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Tryptophan administration increase contractility and change the ultrastructure of mice duodenum

Ç. Özer[1], B. Gönül[1], G. Take[2], D. Erdoğan[3], E. Tong[3], and **Z. S. Ercan[4]**

[1] Department of Physiology, Gazi University, Faculty of Medicine,
[2] Department of Histology and Embriology, Başkent University, Faculty of Medicine,
[3] Department of Histology and Embriology, Gazi University, Faculty of Medicine, and
[4] Department of Pharmacology, Gazi University, Faculty of Medicine, Beşevler Ankara, Turkey

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Summary. Serotonin (5-HT) is a metabolite of tryptophan (TRP). 5-HT has been shown to induce contractions in rat duodenum and ileum. We planned to investigate the *in vivo* effects of TRP administration on duodenal contractility and ultrastructure together.

Two equal groups of adult male Swiss-albino mice were used in the experiments.

Controls (CONT) and TRP treated (100 mg/kg/24 hr in 0.2 ml. saline solution ip, 7 days).

Body weights were recorded at the beginning and at the end of experiments. Duodenum tissues contractility responses to different concentration of KCl and acethycholine (ACh) were recorded on polygraph. The ultrastructural changes in duodenum observed by transmission electron microscopic (TEM) method and 5-HT levels determined by immunohistochemical method.

Body weights decreased and duodenal contractile response of ACh increased significantly by TRP treatment. The duodenal ultrastructural changes in TRP group illustrated partially loss of apical surface and fusion in microvilli. Immunohistochemical detection showed that 5-HT increased by TRP treatment.

There is a relation between duodenal contractility increased by TRP treatment and changes in the duodenal tissue 5-HT level and ultrastructure.

Keywords: Tryptophan – Serotonin – Duodenal contractility – TEM – Immunohistochemistry

1 Introduction

TRP is one of amino acids required for the synthesis of tissue proteins (Peters, 1991). TRP precursor of 5-HT and transported across the blood-brain barrier (Fernstrom, 1991). The conversion of TRP to 5-HT occurs in several tissues throughout the body including the entero chromaffin (EC) cells of the gut, blood platelets and central nervous system (Peters, 1991). 5-HT is a well established neurotransmitter produced and active in nervous tissues and digestive tract (Vander et al., 1998). 5-HT is effective both on brain and gastrointestinal tract (GIT) functions (Pilot et al., 1983). 5-HT is involved in the central regulation of feeding behavior. Increased brain levels of 5-HT result in decrease food intake (Rotter et al., 1996). The GIT represents the largest depot of 5-HT in the body. Within the gut 5-HT is found both in EC cells in epithelium of the mucosa and in neurons of the myenteric plexus (Gershon, 1991). Physiological studies suggest that 5-HT plays important role in mediating GIT sensitivity and motor activity, secretion and more complex activities such as emesis and diarrhoea (Reed and Gwee, 1994). 5-HT involved in the regulation of gastrointestinal motility (Pilot et al., 1983). 5-HT has been shown to induce contractions in rat duodenum and ileum (Salvador et al., 2000). We planned to investigate the *in vivo* effects of TRP administration on duodenal contractility and ultrastructure together.

2 Material and method

Two equal groups of adult male Swiss-albino mice weighing 40 ± 4 g (Gazi University, Medical Faculty, Animal Breeding Unit) were used in the experiments. Animals were fed with chow (Korkuteli-Turkey) ad libitum, daily mean consumption was 15 g/100 g BW. They were used in compliance with the European Community guidelines for the use of experimental animals.

1. Controls (CONT) treated with physiological saline solution (0.9% NaCl) 0.2 ml/24 hr, intraperitoneally-ip, 7 days).
2. TRP group treated with L-TRP (Merck-9770, only one injection 100 mg/kg/24 hr in 0.2 ml. saline solution ip at 9 am for 7 days) (Erikson and Walinder, 1998).

