Damaging effects of intense repetitive treadmill running on murine intestinal musculature

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INTENSE EXERCISE (IE) is associated with accelerated generation of reactive oxygen species (ROS), overwhelming the antioxidant defense mechanisms and leading to oxidative stress in different tissues such as skeletal muscle, heart, liver, kidney, and brain (13). Specific sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain, activation of xanthine oxidase reaction, hemoglobin oxidation, and activation of neutrophils (29). One of the main consequences of free radical accumulation in the tissue is membrane fluidity alterations due to lipid peroxidation in the fatty acid moieties of phospholipid molecules. Because this can lead to membrane structural disorganization and cellular macromolecules, such as proteins and DNA (3), being targets for free radicals, membrane-bound enzymes and receptor function may be affected (2).

Although gastrointestinal symptoms such as diarrhea and intestinal bleeding are commonly observed during prolonged IE (25, 30), the effects of exercise on functional features, except for food malabsorption and mucosal structural damage, are poorly understood. In addition, it has been reported that, in response to maximal exercise training, splenic flow may decrease to critical levels, causing disturbances in gastrointestinal motility, intestinal absorption, and mucosa integrity, probably due to exercise-induced ischemia-reperfusion events (20).

The harmful effects of IE training associated with an inappropriate recovery schedule on the skeletal muscle, cardiac, cardiovascular, and respiratory tissues (6, 16) have been exhaustively explored. In contrast, studies that focus on tissues not directly engaged with movement, such as those from the gastrointestinal tract, remain scarce despite their essential role to body homeostasis. Rosa et al. (27) recently reported that a habitual moderate aerobic program avoided both the structural alterations and the enhanced oxidative stress of C57BL/6 ileum caused by aging. However, as far as IE is concerned, no studies have been conducted on intestinal contractility, particularly on the cell signaling triggered by either depolarization or agonist stimulation. Thus the aim of the present study was to investigate the effects of IE on intestinal oxidative stress and to unravel its relationship with possible morphological and functional impairments of the tissue and the consequences in terms of contraction damages.

MATERIALS AND METHODS

Animals

Inbred male C57BL/6 mice (3 mo old, 28 ± 2 g) were obtained from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia-Universidade Federal de São Paulo animal facility. Mice were housed five animals/cage, with water and food ad libitum. Animals were kept on a 12:12-h light-dark cycle (0600 to 1800) and maintained at 23°C for at least 5 days before any experimental procedure. Animals were divided into three groups: sedentary (control group), 4 days of IE (EX.4D group), and 10 days of IE (EX.10D group). Mice were killed by cervical dislocation 24 h after the second incremental test (IT2), and gastrocnemius muscle and ileum were isolated quickly, frozen, and stored at −80°C for oxidative stress analysis. Ileum segments were also separated for pharmacolog-
Exercise Protocol

Animals were submitted to treadmill running, a type of exercise in which intensity and duration are easily manipulated and quantified in contrast to voluntary wheel or swimming exercises (9). Animal groups were initially acclimated to the treadmill environment by 15-min daily running sessions at 10 m/min, for 5 successive days (adaptation period). The individual maximum velocity was determined by submitting each animal to an incremental test (IT), which consisted of 3 min of warm-up at 5 m/min, followed by progressive increases of 1 m/min every minute until animals reached exhaustion, after adjustment of the treadmill speed at 10 m/min for 1 min, and 3 min of cooling down at 5 m/min. Subsequently, the exercised animal groups were submitted to a bout of intense daily exercise, according to the following schedule: 1) 3 min of warm-up at 5 m/min, 2) running at 85% of maximum running velocity until animals reached exhaustion, and 3) 3 min of cooling down at 5 m/min. The treadmill grade was always set at 0%. Mice were stimulated to run by gentle hand prodding. Electrical shock was avoided as negative reinforcement because this would have added undue stress, which is not usually associated with exercise. The running time until exhaustion, hereafter referred to as exhaustion time, was measured in every session. Control animals were exposed to the same environmental conditions (handling, treadmill motor noise, vibration, and deprivation of food and water) during the period that the experimental groups were performing daily exercise sessions.

After all exercise sessions or ITs, animals were carefully checked for any kind of physical injury, such as bleeding, diarrhea, or abnormal behavior, which could interfere with the results; no animals were discarded because of these problems.

