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# Chocolate Brown HT Inhibits the Contractile Activity of the Small Intestinal Visceral Smooth Muscle in Male Albino Rats

**Soumi Biswas[1], Sandhi Paul[2], Sourapriya Mukherjee[1,3], Kamalesh Das[1,4], Goutam Paul[1]**

[1]Molecular Neurotoxicology Laboratory, Department of Physiology, University of Kalyani-741235, West Bengal, India.
[2]Ashiyan Medical College, University of Dhaka, Dhaka-1219, Bangladesh.
[3]Department of Physiology, KPC Medical College, Kolkata-700032
[4]Department of Physiology, Uluberia College, Uluberia, Howrah-711315

Corresponding Author: Goutam Paul

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## ABSTRACT

Artificial food dyes, especially azo dyes, have raised health concerns due to their physiological effects on various organ systems. Chocolate Brown HT (also known as E155 or Brown HT), a brown synthetic diazo dye has been widely used for food, cosmetic, and pharmaceutical applications. Till date no such studies have reported the effects of Chocolate Brown HT (CBHT) on the contractile activity of small intestinal visceral smooth muscle (SiVSM). So, this study has been designed to examine the effects of graded doses of CBHT on the contractile activity of SiVSM. To evaluate the effect of CBHT on duodenal visceral smooth muscle (dVSM) contractility, *ex vivo* recordings of duodenal movements were conducted using an isotonic transducer (IT-2245) connected to an RMS Polyrite D. CBHT inhibits the contractions of small intestine (duodenum) *ex vivo* as evidenced from the decrease in both the frequency and the amplitude of contractions in a dose-dependent manner. From the results it can be suggested that the CBHT induced inhibition of the contraction of the SiVSM might be due to suppression of the activity of intrinsic cholinergic myenteric efferents and/or facilitation of the activity of nitrergic and/or adrenergic intrinsic myenteric efferents. In conclusion, it can be suggested that CBHT inhibits the contractile activity of SiVSM in dose-dependent manner.

***Keywords:*** Chocolate Brown HT, azo dye, contractile activity, small intestinal visceral smooth muscle, intrinsic myenteric efferents.

## INTRODUCTION

Synthetic food colorants are widely used additives in the global food industry, primarily to enhance the appearance and acceptability of products such as beverages, candies, desserts, and bakery goods. Among these, azo dyes represent the most extensively used class, accounting for over 60% of all food dyes employed in processed food products (Chung, 2016). One such azo dye, Chocolate Brown HT (also known as E155 or Brown HT), is a brown synthetic diazo dye permitted in certain countries for food, cosmetic, and pharmaceutical applications (EFSA, 2010; Chequer et al., 2011). Chocolate brown HT (CI (1975) No. 20285) is a disodium salt of 4,4-[(2,4-

dihydroxy-5-hydroxymethyl1,3-phenylenebisazo) di (naphthalenel-sulphonic acid).

This is used in ice cream, soft drinks, puddings, and sauces, fish and meat spreads, dessert mixes, sugar and flour confectionary, and preservatives (Food Additives and Contaminants Committee, 1979). Despite its approval by food regulatory agencies in parts of the European Union and India, Chocolate Brown HT has been banned in the United States, Canada, Norway, and Japan due to reported toxicological concerns (EFSA, 2010; Islam et al., 2024). Concerns regarding the safety of synthetic dyes stem from a growing body of literature linking their consumption with adverse health outcomes, including hyperactivity in children, allergic reactions, cytotoxicity, genotoxicity, and even carcinogenicity (Chung, 2016). *In vivo* and *In vitro* studies have revealed that many azo dyes undergo azoreductive metabolism in the gut, producing aromatic amines that can be mutagenic or carcinogenic (Rahman et al., 2024). It significantly lowers hemoglobin, leucocyte, and red cell counts, as well as hematocrit and serum urea levels (Drake et al., 1978; Sultana et al., 2023). It exhibits harmful effects on the brain by lowering levels of dopamine, norepinephrine, and gamma-aminobutyric acid (Bawazir, 2012). It suppresses the female reproductive system and impairs the male reproductive system by lowering serum levels of GnRH, LH, FSH, and testosterone (Khatun et al., 2017; Abbas et al., 2019).