Animals body weights were recorded at the beginning and at the end of the 7 days of experimental period. At the end of the experiments mice were killed by decapitation under ether anesthesia. The duodenal (2 cm) parts of intestines were removed immediately.

2.1 Preparation of tissues for concentration-response curves

The experiments were performed on the rapidly removed duodenal tissues. Isolated mice duodenum (10 mm) was suspended in 10 ml organ bath containing warmed (37°C) and aerated (5% CO_2 in O_2) Tyrode solution with 2 g initial tension. The tissues were allowed to equilibrate for 45 min until a steady-state baseline was obtained. Tyrode solution was prepared as follows (mM) NaCl 136.8; $CaCl_2$ 1.8; KCl 2.7; NaH_2PO_4 0.42; MgCl 1.05; $NaHCO_3$ 11.9; glucose 5.6 at pH 7.4. Concentration-dependent contractility responses to KCl (10 mM–80 mM) and ACh (10^{-7} M–10^{-4} M) were obtained in each duodenum and were recorded with Grass Model 7 Polygraph (Grass Instruments Co., Quincy, Massachusetts) by using force displacement transducer (Model FTO3, Grass Instruments). The contractile responses obtained by agonist were estimated either by the change of g-tension or by the % of maximum (Max) response of each preparation.

2.2 Histological studies

2.2.1 Electron microscopical studies

One cm of dissected duodenal parts were used for TEM and immunohistochemical experiments. Tissue samples were prepared for transmission TEM. The pieces were placed in 2.5% glutaraldehyde and then in phosphate buffer saline post fixative 1% osmium tetraoxide. The specimens were fırstly embedded in Dodecyenyl Succinic Anhydride (DDSA) + Araldyt CY 212 (1:1, v/v) for overnight at room temperature, then 24 hr at 40°C and 48 hrs at 60°C. Thin sections were stained with lead citrate and uranyl acetate and were photographed using Carl Zeiss EM 900 electron microscope.

2.2.2 Immunohistochemical experiments

Preparation of tissue samples; Pieces of duodenum were transported immediately 10% formal saline, dehydrated in graded alcohols and embedded in paraffin wax. 4 μm sections were cut on a microtome (Leica SM 2000, Germany) and mounted on polylizine-coated slides.

Antibodies and staining procedure; Slides were de-waxed. Following rehydration through a descending ethanol series, endogenous enzymes were blocked using 1.2% hydrogen peroxide. In order to increase the immunoreactivity of formalin-fixed paraffin wax sections a protease step is necessary. Slides were therefore incubated in 0.1% protease (Protease XXV, Cat # 9006-002, Neomarkers, USA) for 10 min at 37°C. Following a phosphate buffer wash slides were blocked using normal goat serum prior to the application of a 1:100 concentration of monoclonal mouse anti-serotonin primary antibody (Lot # 020-4, Cat # N1530, DAKO Corporation, USA). Two phosphate buffer rinses preceded secondary antibody application, biotinylated antimouse lg (Lot # 080-1, Cat # K0673, DAKO Corporation, USA), 1:300 for 30 min. TRP epitopes were identified using the avidin biotin (ABC) complex method and visualized with the use of DAB (diaminobenzidine tetra hydrochloride, Lot # 080-1, Cat # K0673, DAKO Corporation, USA). Control slides were prepared using the same method omitting either primary antibody. Afterwards, the slides were counterstained with hematoxylin for 1 minute, dehydrated in graded ethanol and mounted with entellan (Mikroskopie Entellan # 740212765, Merck, Germany).

2.3 Statistical analysis

Data are expressed as means ± SEM. Differences between groups were considered significant at $P < 0.05$ by Mann Whitney U test by using Statview computer program because of 2 groups and less than 20 animals in each group.

3 Results

3.1 Body weight and food consumption results

There was no difference in the body weight of controls at the end of 7 days but there was significant decrease in the body weight of TRP treated ones (13.6%). In the TRP treated group, food consumption is 30% of controls (Table 1).

