# Chronic aerobic swimming exercise promotes functional and morphological changes in rat ileum

## Exercise promotes morphofunctional changes in ileum

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**Abstract**

Several studies have reported the gastrointestinal effects promoted by the physical exercise. Thus, we aimed to evaluate the influence of swimming exercise on the contractile reactivity, lipid peroxidation and morphology of rat ileum. Wistar rats were divided into sedentary (SED) and groups exercised for 2 (EX2), 4 (EX4), 6 (EX6) or 8 (EX8) weeks, 5 days/week. Animals were euthanized, the ileum was removed and suspended in organ baths where the isotonic contractions were recorded. Lipid peroxidation was evaluated by MDA measurement with TBARS assay and morphology by histological staining. Cumulative concentration-response curves to KCl were attenuated, as the $E_{max}$ values were changed from 100% (SED) to 63.1 ± 3.9 (EX2), 48.8 ± 3.8 (EX4), 19.4 ± 1.8 (EX6) and 59.4 ± 2.8% (EX8). Similarly, cumulative concentration-response curves to carbachol (CCh) were attenuated, as the $E_{max}$ values were changed from 100% (SED) to 74.1 ± 5.4 (EX2), 75.9 ± 5.2 (EX4) and 62.9 ± 4.6 (EX6), but not in the EX8 (89.7 ± 3.4%). However, CCh potency was increased in this latter, as the $EC_{50}$ was altered from 1.0 ± 0.1 x $10^{-6}$ (SED) to 2.1 ± 0.4 x $10^{-7}$ (EX8). MDA concentration was altered only in EX4 (44.3 ± 4.4) compared to SED (20.6 ± 3.6 μmol/L). Circular layer was reduced in SED when compared to the exercised groups. Conversely, longitudinal layer was increased. In conclusion, chronic swimming exercise reduces the ileum contraction, equilibrates the oxidative damage and promotes changes in tissue size to establish an adaptation to the exercise.

Keywords: aerobic exercise, contractile reactivity, gastrointestinal tract, oxidative stress, rat ileum, swimming.

## Introduction

Exercise is an activity that affects all organs and tissues and can result in many health benefits [1], such as decreasing the vasoconstriction resulting from aging [2] and through changes on cardiovascular system in order to maintain tissue oxygen demand [3-5]. It has been considered an important therapeutic tool in the prevention and treatment of various diseases such as cardiovascular, metabolic syndrome and gastrointestinal (GI) disorders[6-8].

The exercise promotes positives effects on gastrointestinal tract such as reduced incidence of colorectal carcinoma, diverticulitis, cholelithiasis and constipation. Despite this, strenuous exercise may cause gastrointestinal symptoms, such as nausea, vomiting, diarrhoea and intestinal bleeding. Thus, recommendations to reduce exercise-induced GI-symptoms include reduction of exercise intensity and prevention of dehydration[7,9].

It is well described that exercise is responsible for stimulating the reactive oxygen species (ROS) and reactive nitrogen species (RNS) production during muscle contraction, and it plays an important role in muscle metabolism. Studies have reported that in a physiological range, that is low to moderate levels, ROS provide several regulatory functions in the cell, such as the control of gene expression, regulation of signaling pathways and modulation of skeletal muscle contraction force, leading to an increase on muscle strength[10]. However, increased ROS and RNS concentrations promote changes in the lipid matrix and cell membranes, characterizing the oxidative stress[10-13]. In this context, the long-term exercise, namely chronic exercise, in moderate intensity, promotes increase in antioxidant defenses, which remove free radicals (FR) and stabilize the reactive species production[14].

Although intestinal smooth muscle is not directly involved in physical exercises, it is subjected to physiological stress, especially for the ischemia-reperfusion process due to the diversion of blood flow to the skin and the active skeletal muscles[15]. This ischemia can promote motor and intestinal mucosa changes[16]. The effect of exercise has been extensively studied on the intestinal mucosal layer, absorption of nutrients and gastrointestinal permeability[17,18]. However, few studies have reported the effect of exercise on intestinal contractile response, despite the smooth muscle reactivity abnormalities represent one of the pathophysiological processes that characterize intestinal colic, diarrhea and constipation[19].

