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Erythrosine Inhibits the Contractile Function of Duodenal Visceral Smooth Muscle of Rat *Ex Vivo* by Augmenting the Nitrergic Signaling Pathway

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ABSTRACT

Erythrosine, a poly-iodinated xanthene dye, extensively used as a dye in foods, cosmetics and pharmaceuticals. Because of its stability and bright persistent color, it is widely used as a food coloring agent in the food industry. Despite being reported of its toxicity by some researchers, it is widely used in food to attract customers. As a result, people are often exposed to Erythrosine by consuming Erythrosine contaminated food. Therefore, the aim of the study was to determine how Erythrosine affected the contractile activity and motility of the duodenum, an important component of the small intestine. To investigate the effect of Erythrosine on duodenal visceral smooth muscle contractile activity, we observed its effect on duodenal contractions by recording *ex vivo* duodenal movements of control and erythrosine-exposed rats using an isotonic transducer (IT 2245) connected to RMS polyrite D. Erythrosine exposed rats showed a significant suppression of the duodenal contractions through decrease in both frequency and amplitude of contractions compared to control rats in a dose-response pattern. In addition, to investigate possible neurocrine mechanisms involved in Erythrosine - induced suppression of duodenal visceral smooth muscle (dVSM) contraction in response to Erythrosine and nitrergic antagonists, duodenal movements were recorded in response to application of Erythrosine and L-NAME (nitric oxide synthase inhibitor) and Methylene blue (MB) (soluble guanylyl cyclase blocker). The degree of inhibition of dVSM contraction by Erythrosine is counteracted in L-NAME and MB pretreated conditions. Therefore, Erythrosine inhibits the dVSM contractile activity by inhibiting dVSM contractions, thereby facilitating the action of inhibitory nitrergic-myenteric efferents through the nitric oxide-mediated soluble guanylyl cyclase pathway.

Keywords: Erythrosine, contractile function, duodenal visceral smooth muscle, nitrergic-myenteric efferents, soluble guanylyl cyclase pathway

INTRODUCTION

Now a days, a lot of synthetic food colors are used to make the food attractive to the customers due to its low cost, stability and its colorful attractiveness. The impact of food coloring on human health is a serious problem, particularly for children, where hyperactivity has been linked to the coloring of food (Neeta, 2018). Although food regulatory agencies have approved the majority of synthetic food colors, long-term health implications of ingestion remain a major worry. Synthetic food colors are frequently used in greater quantities by food manufacturing businesses than the ADI (Advised daily intake) (Rao et al., 2008).

Significant hazards to the public's health could result from such uncontrolled intake, particularly for young children and teenagers.

Erythrosine (FD & C Red No.-3) is a poly-iodinated xanthene group of dye synthetically produced by iodination of fluorescein, generally it's the condensation product of phthalic anhydride and resorcinol. Erythrosine is primarily used as colorants in various industries such as foods, cosmetics and pharmaceuticals (Ganesan et al., 2011). It is used as a food colorant in food products such as cakes, candies, popsicles, candied cherries, cocktails, cream biscuits and many other food products. Though it is extensively used as a food colorant, still its toxicity has been reported by many researchers. Erythrosine causes endotoxic and mutagenic effects in HepG2 cells (Chequer et al., 2012). Further it possesses cytotoxicity and cytostatic effects on human peripheral blood cells (Mpountoukas et al., 2010). Chronic exposure of erythrosine reported to affect thyroid function (Jennings S et al., 1990), childhood behaviour (Silbergeld K. and Anderson 1982), and inhibits the enzymes that metabolise drugs (Mizutani T, 2009) and mitochondrial respiration (Reyes et al., 1996).

Erythrosine is a common food additive that has already become a part of consumption. It has an Acceptable Daily Intake (ADI) of 0.1mg/kgbw/day, which is strictly set, but if the intake increases it may result in adverse effects especially in mammals. However, some general effects include nausea, vomiting, severe abdominal pain sometimes, blurred vision or even hearing loss and unusual tiredness.

