Research Article

Metanil Yellow Inhibits the Contractile Activity of the Duodenal Visceral Smooth Muscle by Augmenting sGC Mediated Nitrergic Signaling Pathway

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Received: 09-05-2024; **Revised:** 27-07-2024; **Accepted:** 05-08-2024; **Published on:** 15-08-2024.

ABSTRACT

Metanil yellow (MY), a synthetic xanthene dye, though categorized as non-permitted food color is widely used as a food colorant in the food industry because of its persistent yellow color to attract customers. As a result, humans often expose to MY through eating food contaminated with MY. Therefore, the study was aimed to investigate how MY affected the duodenum's contractile activity, which is a crucial component of the small intestine that provide it its motility. In order to examine the effect of MY on the contractile activity of the duodenal visceral smooth muscle, we observed its influence on duodenal contractions by recording the duodenal movement ex vivo of control and MY-exposed rats using an isotonic transducer (IT-2245) connected to RMS polyrite D. Rats exposed to MY showed a significant suppression in the amplitude of duodenal contractions as compared to control rats. Moreover, to investigate the probable neurocrine mechanisms in MY-induced suppression of the duodenal visceral smooth muscle (dVSM) contractions, the movement of duodenum was recorded in response to application in combination with MY and Nitrergic agonists (Sodium Nitroprusside) and antagonists (L-NAME and Methylene Blue (MB)) respectively. MY showed synergistic inhibition of the contraction of the dVSM in presence of SNP whereas MY induced inhibition of the contraction of the dVSM has been counteracted in L-NAME and MB pre-treated condition. In conclusion, it is suggested that MY inhibits the contractile activity of the dVSM by inhibiting the contractions of the dVSM by augmenting soluble guanylyl cyclase (sGC) mediated nitrergic signaling pathway.

Keywords: Metanil yellow, duodenal visceral smooth muscle, intrinsic myenteric efferent, L-NAME and MB.

INTRODUCTION

In the present time, to make food items more attractive to the consumers, several types of synthetic colorants are added to the food. Some of the food colors are permitted to be used in food in a permissible limit while some colorants are not permitted for use in food items at all, but still used due to their stability and cheapness as compared to permitted food colors. Metanil yellow (MY) is one of the widely used food colorants, which is used in sweets, spices etc. for its bright yellow color[1].

MY is a yellow azo dye synthesized by the diazotization of metanilic acid and diphenylamine[2]. Though it is allowed to be used in industries for coloring wool, nylon, silk, paper, aluminum, ink, detergent, etc.[3], it is not a permitted food color according to the prevention of Food Adulteration Act 1954, India. Various studies have concluded that MY is a significant toxicant. MY found to alter the normal neurotransmitter secretion and damages the developing brain and also hampers the learning ability by damaging the granular and purkinjee cell layer[4]. MY exposure results in necrosis of tubular epithelium, disruption of Bowman's capsule and swelling of renal tubular epithelial cells resulting detrimental changes in the convoluted tubule and collecting duct[5]. MY is proven to disrupt normal estrous cycles and folliculogenesis in female rats inducing oxidative stress[1]. On the other hand, in male individuals, MY results in degeneration of seminiferous tubules[6]. Some studies have found that MY can induce oxidative stress in vital organs like heart, kidney and liver[7,8].MY has been considered carcinogenic and mutagenic[9,10].

Though MY is considered as a non-permitted food color, it is extensively used to color food articles viz., laddoo, biryani, jalebi, etc. So, humans are often exposed to MY through consumption of MY tainted foods. Since the gastrointestinal system is the organ that gets primarily exposed to MY on ingestion of MY contaminated food stuff, it might exhibit its toxicity and alters the normal physiologic function of the digestive system.

The digestion of foods and absorption of nutrients are the principal functions of the gastrointestinal system that is regulated by the contractions of the gastrointestinal visceral smooth muscle that provides gastrointestinal motility and helps in mixing and churning of the chyme with enzymes and helps in propulsion of the luminal contents aborally. It is postulated that, MY enters the body by absorption of consumed adulterated food by intestine and is transported to blood. Then the toxic chemical reaches various organs and exerts adverse effects by impairing their metabolic processes. MY is reported to impair nutrient absorption by damaging structural integrity of the gastrointestinal tract[11].