Even though studies have evaluated the reactivity of ileum using the treadmill exercise, the physiological responses of treadmill exercise differs from swimming exercise, as well as the use of swimming exercise shows advantages over the treadmill protocol, because swimming is a natural ability of rats, therefore, is widely used as an appropriate model of physical exercise, while to running, rats must be conditioned, does not representing a natural instinct[20,21]. However, there are many works relating the effects of treadmill in physiological parameters instead swimming exercise, while it is a common human sport practice.

Therefore, in this work we investigated the influence of chronic swimming aerobic exercise on the contractile reactivity of intestinal smooth muscle, oxidative stress and morphology of rat ileum hypothesizing that the regular practice of swimming exercise in a moderate intensity can provide beneficial effects for the gastrointestinal system.

## Materials and Methods

### Ethical approval

All experimental procedures were performed following the principles of animal care of the Guidelines for the ethical use of animals in applied etiology studies[22] and previously approved by UFPB Ethics Committee on Animal Use (Protocol/CEUA no. 0907/13).

### Animals

Wistar rats (*Rattus norvegicus*), initially two months old, weighing 180-200 g, were obtained from the bioterium of the Biotechnology Center (CBiotec)/UFPB. The animals were kept under restricted food control with balanced diet (Labina®), to avoid large differences in body weight and density, and had access to water *ad libitum*. They were maintained in rooms at 21 ± 1 °C and submitted to a 12 h light-dark cycle (light from 6 to 18 h). Forty-eight hours after the last exercise session, the animals were fasted for 18 h (receiving only water *ad libitum* during this period) and were then euthanized by cervical dislocation followed by cervical vessels section to perform the experimental analysis. This time of fasting is important to avoid the influence of substances released by the GI tract during the intestinal transit.

**Drugs**

Calcium chloride bihydrate ($CaCl_2.2H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and glucose ($C_6H_{12}O_6$) were purchased from Vetec (Brasil). Sodium bicarbonate ($NaHCO_3$) was purchased from Fmaia (Brasil). Sodium chloride (NaCl) and potassium chloride (KCl) were purchased from Química Moderna (Brasil). Monopotassium phosphate ($NaH_2PO_4$), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Nuclear (Brasil). Carbamylcholine hydrochloride (CCh) was purchased from Merck (Brasil). Formaldehyde was purchased from Vetec (Brasil). Thiobarbituric acid, tetramethoxypropane, perchloric acid, Mayer's hematoxylin and eosin were purchased from Sigma (Brasil). Carbogen mixture (95% $O_2$ and 5% $CO_2$) was obtained from White Martins (Brazil).

The CCh was used to mimic the cholinergic stimulation that happens on the intestinal smooth muscle promoted by the myenteric plexus[23,24]. The KCl was employed to simulate the pacemaker of interstitial cells of Cajal located at the boundaries and in the substance of the inner, circular muscle layer, from which they spread to the outer, longitudinal muscle layer[25,26].

**Exercise protocol**

In swimming protocols for rats, the animals swim vertically and are submitted to exercise with overloads tied to the thorax[27-30]. Brito et al. (2015) [31] showed that rats submitted to forced swimming exercise for 1 h with a metal ring of 3-6% of theirs body weight attached to their torso present blood lactate levels into the range of aerobic exercise, characterizing a moderate intensity. Thus, based on these evidences, we performed the exercise protocol with rats into a restricted range of age with a metal of 3% of their body weight attached to their body to avoid the inherent ability of rats to remain floating on the water surface.

The animals were divided into five groups (five animals each): sedentary (SED) and exercised for two (EX2), four (EX4), six (EX6) and eight weeks (EX8). Before the experiments, animals were placed in a container with 1.5 cm of water at 23-25 °C for 2 minutes, for the animal acclimation. This procedure was important to prevent the animal stress, especially on start of the exercise[32]. The swimming protocol was adapted from Chies et al. (2003) [33], and was performed in a plastic container measuring 43x63x33 cm, with water at a

temperature of 23-25 °C. The animals of sedentary group (control) were subjected to the same stress of exercised groups, including food deprivation and exposure to noise throughout the training. Exercised animals were subjected to daily forced swimming for 1 h, 5 days per week, between 8 a.m. and 4 p.m, kept attached to a metal ring corresponding to 3% of their body weight on its trunk, which improves the resistance of the animal to exercise and prevents the fluctuation[22,27,34]. Both mouse and rat are accepted in swimming exercise protocols, in this view, we used the rat model precisely by fact that rats TGI exhibit more similarities to humans, being more sensitive to the cholinergic transmission[34,35]. The animals rested for 48 hours at the end of each week of exercise[36-38].

