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Article

Non-invasive biomagnetic assessment of gastrointestinal motility in a loperamide-induced constipation model

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Constipation is a disorder of the gastrointestinal (GI) and some of the main etiological mechanisms are directly related to changes in GI physiology. The capacity to carry out paired assessments and measure GI parameters under the influence of constipation is a relevant point in selecting a suitable methodology. We aimed to perform a non-invasive investigation of gastrointestinal motility in constipated rats using the alternating current biosusceptometry system (ACB). The animals were split into two groups: the pre-induction stage (CONTROL) and post-induction loperamide stage (LOP). We assessed GI motility parameters using the ACB system. Colon morphometric and immunohistochemical analyses were performed for biomarkers (C-kit) for interstitial cells of Cajal (ICC). Our results showed a significant increase in gastrointestinal transit in the LOP group in addition to a reduction in the dominant frequency of gastric contraction and an arrhythmic profile. A change in colonic contractility profiles was observed, indicating colonic dysmotility in the LOP group. We found a reduction in the number of biomarkers for intestinal cells of Cajal (ICC) in the LOP group. The ACB system can evaluate transit irregularities and their degrees of severity, while also supporting research into novel, safer, and more efficient treatments for constipation.

Keywords: Alternating Current Biosusceptometry (ACB)**.** Loperamide. Constipation. Gastrointestinal Transit. Colonic Motility.

INTRODUCTION

Constipation is a disorder of the gastrointestinal (GI) tract characterized by symptoms such as the sensation of incomplete evacuation, difficulties during defecation caused by the presence of hard and dry stools, and different levels of discomfort (Bongers *et al*., 2009; Zhang *et al*., 2018), which may occur either in isolation or secondary to another underlying disorder. Although it is a common condition, studies have shown that constipation significantly affects the health-related quality of life and is a moderate risk factor for colorectal cancer (Tashiro *et al*., 2011).

Some of the main etiological mechanisms of constipation are directly related to changes in GI physiology, such as dysfunction in secretion and absorption, dysmotility, and alterations in innervation and GI inflammation (Ford *et al*., 2014).

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Currently, one of the most common animal models to assess constipation is based on the administration of loperamide to rats (Yan *et al*., 2017). This experimental model allows us to understand the mechanisms underlying the etiology of constipation and how it affects GI motility, in addition to allowing tests for developing new laxatives (Li *et al*., 2021). However, the techniques used to assess the parameters of gastrointestinal motility, such as gastrointestinal transit and contractility of segments of the gastrointestinal tract, are invasive. Nowadays, the activated charcoal technique is considered the gold standard for gastrointestinal transit time and consists of measuring the distance traveled by the marker in the intestine after administration (Padmanabhan *et al*., 2013). To measure the contractility of specific intestinal segments, the organ bath technique is well established in the field of pharmacology because it can determine dose-dependent response curves (Lee *et al*., 2012). However, both techniques require the death of experimental animals, avoid repeated measurements with the same animal at different treatment stages, significantly increasing the number of animals needed for the study, and do not allow paired analyses.

The capacity to carry out paired assessments and measure GI parameters under the influence of constipation is a relevant point in selecting a suitable methodology. Alternating current biosusceptometry (ACB) is a non-invasive technique for assessing gastrointestinal transit and motility. Due to its intrinsic characteristics, the ACB system has been widely used in studies involving GI in animals and humans, in which several validations were carried out using gold-standard techniques to analyze parameters related to gastrointestinal motility, such as gastric emptying, gastrointestinal transit, contractility, gastric and colonic contractility (Gama *et al*., 2023) (Americo *et al*., 2010; Américo *et al*., 2009; Romeiro *et al*., 2006). Furthermore, recent studies have applied ACB as an alternative technique to assess the motility of the gastrointestinal tract in rats subjected to surgical interventions and pathological models (Calabresi *et al*., 2015; Calabresi *et al*., 2019; Hauschildt *et al*., 2018).

In this study, we aimed to perform a non-invasive investigation of gastrointestinal transit and contractility in constipated rats using ACB system monitoring in real time.

MATERIAL AND METHODS

Experimental design of the loperamide-induced model

In this study, 30 males (*Rattus norvegicus*, Wistar, weighing 250–300 g) were used. All animal experiments were conducted according to the Animal Use Ethics Committee approval (protocol no. CEUA – IBB 5587230421).

The animals were randomly divided into two groups: one corresponding to the moment before loperamide hydrochloride induction (CONTROL group) and the other corresponding to the moment after loperamide hydrochloride induction (LOP group). Thus, the CONTROL and LOP groups correspond to the same animal at different moments. Loperamide was purchased from Sigma-Aldrich (St.Louis, MO, USA). The constipation was induced in the LOP group via oral administration of loperamide hydrochloride (5 mg/kg) once daily for seven consecutive days, based on a previously published protocol (Eor *et al*., 2019).

Metabolic Parameters

During the study, animals from all experimental groups were kept in metabolic cages to collect stools without contamination and to avoid coprophagia, either pre- or post-induction protocol (loperamide). Metabolic parameters (collection of droppings from fecal pellets) were based on assessments conducted every hour for 24 hours, both pre-and post-induction, as recommended by other studies (Lee *et al*., 2012).

We weighed the feces three times per sample using an electric balance, while water content was determined by the difference between wet and dry weights of feces, as previously described (Eor *et al*., 2019).

Fecal parameter analysis

All fecal pellets collected over 24 hours were counted and weighed for later storage in volume-controlled tubes. The fecal pellets were dried using a FreeZone 2.5 freeze dryer (Labconco, Kansas, USA),

and the fecal moisture content (FMC) was calculated according to the formula:

$$FMC(\%) = \frac{wetweight - dryweight}{wetweight} * 100$$

The wet and dry weights refer to fecal pellets during collection and after lyophilization, respectively.

To complement the data obtained from evaluating the parameters described above, fecal pellet morphology was observed and recorded. Some pellets were chosen and collected randomly before and after induction (loperamide).

Alternating current biosusceptometry (ACB)

The ACB system is a biomagnetic technique for detecting magnetic materials, and consists of two pairs of coils separated by a distance of 15 cm. The system works as a double magnetic flux transformer, providing two pairs of coils (excitation/pick-up coils). The pair of coils furthest from the sample provides the reference signal, whereas the other acts as a detector closer to the sample studies recognizing it. The excitation coils generate an AC magnetic field that induces a current into the detection coils. When no magnetic material is positioned close to the pick-up, the magnetic response is minimized due to the balance of the gradiometer. A magnetic material close to the detector pick-up coil creates an imbalance in the magnetic flux, generating an electrical signal in the pick-up coils. The signal strength obtained in the system is proportional to the amount of magnetic material and inversely proportional to the distance between the sensor and magnetic material.