So, humans are often exposed to erythrosine through consumption of erythrosine tainted foods. On exposure to erythrosine, the small intestine gets primarily exposed to it. As we know, the small intestine plays a pivotal role in regulating the digestive and absorptive functions through its motor function which is being provided as a result of contractions of the visceral smooth muscle cells located at the wall structure of the small intestine. Different motor patterns help the luminal contents to mix with succus entericus and other enzymes and helps to move aborally for excretion. It is hypothesized that erythrosine on consumption might exert its toxicity and alter the motor functions of the small intestine resulting in improper digestion and malabsorption. Since, the effect of erythrosine on the contractile (motor) function of the small intestine, the organ exposed during digestion and absorption of erythrosine contaminated foodstuffs is not well established so far, the present aim of the study was to elucidate the pharmacodynamics in erythrosine induced effects on the contractile functions of small intestinal visceral smooth muscle (SiVSM) in albino rat.

MATERIALS & METHODS

Chemicals and Reagents

All the Chemicals and reagents used were of analytical grade. The test chemical, Erythrosine, 3,4,5,7-tetra-iodofluorescein (CAS No.- 16423-68-0) and N-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) was acquired from Sigma Aldrich, USA. Methylene blue (MB), potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), and glucose, were procured from E-Merck, India.

Animals

For the experimental setup, Male albino rats of Charles foster strain were selected weighing about 130-150 grams. The animals were kept at departmental animal house at about 25-27°C temperature and 24-hour light-dark cycle. They were fed with laboratory chow and water *ad libitum*. All the experiments were carried out following the animal ethics committee's guidelines from Kalyani University.

Preparation of Doses of Erythrosine

Erythrosine was dissolved in double distilled water. Four different doses of erythrosine viz., 1.6 µM, 2.4 µM,3.2µM and 4.0 µM were used in the present study.

Experimental Design

The animals were exposed to different doses and exposure conditions as mentioned in Table 1.

Table 1. Experimental Design

Groups	Exposure conditions
Set 1	Application of graded doses of Erythrosine (1.6, 2.4, 3.2, 4.0µM) on the duodenal segments
Set 2	Application of single dose of L-NAME (200 µM) on the duodenal segments
Set 3	Application of effective dose of Erythrosine (3.2 µM) on duodenal segments pretreated with L-NAME (200 µM)
Set 4	Application of single dose of MB (200 µM) on the duodenal segments
Set 5	Application of effective dose of Erythrosine (3.2 µM) on duodenal segments pretreated with MB (200 µM)

Animal Sacrifice

The night before the sacrifice, the animals were maintained in a state of fasting. To have a painless sacrifice, the animals underwent cervical dislocation following the guidelines established by Kalyani University's Animal Ethics Committee.

Tissue Collection

Just after cervical dislocation, the abdominal cavity of the sacrificed animal was cut in order to isolate the small intestine. The duodenal portion of the small intestine was isolated after surgically separating it from mesentery. The duodenum was selected for the experimental purpose as the motility of duodenum is more prominent than that of the other portions. Soon after isolation, the lumen was cleared with flushing out process gently and carefully. By this cleaning process, emptying of luminal content was ensured. For the recording of the duodenal motility, the isolated tissue segment was immediately mounted within the organ bath of Dale's apparatus using the lowest time duration possible, starting from the moment of animal sacrifice.

Tissue Mounting for Recording of Movement of Duodenum

To record the spontaneous pattern of motility of duodenal segment of small intestine *ex vivo*, about 3 cm long (approx) duodenal segment was taken and positioned vertically with the help of two metal hooks piercing through the two opposite ends of the tissue segment and was soaked into an organ bath filled with 45 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 having a pH of 7.4. The organ bath was given the facility to deliver a continuous oxygen supply (95% O_2 and 5% CO_2) to the tissue with the help of an oxygen bubbler. The temperature of the isolated tissue segment, within the organ bath, was maintained at 37±0.5°C with the help of an automatic thermostat machine attached with the Dale's apparatus.