**Contractile reactivity measurement**

Animals were euthanized by cervical dislocation followed by exsanguination. The ileum was immediately removed, cleaned of fat and connective tissue, immersed in physiological solution at room temperature and bubbled with carbogen mixture. To register the isotonic contractions, ileum segments (2-3 cm) were suspended by cotton yarn in organ bath (5 mL) and recorded on smoked drum through levers coupled to kymographs (DTF, Brazil) under resting tension of 1.0 g at 37 °C[39]. The organ baths were warmed by a thermostatic pump Polystat 12002 Cole-Palmer (Vernon Hills, IL, USA). The physiological solution used was Tyrode solution, whose pH was adjusted to 7.4, and the composition (in mM) was: NaCl (150.0), KCl (2.7), $CaCl_2$ (1.8), $MgCl_2$ (2.0), $NaHCO_3$ (12.0), $NaH_2PO_4$ (0.4), D-glucose (5.5). After 30 min of stabilization period, an isotonic contraction was induced with 30 mM KCl to verify the functionality of the organ, 15 min after, two similar cumulative concentration-response curves to KCl ($10^{-3}$ x $10^{-1}$ M) and CCh ($10^{-9}$x $10^{-4}$ M) were obtained. The contractile reactivity was assessed based on the values of the concentration of a substance that produces 50% of its maximal effect ($EC_{50}$) and the maximum effect ($E_{max}$) of contractile agents to the control and exercise groups. Tyrode's solution, KCl and CCh were diluted in freshly prepared distilled water time of the experiment.

**Lipid peroxidation assay**

Lipid peroxidation in tissue was determined measuring the chromogenic product of 2-thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), which is one of the products formed as a result of membrane

lipid peroxidation[40]. Ileum segments of each animal were homogenized with KCl (1:1), and 250 µL of tissue homogenate were incubated at 37 °C for 60 min. Then, the mixture was precipitated with 35% perchloric acid and centrifuged at 14.000 rpm for 20 min at 4 °C. The supernatant was transferred to eppendorffs and 400 µL of 0.6% thiobarbituric acid were added and incubated at 95-100 °C for 1 h. After cooling, the samples were read in spectrophotometer at a wavelength of 532 nm. The results were expressed in µmol/L per gram of dry tissue.

**Histological analysis**

Samples of ileum were fixed in 10% formaldehyde solution, subjected to paraffin embedding standard histological procedures. This procedure included the following steps: 1) dehydration of the tissue at increasing alcohol series, 70% for 24h, 80, 96 and 100% (third bath) for 1 h each; 2) diaphanization or bleaching in which the tissue was immersed in 100% xylene alcohol (1:1) for 1 h followed by two immersions in pure xylene for 1 h each; 3) impregnation in paraffin, wherein the sample was immersed in two baths of liquid paraffin (heated to 50 °C) for 1 h each. The blocks obtained were cut to 5 µm thick in cross-section of the ileum using a rotary microtome. The sections were stained with Mayer's hematoxylin and eosin[41]. The slides were analyzed with an optical microscope with an attached camera, where two cross sections per animal were photographed and analyzed. The second quadrant of the ileum circumference was analyzed, and measurements of the circular and longitudinal muscle layers were obtained in analyzer program images[42].

**Statistical analysis**

Data were expressed as mean ± standard error of the mean (S.E.M.). Cumulative concentration-response curves were fitted and $EC_{50}$ values were obtained by nonlinear regression[43]. Comparison of two groups was performed using the Student's *t* test and multiple comparisons by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test. The differences were considered significant when $p < 0.05$.

All data were analyzed using GraphPad Prism® software version 5.01 (GraphPad Software Inc., San Diego CA, USA).