Hence, for the assessment of adverse effects of MY on the physiological functionality of the digestive system, contractile activity of the small intestine, especially of the duodenal visceral smooth muscle (dVSM) has been examined. Till date, no study has been conducted to examine the effect of MY on the contractile activity of the

small intestine. Hence, the present aim of the study is to examine the effect of MY on the contractile activity of the dVSM and also to examine the probable pharmacodynamics involved in the MY induced alteration of the contractile activity of the dVSM.

MATERIALS AND METHODS

Chemicals and Reagents

All the reagents and chemicals that were used to conduct this study were of analytical grade. The test chemical-Metanil Yellow; 3-((4-(Phenylamino)phenyl)azo) benzenesulfonic acid monosodium salt;and N-ω-nitro-L-arginine methyl ester (L-NAME) hydrochloride has been purchased from Sigma Aldrich, USA. Methylene blue (MB) and sodium nitroprusside (SNP), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), glucose, etc. were procured from E.Merck, India.

Experimental Animals and Care

As the experimental model, adult female 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 laboratory chow and water and were kept in the animal house in accordance with the animal ethics committee's guidelines from Kalyani University.

Experimental Design

The animals were treated to different exposure conditions as mentioned in Table 1.

Table 1: Experimental Setup for the study

Groups	Exposure conditions
Set 1	Application of graded doses of MY (4, 8, 16, 32 µM) on the duodenal segments
Set 2	Application of single dose of SNP (2.5µM) on the duodenal segments
Set 3	Application of effective dose of MY on duodenal segments pretreated with SNP
Set 4	Application of single dose of L-NAME (200 µM) on the duodenal segments
Set 5	Application of effective dose of MY on duodenal segments pretreated with L-NAME
Set 6	Application of single dose of MB (200 µM) on the duodenal segments
Set 7	Application of effective dose of MY on duodenal segments pretreated with MB

Animal Sacrifice

The animals selected for the experiment were kept at fasting conditions overnight, prior to sacrifice. For sacrificing, cervical dislocation process was performed to ensure minimum pain during sacrifice following the guidelines of the Animal Ethics Committee of Kalyani University.

Collection of the Organ

After performing cervical dislocation and ensuring its effect, the abdominal cavity of the animal was cut open. Then the small intestine was isolated by surgically dissecting it from the mesentery, stomach and the large intestine by transverse incision. After that the portion of the small intestine adjacent to the stomach, comprising the duodenal part, was isolated from the collected section of the small intestine and was collected. Then the section was immediately transferred to temperature-controlled Tyrode's fluid in a beaker. Only the duodenum segment was selected for the representation of the small intestine as the motility of the duodenum is most prominent in the small intestine. Following the collection of the duodenal segment, the lumen was rinsed carefully by a gentle flushing out process ensuring elimination of any luminal contents present inside. The isolated tissue segment was immediately mounted in the organ bath of Dale's apparatus for the recording of duodenal motility.

Recording of the Movement of the Duodenum

For recording of the spontaneous motility of DVSM *ex vivo*, an approximately 3 cm long segment of duodenum was hanged vertically by two metal hooks piercing through the two opposite ends of the tissue segment and was immersed into an organ bath filled with 40 ml of Tyrode's solution. The composition for Tyrode's solution was 8.0g sodium chloride (NaCl), 0.2g potassium chloride (KCl), 0.2g calcium chloride ($CaCl_2$), 0.1g magnesium chloride ($MgCl_2$), 0.05g sodium di-hydrogen phosphate (NaH_2PO_4), 1.0g sodium bi-carbonate ($NaHCO_3$) and 1.0g dextrose into a final volume of 1L (pH of 7.4). A continuous supply of oxygen with a flow rate of 2-3 air bubbles/second into the immediate vicinity of the tissue was maintained with the help of an oxygen bubbler. The temperature of the organ bath was maintained at 37±0.5°C by an automatic thermostat attached with the Dale's apparatus.