For recording of the motility of vertically isolated tissue segment, its lower end was attached to the bottom side of the organ bath with the help of a metal hook and its upper end was attached in a similar way with the help of the lever of an isotonic transducer apparatus (IT-2245) was coupled to RMS

Polyrite D (RMS, Chandigarh, India). Each segment was given the time of about 30 mins to stabilize under the given experimental set up and was washed frequently with fresh Tyrode's solution for removal of accumulated metabolites. Final, recording of isotonic contraction, resulting from the spontaneous rhythmic movement of the isolated tissue segment was acquired continuously with the application of different doses of erythrosine and then some blockers.

Statistical Analysis

The data values for every experimental group were presented as means ± SEM. The force of contractions was computed using the frequency and amplitude of the movement records. The percentage change from the basal (or control) values represented the values of the treated preparations for the functional tests. To evaluate any significant differences between the groups, a one-way ANOVA was performed (GraphPad Prism 8). $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of Erythrosine on the Contractile Functions of the dVSM *Ex Vivo* of Rat

The contractile function of the dVSM in response to various doses of Erythrosine was assessed by recording the movement of the duodenum (*ex vivo*) in single dose acute experiments. Analysis of the tracings indicate that exposure to different concentrations of Erythrosine resulted in a dose-dependent decrease in the amplitude of the isolated duodenal segments. Additionally, the frequency of contractions of the dVSM did not decreased in a dose-dependent manner upon exposure to Erythrosine (Figure 1). The suppression of duodenal motility remained constant for the duration of the 10-minute experiment period. The duodenal motility was inhibited by more than 80% compared to the control at the 4µM dose, leading to the cessation of contractile activity for the remaining duration.

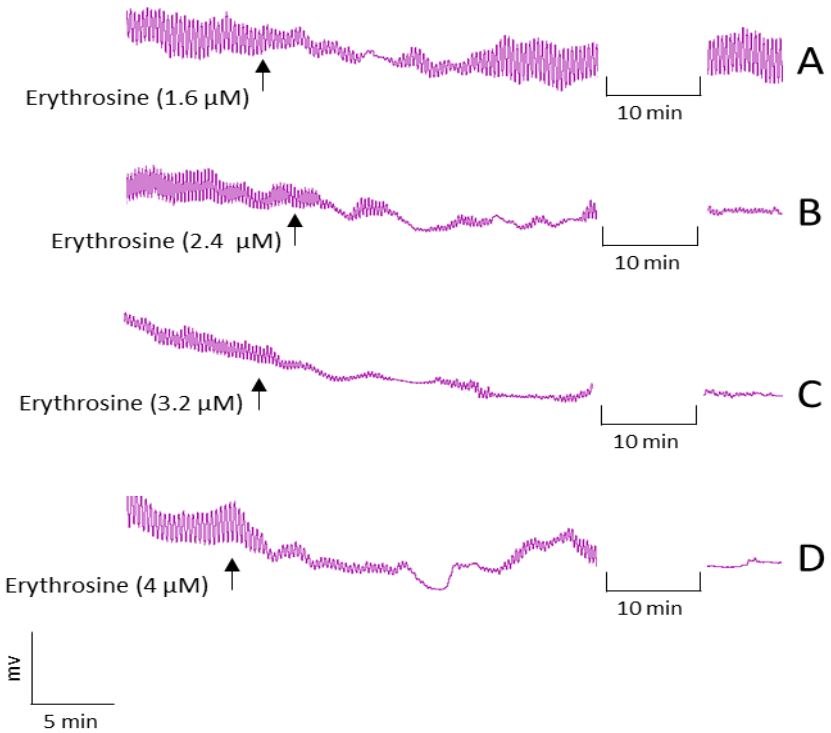

Figure 1. Tracings showing representative records of the effect of graded concentrations of erythrosine on the movement of duodenum of rat in tissue organ bath obtained with an isotonic transducer coupled to RMS Polyrite-D.