Two arrays of ACB sensors were used in this study. The ACB mono-channel (Mono-ACB) system was developed specifically for in vivo measurements (Figure 1A). The Cavity ACB system was developed and designed for sample ex vivo quantification assessment (Figure 1B). It was developed with a dedicated geometry, in which the samples can be positioned at the center of the detection coils, increasing the sensitivity by almost an order of magnitude compared to the previous ACB system.

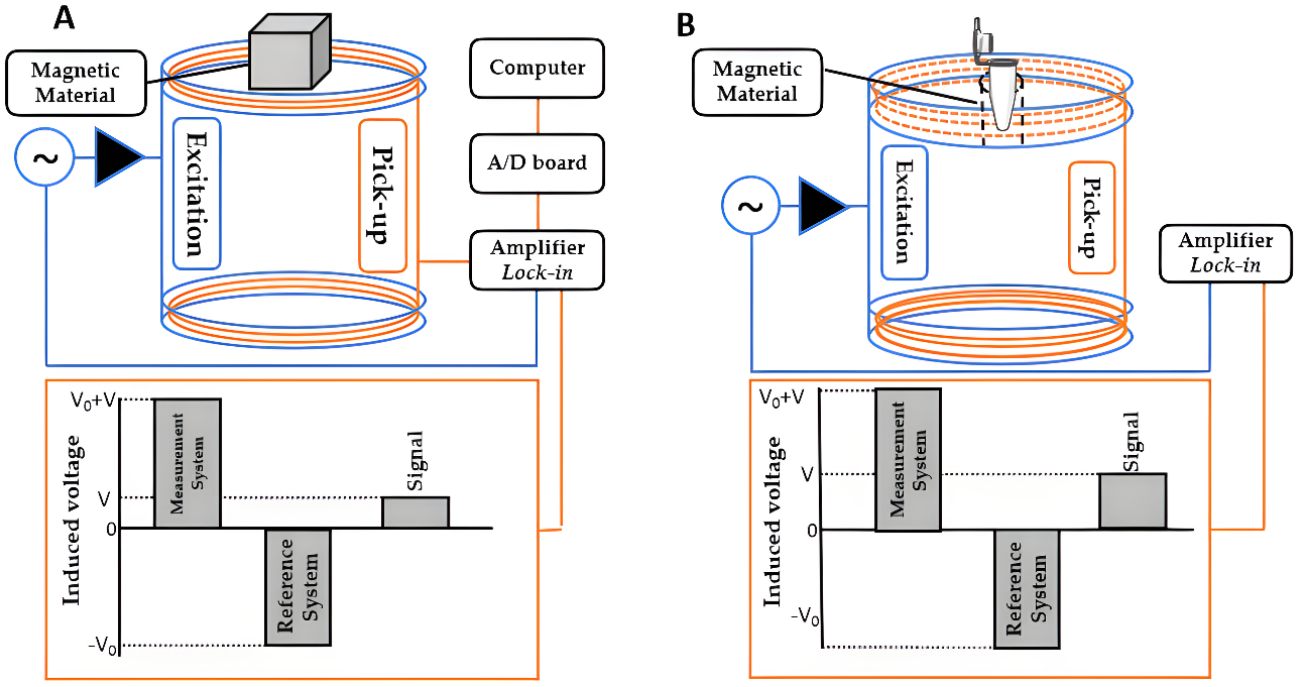

FIGURE 1 - (A) Schematic diagram of operation the Alternate Current Biosusceptometry (ACB) sensor MonoACB in the presence of magnetic material. (B) Schematic diagram of operation the Alternate Current Biosusceptometry (ACB) sensor Cavity-ACB in the presence of magnetic material. The blue arrows indicate the excitation windings of the coils, and the orange arrows indicate the pick-up windings of the coils.

Magnetic Measurements by ACB

Magnetic monitoring was performed by measuring the intensity values recorded using the Mono-ACB system positioned on the abdominal surface. The animals were handled gently by their neck, and the sensor was positioned on their gastric and cecum projections after ingesting solid magnetic meals (Figure 2). The stomach was determined as the region in which the highest signal and cecum region were determined through the anatomical references defined in previous studies (Huizinga, 2001). This system enabled the monitoring gastric emptying (GE) and orocecal transit (OCT) and the detection of gastric and colonic contractility profile frequencies. In contrast, the Cavity ACB system allowed quantified gastrointestinal transit parameters, such as the oroanal transit time (OATT) and fecal pellet elimination rate (FPER).