To record the motility or movement of the isolated tissue segment, its lower end was attached at the bottom end of the organ bath and its upper end was attached similarly with the lever of an isotonic transducer apparatus (IT-2245), the apparatus for recording of the continuous movement of the isolated tissue segment, was coupled with analyzing software, RMS Polyrite-D (RMS, Chandigarh, India). Each new segment was initially allowed to stabilize for at least 35 mins under the given experimental set up and was washed repeatedly with fresh Tyrode's solution for removal of accumulated metabolites. Finally, recording of isotonic contraction, resulting from the spontaneous rhythmic movement of the isolated tissue segment was acquired continuously following administration of different doses of MY and experimental blockers.

Statistical Analysis

The values of each experimental group were expressed as means ± SEM for the data. The frequency and amplitude of the movement recordings were used to calculate the force of contractions. The values of treated preparations for functional tests were given as the percentage change from the basal (or control) values. One way ANOVA was used to assess any statistical differences between the groups (GraphPad Prism 8). It was deemed significant when P < 0.05.

RESULTS AND DISCUSSION

Effect of Graded Doses of Metanil Yellow on the Movement of Isolated Duodenum *Ex Vivo* of Rat

In order to examine the effect of MY on the contractile activity of the dVSM, the movement of the duodenum (*ex vivo)* were recorded in response to graded doses of MY in single dose acute experiments. From the tracings, it is suggested that exposure to graded concentrations of MY on the isolated duodenal segments showed decreased amplitude in a dose dependent manner. Further, the frequency of the contractions of the dVSM was also decreased on exposure to MY dose dependently (Figure 1). The suppression of duodenal motility was unchanged for the rest of the experiment period (20 min). In the case of the 32 µM dose, the duodenal motility was inhibited >80% compared to control, where the contractile activity has been ceased for the rest of the duration (Figure 2).

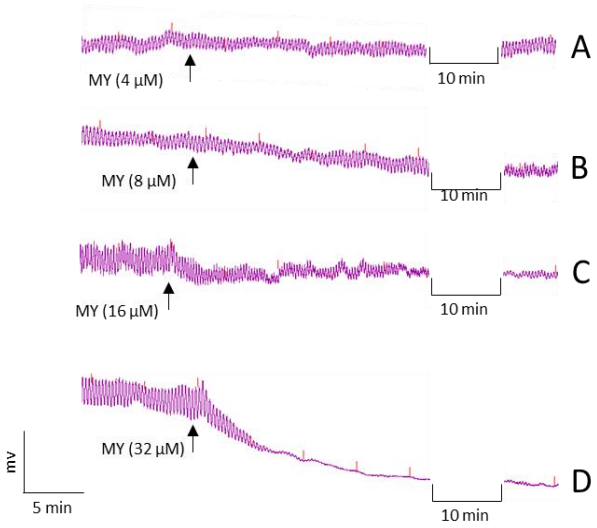

Figure 1: Tracings showing representative records of the effect of graded concentrations of MY on the isolated duodenal movement of rat in tissue organ bath obtained with an isotonic transducer coupled to RMS Polyrite-D.