Training Intensity Markers

Physical performance. Physical performance was assessed by the treadmill maximum velocity each animal reached in the IT. Two ITs were performed by each animal: the first one 24 h after the last day of the adaptation period (IT1) and the second one 24 h after the last day of exercise (IT2).

Blood lactate concentration. Blood samples (25 µl) were collected from the caudal vein of the animals at rest, immediately after IT1, and again after the 4th and 10th day of exercise training. Blood samples were stored in 50 µl of 1% natrium fluoride and frozen at −20°C. Blood lactate concentrations were measured in a lactate analyzer (YSI model 1500 Sport Lactate Analyzer) according to the manufacturer’s technical specifications.

Morphological Studies on Tissue

Fresh ileum sections were isolated and appropriately stained with hematoxylin and eosin. In brief, tissue samples were fixed with 2% osmium tetroxide in cacodylate buffer, pH 7.2, for 1 h at 25°C, dehydrated in graded ethanol, treated with uranyl acetate and lead citrate and examined in a Jeol EXII transmission electron microscope (Tokyo, Japan).

Isometric Contraction Assays

Distal segments of the ileum, 2.0 cm long, were tied up to a steel hook support, suspended in a 5-ml perfusion chamber containing Tyrode solution at 37°C, pH 7.4, and bubbled with air. Isometric tension was recorded by means of a force transducer (TRI 210, Letica, Barcelona, Spain) connected to an amplifier (model AECA-0804, Solucao Integrada, Sao Paulo, Brazil). Acquisition and analysis of the isometric contractions were conducted with KitCad8 software (Software & Solutions, Sao Paulo, Brazil). Three ileum strips were obtained from each animal and allowed to equilibrate under 0.5-g basal tension for at least 30 min before any experimental procedure. Throughout the resting period, chamber solution was renewed with fresh Tyrode solution every 10 min. Intestinal tissue responsiveness was evaluated by the construction of noncumulative concentration-response curves elicited by carbachol (CCh), bradykinin (BK), or KCI. Tissue strips were exposed to each stimulant concentration for 1.5 min, and 5-min intervals were allowed between two successive challenges. The potency, assessed as the concentration of the agonist that causes 50% of the maximum response or the concentration of KCl that causes 50% of the maximum response (EC50), and the efficacy, the maximum contractile response (Emax), were determined. Only one concentration-contractile response curve was produced per ileum strip.

Oxidative Stress Levels

Lipid peroxidation assay. Peroxidative damage to membrane lipid constituents from both the gastrocnemius muscle and the ileum was determined by measuring the chromogen reaction product of 2-thiobarbituric acid (TBA) with one of the products of membrane lipid peroxidation, malondialdehyde, according to the technique described by Winterbourn et al. (33) and adapted by Rosa et al. (27). In brief, homogenized ileum pools from six animals were incubated for 30 min with the reaction mixture at 95°C. The chromogen reaction product was extracted in n-butanol, and its concentration was determined spectrophotometrically (U-2000; Hitachi, Tokyo, Japan) at 532 nm. Results are expressed as nanomoles per milliliter per gram of dry tissue.

Carbonyl assay. Oxidative damage to intestinal proteins was spectrophotometrically determined by quantifying ileum carbonyl content according to the method described by Reznick and Packer (26). Briefly, 10 mM 2,4-dinitrophenylhydrazine (DNPH), dissolved in 2.5 M HCl, was added to ileum homogenate pools from each animal group to generate chromophoric dinitrophenylhydrazones. After the DNPH reaction time, proteins were precipitated in 20% (wt/vol) TCA, followed by successive washings with ethanol-ethyl acetate mixture (1:1) and centrifugation at 6,000 g. The last pellet was dissolved in 6 M guanidine-HCl solution. The protein carbonyl content was assessed spectrophotometrically (U-2000; Hitachi) at 370 nm, using the molar extinction coefficient of DNPH, ε = 22,900 M−1·cm−1. Total protein content was spectrophotometrically measured compared with a BSA standard curve (0.25–2.0 mg/ml) at 280 nm. Total antioxidant assay. Total antioxidant capacity was assessed by the antioxidant assay kit from Cayman Chemical (Ann Arbor, MI). In brief, tissue samples were homogenized in phosphate buffer containing anti-protease cocktail. The assay relies on the ability of the antioxidants presented in the tissue homogenate to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (ABTS) to ABTS+ (ε = 22,000/M) promoted by metamyoglobin. The amount of ABTS+ produced was assessed spectrophotometrically at 750 nm. The sample antioxidant capacity was compared with the Trolox standard curve, a
water-soluble tocopherol analog that prevents ABTS oxidation. Results are expressed as nanomoles of antioxidant per milligram of total protein.