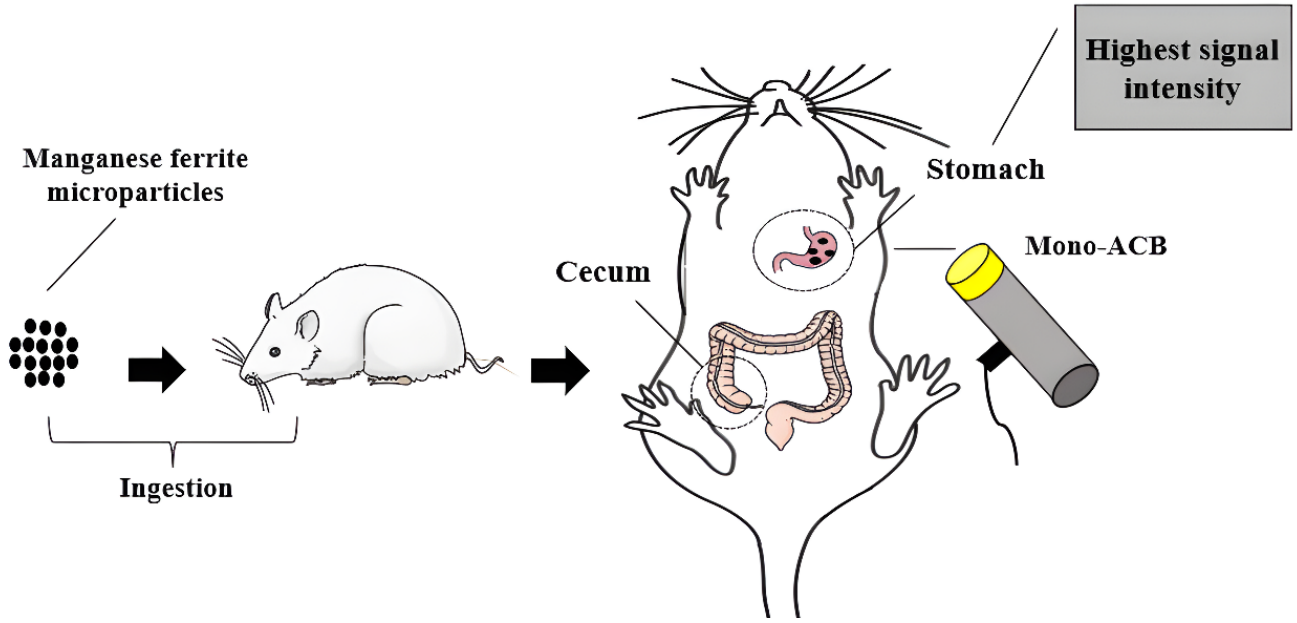

FIGURE 2- A representative diagram of the experimental protocol setup. In this setup, the animal consumes food containing manganese ferrite microparticles, following which the stomach is identified as the region with the highest magnetic signal intensity. The cecum region is determined based on anatomical references. After establishing these reference regions, the changes in magnetic signal intensity over time are registered.

The magnetic meal (Pellet 2 g) comprised powder ferrite (0.5 g) and laboratory chow (1.5 g). Manganese microparticles (ferrite, $MnFe_2O_4$, 50–100 µm, Ferroxcube, El Paso, USA) provide an excellent magnetic response and do not require previous magnetization. Furthermore, it is non-absorbable and inert to the body (Soares *et al*., 2021).

Gastrointestinal transit

After ingesting the solid magnetic meals, GE and OCT were obtained by recording the signal intensity from subsequent measurements at 30-minute intervals for 6 h in the anatomical regions of the stomach and cecum by Mono-ACB, respectively. The following measurements were performed at the described interval when the animals were awake by manipulating the animals by the neck. The raw signals obtained in each anatomical region were analyzed by visual inspection and statistical moment analysis. The statistical moment was defined from the magnetic intensity curves' temporal average and normalized by the area under the curve. Therefore, two relevant parameters were quantified. The Mean Gastric Emptying Time (MGET) was defined as the time t (min)

when the mean amount of magnetic meal was emptied of the stomach. This was quantified using the area under the emptying curve (Calabresi *et al*., 2015; Podczeck *et al*., 2007). The Mean Cecum Arrival Time (MCAT) was defined as the time t (min) when an increase in the mean amount of magnetic meal arrived in the cecum and was calculated by the area between the cecum arrival curve until maximal cumulative values (Calabresi *et al*., 2015). Furthermore, we quantified the OATT and FPER by Cavity-ACB collecting fecal pellet from each group and storing it in a volume-controlled flask. The quantification of the first pellet with a magnetic signal more significant than the baseline (empty flask) was defined as the OATT (Gama *et al*., 2020). As the collection of fecal pellets continued every hour, the pellets excreted over time were quantified, and their respective magnetic intensity values were recorded until there was no more magnetic material in the feces (Gama *et al*., 2020). The FPER was defined as the time in hours that an average amount of magnetic material was excreted in the feces, calculated through statistical moments, in which the area under the magnetic signal intensity curve is normalized by the time (Podczeck, Newton, Yuen, 1995).

Contractility Measurements

Each animal was monitored at two different moments to assess contractility patterns. The animals were anesthetized using isoflurane (4.0% induction and 1.5% maintenance) 15 minutes and 18 hours after the solid magnetic meal ingestion. While the animals remained laid supine, the Mono-Channel ACB system was positioned in the abdominal region, in which the gastric and colonic contractility were recorded, respectively on a Biopac A/D system (MP100 System; BIOPAC, CA, USA) at a sampling rate of 20 Hz for 15 minutes.

After the ACB monitoring, the animals returned to the metabolic box cages. After gastric and colonic contractility measurements, fecal pellets continued one hour apart, until no magnetic material was detected. Gastric and colonic contractility data were analyzed using MatLab® (R2015a, Natick, MA, USA) by signal visual inspection and Fast Fourier Transform (FFT), in which we considered the highest peak of frequency for each FFT as the gastric/colonic dominant frequency and the lowest as the intrinsic noise of the signal. The frequencies of both regions were expressed in millihertz (mHz). We also performed a distribution histogram of these values to analyze gastric contractility after quantifying the frequency values obtained using FFT. The histogram helped to visualize the frequency-distribution profile. From the histogram, a Gaussian adjustment was implemented that allowed us to quantify the value of sigma and Full Width at Half Maximum (FWHM), showing how these values are dispersed from the central value (Huttunen, Törmä, 2005). In order to complement the visualization of the frequency profile over time and identify possible arrhythmias and variations in the stationary profile of the signals, the Running Spectrum Analysis (RSA) was implemented, which shows us the frequency peaks and their intensity as a function of time (Van der Schee, Grashuis, 1987). For colonic contractility, FFT analysis allowed us to identify the two frequencies of colonic contraction activity, classified into rhythmic propulsive motor complexes (RPMCs) and rhythmic propagation ripples (RPRs) according to ex vivo and in vivo studies dated in the literature, respectively (Calabresi *et al*., 2019; Huizinga, 2001).

Immunohistochemistry and Morphometry

Samples of intestine from CONTROL and LOP groups (n=5/group) were fixed for 4 h in Methacarn (70% methanol + 20% chloroform + 10% acetic acid) (Puchtler *et al*., 1970). The samples were dehydrated in ethanol, diaphanized in xylene, and embedded in Paraplast (Sigma Co, Saint Louis, MO). Histological sections of 5 µm were deparaffinized and subjected to Hematoxilin-Eosin (HE) staining for morphometric analysis or to immunohistochemistry reaction.

The morphometric analyses were performed from 12 random images of histological slides stained with HE (n=5/group, totaling 60 photos per group), and the diameter of the lumen, mucosa, submucosa, and external muscular layer was evaluated, using the ImageJ software.

To do this, the slides were submitted to antigen retrieval in a pressure cooker with 10 mM sodium

citrate buffer pH 6.0 for 30 min. Then, the slides were immersed in H_2O_2/methanol solution to block endogen peroxidase for 10 min, following by blockage of non-specific protein-protein interaction in 5% nonfat milk diluted in PBS and incubated with primary antibody anti-c-Kit (Santa Cruz, SC168, 1:200) overnight at 4°C. Slides were washed in PBS and incubated for 1 hour at room temperature with HRP-conjugated secondary antibody (anti-mouse, Abcam, 1:400). The slides were washed, and the reaction was developed using 3,3′-Diaminobenzidine (DAB, Sigma), counterstained for 30 seconds in Hematoxylin, and analyzed using a Leica DMLB 80 microscope.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.05 (GraphPad Software, La Jolla, California, USA). Data were presented as the mean ± standard deviation. The comparison was performed between different moments within the same group (paired t-test).