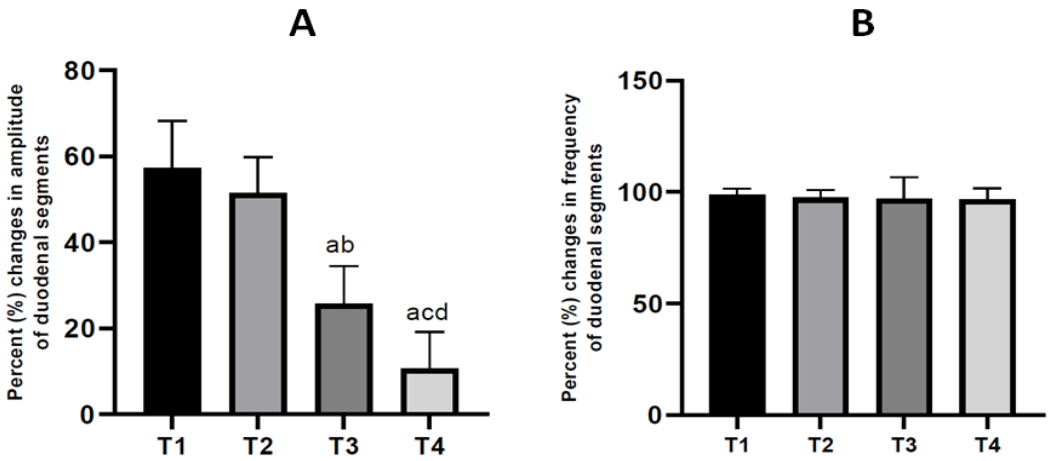

Figure 2: Bar diagram showing percent changes in amplitude and frequency of the contractions of isolated duodenum in response to the application of graded doses of MY. The data represented were mean ± SEM for the entire group. [a]*P*< 0.0001 Vs T1; [c,b]*P*<0.0001, 0.01 Vs T2; [d]*P*<0.05 Vs T3(A).

From the results, it can be suggested that MY suppresses the contractile activity of the dVSM by decreasing the amplitude and frequency of the contractions of the smooth muscle located in the wall structure of the duodenum. Further, we have found no significant alteration in the frequency of contraction of the dVSM by MY. Since, the intrinsic myenteric efferents control the contractions of the visceral smooth muscle of the GI system through its respective innervations mainly excitatory cholinergic, inhibitory adrenergic and inhibitory nitrergic (NANC, non-adrenergic non-cholinergic) myenteric efferents. So, it is expected that the MY induced suppression of the contractile activity of the dVSM might be due to inhibition of cholinergic myeneteric efferents and/or activation of adrenergic/nitrergic (NANC) intrinsic myenteric efferents innervating the dVSM.

Effect of Metanil Yellow on the Movement of Isolated Duodenum Pre-incubated With SNP

In order to examine the probable pharmacodynamics involved in the MY induced inhibition of the contractile activity of the dVSM, the involvement of nitrergic intrinsic myenteric efferents were examined as the nitrergic myenteric efferents is principally inhibitory to the contractions of the visceral smooth muscle located at the wall of the duodenum by releasing the inhibitory neurotransmitter Nitric Oxide (NO) that induces relaxation of the visceral smooth muscle.

So, to ascertain the nitrergic influences in the MY induced suppression of the contractile activity of the dVSM, the movement of the duodenum *ex vivo* in single dose acute experiment was recorded in response to the application in combination of MY and Sodium nitroprusside (SNP), a nitrergic agonist (NO donor). From the tracings (Figure 3), it is suggested that the degree of inhibition of the contraction of the dVSM was increased in SNP pre-treated duodenal preparation compared to MY alone. This synergistic inhibition of MY in combination with SNP surely suggested a possible activation of nitrergic myenteric efferents and results in the inhibition of the contractile activity of the dVSM.

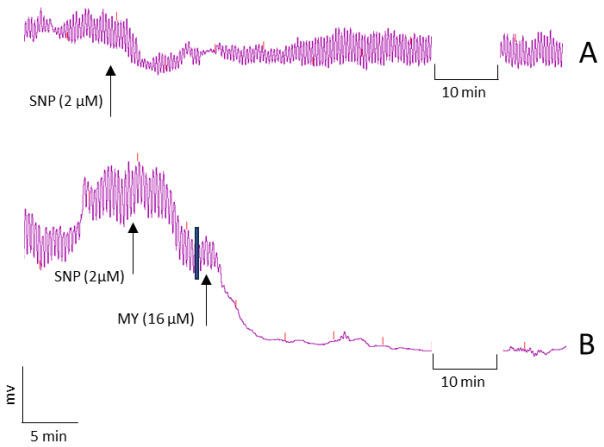

Figure 3: Tracings showing representative records of the effects of MY on the movement of duodenum in SNP pre-treated duodenal preparations *ex vivo*. A: Tracing of effect of SNP (2 µM) on the movement of duodenum. B: Tracing of the effect of MY (16µM) on the movement of duodenum in SNP (2 µM) pre-treated duodenal preparations obtained with an isotonic transducer coupled to RMS Polyrite-D.