3.2 Duodenal contractility results

Duodenal contractilities induced with KCl didn't changed in TRP group compared to control group. Duodenal contractile response of ACh at the concentration of 10^{-4} M as g-tension change increased significantly by TRP treatment (0.99 ± 0.10 for control, 1.34 ± 0.10 for TRP treated group, $p < 0.05$). Duodenal contractilities induced with ACh are shown in Fig. 1 as % Max. Contractile responses of duodenum to ACh with % Max at the concentrations of 10^{-5} M-10^{-4} M were found to be significantly increased in TRP treated groups ($p < 0.01$, $p < 0.001$).

3.3 Histological results

3.3.1 Electron microscopical results

The ultrastructural changes in duodenum of control and TRP treated mice are shown and explained in Fig. 2a, b

Table 1. The effects of TRP treatment on the body weight and daily food intake

Treatment	Body weight changing		Daily food intake
	g	%	g
Control (n = 12)	$+1.7 \pm 0.46^{a}$	+2.3	9.43 ± 0.16^{a}
TRP treated (n = 12)	-4.3 ± 0.66^{b}	−13.6	7.30 ± 0.20^{b}

Each value is the mean ± SEM. The mice treated with TRP 100 mg/kg/daily, ip, 7 days. Control groups received isovolumetric amount of serum physiologic in the same manner. Animals were weighted in the beginning and at the end of experimental period. Control and TRP groups were allowed to ad libitum, Meal parameters were analysed for daily
Body weight changing; difference statistically significant $p < 0.05$ a–b
Daily food intake of groups; difference statistically significant $p < 0.01$ a–b by Mann-Whitney U test

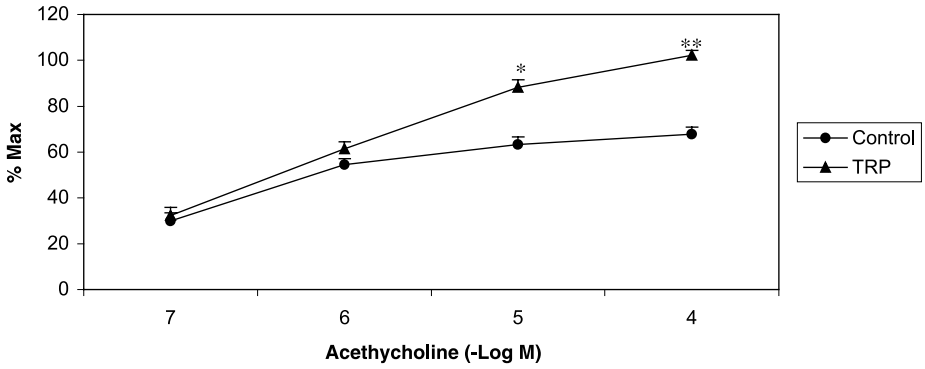

Fig. 1. % Maximum contraction of ACh in the isolated mice duodenum. Each value is mean ± SEM. The mice treated with TRP 100 mg/kg/daily, i.p., 7 days. Concentration-dependent contractility responses to KCl (10 mM–80 mM) and ACh ($10^{-7}-10^{-4}$ M) were obtained in each duodenum and were recorded with Grass Model 7 Polygraph. Difference statistically significant $^{*}p<0.01$, $^{**}p<0.001$ by Mann-Whitney U test