RESULTS

Metabolic Parameters

(Figure 3) represents the assessment of the metabolic parameters of the collected fecal pellets on day 0 (CONTROL) and day 9 (LOP). The results of the FMC quantification are presented in (Figure 3A), while (Figure 3B) shows the number of pellets collected from each group. A reduction of about 50% was observed in FMC (69.81 ± 3.7 vs 36.21 ± 5.6, $p<0.001$) and in the number of pellets (59 ± 5 pellets vs 30 ± 10 pellets, $p<0.001$) excreted for the LOP group concerning the CONTROL group.

Alterations in fecal morphometry (Figure 3C) were observed following the induction with loperamide, as compared to the CONTROL group. In addition, this was also observed by the FMC and the quantity of pellets. The feces presented were of a reduced size and harder consistency. Upon drying, it was indicated that the constipation model significantly impacted the animals subjected to the loperamide induction protocol.

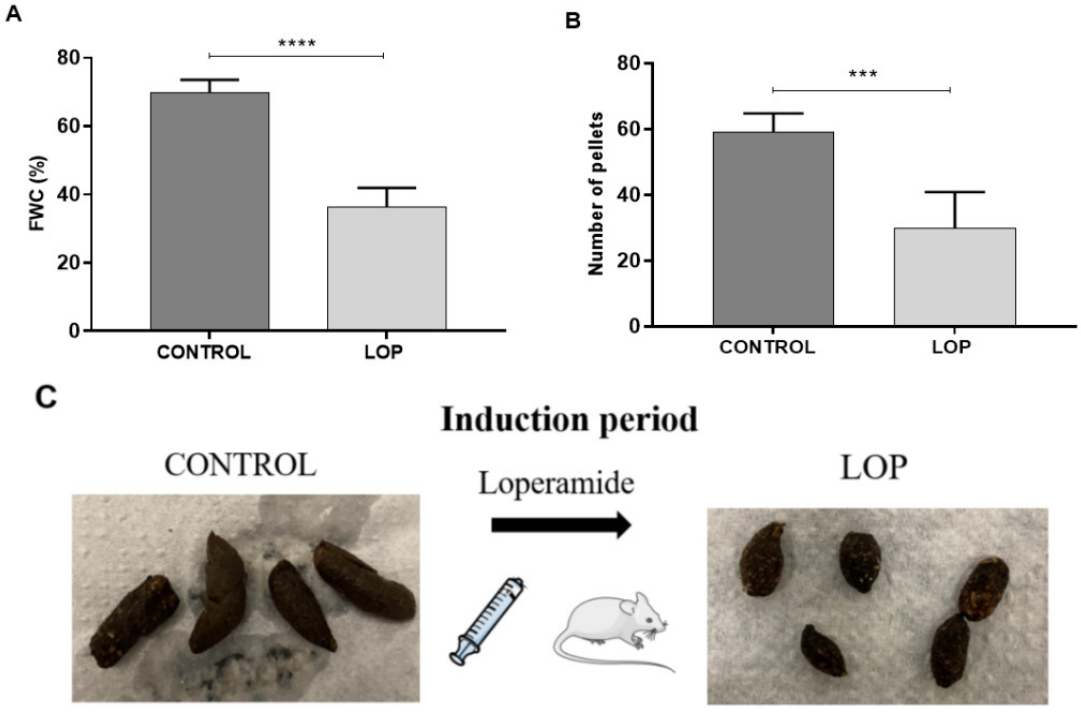

FIGURE 3 – (A) Comparison of the fecal moisture content (FMC) between the CONTROL and LOP groups, indicating a statistical difference of (****) $p < 0.001$; (B) Comparison of the number of fecal pellet between the CONTROL and LOP groups, demonstrating a statistical difference of (***) $p < 0.001$; and (C) Examples illustrating the appearance of fecal pellet before constipation induction (CONTROL) and after induction with loperamide (LOP).

Gastrointestinal Transit

Gastric emptying (GE) and Orocecal transit (OCT)

(Figure 4) summarizes the gastrointestinal transit time parameters in the animal groups assessed by the ACB. The gastric emptying time assessment noted differences between the groups assessed during the protocol. The constipation increased MGET in the LOP group by 12% (118.4 ± 9.592 min vs 105.1 ± 7.96 min, $p < 0.005$) (Figure 4A).

Regarding the Mean Cecum Arrival Time (MCAT), we also observed a significant increase by 5% in the LOP group (247.4 ± 8.38 min vs 237.5 ± 8.26 min, $p<0.005$) (Figure 4B).

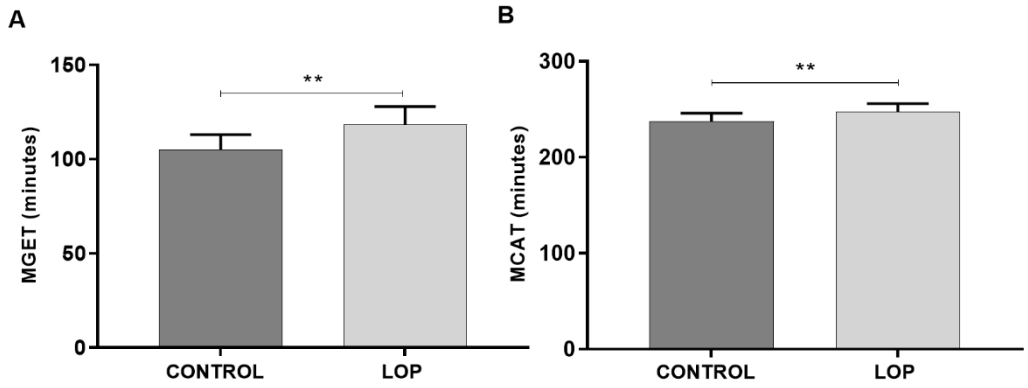

FIGURE 4 – (A) Comparison between the Mean Gastric Emptying Time (MGET) between the CONTROL and LOP groups, and (B) Comparison between the Mean Cecum Arrival Time (MCAT) between the CONTROL and LOP groups. In both analyzes the groups showed a statistical difference with (**) $p<0.005$.