Effect of Metanil Yellow on the Movement of Isolated Duodenum Pre-incubated With L-NAME

In order to ascertain the activation of nitrergic myenteric effrents in the MY induced suppression of the contractile activity of the dVSM, the movement of the duodenum *ex vivo* in single dose acute experiment was recorded in response to the application in combination of MY and L-NAME, a nitrergic antagonist (Nitric Oxide Synthase (NOS) inhibitor). It was observed from the tracings that L-NAME when administered alone does not produce any significant alteration in the contraction of the dVSM whereas the MY induced inhibition of the contraction of the dVSM was counteracted on application of MY in combination with L-NAME as observed compared to the inhibition of the contraction of the dVSM exhibited by MY alone.

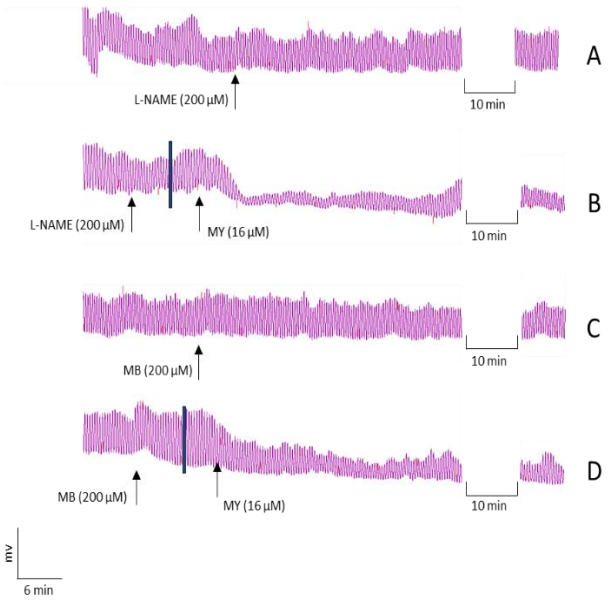

Figure 4: Tracings showing representative records of the effects of MY on the movement of duodenum in L-NAME and MB pre-treated duodenal preparations *ex vivo*. A: Tracing of effect of L-NAME (200 µM) on the movement of duodenum. B: Tracing of the effect of MY (16 µM) on the movement of duodenum in L-NAME (200µM) pre-treated duodenal preparations. C: Tracing of the effect of MB (200 µM) on the movement of duodenum. D: Tracing of the effect of MY (16 µM) on the movement of duodenum in MB (200 µM) pre-treated duodenal preparations obtained with an isotonic transducer coupled to RMS Polyrite-D.