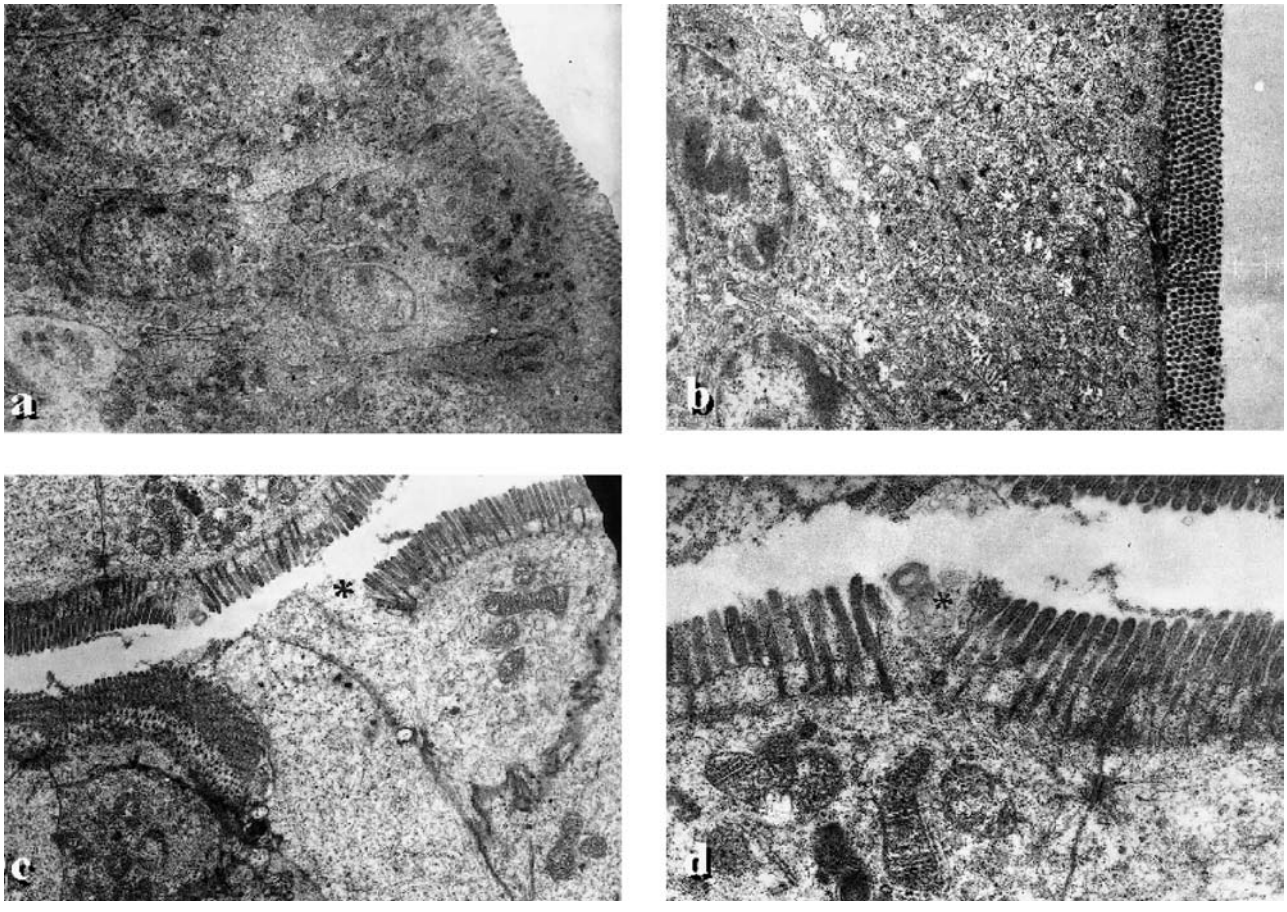

Fig. 2. **a**, **b** Electron microscopy of mice duodenum tissues in control group. TEM of cross-section through control groups duodenum illustrating the normal structure. (Uranyl acetate-Lead citrate, ×3000, ×4400 respectively). **c**, **d** Electron microscopy of mice duodenum tissues in TRP treated group TEM of cross-section through TRP groups duodenum illustrating partially loss of apical surface and fusion in microvilli and secretory release (∗) (Uranyl acetate-Lead citrate, ×4400, ×12000 respectively)

(TEM of cross-section through control groups duodenum illustrating the normal epithelial cells, organels and microvilli) and Fig. 2c, d (TEM of cross-section through TRP treated groups duodenum illustrating partially loss of apical surface with granular fusion in microvilli and secretory release) respectively.

