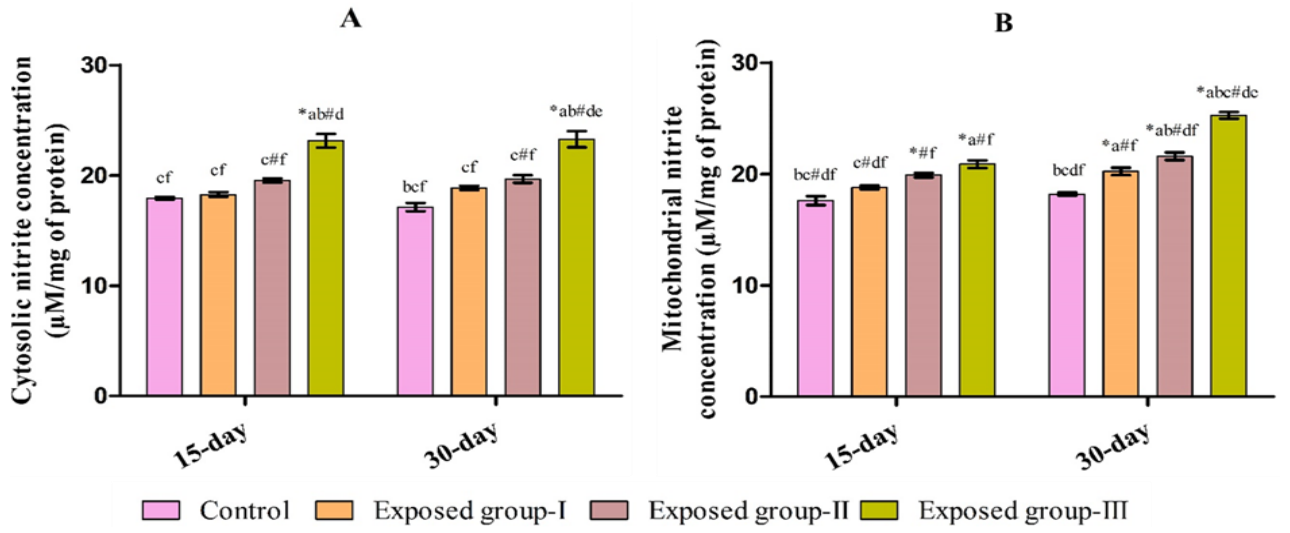

Figure 4: Bar diagram showing the changes in cytosolic and mitochondrial nitrite concentration in heart ventricular muscle of both the control group and metanil yellow (MY) exposed groups of rats for 15-day and 30-day exposure durations. A: represents the cytosolic nitrite concentration of ventricular muscle homogenate of metanil yellow (MY) exposed groups of rats for 15-day and 30-day exposure duration; B: represents the mitochondrial nitrite concentration of ventricular muscle of metanil yellow (MY) exposed groups of rats for 15-day and 30-day exposure duration. Value of nitrite concentration is expressed as mean±SEM of n=6 rats. Statistical significance denoted as $^{*}p<0.001$ vs. control of 15-day exposure duration; $^{a}p<0.001$ vs. exposed group-I of 15-day exposure duration; $^{b}p<0.001$ vs. exposed group-II of 15-day exposure duration; $^{c}p<0.001$ vs. exposed group-III of 15-day exposure duration and $^{\#}p<0.001$ vs. control of 30-day exposure duration; $^{d}p<0.001$ vs. exposed group-I of 30-day exposure duration; $^{e}p<0.001$ vs. exposed group-II of 30-day exposure duration; $^{f}p<0.001$ vs. exposed group-III of 30- day exposure duration.

Furthermore, the result of biochemical experiment shown in Figure 4 indicates significant ($p<0.001$) increase in cytosolic as well as mitochondrial nitrite concentrations in heart ventricular muscle homogenate of MY-exposed groups of rats in a dose dependent manner in case of both the 15-day and 30-day exposure durations in comparison to the control group of rats also following a significant ($p<0.001$) duration dependent pattern.