**Results**

**Contractile reactivity measurement**

Cumulative concentration-response curves to KCl (n = 5) were attenuated, with reduction on $E_{max}$ value from 100% (SED) to 63.1 ± 3.9; 48.8 ± 3.8; 19.4 ± 1.8 and 59.4 ± 2.8% in the groups exercised by 2, 4, 6 and 8 weeks, respectively. However, the $EC_{50}$ values of the exercised groups ($EC_{50}$ = 1.5 ± 0.1; 1.9 ± 0.4; 2.6 ± 0.3 and 2.4 ± 0.2 x $10^{-2}$ M, respectively) showed no statistical difference when compared with SED group ($EC_{50}$ = 1.7 ± 0.1 x $10^{-2}$ M) (Figure 1, table 1). Similarly, cumulative concentration-response curves to CCh (n = 5) were attenuated due to the exercise, with reduction of $E_{max}$ value from 100% (SED) to 74.1 ± 5.4; 75.9 ± 5.2; 62.9 ± 4.6 and 89.7 ± 3.4% in the groups exercised by 2, 4, 6 and 8 weeks, respectively. In contrast, the $EC_{50}$ values of the exercised groups ($EC_{50}$ = 1.5 ± 0.5; 1.3 ± 0.2; 1.5 ± 0.3 x $10^{-6}$ and 2.1 ± 0.4 x $10^{-7}$ M, respectively) have showed no statistical difference when compared to the SED group ($EC_{50}$ = 1.0 ± 0.1 x $10^{-6}$ M). However, differences in the values of $EC_{50}$ between EX2 *vs.* EX8 and EX6 *vs.* EX8 groups were observed (Figure 2, table 2).

**Lipid peroxidation assay**

The MDA concentration (n = 5) in rat ileum was increased from 20.6 ± 3.6 (SED) to 44.3 ± 4.4 µmol/L/g of dry tissue (EX4), but did no differ on groups exercised by 2, 6 and 8 weeks (28.1 ± 4.8; 20.0 ± 3.6 and 17.2 ± 3.6 µmol/L/g of dry tissue, respectively) when compared to the control (Figure 3).

**Histologic analysis**

The smooth circular muscle layer (n = 5) was decreased from 50.9 ± 0.3 (control) to 44.0 ± 1.8, 43.5 ± 1.3, 35.5 ± 1.4 and 41.6 ± 0, 6 µm in groups exercised by 2, 4, 6 and 8 weeks, respectively (Figures 4 and 5). However, longitudinal smooth muscle layer (n = 5) was increased from 21.6 ± 0.3 (control) to 31.8 ± 1.0, 36.2 ± 2.5, 29.6 ± 1.8 and 30.8 ± 1, 3 µm in groups exercised by 2, 4, 6 and 8 weeks, respectively (Figures 4 and 6).

**Discussion**

In this study we investigated the influence of chronic aerobic swimming exercise on the contractile reactivity, oxidative stress and morphology on rat ileum, being shown that this type of exercise decreases the contractile response, the tissue lipid peroxidation and modifies the thickness of intestinal smooth muscle layer.

Abnormalities on intestinal contractility represent one of the pathophysiological processes that characterize intestinal disorders, such as colic, diarrhea and constipation[20]. Thus, physical exercise has become a growing practice in development, which has been considered an important therapeutic tool in the prevention and treatment of diseases affecting the gastrointestinal tract[7].

Swimming exercise can be used to identify physiological, biochemical and molecular responses against the adjustments caused by chronic training[44-46] similar to found in experiments with human[27-30]. Regarding the gastrointestinal tract, individuals who practice swimming exercise can present gastrointestinal alterations[47], however, the precise mechanism involved in these effects remains unclear.