**Solutions**

Contractile experiments were carried out with tissue strips bathed in Tyrode solution (in mM): 135 NaCl, 2.68 KCl, 1.36 CaCl$_2$, 2H$_2$O, 0.49 MgCl$_2$, 6H$_2$O, 12 NaHCO$_3$, 0.36 NaH$_2$PO$_4$, and 5.5 d-glucose, pH 7.4. The following solutions were used for lipid peroxidation assays: phosphate buffer solution containing (in mM) 20 KH$_2$PO$_4$, pH 7.4. The following solutions were used for lipid peroxidation assays: phosphate buffer solution containing (in mM) 20 KH$_2$PO$_4$, 150 KCl, and 40 HEPES and reaction mixture containing 20 mM phosphate buffer, 11% acetic acid, 0.1% tungstophosphoric acid, 0.5% SDS, and 0.2% TBA. For carbonyl assays, homogenizing buffer, containing 50 mM NaH$_2$PO$_4$, 0.1% digitonin, a cocktail of antiproteases (5 mg/ml leupeptin, 7 mg/ml pepstatin, and 5 mg/ml aprotonin), and 1 mM EDTA, was used.

**Chemicals**

All chemicals were analytical grade. Salts, d-glucose, n-butanol, TBA, tungstophosphoric acid, SDS, ethylic alcohol, acetic acid, and xylene were purchased from Merck (Darmstadt, Germany); CCh, BK, EDTA, DNPH, TCA, antiprotease cocktail, digitonin, guanidine, and HEPES were from Sigma (St. Louis, MO); osmium tetroxide, glutaraldehyde, 812 epoxy resin, formaldehyde, propylene oxide, uranyl acetate, and lead citrate were from Electron Microscopy Sciences (Hatfield, PA); hematoxylin and eosin were from Nuclear (Diadema, Brazil); and the antioxidant assay kit was from Cayman Chemical.

**Statistical Analysis**

Data are presented as means ± SE, with n representing the number of experiments. Statistical significance was analyzed by one-way ANOVA followed by Tukey’s test. P values of <0.05 were considered statistically significant.

**RESULTS**

**Training Intensity Markers**

Animal adaptation to the exercise protocol was assessed through two well-known IE markers: exhaustion time and blood lactate concentration (5, 18). Exhaustion time throughout the training period decreased exponentially, approaching stabilizing levels within 8–10 days (Fig. 1A). Thus, exhaustion time fell from 186 s before the initiation of the training period to 103 s and 53 s after the 4th and 10th day of exertion, respectively (Fig. 1A). From the observed 50% and 30% decreases in exhaustion time (Fig. 1A), we selected the 4th and 10th day, respectively, of IE training to evaluate the exercise-induced effects on ileum morphology, oxidative stress, and contractility. Interestingly, the maximum velocity reached by the animals at day 4 of IE training decreased by 56%, whereas a partial recovery of up to 19 m/min was seen at day 10 (Fig. 1B).

Consistent with the high-intensity training, a growing accumulation of lactate in blood from exercised animals throughout the IE period was detected, ranging from 2 mM before the training initiation (control) up to 5 mM after IT1 and reaching values higher than 6 mM after the 4th and 10th days of IE (Fig. 1C).

**Cellular and Ultracellular Morphological Studies**

Previous research by our group (27) has shown that aging-induced hypertrophy of the murine ileum muscular layer was avoided by the performance of a moderate exercise program throughout the animals’ life span, thus raising the possibility that intestinal musculature morphology might be influenced by exercise intensity. We thus explored this possibility by evaluating the cellular and ultracellular structures of the ileum.

