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Effect of diclofenac on colonic motility in experimental irritable bowel syndrome

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The goal of the study was to investigate the effect of non-selective COX inhibitor diclofenac on colonic motility in a rat model of irritable bowel syndrome (IBS) in a tissue bath setup. IBS was provoked in rats by intracolonic injection of 0.5% acetic acid on postnatal days 8-21. At the end of the 8 weeks, tissue bath tests were carried out on distal and proximal colons. Diclofenac significantly lowered the mean pressure value (MPV) of distal colon in control rats (P<0.05) when compared in rats with IBS. Additionally, diclofenac had no significant effect on the MPV of spontaneous contractions in proximal colonic segments in both control and IBS. Histological examination revealed no significant differences between the groups. Immunoreactivity for COX-2 was significantly increased in distal colon of IBS rats compared with controls, while immunoreactivity for COX-1 was not different between groups (P<0.05). Increased COX-2 products may responsible from decreased diclofenac responses in distal colon of rats with IBS.

Keywords: Intestinal inflammation, Non-selective COX inhibition, Smooth muscle contraction, Irritable bowel syndrome model, Immunohistochemistry analysis, Diclofenac response

Nonsteroidal anti-inflammatory drugs (NSAIDs) hold a paramount position among medicinal agents owing to their anti-inflammatory and analgesic attributes. However, the potential for adverse events within the upper gastrointestinal tract escalates with chronic and high-dose administration of these agents[1]. Furthermore, the significance of severe adverse outcomes such as perforation, overt bleeding, and constriction in the lower gastrointestinal tract is progressively gaining[2,3]. As conventional, anti-inflammatory and analgesic actions of NSAIDs have been explained on inhibition of cyclooxygenase (COX) enzyme, which synthesizes prostaglandins[4]. The COX enzyme comprises at least two isoforms, namely COX-1 and COX-2. COX-1, consistently active, produces vital prostaglandins for organism continuity. It regulates processes like gastric mucosal protection, vascular balance, platelet aggregation, reproduction, and renal function. On the other hand, COX-2 is rapidly up-regulated in response to cytokines and growth factors in injury, ischemia, and oxidative stress, generating prostaglandins inducing inflammation and pain[5]. Gastrointestinal tract injury due to commonly used NSAIDs is not only caused by COX-1 inhibition but also by the concomitant inhibition of COX-2[6]. This is because COX-1 inhibition induces COX-2 gene expression, producing of prostaglandins that can cause gastrointestinal damage[7].

Irritable bowel syndrome (IBS), which belongs to the category of functional bowel disorders, is characterized by a range of symptoms including abdominal pain or discomfort, diarrhea/constipation, cramps, bloating, altered bowel habits, and a sensation of incomplete bowel evacuation[8]. Dysmotility in the form of increased frequency and amplitude of contractions in the colon has been reported to be the cause of these symptoms in IBS[9,10]. Although the exact mechanisms of disordered colonic motility in IBS are still under investigation, some studies suggest that patients may benefit from COX inhibition[11]. IBS patients have been found to have visceral hypersensitivity, increased permeability and sensitivity to NSAIDs in the intestines[12]. However, studies on the causes of IBS and the mechanisms of these conditions are still ongoing[13].

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The experimental models developed for IBS research have contributed significantly to understanding the mechanisms and demonstration of changes in colonic motor function, visceral hypersensitivity, visceral permeability and the factors affecting them, such as NSAIDs.

As a consequence of all this, this study aimedto investigate the mechanism of effect and acute effect of non-selective COX inhibitor diclofenac on colonic motor function in experimental IBS.

Materials and Methods

Animals

Experimental protocol was conducted using neonatal, male Wistar-Albino rats in Dokuz Eylul University Faculty of Medicine Multidisciplinary Animal Experiments Research Laboratory. The rats were provided with *ad libitum* standard pellet rat food and water in a rodent cage at 22±2°C room temperature within a controlled 12 h lightand dark cycle room. All neonatal rats in the experiment were maintained per cage with an adult female rat in each cage until one month old. The Dokuz Eylul University Animal Experiments Local Ethics Committee approved the protocol of experiment.