Oroanal Transit Time (OATT) and Fecal Pellet Elimination Rate (FPER)

The Oroanal transit time (OATT) and the Fecal pellet elimination rate (FPER) are shown in (Figure 5A) and (Figure 5B), respectively. The OATT values indicated an intense increased by 90.5% in the LOP group (16.0 ± 1.9 hours vs 8.4 ± 1.4 hours, $p < 0.001$). Furthermore, in (Figure 5B), we observed that the LOP group had a longer mean time to eliminate half of the ingested magnetic material (FPER), (47.5 ± 1.5 hours vs 22.1 ± 0.7 hours, $p < 0.001$). In general, it can be seen that the LOP group presented a prolonged elimination of fecal pellets.

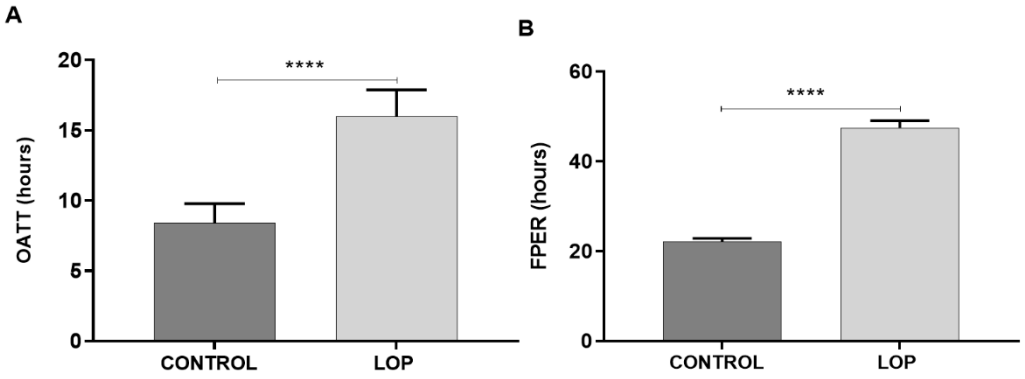

FIGURE 5 - (A) Comparison between the Oroanal Transit Time (OATT) between the CONTROL and LOP. For Review Only groups, and (B) Comparison between the Fecal Pellet Elimination Rate (FPER) between the CONTROL and LOP groups. In both analyzes the groups showed a statistical difference with (****) $p < 0.001$.

Contractility Measurements

Gastric Contractility

The dominant frequencies of gastric contraction obtained through FFT did not show a significant difference between the groups, which were (71.2 ± 4.5 mHz vs 67.2 ± 9.8 mHz) for the CONTROL and LOP groups, respectively.

Despite the mean frequency values not being significantly different, it was observed that there was a greater oscillation in the frequencies between the measurements. (Figure 6) presents two gastric contractility signals and their respective RSA for the same animal, in which (Figure 6A) represents the CONTROL group and (Figure 6B) represents the LOP group.

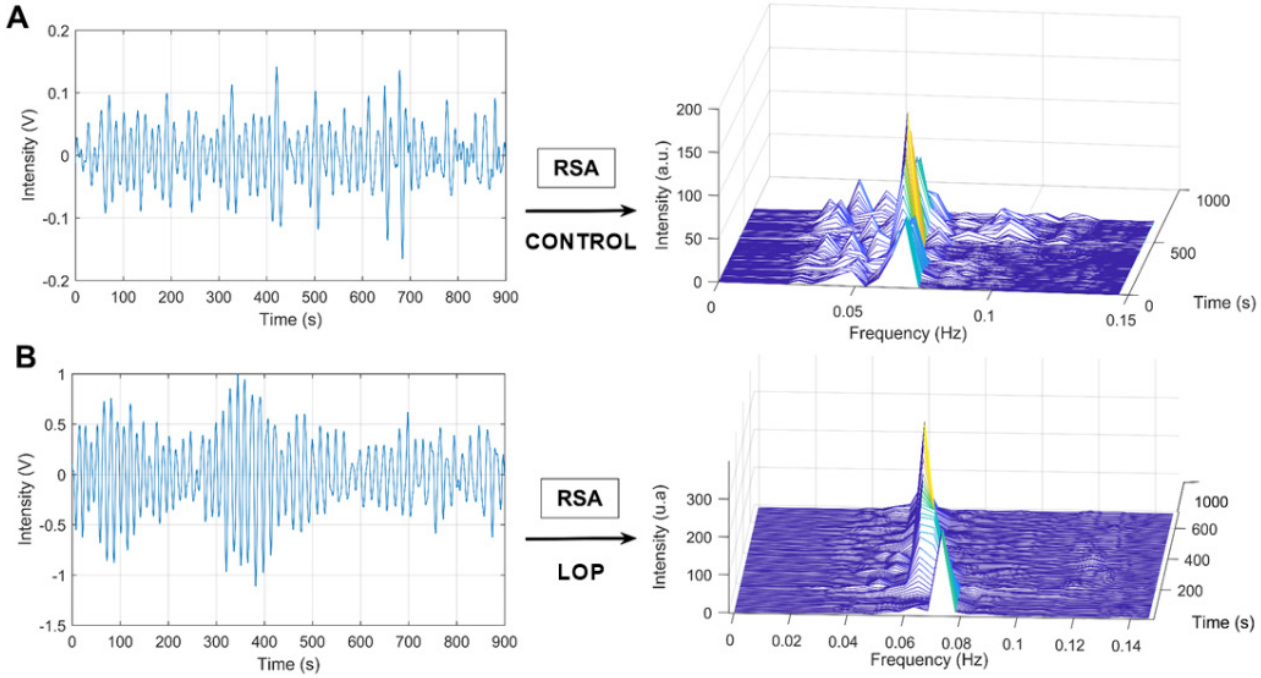

FIGURE 6 - (A) Example of signal recorded from activity contraction stomach and its respective Running Spectrum Analysis (RSA) for CONTROL group, and (B) Example of signal recorded from activity contraction stomach and its respective Running Spectrum Analysis (RSA) for LOP (Loperamide) group.

The RSA spectrum showed a non-stationary frequency profile for the LOP group showing changes in the frequency profile throughout the acquisition. This characterizes it as a non-stationary signal. This can be observed by quantified FWHM analysis and we obtained higher values for the LOP group compared to the CONTROL group (22.69 ± 2 vs. 10.59 ± 1), (Figure 7).