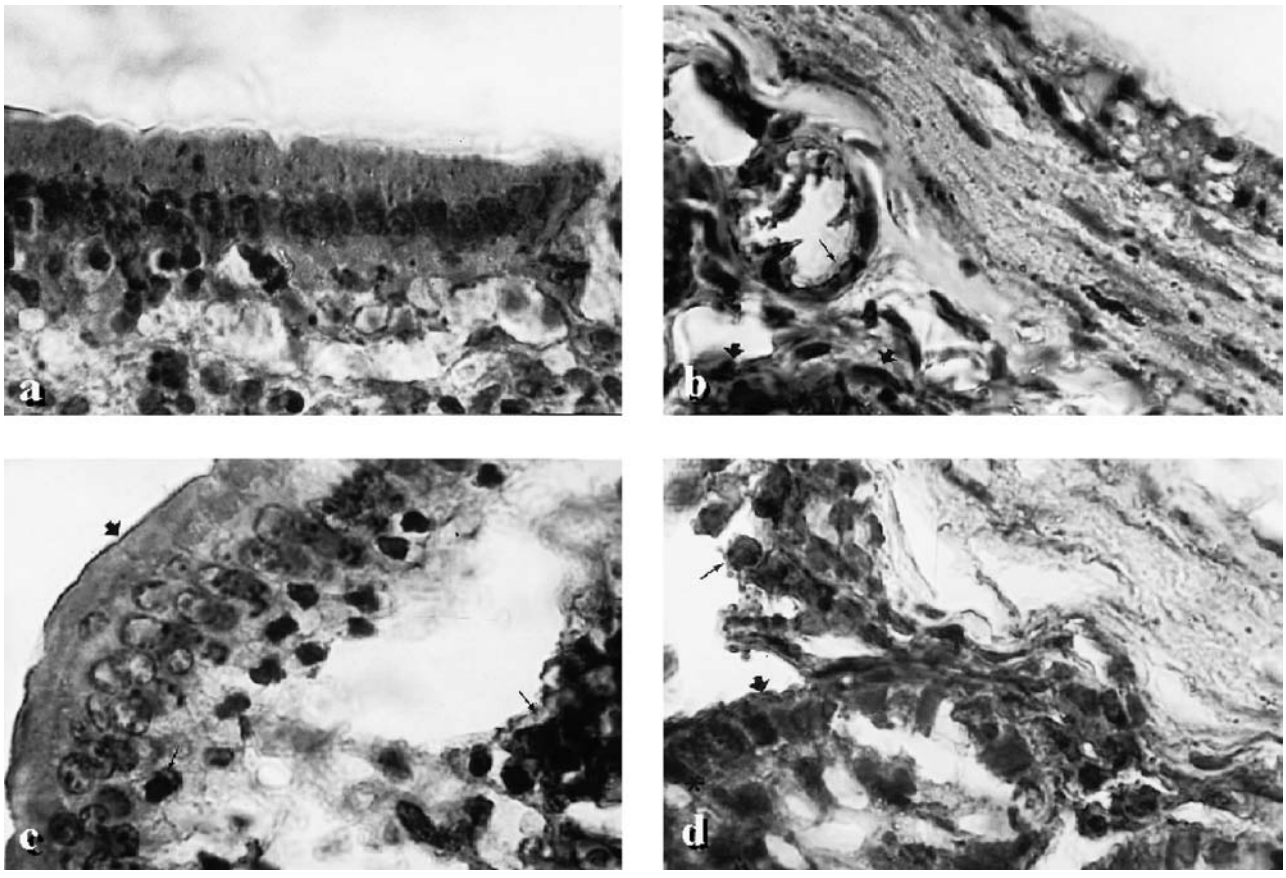

Fig. 3. **a**, **b** Serotonin immunoreactivity of mice duodenum tissues in control group. **a** Generally negative to weak immunoreactivity was seen absorptive cells and connective tissues. (Immunoperoxidase hematoxylen ×100). **b** Strong immunoreactivity was observed in the basal parts of the intestinal gland (⬆) and widespread cytoplasmic reactivity was seen in the blood vessel of tunica submucosa (↑) (Immunoperoxidase hematoxylen ×100). **c**, **d** Serotonin immunoreactivity of mice duodenum tissues in TRP treated group. **c** Strong staining was seen in absorptive cells (⬆) and connective tissue cells (↑). (Immunoperoxidase hematoxylen ×100). **d** Strong immunoreactivity was observed in basal parts of intestinal gland (⬆), enteroendocrine cells of glands (∗) and some cells in submucosa (↑). (Immunoperoxidase hematoxylen ×100)

3.3.2 Immunohistochemical results

Control group: Weak 5-HT immunoreactivity was observed in the control group duodenum sections when compared to TRP group (Fig. 3a, b). There were no staining in the surface absorptive cell membranes, however in cytoplasm, weak staining was detected. No immunoreactivity was seen in the connective tissues (Fig. 3a). Strong membranous and widespread cytoplasm staining was observed in the basal parts of intestinal glands. Widespread cytoplasmic immunoreactivity was seen in the blood vessels of tunica submucosa. There was not 5-HT immunoreactivity in the muscle layer muscle cells (Fig. 3b).

TRP group: In TRP treated group, strong 5-HT immunoreactivity was observed in duodenum when compared to the control group (Fig. 3c, d). In the surface absorptive cells, strong staining was dedected. In some cells weak immunostaining was seen in apical cytoplasm. Strong staining was determined in some connective tissue cells (Fig. 3c). In generally, strong immunoreactivity was found in the basal of intestinal glands. Strong staining was determined in some of the cells which were seemed to be enteroendocrine cells. Strong reactivity was seen in some of the cells in submucosa. No reaction was found in smooth muscle cells (Fig. 3d).