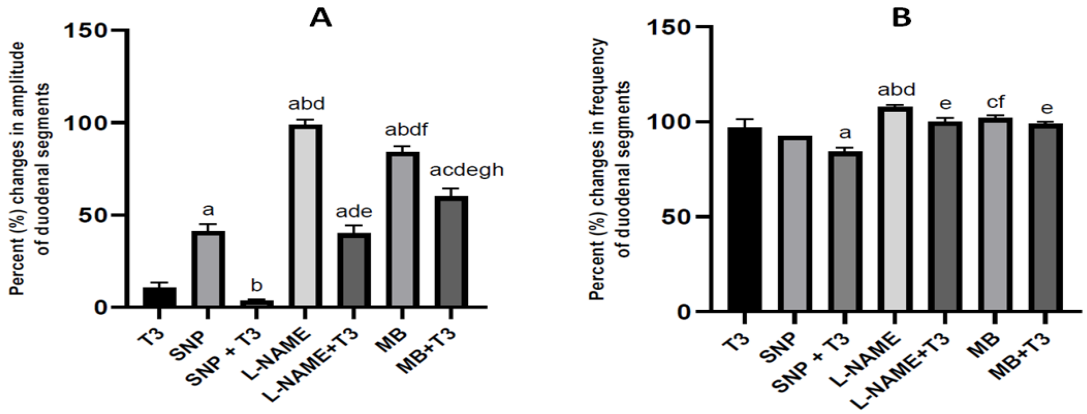

Figure 5: Bar diagram showing percent changes in amplitude and frequency of the contractions of isolated duodenum in response to the application of MY in combination with nitrergic agonist and antagonist(s). The data represented were mean ± SEM for all the group. [a]*P*< 0.0001 Vs T3; [c,b]*P*< 0.01,0.0001Vs SNP; [d]*P*<0.0001 Vs SNP+T3; [e]*P*< 0.0001 Vs L-NAME; [g,f]*P*< 0.01, 0.0001 Vs L-NAME+T3; [h]*P*< 0.001Vs MB (A). [a]*P*< 0.05 Vs T3; [c,b]*P*< 0.05, 0.01Vs SNP; [f,e,d]*P*< 0.001, 0.01, 0.0001 Vs SNP+T3 (B).

Effect of Metanil Yellow on the Movement of Isolated Duodenum Pre-incubated With MB

In order to ascertain the activation of nitrergic signaling pathway in the MY induced suppression of the contractile activity of the dVSM, the movement of the duodenum *ex vivo* in single dose acute experiment was recorded in response to the application in combination of MY and MB, a nitrergic antagonist (soluble guanylyl cyclase (sGC) blocker). It was observed from the tracings that MB when administered alone does not produce any significant alteration in the contraction of the dVSM whereas the MY induced inhibition of the contraction of the dVSM was counteracted on application of MY in combination with MB as observed compared to the inhibition of the contraction of the dVSM exhibited by MY alone (Figure 4 and 5).

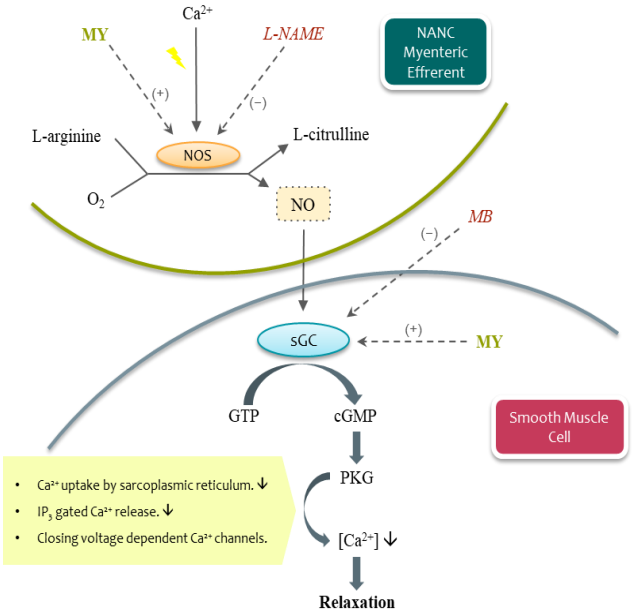

Figure 6: Schematic representation of the probable neurocrine mechanisms involved in the MY induced suppression of the contractile activity of the dVSM. (+); indicates stimulation, (-); indicates inhibition, indicates decrease in levels.

From the results, it was observed that both the nitrergic antagonists (L-NAME and MB) counteracted the inhibition of the contraction of the dVSM exhibited by MY, clearlysuggests that MY suppresses the contractions of the dVSM through nitric oxide mediated soluble guanylyl cyclase pathway by augmenting the activity of intrinsic nitrergic myenteric efferents (Figure 6).

CONCLUSION

MY is a non-permitted food color and it is used as an adulterant in food industry despite of being its toxicity. In conclusion, it is suggested that MY inhibits the contractile activity of the dVSM by suppressing the contraction of the visceral smooth muscle located at the wall of the duodenum that provides motility. We found that MY suppresses the contractile activity of the dVSM probably by augmenting the soluble guanylyl cyclase mediated nitrergic signaling pathway. The extrapolations from the study reveal that metanil yellow on chronic consumption might inhibit the contractile activity of the dVSM resulting in impaired digestion and absorption in humans.

Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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