The small intestinal visceral smooth muscle (SiVSM), plays a vital role in peristalsis, digestion, and nutrient absorption through its contraction and relaxation that provides motility to the small intestine. GI motility is governed by the intricate interplay of enteric neurons, interstitial cells of Cajal, smooth muscle contractile proteins, and neurotransmitters such as acetylcholine, nitric oxide, and serotonin (Grundy et al., 2006; Sanders et al., 2006; Furness, 2012). Disruption in this neuromuscular coordination, either by pharmacological agents or toxic compounds, may result in GI dysmotility, constipation, diarrhea, or abdominal discomfort (Rao & Gershon, 2016). Previous research has demonstrated that certain food dyes such as metanil yellow significantly alter GI motility by modulating neurotransmitter pathways, affecting calcium homeostasis, or inducing oxidative stress in smooth muscle tissues (Rahman et al., 2019; Ramchandani et al., 1997)

Thus, disruption in this neuromuscular coordination, either by pharmacological agents or toxic compounds, may result in impaired digestion and malabsorption and also led to onset of various diseases such as GI dysmotility, constipation, diarrhea, or abdominal discomfort (Rao & Gershon, 2016). Despite the biological plausibility and established risks associated with several azo dyes, the impact of Chocolate Brown HT on the contractile activity of the SiVSM remains largely unexplored till date. Therefore, the present study was designed to understand the effect(s) of Chocolate Brown HT on the contractile activity of the isolated duodenal (small intestinal) VSM in male albino rat *ex vivo* in rats.

## MATERIALS & METHODS

### Chemicals and Reagents

All the reagents and chemicals that were used to conduct this study were of analytical grade. The test chemical- Chocolate Brown HT (E155 or Brown HT) was procured from Sigma-Aldrich. 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 male albino rats of Sprague Dawley strain with body weight ranging around 130-150 g and age around 2-3 months were selected. They were kept in the room temperature of 25-27°C at the departmental animal care room

with 24 hours light-dark cycle and were fed with 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 condition |
|---|---|
| Set 1 | Application of 100 µM Chocolate Brown HT on the duodenal segments |
| Set 2 | Application of 200 µM Chocolate Brown HT on the duodenal segments |
| Set 3 | Application of 300 µM Chocolate Brown HT on the duodenal segments |
| Set-4 | Application of 400 µM Chocolate Brown HT on the duodenal segments |

**Animal Sacrifice**

The selected animals were subjected to overnight fasting prior to sacrifice to standardize physiological conditions. Euthanasia was performed using cervical dislocation, ensuring minimal pain and distress, in strict accordance with the ethical guidelines approved by the Animal Ethics Committee of the University of Kalyani.

**Collection of the Organ**

Following cervical dislocation and confirmation of death, the animal's abdominal cavity was opened. The small intestine was carefully dissected free from the mesentery, stomach, and large intestine via transverse incisions. The proximal portion of the small intestine, specifically the duodenum, was isolated for the study, as it exhibits the most prominent motility among intestinal segments. The collected duodenal segment was immediately transferred to a beaker containing temperature-controlled Tyrode's solution. The lumen was gently flushed to remove any residual contents. The cleaned segment was then promptly mounted in the organ bath of Dale's apparatus for *ex vivo* recording of spontaneous duodenal motility.

**Recording of the Movement of the Duodenum**

To record the spontaneous *ex vivo* motility of duodenal visceral smooth muscle (dVSM), a duodenal segment approximately 3 cm in length was vertically suspended in an organ bath containing 40 ml of Tyrode's solution. The segment was secured using two metal hooks inserted at both ends of the tissue. The composition of Tyrode's solution included: 8.0 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.1 g $MgCl_2$, 0.05 g $NaH_2PO_4$, 1.0 g $NaHCO_3$, and 1.0 g dextrose per liter, adjusted to pH 7.4. Oxygenation was maintained using a continuous flow of oxygen at a rate of 2–3 bubbles per second, delivered directly into the organ bath via an oxygen bubbler. The bath temperature was kept at 37 ± 0.5°C using an automatic thermostat integrated with Dale's apparatus. The lower end of the duodenal tissue was anchored to the base of the organ bath, while the upper end was connected to the lever of an isotonic transducer (IT 2245). The transducer was interfaced with RMS Polyrite-D software (RMS, Chandigarh, India) to enable continuous recording of tissue contractions. Each tissue segment was allowed to stabilize for at least 35 minutes under these experimental conditions and was rinsed multiple times with fresh Tyrode's solution to remove metabolic residues. Isotonic contractions representing spontaneous rhythmic motility were recorded continuously following the administration of various concentrations of coumarin and selected pharmacological blockers.