Exercise taken in long- term is a stimulus for physiological adaptations. These adjustments are reflected by changes in contractile proteins, mitochondrial function, metabolic changes, intracellular signaling and transcriptional response[48]. In a study performed by Rosa et al. (2005) [49], it was observed that chronic aerobic treadmill exercise does not affect the contractile reactivity of old mice ileum. In addition, Lira et al. (2008) [42] showed that the same chronic exercise alters the ileum contraction reactivity of young mice, decreasing the response to acetylcholine. This effect was associated to changes in plasma concentration of hormones that regulate the function of gastrointestinal tract. The authors analyzed the influence of treadmill exercise on contractile reactivity of mouse ileum in exercise periods of 10, 25, 40 and 55 days and it was verified that the ileum contractile reactivity was increased from 10 days training, but it was decreased from 55 days, highlighting the importance of chronic evaluations. In fact, the time necessary to these adaptations occur varies depending on type and time of exercise[50]. Additionally, differences in physiological responses have been demonstrated in rats treadmill or swimming exercised, being observed that treadmill exercise is more effective in modulating peripheral serotonergic system, while swimming has greater influence on sympathetic nervous system[51], increasing the adrenaline release[52].

Therefore, since there are differences in physiological changes obtained in various modalities of exercise, as well as studies show that treadmill exercise reduces mouse ileum contractile reactivity, it was

decided to investigate whether chronic aerobic swimming exercise could also alter rat ileum contractile reactivity.

It was verified that the exercise attenuated the cumulative concentration-response curves to KCl, an electromechanical contractile agent[53], decreasing its efficacy without changing the contractile potency (Figure 1). Similarly, the exercise attenuated the cumulative concentration-response curves to CCh, which acts by a pharmacomechanical coupling[54], decreasing its efficacy with increasing on the EX8 potency, which can be associated to the adaptation process to the exercise (Figure 2). In addition, it was verified that exercise altered the rat ileum contractile reactivity from second to sixth week, promoting a decrease on contractile responsiveness to both contractile agents. In contrast, in the eighth week of exercise (EX8), the cumulative curve to KCl was increased compared with the exercised group for six weeks, do no differing to groups exercised for 2 and 4 weeks, but not completely restored to the control group, while the cumulative curve to CCh for this group was similar to that achieved in the sedentary group, representing a complete restauration of the contractile response to this agonist (Figure 1 and 2). Thus, the rat ileum contractile reactivity undergoes adaptive responses from the first few weeks of exercise and attains a stabilization period on the eighth week. This data set indicate that chronic aerobic swimming exercise decreases rat ileum responsiveness front both contractile agents, possibly due to an increase on the noradrenaline release during the swimming exercise[51]. Furthermore, we cannot discharge that, perhaps, the swimming exercise alters the cholinergic transmission and the resting membrane potential of intestinal smooth muscle cell, which could be the responsible for the contractile response attenuation.

The physiological changes produced on chronic exercises promote increase of antioxidants that remove free radicals and stabilize the production of reactive species[15]. Silva et al. (2009) [55] demonstrated a decrease in MDA levels in mice skeletal muscle after 8 weeks of treadmill exercise. However, in a study developed by Rocha et al. (2010) [56], it was observed that a period of eight weeks of swimming exercise did not decreased the levels of lipid peroxidation in rat aorta. In contrast, it was observed an increased expression of the antioxidant superoxide dismutase enzyme, but without changing the concentration of catalase, leading to an imbalance in the SOD/CAT. It is related that this imbalance in SOD/CAT can explain the increase in tissue lipid peroxidation, leading to oxidative damage, promoting changes in their structure and function. However, it is

reported that minor damage on tissue structure is needed to promote adaptation, functioning as a cellular stimulus.

To verify whether the swimming exercise reduces oxidative damage on rat ileum, it was investigated the production of MDA, indicating possible alterations in the lipid matrix and cell membranes resulting from the indirect effect of ROS production[13]. We showed that chronic aerobic swimming exercise increases lipid peroxidation after four weeks of exercise, and decreases after six weeks (Figure 3). These data support the hypothesis that chronic aerobic exercise increases lipid peroxidation in this tissue after four weeks of exercise and it served as a cellular stimulus for adaptation to exercise and to stimulate the cellular production of antioxidant to remove free radicals and reduce oxidative damage promoted by the exercise itself.

Since there was an increase in lipid peroxidation, indicating an increase of free radicals production on rat ileum during four weeks of exercise, and knowing that the increase in free radicals causes oxidative stress with potential damage to tissues and organs[15,57,58], we hypothesized that this increase would be changing the architecture of intestinal smooth muscle. To assess this hypothesis, we measured the muscle layer thickness through histological sections of rat ileum, and we verified a decrease on the circular muscle layer thickness (Figures 4 and 5) and an increase on the longitudinal muscle layer thickness (Figures 4 and 6) in all exercised groups.