Chemicals and Drugs

The experiments were carried out using a modified Krebs bicarbonate solution with the following composition (in millimolar (mM)) NaCl (120), $NaHCO_3$ (22), KCl (4.6), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.14) and D-glucose (11.5). All components of the Krebs solution, as well as acetic acid and diclofenac used in the experimental procedures, were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Induction of IBS

A total of 12 male Wistar-Albino rats were included in the study and were randomly divided into two groups. Each animal underwent colonic infusion as part of the experimental procedure. Group 1, designated as the control group, received a colonic infusion of 0.9% saline solution. Group 2, on the other hand, was subjected to a colonic infusion of 0.5% acetic acid solution (0.3 mL) in saline. This infusion was administered into the colon, approximately 2 cm from the anus, once daily during the postnatal days 8 to 21[14]. Experiments were conducted in these rats at the end of the 8 weeks.

Surgical procedure and colonic motor function recording

On the day of the experiment, rats were sacrificed by cervical dislocation and their abdominal cavities were opened by making a longitudinal incision in their abdominal walls. Distal and proximal colon parts, each 2 cm long, were removed separately and cut in a ring shape. Tissue baths (20 mL) were continuously bubbled with carbogen gas (95% O2 and 5% CO2) before and during experiment. The parts were placed in one of the tissue baths, which was filled Krebs bicarbonate solution at 37°C. Krebs bicarbonate solution was containing in mM: 120 NaCl; 22 $NaHCO_3$; 4.6 KCl; 2.5 $CaCl_2$; 1.2 $MgCl_2$; 1.14 NaH_2PO_4; 11.5 D-glucose. The superior end of the segment was secured to an isometric force-displacement transducer (FDT-05, MAY, Turkey) and a tension of 0.6 g was applied as a preload. The opposite end was attached to a stationary hook located at the bottom of the bath. All intestinal segments were washed every 10 min and allowed to equilibrate for at least 30 min at beginning.

At the end of the adaptation period, diclofenac, a non-selective COX inhibitor, were added cumulatively at concentrations of 1×10^{-5} and 1×10^{-4} mol/L to the tissue bath to investigate their direct effect on distal and proximal colonic segments in terms of non-selective COX inhibitors. Fresh preparations of diclofenac and Krebs bicarbonate solution were made on the day of the experiment.

Following the surgical procedures, proximal (n = 6 for IBS; n = 5 for control) and distal (n = 6 for IBS; n = 4 for control) colon segments were obtained from the IBS (n = 6) and control (n = 5) groups. One sample was excluded from analysis in the control-distal group due to inadequate oxygenation.

In both control and IBS groups, the mean pressure values (MPV) of spontaneous colonic contractions were compared as a percentage of the initial MPV. The MPV was calculated by averaging the maximum contraction values measured at 5 min intervals. At the end of the experimental procedure for each sample in the groups, the contractile state of bowel smooth muscle was tested with KCl (80 mmol/L).

Histological and morphological examination

Histopathological and morphological examination was performed using hematoxylin-eosin (H&E) stain. To this end, all tissues were initially fixed in 10% formalin for 24 h. Subsequently, they were embedded in paraffin wax and 5 µm sections were obtained using a microtome (Leica RM2245). Finally, the

samples were stained with H&E. Also, the thickness of the tissues were measured with light microscope and its software (Olympus labSens İmaging Software, USA).