## STATISTICAL ANALYSIS

Data from each experimental group were expressed as mean ± SEM. The frequency and amplitude of the recorded duodenal movements were analysed to determine the contractile force. For functional assessments, the responses of treated tissues

were calculated as percentage changes relative to their respective basal (control) values. Statistical comparisons among groups were performed using one-way ANOVA with GraphPad Prism 8 software. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

Exposure to graded doses of CBHT on isolated duodenal segments in an organ bath setup significantly reduces both the amplitude and frequency of duodenal contractions in a dose-dependent manner as obtained from the recorded progressive decline in contractile activity with increasing concentrations of CBHT (Figure 1, Figure 2 and Table 2).

The small intestine performs various digestive, absorptive, secretory, and immunological functions, which are coordinated by the contractile activity of visceral smooth muscles (VSM) situated in the muscularis externa layer. These contractions are essential for mixing luminal contents with pancreatic and biliary secretions and for propelling the chyme distally, thus facilitating digestion and absorption. The regulation of intestinal motility is orchestrated by the interstitial cells of Cajal (ICCs), which are located within the intestinal musculature. ICCs generate and propagate electrical slow waves (ESWs) that determine the excitability of smooth muscle cells. Moreover, they mediate neural signals from the enteric nervous system (ENS), thus integrating myogenic and neurogenic control of motility.

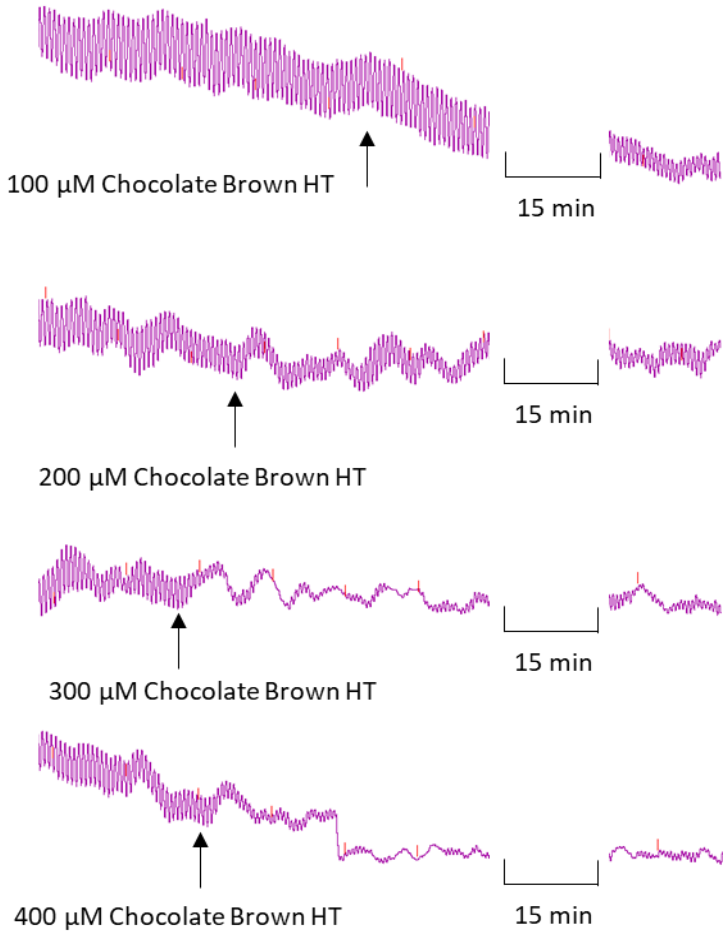


**Figure 1.** Tracings showing representative records of the effect of graded concentrations of Chocolate Brown HT (CBHT) on the isolated duodenal segment in order to examine the effect of CBHT on the contractile activity of the SiVSM in rat *ex vivo* obtained with an isotonic transducer coupled to RMS Polyrite-D.