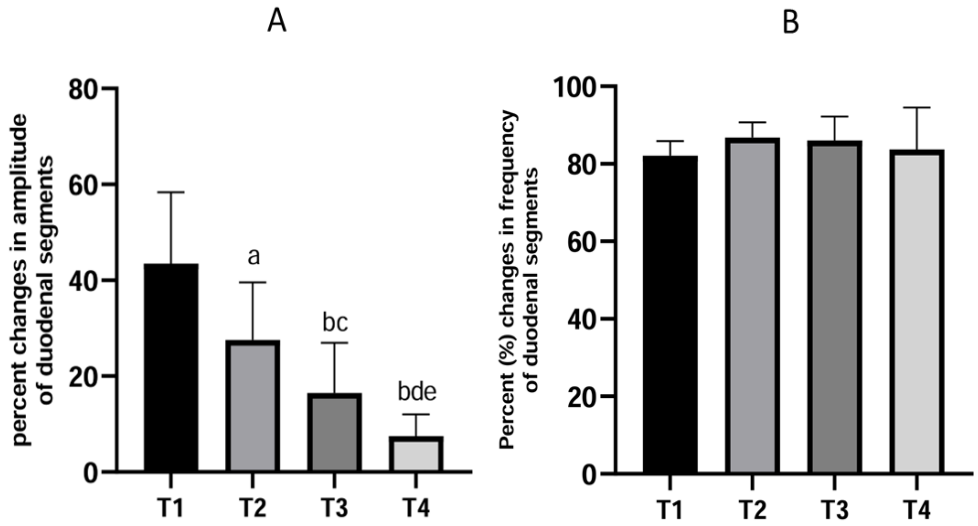

Figure 2. Bar diagram showing percent changes in amplitude and frequency of the contractions of isolated duodenum in response to the application of graded doses of erythrosine. The data represented were mean ± SEM for all the group. $^{b,a}P$< 0.0001, 0.001 Vs T1; $^{d,c}P$< 0.0001, 0.05 Vs T2; ^{e}P<0.05 Vs T3 (A).

The findings suggest that Erythrosine reduces the contractile activity of the dVSM by diminishing the force (amplitude) of the contractions in the smooth muscle within the duodenum's wall structure. The contractions of the visceral smooth muscle in the GI system are primarily regulated by its intrinsic myenteric efferents through excitatory cholinergic, inhibitory adrenergic, and inhibitory nitrergic (NANC) innervations. It is expected that the suppression of the contractile function of the dVSM by Erythrosine is likely a result of inhibiting the activity of cholinergic myenteric efferents and/or stimulating the activity of adrenergic/nitrergic (NANC) intrinsic myenteric efferents that innervate the dVSM.

Effect of Erythrosine and L-NAME in Combination on the Contractile Function of the dVSM *Ex Vivo* of Rat

To investigate the potential pharmacodynamic effects of Erythrosine -induced inhibition on the contractile function of dVSM, we studied the role of nitrergic intrinsic myenteric efferents through combination study. Nitrergic myenteric efferents primarily inhibit contractions of the visceral smooth muscle in the duodenal wall by releasing the inhibitory neurotransmitter Nitric Oxide (NO), which promotes relaxation of the visceral smooth muscle.

In order to investigate the impact of nitrergic influences on Erythrosine -induced suppression of dVSM contractile activity, we conducted a single dose acute experiment to record the movement of the duodenum *ex vivo* in response to the application of Erythrosine and L-NAME, a nitrergic antagonist (Nitric Oxide Synthase (NOS) inhibitor). The tracings showed that administering L-NAME alone did not cause any significant change in the contraction of the dVSM. However, the inhibition of dVSM contraction by Erythrosine was reversed when Erythrosine was combined with L-NAME compared to the inhibition caused by Erythrosine alone. This clearly suggests that Erythrosine augments the activity of intrinsic nitrergic myenteric efferents by promoting the activity of nitric oxide synthase, the enzyme responsible for NO synthesis in the myenteric neurons.