Immunohistochemistry examination

The tissues which were formalin-fixed and paraffin-embedded were used for immune-histochemical staining as well. The rat tissue samples were kept overnight at 60°C and then were deparaffinized with xylene for 30 min. After dehydration with ethanol, the bowel segments were rinsed with distilled water. To suppress endogenous peroxidase activity, the bowel tissues were placed in 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 min and followed by 15 min in 3% H_2O_2 solution. Subsequently, the segments underwent incubation with anti-COX-1 primary antibody (sc-48143, Santa Cruz Biotechnology, Inc.) and anti-COX-2 primary antibody (sc-23983, Santa Cruz Biotechnology, Inc.) at a 1/100 dilution for 18 h at +4°C. After additional serial washing in three times in PBS for 5 min each followed by incubation with biotinylated IgG and streptavidin peroxidase (Histostain Plus kit, cat. no. 85-9043, Invitrogen). After three 5 min rinses in PBS, the sections were subjected to stain with the DAB Substrate system containing diaminobenzidine (DAB-plus substrate kit, Invitrogen) for immunoreactivity detection, followed by counterstaining with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). The slides were then sealed with mounting medium (ClearMount™, Mounting Medium Cat. No.: 00-8110 Invitrogen, Carlsbad, CA, USA) and examined using light microscopy (Olympus BX-43, Tokyo, Japan). Two observers, who were independent and blinded, assessed the staining scores.

The staining intensity of COX-1 and COX-2 was scored on a scale of 0 to 3, with 0 indicating no staining, 1 indicating mild staining, 2 indicating moderate staining, and 3 indicating intense staining.

Statistical analysis

Statistics Kingdom website (https://www.statskingdom.com/ Access Date: September 15th, 2022) was used for statistical analyses of obtained data. All obtained data were presented as mean ± standard error of mean (mean ± SEM). Repeated measures within groups were compared using the Friedman test, followed by Wilcoxon signed rank tests to identify significant differences between groups. Between-group comparisons were performed using the Mann-Whitney U test. Statistical significance was considered when P value was under 0.05.

Results

Influence of diclofenac on spontaneous colonic motor function in isolated distal and proximal segments

The distal and proximal colonic isolated segments exhibited intrinsic spontaneous contractions during the resting state. No significant difference was observed in the spontaneous contractions between IBS and control rats. The MPV of the spontaneous contractions in isolated distal colon segments were measured as 518.60 ±15.43 mmHg in control (n = 4) and 531.40 ± 14.22 mmHg in IBS group (n = 6), revealing no significant difference between the two groups (P>0.05). As for the proximal colon segments, the MPV were recorded as 422.80 ±12.30 mmHg in control group (n = 5) and 417.20 ± 11.20 mmHg in IBS group (n = 6) rats, with no significant difference observed between groups (P>0.05).

Proximal and distal colon sections from control and IBS rats reacted to diclofenac administration into the organ bath (first 10^{-5} and then 10^{-4} mol/L) by exhibiting a gradual reduction of spontaneous colonic contractions in a manner dependent on concentration (Fig. 1A-1D). Diclofenac did not have any significant effect (P>0.05) on the MPV of spontaneous contractions in the proximal colon of either control (n = 4) or IBS (n = 6) rats (Fig. 2A). Diclofenac significantly reduced distal colonic (n = 6) MPV of spontaneous contractions in control group (n = 5) compared to base line MPV at 10^{-4} mol/L concentration (P<0.05) (Fig. 2B). The MPV of the control group was significantly decreased compared to IBS group in the distal colon and at 10^{-4} mol/L concentration (P<0.05) (Fig. 2B).

Upon completing the study protocol for each individual sample, a solution of KCl (80 mmol/L) was introduced into all tissue baths, and the contractile responses of the distal and proximal colonic segments were recorded and analysed. The statistical analysis showed that there were no statistically significant differences in the contractile responses between the control and IBS groups (P>0.05) (Fig. 3A).

Histopathological and morphological findings

H&E staining did not reveal any differences in morphology between the control and IBS groups. Proximal and distal segments had no structural