Given these results, we concluded that lipid peroxidation is not related to alterations in muscle layer thickness, since there was an increase in lipid peroxidation in the group exercised for 4 weeks, while the alterations of the smooth muscle layers occurred in all the exercised groups. Thus, chronic aerobic swimming exercise alters the structure of the tissue, probably not due to the increase of reactive species production, however, other mechanism seems to be related to this change in the structure of smooth muscle acting as a stimulus to the organ adaptation.

The contractile reactivity of circular and longitudinal smooth muscle layers of rat ileum is affected differently by the hypertrophic process. Hypertrophy of the circular layer shows an increase in contractile efficacy, while hypertrophy of the longitudinal layer exhibits a greater sensitivity to relaxing factors, leading to decreased contraction[59,60]. These data corroborate our results that showed a decrease in circular and increase in longitudinal layer, with reduction in contractile reactivity.

It is well known that the two most common symptoms in patients affected by chronic diseases, such as Crohn's Disease, are diarrhea and constipation[61]. It has been suggested that physical exercise has beneficial effects on the gastrointestinal tract, mainly due to decreased gastrointestinal blood flow, neuroimmuno-endocrine alternations and increased gastrointestinal motility[7]. Additionally, some studies have shown that physical activity can accelerate orocecal transit time in different populations and improve symptoms of constipation in irritable bowel disease patients[62].

Therefore, our results provide some initial evidences that the swimming physic exercise, in animal model, modifies the intestinal motility due to mechanisms involving the cholinergic transmission as well as affecting the resting membrane potential. To what extent these findings can be extrapolated to human to explain the reducing in intestinal motility is a point that need more research.

**Competing interests**

The authors declare no conflict of interest.

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**Tables**

**Table 1 -** Values of $EC_{50}$ (M) and $E_{max}$ (%) of KCl in the SED, EX2, EX4, EX6 and EX8 groups on rat ileum.

| KCl (M) | $EC_{50}$ (M) | $E_{max}$ (%) |
|---|---|---|
| SED | $1.7 \pm 0.1 \times 10^{-2}$ | 100 |
| EX2 | $1.5 \pm 0.1 \times 10^{-2}$ | $63.1 \pm 3.9^{****}$ |
| EX4 | $1.9 \pm 0.4 \times 10^{-2}$ | $48.8 \pm 3.8^{**** \#}$ |
| EX6 | $2.6 \pm 0.3 \times 10^{-2}$ | $19.4 \pm 1.8^{**** \#\#\#\# ¥¥¥¥}$ |
| EX8 | $2.4 \pm 0.2 \times 10^{-2}$ | $59.4 \pm 2.8^{**** ££££}$ |

One-way ANOVA followed by Bonferroni's post-test (n = 5). $^{\#}p < 0,05$ (EX2 *vs.* EX4); $^{****}p < 0,0001$ (SED *vs.* EX2/EX4/EX6/EX8); $^{\#\#\#\#}p < 0,0001$ (EX2 *vs.* EX6); $^{¥¥¥¥}p < 0,0001$ (EX4 *vs.* EX6); $^{££££}p < 0,0001$ (EX6 *vs.* EX8).

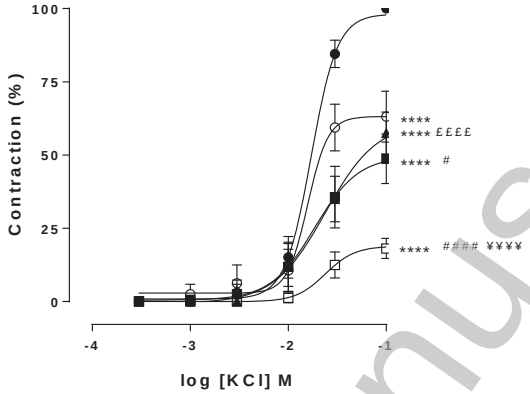
**Table 2 -** Values of $EC_{50}$ (M) and $E_{max}$ (%) of CCh in the SED, EX2, EX4, EX6 and EX8 groups on rat ileum.