**Table 2. Table showing the percent alterations in amplitude and frequency of contractions of the dVSM as a result of exposure to CBHT**

| Concentration (M) | % Inhibition of Amplitude | % Inhibition of Frequency |
|---|---|---|
| 100 µM CHBT | 73.17 ± 3.002 % | 103.7 ± 0.651% |
| 200 µM CHBT | 48.96 ± 1.579 % | 98.45 ± 0.729 % |
| 300 µM CHBT | 35.85 ± 3.387 % | 93.34 ± 1.514 % |
| 400 µM CHBT | 23.92 ± 1.992 % | 82.59 ± 2.709 % |

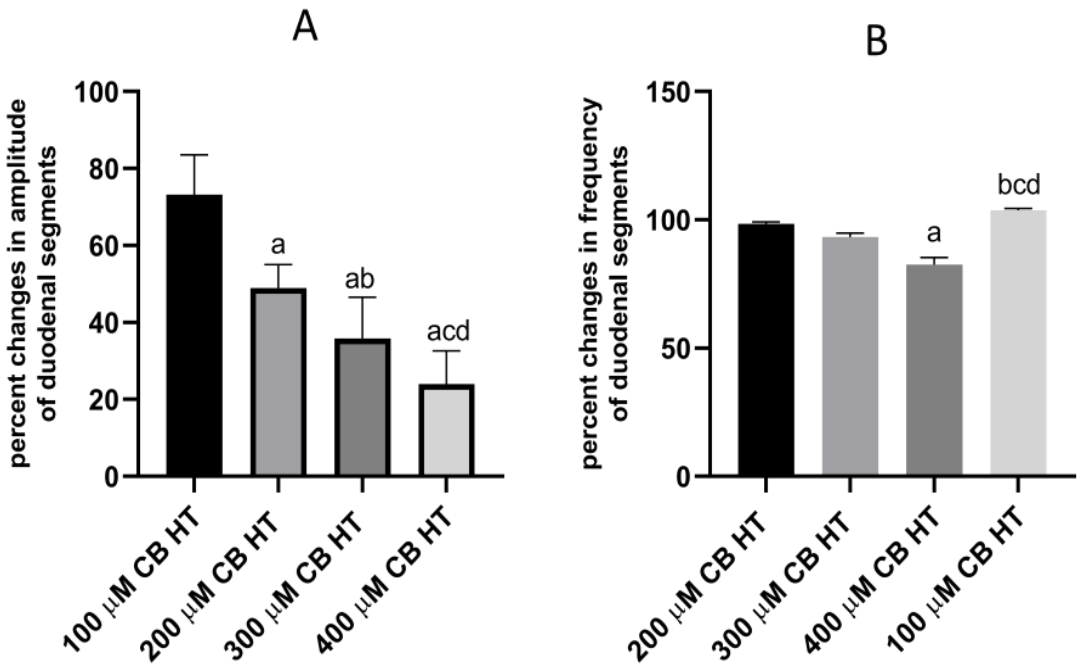


**Figure 2.** Bar diagrams showing the percent changes in the amplitude (A) and frequency (B) of contraction of the duodenum in CBHT exposed groups (100 µM, 200 µM, 300 µM, 400 µM) compared to control. The data were represented as mean ± SEM for all the groups. [a]$p$<0.001 vs. 100 µM CBHT, [b,c]$p$< 0.01, 0.001 vs. 200 µM CBHT and [d]$p$<0.01, vs. 300 µM CBHT (A). [a,b]$p$<0.01,0.0001 vs. 100 µM CBHT, [c]$p$<0.0001 vs. 200 µM CBHT and [d]$p$<0.01, vs. 300 µM CBHT (B).

Based on these findings, it is plausible to hypothesize that CBHT may inhibit the frequency of the contractions of the dVSM probably through decrease in the generation and/or propagation of ESWs by disrupting ICC function. It may also interfere with the transduction of excitatory neural inputs from the ENS, potentially through alterations in neurotransmission pathways or disruptions in the membrane potential gradients of smooth muscle cells, thereby impairing the basal electrical rhythms (BERs). Furthermore, the CBHT induced inhibition of the amplitude of the contraction of the dVSM might result from the suppression of excitatory cholinergic myenteric efferents and/or facilitation of inhibitory adrenergic or nitrergic (non-adrenergic, non-cholinergic; NANC) intrinsic myenteric efferents innervating the dVSM (Figure 3).