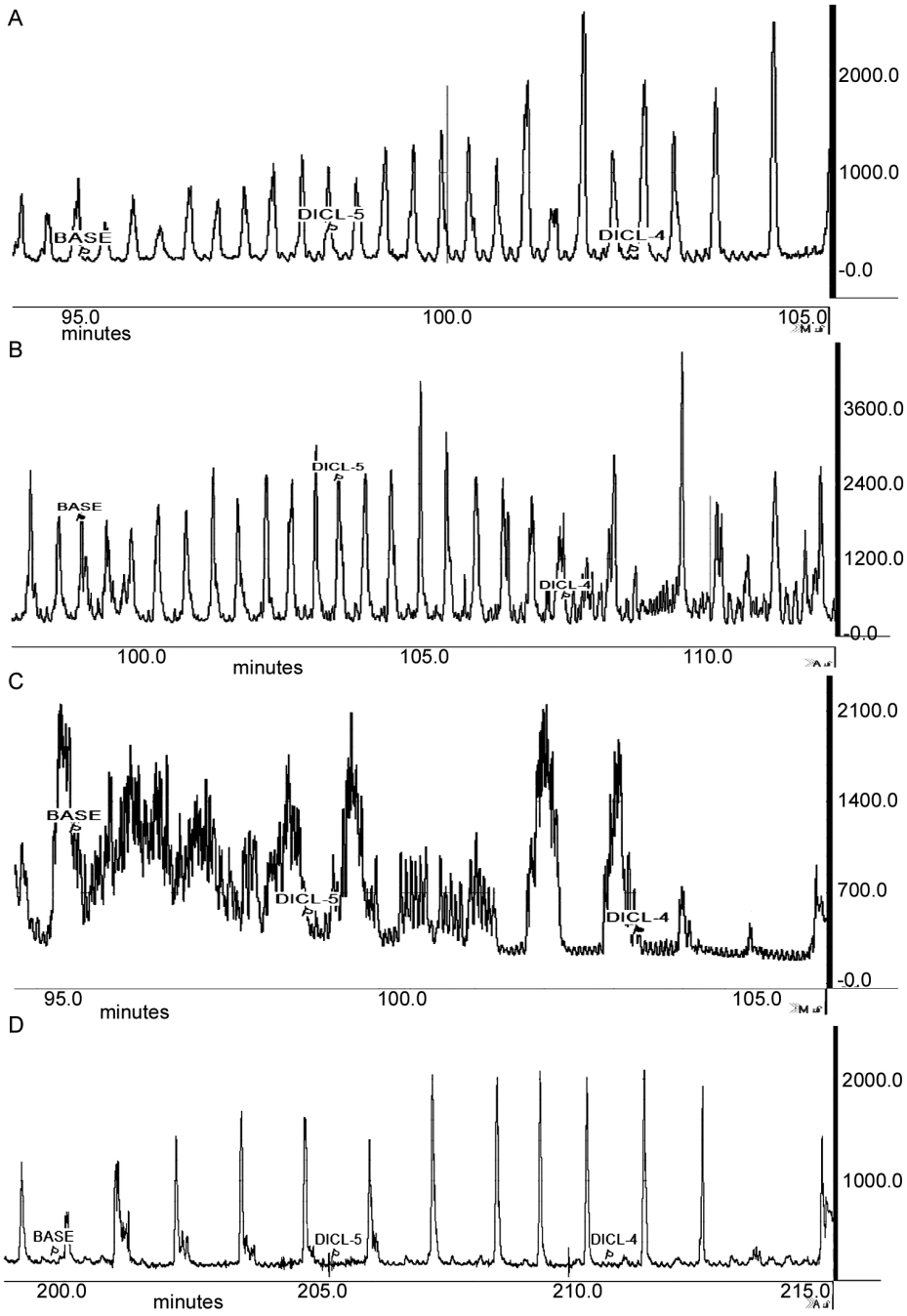

Fig. 1 — Representative traces of the effects of diclofenac on spontaneous motor contractions of proximal and distal colonic tissues isolated from control and IBS rats.

injury. In particular, when the thickness of the smooth muscle layer was measured using light microscopy, it was found to be similar in both tissues. (Fig. 4A & 4B).

Immunohistochemical analysis

Anti-COX-1 and anti-COX-2 primary antibodies were utilized for immunohistochemical analysis. COX-1 immunoreactivity was observed moderate (2) to intense (3) immunoreactivity with no statistically significant distinction between both groups (Fig. 5A & 5B) (P>0.05). COX-2 immunoreactivity was detected moderate (2) to intense (3) staining in the control group (Fig. 5C) and intense (3) staining in the IBS group (Fig. 5D) (P<0.05).