Nitrite being a reactive nitrogen species (RNS) has been found to play a dual role in the context of cellular functioning depending on its concentration. While being present at low concentrations it acts as the signalling molecule during execution of a number of cellular processes. However, a high nitrite concentration within cellular milieu shows a deteriorative effect on the structure of cellular components[41]. At very high concentrations, nitrite can cause oxidative damage to cellular compartments as well[42,43]. Elevated nitrite levels activate the pyruvate cycle (P cycle) within mitochondria which supplies excess NADH to the electron transport chain (ETC), further increasing the production of ROS within cellular system. Thus, nitrite-induced ROS production has been correlated with altered gene expression of several antioxidant enzymes, such as SOD and CAT, which further potentiates generation of ROS by decreasing the activity of the cellular antioxidant defence system. The altered antioxidant defence mechanism increases the excessive formation of reactive species, which ultimately attacks cellular membrane lipids and causes the destruction of the architectural organization of muscle cells[4,44]. Alongside, excessive nitrite hinders the immune system and suppresses the production of IgM and lysozyme (LZM) which exerts a cellular condition enormously prone to several oxidative damages through the production of reactive species. The resulting excessive ROS entities act as potent stressors, causing damage to cellular membranes and bio- molecules leading to deterioration of cellular structure[45,46]. Moreover, nitrite also affects the activity of cellular antioxidant enzymes by disrupting the redox homeostatic balance, further causing an overabundance of oxidative radicals that induce cellular damage. Our previous findings concerning the MY-induced elevated oxidative stress supports the current observation and hypothesis that MY exposure leads to an excessive formation of nitrite in both the cytosolic and mitochondrial portion, probably resulting in cellular oxidative stress through the generation of excessive reactive species that might damage cells through lipid peroxidation and alteration of architecture of membrane system.

Effects of MY on the morphology of heart ventricular muscle:

Therefore, the next section of the study is aimed towards the evaluation of the effects of MY on the morphology of heart ventricular muscle. The morphology of heart ventricular muscle is evaluated by histological analysis of cyto-architecture using hematoxylin and eosin (H&E) staining method followed by visualization through bright-field microscopy along with ultra- structural analysis of surface architecture visualized through scanning electron microscopy. The cyto-architectural observation performed on heart ventricular muscle shown in Figure 5 reveals MY-induced dose and duration dependent morphological alterations in the form of presence of distorted arrangement of muscle fibres in MY-exposed groups of rats in comparison to the control group of rats as has been visualized microscopically in ventricular tissue sections. The morphological alterations prominently indicate towards myo- degeneration of heart ventricular muscle, denoted by the presence of deteriorations of muscle fibres, several laceration and destruction of interconnecting myofibrillar tissue.

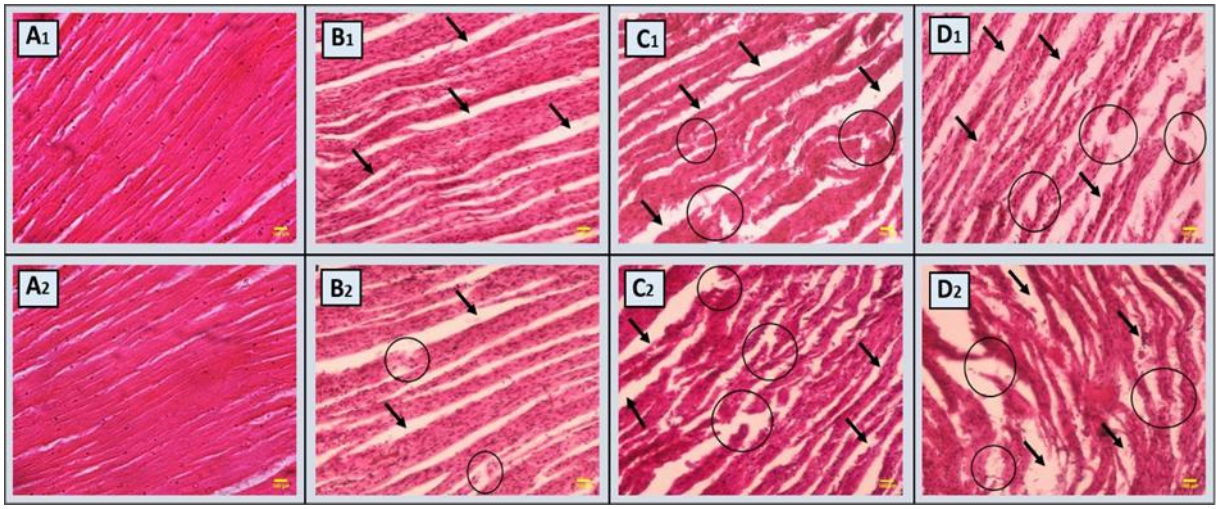

Figure 5: Representative photomicrographs showing hematoxylin and eosin (H&E)- stained heart ventricular tissue section of rats subjected to MY exposure for 15-day and 30-day exposure durations. A1 and A2: heart ventricular tissue sections of control rats for 15-day and 30-day exposure duration respectively; B1, C1, D1: heart ventricular tissue sections of 15-day MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively); B2, C2, D2: heart ventricular tissue sections of 30-day MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively). Black circles prominently

indicate the site of destruction of muscle fibres, while the arrowheads indicate lesions and loss of muscle fibres. Images captured by digital SLR Olympus camera (E-620) attached to Olympus bright-field light microscope (CH20i) at the magnification of 400X.