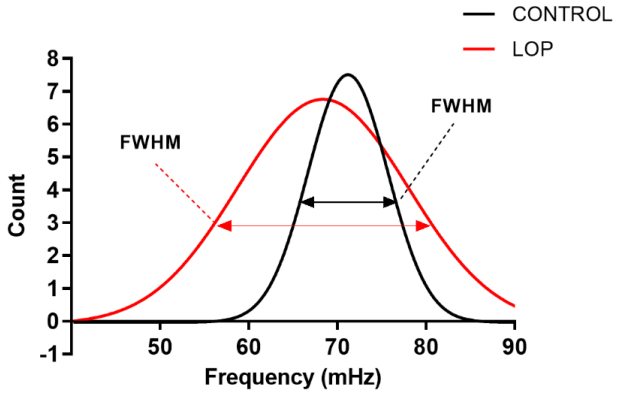

FIGURE 7 - Distribution of quantified values for gastric contraction frequency and total width at half-maximal (FWHM) for both the CONTROL and LOP (Loperamide) groups, represented as black and red lines, respectively.

Colonic contractility

We analyzed the frequency variation of each contraction activity throughout the experiment. Table I shows the mean values of the dominant frequencies of colonic contraction for each of the two frequency profiles for the CONTROL and LOP groups.

TABLE I - Quantification of dominant colonic contraction frequencies: The rhythmic propulsive motor complexes (RPMCs) and rhythmic propagation ripples (RPRs) for the CONTROL and LOP groups expressed as mean ± standard deviation

Groups	Frequency of Colonic Contraction (mHz)	
	Low frequency (RPMC)	High frequency (RPR)
CONTROL	13.4 ± 3.5 [a]	56.8 ± 8.3 [a]
LOP	12.2 ± 5.7 [b]	39.4 ± 3.3 [b]

**Different letters indicate significant differences within columns ($p > 0.005$)*

The quantification of dominant frequency peaks for each identified profile showed a significant difference in high frequencies (RPR) and a decrease in value in the LOP group (56.8±8.3 mHz vs 39.4±3.3 mHz, $p < 0.005$). Approximately 43% of the animals in the LOP group evaluated did not show RPR. The quantification of low frequencies (RPMC) showed no significant difference which was (13.4 ± 3.5 mHz vs 12.2 ± 5.7 mHz) for the CONTROL and LOP groups, respectively. (Figure 8) presents an example of the signal and its respective FFT for the same animal, in which (Figure 8A) refers to the CONTROL group and (Figure 8B) refers to the LOP group.

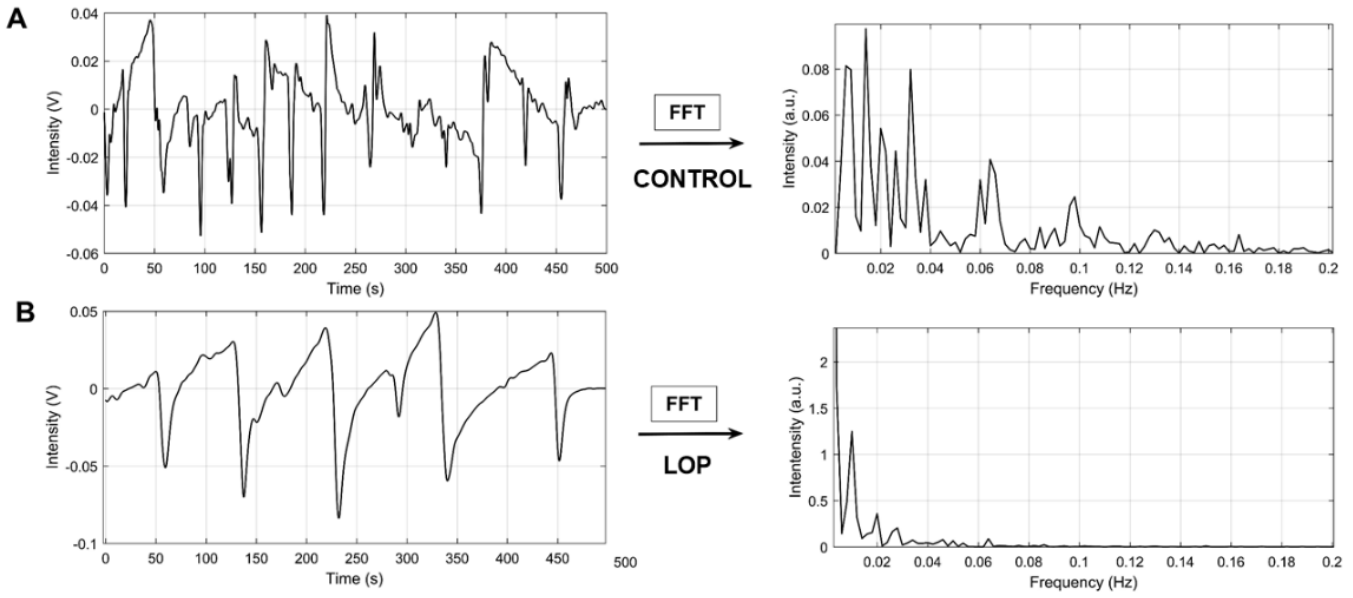

FIGURE 8 - (A) The sign referring to colonic contractility and its respective Fast Fourier Transform (FFT) for the CONTROL group, and (B) The signal related to colonic contractility and its corresponding Fast Fourier Transform (FFT) for the LOP group.

In the CONTROL group signal, we observed two colonic frequency profiles. An analysis of the FFT frequency profile revealed two well-defined dominant frequency peaks. However, in the LOP group, it was hard to identify the high-frequency profile, either in the signal or in the spectrum of dominant frequencies obtained by FFT. Our results, as shown by FFT, corroborate the previously shown statistical decrease of high frequencies (RPR) in the LOP group.

Immunohistochemistry and Morphometry

Morphometric analyses were performed on histological sections of the intestines of animals from the CONTROL and LOP groups to quantify the thickness of the intestinal layers. The slides were stained with HE, and the histological images representing the results are shown in Figures 9 A and C. In Figures 9B and 9D, the myenteric complexes between the smooth muscle layers of the intestinal tube are shown. When we performed morphometric analyses, to quantify the layers of the intestine, no changes were observed in the luminal ($p= 0.372$), mucosa ($p=0.572$), submucosa ($p= 0.941$), and muscular ($p=0.056$) compartments, when we compared the LOP group with the CONTROL group. It should be mentioned that these analyses were carried out using the ImageJ software, and the quantification data are represented in Figure 9E.