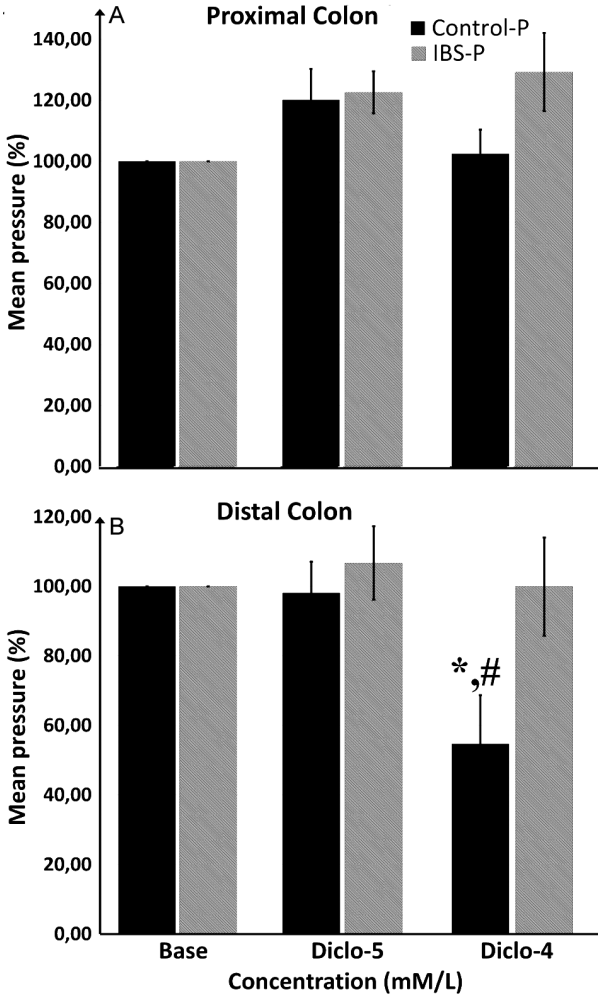

Fig. 2 — Diclofenac's effects on mean pressure of spontaneous contractions of control and IBS-rats: proximal and distal colon.

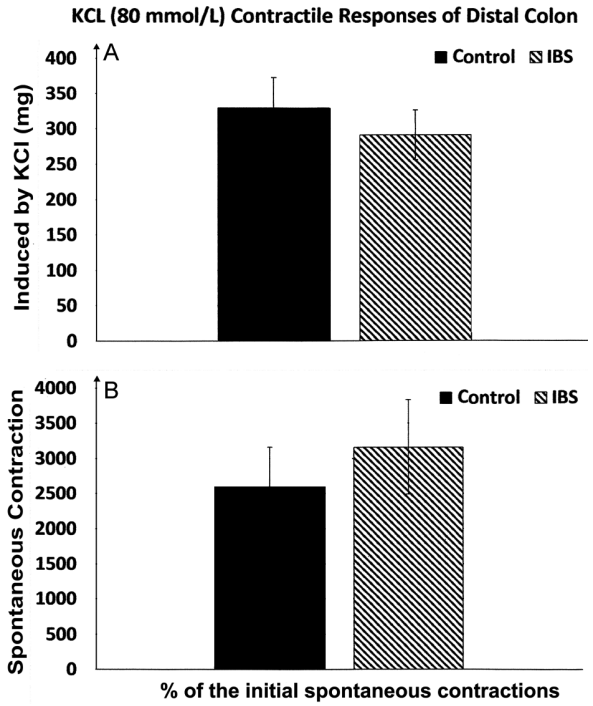

Fig. 3 — KCl (80 mmol/L) contractile responses on proximal and distal colonic tissues colonic tissues isolated from control and IBS-rats.