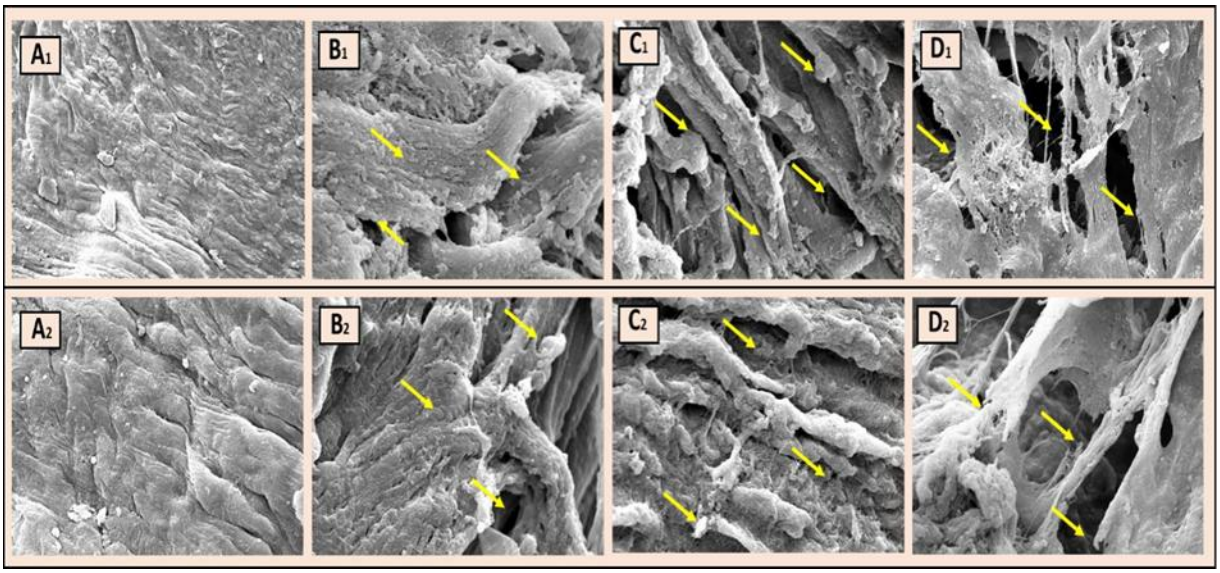

Figure 6: Representative electron micrographs showing the ultra-structure of ventricular muscle of both control and metanil yellow (MY) exposed groups of rats for 15-day and 30-day exposure duration. A1 and A2: represent the heart ventricular muscle of control rats for 15-day and 30-day exposure duration respectively; B1, C1 and D1: represent the heart ventricular muscle of MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively) for 15-day; B2, C2 and D2: represent the heart ventricular muscle of MY exposed groups of rats (250, 500, 750 mg/kg BW/day respectively) for 30-day. Arrowheads indicate the formation of blebs and destruction of muscle fibres. Electron micrographs captured using scanning electron microscope at 5000X magnification.