Next, we performed immunolocalization analyses of the transmembrane receptor tyrosine kinase c-Kit (c-Kit) in intestinal histological sections from animals in the CONTROL and LOP groups. c-kit was chosen as a biomarker for interstitial cells of Cajal (ICC), which constitute the myenteric plexus of the intestinal wall. The reaction data are shown in Figure 9 F-I, in animals from both groups. We observed a decrease in the number of cells immunostained for c-kit in animals from the LOP group compared to histological sections from the CONTROL group. Taken together, these data suggest that treatment with loperamide does not change the morphological structure of animals; however, it affects the blockade of c-kit receptors, which has a direct effect on colonic motility.

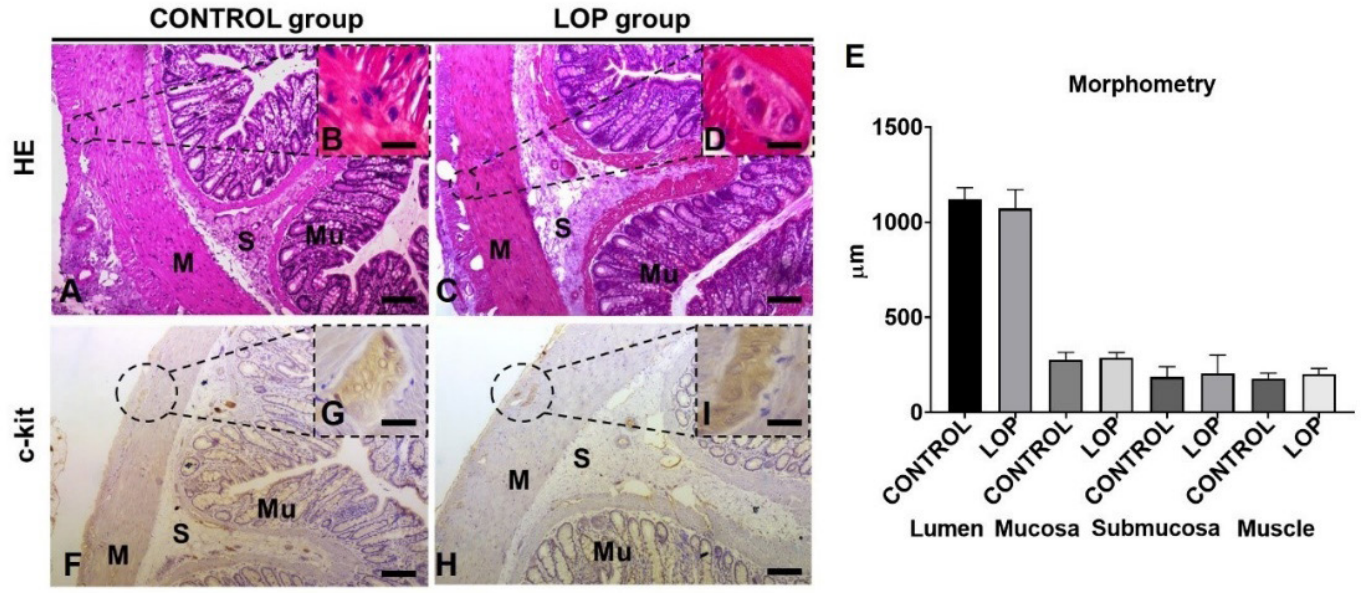

FIGURE 9 - Morphological and morphometric data of the intestine of animals from both experimental groups. Representative histological sections of the intestine from the CONTROL (A-B and F-G) and LOP (C-D and HI) groups stained with Hematoxylin-Eosin (HE) (A-D) and immunostaining for Tyrosine Kinase Receptor c-Kit (c-kit) (F-I). Representation of the morphometric quantifications of the intestinal layers, it is possible to observe that there were no changes in the luminal (p=0.372), mucosa (Mu) (p=0.572), submucosa (S) (p=0.941) and muscular (M) (p=0.056) compartments in the animals in the LOP group when compared to CONTROL (E). In detail, the myenteric plexuses stained with HE (B and D) and the ICC immunostained for cKit (G and I) are represented. Data are expressed as mean ± SD. Scale bar: A, C, F, and H: 200 µm; B, D, G, and I: 10 µm.

DISCUSSION

Using the ACB system, we conducted a comprehensive and sequential evaluation of gastrointestinal motility and its alterations in a loperamide-induced constipation model in the same rat. This technique has several advantages over other methods, such as the need for radiotracers or contrast agents, and the ability to measure both the GI tract transit time and its contractility profile simultaneously.

Assessing gastrointestinal motility is crucial for understanding the effects of drugs and interventions on the digestive system. Two techniques used to evaluate gastrointestinal motility are described, including the activated charcoal technique and the organ bath technique.

The activated charcoal technique is a well-established method for measuring gastrointestinal transit time, and it is considered the gold standard. The technique involves administering a marker and then measuring the distance it travels in the intestine. This technique provides a quantitative measure of transit time, which is essential for assessing the effects of different treatments on the digestive system. However, this technique is invasive and uses one animal each time, involving larger groups and not evaluating the control and response for the same animal.

The organ bath technique is another established method used to measure the contractility of specific intestinal segments. This technique involves dissecting the intestinal tissue and mounting it in an organ bath where it can be exposed to different pharmacological agents, and its response recorded as a dose-dependent curve. The technique allows for the measurement of contractility in specific segments of the intestine, which is useful for evaluating the effects of different treatments on the function of the digestive system. However, this technique is also invasive and is used only for ex-vivo studies.

To overcome the limitations of both techniques, further research is needed to develop non-invasive techniques that allow for repeated measurements within the same animal, while controlling for individual variability. The development of such techniques would

significantly advance the field of gastrointestinal research and pave the way for more effective treatment of gastrointestinal disorders (Calabresi *et al*., 2019). Establishing a methodology that allows the sequential assessment of GI motility in the same rat in a constipation model is promising for studies involving new laxatives and the mechanisms of action of constipation and loperamide. The ACB presents intrinsic characteristics suitable for gastroenterology due to its ability to detect the contraction frequency of TGI segments, such as the stomach and colon.

In this context, the ACB system presents a promising solution. It has intrinsic characteristics suitable for gastroenterology due to its ability to detect the contraction frequency of TGI segments, such as the stomach and colon. This capability allows for a more detailed analysis of GI motility, which is crucial in understanding and treating gastrointestinal disorders. Several previous papers have demonstrated the non-invasive ability of the ACB system to measure the contraction frequency of various segments of the gastrointestinal tract, which is particularly beneficial in chronic conditions where long-term monitoring is essential. In addition, the ACB system's ability to provide real-time feedback makes it an excellent tool for assessing the immediate effects of therapeutic interventions. Thus, we expect that the next logical step as a prospect is to use this methodology based on ACB system evaluations to monitor cases of constipation in humans, including children, which could be a valuable and possible actual pre-clinical application. Strengthening the ACB system and eventually consolidating it in the clinical environment is essential to advancing our understanding and treatment of gastrointestinal disorders.