Discussion

Irritable bowel syndrome is a common public disorder which is demonstrated by recurrent abdominal pain, increased contraction of the distal colon and changes in the defecation pattern in the absence of any organic cause. The pathophysiology of IBS remains indefinite[15]. Gastrointestinal motility and visceral hypersensitivity are the most important point in the areas of researchers. The Rome IV criteria represent an updated classification system founded on patients' bowel habits, which define four subtypes of IBS in functional bowel disorders. These include IBS with predominant diarrhea (IBS-D), IBS with predominant constipation (IBS-C), IBS with mixed bowel habits (IBS-M), and an IBS subtype that is undetermined (IBS-U)[8,16]. The changes in the bowel habit of IBS subtypes may be related to COX activity in colonic smooth muscle[17].

Diclofenac sodium is a frequently used NSAIDs that interferes with the synthesis of prostaglandins and decreases the chemotaxis of neutrophils as well as the production of superoxide. *In vivo* and *in vitro* studies have shown that diclofenac is a potent inhibitor of COX enzymes, which makes it efficacious in treating pain and reducing inflammation in a variety of inflammatory conditions[18]. Different studies have reported that diclofenac exerts various effects on ion channels, including voltage-gated channels such as those for sodium (Na^+), calcium (Ca^{2+}), or potassium (K^+), ligand-gated K^+ channels, transient receptor potential channels, as well as other cation channels and chloride channels in specific cell types[19]. These effects may result in channel inhibition, activation, or alterations in their expression profiles. It is widely acknowledged that diclofenac has a significant influence, particularly on K^+ channels. Additionally, It was demonstrated that this may enhance anti-nociceptive efficiency potentially through the activation of ATP-sensitive K^+ channels (K_{ATP})[20]. Different tissues exhibit variations in the functional and pharmacological characteristics of K_{ATP}. Activation of K_{ATP} induces membrane hyperpolarization, resulting in the relaxation of gastrointestinal smooth muscle[21].

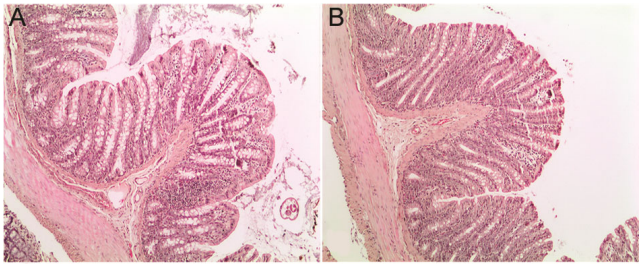

Fig. 4 — Photomicrographs of H&E staining within the control and IBS tissues (×10). There was no difference in morphology between control (A) and IBS (B) tissues in H&E staining.

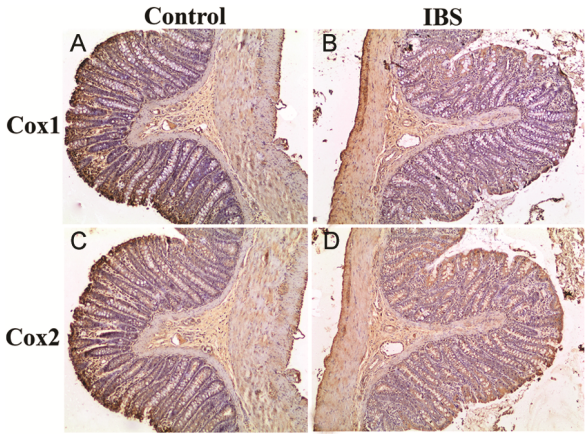

Fig. 5 — Photomicrographs of COX-1 and COX-2 staining of control and IBS tissues (×10). The immunoreactivity of sections stained with (A, B) COX-1 and (C, D) COX-2 primary antibodies is shown. Intensity of reactivity (score: 0= no staining; 1= mild; 2= moderate, and; 3= intense).

We induced IBS-like visceral hypersensitivity in rats using intra-colonic infusion of acetic acid to evaluate the direct effects of the non-selective COX inhibitor diclofenac on colonic smooth muscle. Distal and proximal colonic segments were then isolated and their contractile activity was assessed *in vitro* in a tissue bath. The initial spontaneous motor activities did not significantly differ between the control and IBS groups. Given the extremely variable amplitude and frequency of the spontaneous contractions, we calculated the mean pressure value (MPV) to quantify the direct effects of diclofenac on initial spontaneous colonic contractions. We observed no significant differences between the control and IBS groups in MPV, KCl contractile responses, or colonic smooth muscle histopathology. These findings indicate that colonic smooth muscle contractile mechanisms remain intact in tissues with IBS.