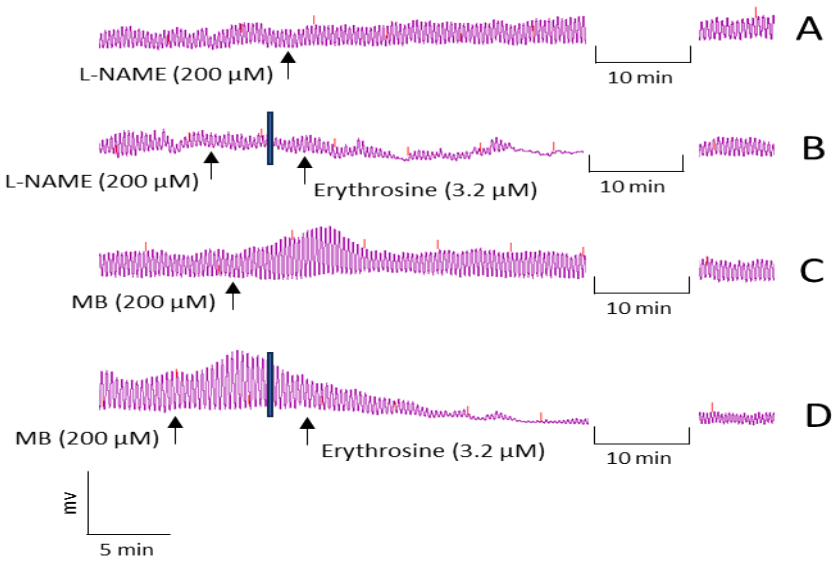

Figure 3. Tracings showing representative records of the effects of erythrosine on the movement of duodenum in L-NAME and MB pre-treated duodenal preparations *ex vivo*. A: Tracing of effect of L-NAME (200µM) on the movement of duodenum. B: Tracing of the effect of erythrosine (3.2µM) on the movement of duodenum in L-NAME (200µM) pre-treated duodenal preparations. C: Tracing of effect of MB (200µM) on the movement of duodenum. D: Tracing of the effect of erythrosine (3.2µM) on the movement of duodenum in MB (200µM) pre-treated duodenal preparations obtained with an isotonic transducer coupled to RMS Polyrite-D.

Effect of Erythrosine and MB in Combination on the Contractile Function of the dVSM *Ex Vivo* of Rat

The duodenum's movement was recorded in a single dose acute experiment to determine if the nitrergic signaling pathway was activated in Erythrosine -induced suppression of the contractile function of dVSM. The experiment carried out by applying Erythrosine and MB, a nitrergic antagonist (sGC blocker) in combination, and observing the response *ex vivo*. From the tracings, it can be stated that when MB was given alone, it did not cause any notable changes in dVSM contraction. However, when Erythrosine was applied in combination with MB, the inhibition of dVSM contraction caused by Erythrosine was reversed compared to the inhibition caused by Erythrosine alone.