| CCh (M) | $EC_{50}$ (M) | $E_{max}$ (%) |
|---|---|---|
| SED | $1.0 \pm 0.1 \times 10^{-6}$ | 100 |
| EX2 | $1.5 \pm 0.5 \times 10^{-6}$ | $74.1 \pm 5.4^{**}$ |
| EX4 | $1.3 \pm 0.2 \times 10^{-6}$ | $75.9 \pm 5.2^{**}$ |
| EX6 | $1.5 \pm 0.3 \times 10^{-6}$ | $62.9 \pm 4.6^{****}$ |
| EX8 | $2.1 \pm 0.4 \times 10^{-7 * \#}$ | $89.7 \pm 3.4^{\#\#}$ |

One-way ANOVA followed by Bonferroni's post-test (n = 5). $^{*}p < 0,05$ (EX2 *vs.* EX8); $^{\#}p < 0,05$ (EX6 *vs.* EX8). $^{**}p < 0,01$ (SED *vs.* EX2/EX4); $^{\#\#}p < 0,01$ (EX6 *vs.* EX8), $^{****}p < 0,0001$ (SED *vs.* EX6).

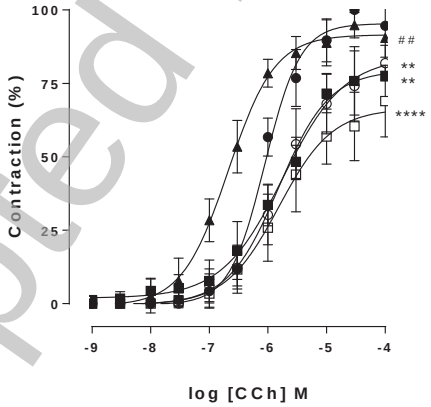
**Figures and legends**

**Figure 1 -** Cumulative concentration-response curves to KCl in the SED (●), EX2 (○), EX4 (■), EX6 (□) and EX8 groups (² ) on rat ileum.



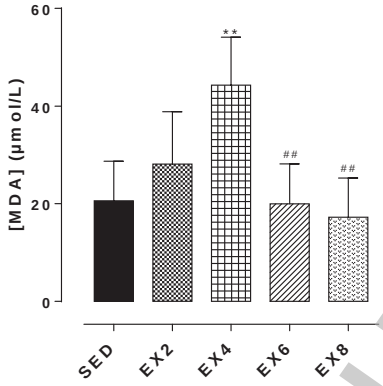

The symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test. $^{\#}p < 0.05$ (EX4 *vs.* EX2), $^{****}p < 0.0001$ (SED *vs.* EX2/EX4/EX6/EX8); $^{\#\#\#\#}p < 0.0001$ (EX2 *vs.* EX6); $^{¥¥¥¥}p < 0.0001$ (EX4 *vs.* EX6); $^{££££}p < 0.0001$ (EX6 *vs.* EX8).

**Figure 2 -** Cumulative concentration-response curves to CCh in the SED (●) and EX2 (○), EX4 (■), EX6 (□) and EX8 (² ) groups on rat ileum.