The enzyme COX catalyzes the major rate-limiting step of synthesising prostaglandins (PGs), which play an important role in smooth muscle function, in the gut[22,23]. Since COX-derived PGs are well known to affect smooth muscle contractility in the colon of humans and animals[18,20-26], the present study focused on the direct effect on colonic motility of non-selective COX inhibitor diclofenac in a rat model of IBS. Our data demonstrated that the non-selective COX inhibitor diclofenac significantly decreased the MPV in distal colon of control rats, whereas MPV in distal colon of rats with IBS did not change significantly. Inhibitor effect of diclofenac was significantly lower in distal colon IBS rats compared to control rats. But, diclofenac responses in proximal colon did not significantly change both control and IBS groups. Fornai *et al.* showed that the COX isoforms (COX-1 and COX-2) enhance tachykininergic motor activity in normal human colon[27]. It has been suggested that COX-1 inhibitor reduced the basal motility index of guinea pig colon and lowered PGF2α levels. This decrease in the basal motility index induced by the COX-1 inhibitor was attributable to reductions in PGF2α levels[28]. These results are compatible with our findings that non-selective COX inhibitor diclofenac reduces the MPV in distal colon of control rats.

Analysis of immunohistochemical staining demonstrated moderate/intense immunoreactivity for COX-1 and COX-2 in control group, but COX-2 immunoreactivity was intense in IBS group; these differences were statistically significant. Increase immunoreactivity to COX-2 may be due to an increase in COX-2 products in distal colon of rats with IBS. On the other hand, we did not find significant difference in immunoreactivity for COX-1 between two groups. All these findings suggest that the COX-2 may have a substantial role for alterations of colonic motility in IBS. Therefore, we have supposed that increased COX-2 level may be responsible from changes in intestinal motility in IBS.

Mahadevan *et al.*[29] and Miao *et al.*[30] evaluated 27 patients with Crohn's disease and Ulcerative colitis (UC) and pouchitis receiving rofecoxib or celecoxib. Treatment was shown to be both beneficial and safe. For this reason, selective COX-2 inhibitors, by reducing the increased COX-2 level in IBS, are expected to be safe and effective drugs for treatment of abdominal pain and alterations in gastrointestinal motility associated with IBS. In a case-control study by Felder *et al.*, patients with Crohn's disease or ulcerative colitis (inflammatory bowel disease) were compared with IBS patients (control) in terms of

NSAIDs use. These authors have claimed that increased NSAIDs usage has increased in aggravated inflammatory bowel disease. However, all (n=62) of the IBS patients included in the study used NSAIDs and 92% (n=57) of them reported that their NSAIDs use did not affect the occurrence of disease symptoms. Although this study is not directly on IBS, it is noteworthy in terms of the high use of NSAIDs in IBS patients[31].

Conclusion

In conclusion, our results suggest that underlying mechanisms of IBS reduced inhibitor effect of diclofenac in rat distal colon, probably through increasing COX-2 levels. On the other hand, decreased diclofenac response may be due to down-regulation of K_{ATP} channels in distal colon of rats with IBS. Selective COX-2 inhibitors cause less gastrointestinal toxicity compared to conventional NSAIDs such as diclofenac. Additional studies are necessary to determine the exact mechanism of alterations in the bowel motility in IBS.

Author contributions

Omer Demir, Tijen Kaya Temiz, Gokhan Koyluoglu designed research; Tijen Kaya Temiz, Omer Demir, Fatma Simsek, Selen Bahceci performed research; Yusuf Cem Kaplan, Barıs Karadas, Aslı Celik, Gokhan Koyluoglu creating experimental models; Tijen Kaya Temiz, Omer Demir, Fatma Simsek analyzed data; Tijen Kaya Temiz, Fatma Simsek and Yusuf Cem Kaplan wrote the paper.

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Conflict of interest:

The authors declare no conflicts of interest.

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