4 Discussion

TRP is essential for protein synthesis and precursor of 5-HT (Peters, 1991). Fernstrom and Wurtman have shown that when plasma TRP concentrations rise in rats receiving TRP, brain TRP and 5-HT concentrations also increase. Eriksson and Walinder showed that i.p. administration of L-TRP alone ($100\,mg\,kg^{-1}$) resulted in an increase in the concentration of TRP in the rat brain

from 14 ± 0.7 to $100 \pm 4.3\,\mathrm{nmol\,g^{-1}}$ compared to rats given saline only and also administration of L-TRP alone resulted in an increase in rat plasma TRP ratio (concentration of L-TRP/total concentration of large neutral amino acids) from 0.14 ± 0.003 to 0.42 ± 0.011 compared to rats given saline only (Eriksson and Walinder, 1998). Such variations in amine concentration reflect the general dependence of the rate of 5-HT formation on the degree of saturation of tryptophan hydroxylase (TPH). Fernstrom and Wurtman suggested that the brain TRP elevations were direct responses to the increases in plasma TRP and that, generally, any perturbations which increased plasma TRP would similarly increase brain TRP and 5-HT (Fernstrom and Wurtman, 1972). This results were also supported by Pietroszek and coworkers (Pietroszek et al., 1992). We suggested that GIT, TRP and 5-HT elevations are also related by plasma TRP levels and we demonstrated immunohistologically that ip TRP supplementation increased 5-HT levels of intestine (Özer et al., 2002b). Yu and coworkers supported our results by showing TPH immunoreactivity in GIT (Yu et al., 1999). It is generally accepted that TPH which catalyzes the formation of 5-hydroxy-L-tryptophan, is the initial and rate-limiting enzyme in the biosynthesis of 5-HT. Meyer and Brinck also demonstrated high TPH levels in the small intestine by immunohistochemical method and they suggested that intestinal epithelium has a functional role in the biosynthetic pathway of 5-HT (Meyer and Brinck, 1999).