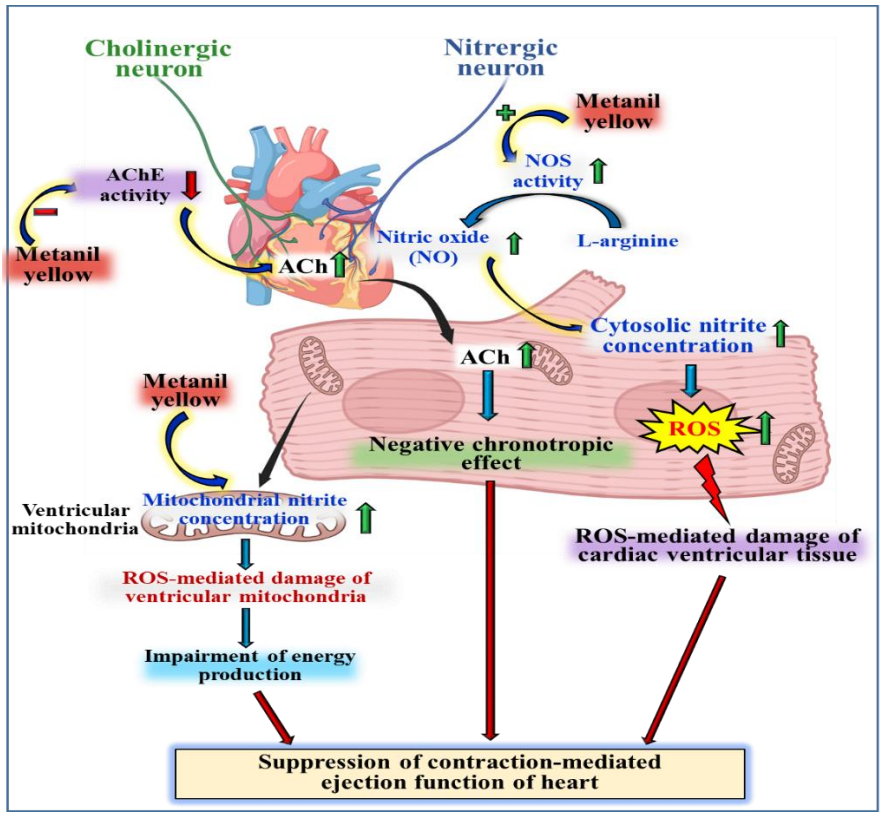

Figure 7: Schematic representation showing the probable mechanism of action of metanil yellow (MY) on heart ventricular muscle and ventricular mitochondria. Here upward arrowheads indicate an increase in the activity; the plus sign indicates potentiation and the minus sign indicates a reduction effect. MY: metanil yellow; ROS: reactive oxygen species; AChE: acetylcholinesterase, ACh: acetylcholine; NOS: nitric oxide synthase; NO: nitric oxide.

Furthermore, the results obtained through using high-resolution scanning electron microscopy in heart ventricular muscle also supports the results of histological experiment. The ultra- structural observation performed on heart ventricular muscle shown in Figure 6 reveals MY- induced dose and duration-dependent topological alterations in the form of presence of disintegrated arrangement of muscle fibres in MY-exposed groups of rats in comparison to the control group of rats as has been visualized microscopically in ventricular tissue sections. The topological alterations can be denoted by the presence of disintegration and lesion of muscle fibres along with the formation of multiple blebs and cavities. These serve as the prominent signs of destruction of cardiac muscle by MY exposure.

Therefore, on the basis of our above observations, it can be suggested that exposure to MY leads to significant deterioration of the architectural organizations of heart ventricular muscle. This destruction is primarily attributed to alterations in the activity of two crucial enzymes: acetylcholinesterase (AChE) and nitric oxide synthase (NOS) within the ventricular neuromuscular junction, responsible for regulating the concentration of ACh and nitric oxide (NO). The disruption in enzyme activity results in an accumulation of ACh molecules, which is due to decreased breakdown by acetylcholinesterase, and an increase in nitrite ions from altered NOS activity. This biochemical imbalance adversely affects the heart's contraction-mediated ejection functions by impairing both cholinergic and nitrergic signaling pathways that are essential for proper cardiac function. Additionally, the elevated levels of nitrite ions contribute to mitochondrial dysfunction exacerbated by oxidative stress, leading to oxidative damage within the ventricular muscle.

The presumable mechanisms involved in these processes are encapsulated below in Figure 7.

CONCLUSION

Based on the comprehensive observations of this study, it can be concluded that MY exerts a suppressive influence on the cardiac ventricular function in rats. This impairment appears to be associated with the involvement of cholinergic and nitrergic signalling pathways through altering the enzymatic activities of acetylcholinesterase (AChE) and nitric oxide synthase (NOS) respectively. In addition to these enzymatic changes, signalling of the nitrergic pathway is also influenced by the enhancement of the concentration of nitrite ions, a by-product associated with nitric oxide metabolism. These combined factors suggest a complex interplay of biochemical processes and signalling pathways of heart function that ultimately leads to compromised overall cardiac function.

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CONFLICT OF INTEREST

There is no conflict of interest.

CONSENT FOR PUBLICATION

All the authors have given their consent for this publication.

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