Regarding the metabolic parameter results, we found a significant reduction in the number of pellets, a reduction in fecal moisture content, and a change in fecal morphometric. Furthermore, the LOP group presented morphometric changes in the feces, which were found as harder and drier to the LOP group. The decreased stool frequency and reduction in the number of pellets can be attributed to the inhibitory action of loperamide on smooth muscle, which reduces bowel movement frequency and increases intestinal transit time and colonic retention (Shimotoyodome *et al*., 2000). According to previous studies, loperamide inhibits intestinal water secretion, which would explain the reduction in the moisture content (FWC) of LOP's group fecal pellets (Liu, Zhi, 2021). Additionally, a longer residence time in the colon, as previously related (Narita *et al*., 2020) and observed by our results, contributes significantly to the reduction of the water content of the fecal content once the colonic epithelium is more efficient at absorbing water.

It is known that the autonomic nervous system innervates intestinal motility. The interstitial cells of Cajal are fundamental in regulating gastrointestinal motility, transmitted as electrical pacemakers, generating and propagating electrical signals, mediating communication between neurons and smooth muscle cells, and modulating muscle tone. Its function is essential to ensure proper coordination of peristaltic muscle contractions and proper movement of food contents along the gastrointestinal tract (Foong *et al*., 2020; Hirst, Edwards, 2004; Huizinga, Hussain, Chen, 2021).

The quantification parameters expressed by the MGET, MCAT, OATT, and FPER, indicated a significant increase in the GI transit of the LOP group, which is related to the fact that loperamide-induced constipation causes inhibition of smooth muscle contractions (Senez, 2015).

We evaluated gastric and colonic contractility by analyzing the signal profile and frequency distribution of the contractions. Using FFT, we found no significant difference in gastric contraction frequencies between the CONTROL and LOP groups. However, we used a histogram and Gaussian fit to further analyze the frequency distribution (Figure 7). We found that the FWHM and sigma values were higher for the LOP group than for the CONTROL group, indicating a wider and more irregular frequency distribution. As shown in (Figure 6) (RSA assessment), the LOP group (Figure 6B) showed signs of non-stationary profiles over time with more significant irregularity in the frequency of gastric contractions than the CONTROL group (Figure 6A), which allowed us to visualize and explain the increase in FWHM and sigma values quantified through the histogram of the frequency distribution in the LOP group compared to the CONTROL group.

Unlike many studies that evaluated colonic mechanical activity by determining contraction frequency through ex vivo assessments (Ma *et al*., 2021), we employed a protocol that allowed us to monitor and determine colonic mechanical activity in a rat constipation model in vivo using the ACB technique. Previous studies have classified colonic mechanical activity patterns into propulsive motor complexes (RPMCs) and rhythmically propagating ripples (RPRs)(Calabresi *et al*., 2019; Huizinga, 2001). RPRs are defined as permanent oscillations with high frequency and low amplitude, characterized by spontaneous motor patterns, where functions are related to food mixing and absorption. On the other hand, RPMC is characterized by intense contractions with low frequency and high amplitude and is associated with stool propulsion through bursts.

According to our results, we found a non-statistically significant decrease in RPMC and a very significant change in the pattern of frequencies referring to RPR associated with high frequencies in the LOP group compared to the CONTROL group. We also note that 43% of the animals in the LOP group did not present a dominant RPR frequency peak, indicating a substantial change in colonic mechanical activity. Although the difference in the PRMC profile was not significant, a specific decrease and relevant change were observed in the LOP group compared to the CONTROL. Additionally, significant and robust alterations in RPR strongly indicate a mechanical change in functional activity associated with colon dysmotility and arrhythmicity in the LOP group caused by loperamide-induced constipation.

The inhibition of smooth muscle motor activity is related to decreased levels of ICC biomarkers (C-Kit and ANO1) in intestinal tissues (Balasuriya *et al*., 2021). Several studies that assessed the histological effects on the colon in loperamide-induced constipation models reported significant changes in the colonic mucosa and decreased levels of ICC biomarkers (C-Kit and ANO1) (Jaladanki, Wang, 2011). These alterations include a decrease in the depth of the crypt, a reduction in the thickness of the mucosa and muscle layers, and infiltration of inflammatory cells in the damaged mucosa in rats treated with loperamide. (Choi *et al*., 2014; Diener, Knobloch, Rummel, 1988; Eor *et al*., 2019; Kim *et al*., 2013; Shimotoyodome *et al*., 2000).

Our study found no significant difference in layer thickness between the LOP and CONTROL groups. However, we did observe a reduction in ICC biomarkers in the LOP group samples. This may be linked to changes in gastric and colonic contractions. Such changes can affect the colon's mechanical activity, altering contraction frequency patterns and causing arrhythmias. This suggests colonic dysmotility in rats treated with loperamide, resulting in slow transit as seen in increased GI transit parameters, MGET, MCAT, OATT, and FPER. Although the ACB system was effective in determining the frequency of gastric and colonic contractions and determining the complete gastrointestinal transit, the system has limitations to be improved, such as the intrinsic noise and the ability to determine the amplitude of the analyzed mechanical contractions, as for example determined in the organ bath technique. New ACB arrangements are being developed to address these limitations.

Furthermore, we believe this methodology is adequate for investigating several organs and their functions in normal circumstances or under dysfunction, including future human clinical research. We recognize that substantial improvements are still required to offer a suitable methodology to reach a real clinical translational potential.

CONCLUSION

In this study, we determined gastric and colonic contraction frequencies and GI transit by ACB in loperamide induced model rats. Alterations in the frequency patterns of gastric and colonic contractions were found in animals from the LOP group that may be directly related to the reduction of ICC biomarkers also found in this study. These factors significantly contributed to the increase in GI transit time observed in this study in LOP group, which were expressed by parameters MGET, MCAT, OATT and FPER.

The ACB system has the potential to assess transit abnormalities and their severities, as well as to assist in the research and development of new, safer, and

more effective laxatives for constipation treatment. Substantial improvements can enhance the quality of ACB measurements, making it a suitable technique for achieving real clinical translational potential in human research.

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CONFLICT OF INTEREST:

The authors state that there is no conflict of interest.

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