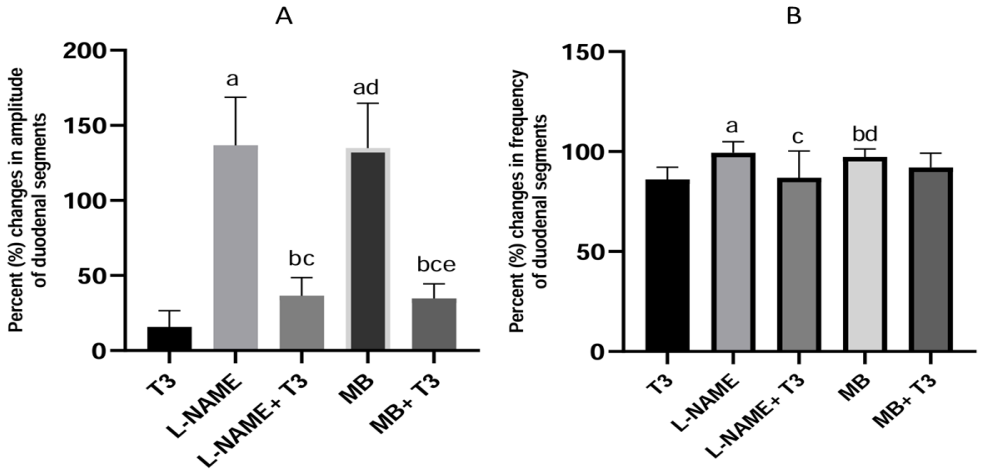

Figure 4. Bar diagram showing percent changes in amplitude and frequency of the contractions of isolated duodenum in response to the application of erythrosine in combination with L-NAME and MB. The data represented were mean ± SEM for all the group. $^{b,a}P$<0.05, 0.0001 Vs T3; ^{c}P< 0.0001 Vs L-NAME; ^{d}P< 0.0001 Vs L-NAME+T3; ^{e}P< 0.0001Vs MB (A). $^{a,b}P$<0.01, 0.05 Vs T3; ^{c}P< 0.01 Vs L-NAME; ^{d}P<0.05 Vs L-NAME+T3 (B).

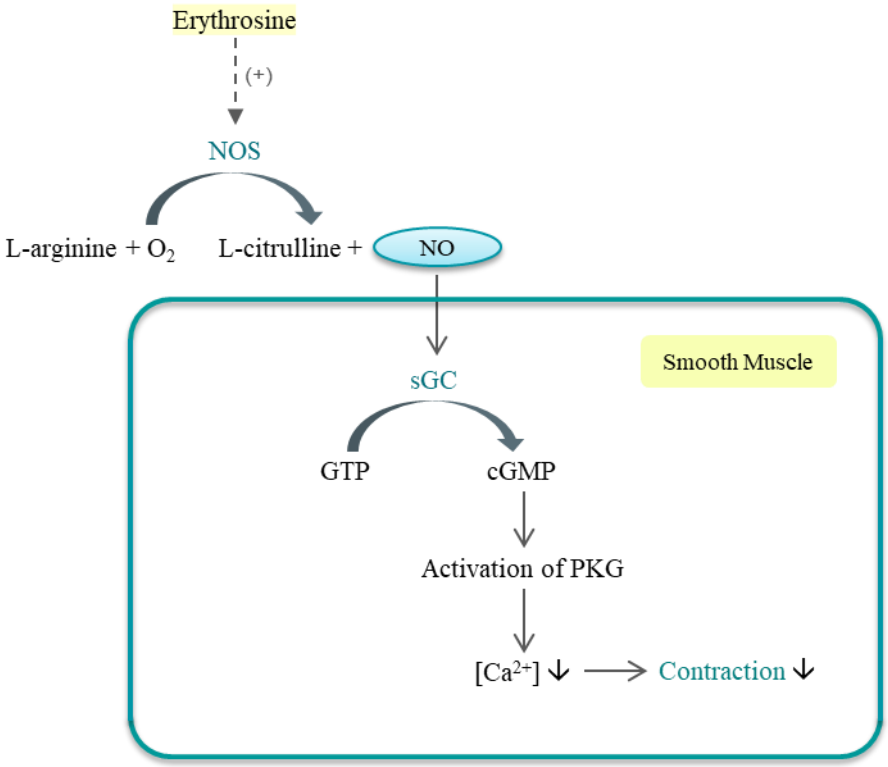

Figure 5. Schematic representation of the probable neurocrine mechanisms involved in the erythrosine induced suppression of the contractile function of the dVSM. (+); indicates stimulation, (-); indicates inhibition, ↓ indicates decrease in levels.

The findings indicated that both L-NAME and MB, which are nitrergic antagonists, restricted the inhibition of dVSM contraction induced by Erythrosine. This strongly indicates that Erythrosine inhibits the dVSM contractions by facilitating the activity of intrinsic nitrergic myenteric efferents that releases nitric oxide.

CONCLUSION

Erythrosine is a synthetic dye extensively used as a food colorant to make the food stuff attractive. It can be concluded that Erythrosine, hinders the contractile function of the dVSM by inhibiting the contractions of the visceral smooth muscle cells in the duodenal wall responsible for motility. The pharmacodynamic study revealed that Erythrosine inhibits the contractile function of the dVSM probably by facilitating the soluble guanylyl cyclase mediated nitrergic signaling pathway. So, it can be extrapolated that erythrosine might inhibit the contractile function of the dVSM in humans on consumption resulting in impaired digestion and absorption.

Declaration by Authors

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Conflict of Interest: The authors declare no conflict of interest.

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