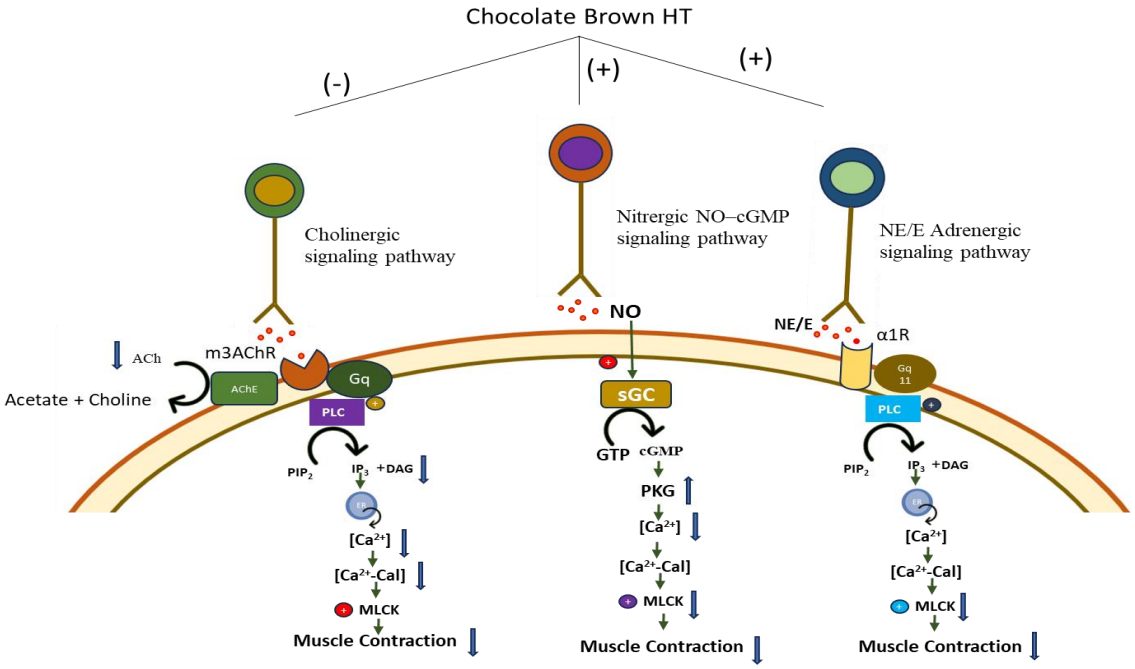


**Figure 3.** Schematic representation showing the probable intrinsic myenteric efferents involved in the CBHT induced inhibition of the contraction of the dVSM. CBHT- chocolate brown HT; ACh-acetylcholine; NO-Nitric Oxide; NE/E- Norepinephrine/Epinephrine; AChE- Acetylcholinesterase; sGC- soluble guanylyl cyclase; [$Ca^{2+}$]-Intracellular calcium concentration; Cal- Calmodulin; MLCK- Myosin light chain kinase. ⬇, indicates decrease in activity or production; ⬆, indicates increase in activity or production. +, indicates facilitation; -, indicates inhibition.

## CONCLUSION

In conclusion, CBHT could be considered as a potent toxicant and it should be recognised as food adulterant rather than food additive. CBHT on exposure significantly suppresses the contractile activity of small intestinal visceral smooth muscle (SiVSM) located in the muscularis externa layer of the rat small intestine by decreasing the frequency and amplitude of contractions of the duodenum, the integral part of the small intestine. This inhibitory effect may result from the suppression of the activity of excitatory cholinergic myenteric efferent and/or the enhancement of the activity of inhibitory noradrenergic and/or non-adrenergic non-cholinergic (NANC) myenteric efferent that innervate the SiVSM. Consequently, chronic or repeated exposure to CBHT-contaminated food may impair key physiological functions of the small intestine—such as digestion, absorption, and secretion—posing potential health risks to humans.

### *Declaration by Authors*

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

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