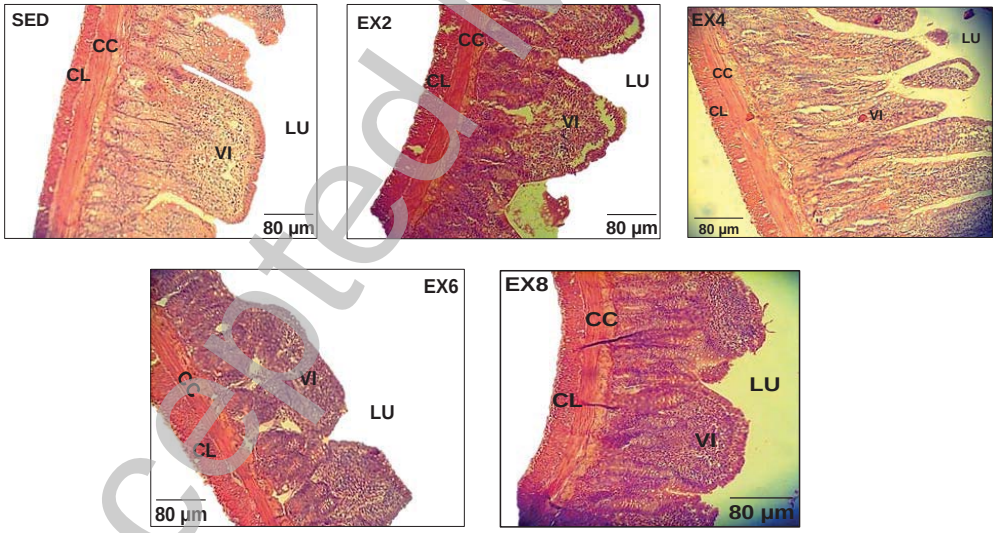

The symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test. $^{**}p < 0.01$ (SED vs. EX2/EX4); $^{\#\#}p < 0.01$ (EX6 *vs.* EX8); $^{****}p < 0.0001$ (SED *vs.* EX6.).

**Figure 3 -** Concentration of MDA in the SED, EX2, EX4, EX6 and EX8 groups on rat ileum.



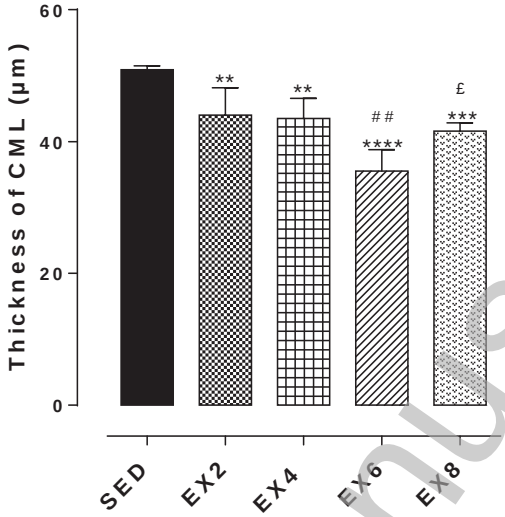

The columns and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test. $^{**}p < 0.01$ (SED *vs*. EX4); $^{\#\#}p < 0.01$ (EX4 *vs*. EX4 and EX6 *vs*. EX8).

**Figure 4 -** Histological section of the rat ileum from the SED, EX2, EX4, EX6 and EX8 groups.



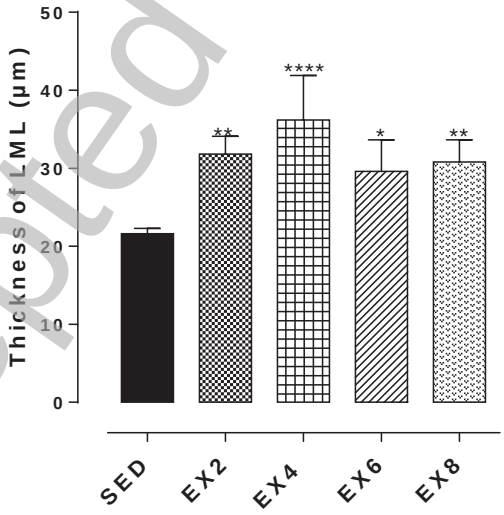

Increased lens: 40x; LL: longitudinal layer; CL: circular layer; VI: villi; LU: lumen.

**Figure 5 -** Circular muscle layer (CML) thickness of the SED, EX2, EX4, EX6 and EX8 groups.




The columns and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test. $^{£}p < 0.05$ (EX6 *vs*. EX8), $^{**}p < 0.01$ (SED *vs*. EX2/EX4); $^{\#\#}p < 0.01$ (EX2/EX4 *vs*. EX6); $^{***}p < 0.001$ (SED *vs*. EX8); $^{****}p < 0.0001$ (SED *vs*. EX6).

**Figure 6 -** Longitudinal muscle layer (LML) thickness of the SED, EX2, EX4, EX6 and EX8 groups.




The columns and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Bonferroni's post-test. ); $^{*}p < 0.05$ (SED *vs*. EX6); $^{**}p < 0.01$ (SED *vs*. EX2/EX8); $^{****}p < 0.0001$ (SED *vs*. EX4).