Serotonergic mechanisms are also important in the control of appetite. Injection of 5-HT into paraventricular nucleus inhibits feeding, causing a decrease in the size and duration of meals (Leibowitz and Shor-Postner, 1986). In the present study decreasing body weight in TRP treated group shows the 5-HT increase in the brain and its effect on the eating behaviour. We demonstrated 5-HT increase by immunohistochemical method in the mice brain by ip TRP administration (Özer et al., 2002a).

The regulation of GIT activity by 5-HT is well documented. Experimental evidence indicates that 5-HT may serve as local regulator of gastrointestinal motility. Release of 5-HT from EC cells of the mucosal epithelium may induce a local secretagogue effect and inhibition of absorption of nutrients together with an augmentation of spontaneous and peristaltic activity (Salvador et al., 2000).

Although the GIT is an important source of melatonin, synthesized from TRP (Bubenik et al., 1996) and exerts relaxing effect on rat ileal smooth muscle via apamin-sensitive K^+ channels (Reyes-Vazquez et al., 1997). The enzymes for the synthesis of melatonin from TRP have also been found in the gastrointestinal system (Lee and Pang, 1993). In this study the effect of melatonin in the isolated mice duodenum was not studied. Because it has been shown that following TRP administration no detectable melatonin synthesis is found by HPLC measurement in the duodenum (Brammer, 1994).

GTP binding proteins such as ACh, histamine and 5-HT are involved in regulating cellular activity (Greger and Windhorst, 1996). *In vivo* microdialysis experiments determined that activation of the 5-HT_4 receptor located in the GIT, stimulated intestinal motor activity associated with local increase in ACh release from the intestinal cholinergic neurons (Taniyama et al., 2000). Tissue amounts of 5-HT in mammalian intestine tend to be highest in the duodenum. In the present study, duodenal contractility induced by ACh, has increased by TRP treatment. Contractile effect of ACh induced by TRP treatment is not mediated by a decrease in the food intake. Since the food intake was diminished to half of controls the contractility of ACh in the mice duodenum did not change when compared with controls (unpublished data).

Teichberg and coworkers have shown that L-TRP suppressed intestinal fluid flow and increased electrolyte transport *in vitro* and produced morphologic alteration in absorptive epithelial cells. Morphologically, L-TRP treatment lend to the formation of clear basal vacuolization in absorptive epithelial cells and loss of the lumenal permeability barrier to macromolecules (Teichberg et al., 1989). Fujimiya and coworkers showed that luminal release of 5-HT increased with elevated intraluminal pressure by immunoelectron microscopic study. Their findings indicate that luminal 5-HT release after raising the intraluminal pressure and 5-HT normally stored in the secretory granules of enterochromaffin cells, appears to be released into the cytoplasmic matrix and diffuses or is transported into the intestinal lumen (secretory granules are no longer dense and immunogold particles are localized over the cytoplasmic matrix and microvilli) (Fujimiya et al., 1997).

In the present study the luminal secretory release is also increased in TRP group (Fig. 2c, d).

TEM of cross-section through TRP groups duodenum illustrating aggregation of secretory granules and fusion in microvilli (Fig. 2c, d) is similar with Fujimiya and coworkers, Teichberg and coworker's results. We also studied 5-HT level in TRP treated group's duodenum with immunohistochemical method, and 5-HT increased with TRP treatment compared to controls duodenal tissue cells (Fig. 3c, d).

In conclusion; increased duodenal contractions in TRP group is related by elevated 5-HT levels in duodenal tissue. Administration of TRP causes an increase in brain as well as peripheral 5-HT levels. According to the results of the present study decrease in the food intake by TRP is not the only factor for the loss of body weight but also the increase in the contractility of the duodenum might be another factor.

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Authors' address: Bilge Gönül, PhD, Prof. Dr., Gazi Ün. Tıp Fak. Dekanlık Bin. Kat 4., Fizyoloji AbD 06510 Beşevler/Ankara, Turkey, Fax: +90 0312 212 46 47, E-mail: ozercigdem@yahoo.co.uk