Figure 2 illustrates representative light micrographs of hematoxylin-eosin-stained ileum sections isolated from the three animal groups. In the ileum isolated from EX.4D animals, there was moderate to high damage of the mucosa layer, with considerable lifting of the epithelial layer, the presence of few denuded villi, and architectural distortion (Fig. 2B). However, these structural alterations, classified as grade 2 or 3 (7), were not seen after 10 days of IE training (Fig. 2C). In addition, the thickness of the ileum muscular layer was reduced by 37% after 4 days of IE, whereas in EX.10D animals this layer was reduced by only 11% (Fig. 2D).
Considering the above morphological findings, it was interesting to perform ultrastructure analysis, mainly to evaluate the influence of IE on cellular organelles. Figure 3 illustrates representative electron micrographs of ileum myocytes isolated from control, EX.4D, and EX.10D animal groups. The intestinal muscle cells presented a central euchromatic nucleus and mitochondria with different forms and sizes localized nearby the nuclear poles in both control and EX.4D animals (Fig. 3). In contrast, muscle fibers with a central voluminous euchromatic nucleus and some mitochondria with various levels of internal membrane derangement were clearly seen in the electron micrographs of ileum myocytes obtained from EX.10D animals (Fig. 3).

**Ileum Reactivity**

The influence of IE on ileum reactivity was investigated by testing both electro- and pharmacological signaling results. As illustrated in Fig. 4, no differences were verified in the strength of CCh signaling for the three animal groups, as the EC$_{50}$ values were $\sim 0.5$ $\mu$M (Fig. 4B and Table 1). Similarly, the EC$_{50}$, which was $\sim 13$ mM, for KCl depolarization was not altered by IE (Fig. 4B and Table 1). On the other hand, the concentration-response curve in response to BK was shifted to the right after 10 days of IE, leading to a significant increase of 0.7 log unit in the EC$_{50}$ value (Fig. 4C, Table 1). In contrast, IE drastically impaired ileum efficacy for both types of couplings. Four days of IE caused a significant reduction of 43% in the maximum contractile response triggered by addition of either KCl or CCh and of 57% for BK-induced maximum contractile response (Fig. 4, Table 1). However, the maximum contraction for all stimulants after 10 days of IE was similar to that observed in the control animals (Fig. 4, Table 1).

**Oxidative Stress Levels**

To verify the relationship among exercise-induced morphological and functional changes and tissue oxidative stress, we evaluated lipid peroxidation, protein carbonyl content, and antioxidant capacity of the whole ileum and also lipid peroxidation of the gastrocnemius muscle as a control for muscles directly engaged in running. The malondialdehyde concentration in the isolated gastrocnemius muscle and ileum isolated from control animals was close to 350 nM ml$^{-1}$ g dry tis-

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Fig. 2. Representative ileum thin-section light micrographs from control (A), EX.4D (B), and EX.10D (C) animal groups. D: histogram of the muscular layer thickness of the ileum from control (n = 5), EX.4D (n = 5), and EX.10D (n = 5) animals. *Significant difference in relation to control animals (P < 0.05). #Significant difference in relation to EX.4D mice (P < 0.05).

Fig. 3. Representative ultramicrographs from ileum isolated from control (n = 3), EX.4D (n = 3), and EX.10D (n = 3) animals. F, muscular fiber; N, nucleus; m, mitochondria; dm, disorganized mitochondria.
We provide evidence for morphological and functional damaging effects of intense and exhaustive exercise on isolated murine ileum. These effects were time dependent, with histological and contractility impairments occurring earlier than in ultrastructure. In fact, there was a significant decrease in the efficacy of both depolarization and agonist signaling, a relevant destruction of the mucosa layer, and a significant decrease in muscular layer thickness in ileum isolated from animals submitted to 4 days of IE. On the other hand, partial mitochondrial deterioration, concomitant with an increase in the level of tissue oxidative stress, was shown only after 10 days of IE when the majority of the earlier histological and structural reactivity of the ileum was almost recovered.

The protocol herein applied can be considered intense and exhaustive because it caused two of the well-known physiological responses expected for this type of exercise: reduction of exhaustion time in each exercise session (Fig. 1A) and decreased maximum velocity reached in the IT2 (Fig. 1B), accompanied by a significant increase in blood lactate concentration (Fig. 1C). In fact, according to Billat et al. (4), any exercise protocol leading to blood lactate higher than 5 mM is classified as intense. It should be stressed that continuous IE sessions are a more stressful stimulus than interval training sessions, where periods of lower intensity exercise are alternated with periods of high intensity, as in the protocol described by Hamada et al. (11), thus causing better adaptive responses. The exercise training markers above also strongly suggest that a 24-h resting period between each exercise session is insufficient to allow animals to have complete recovery from the previous exercise session, thus leading to fatigue and impaired physical performance. These two later physiological alterations are clearly associated with intense and exhaustive exercise (10, 14). As expected, and in accordance with other authors (17, 31), the level of oxidative stress increased in gastrocnemius muscle, one of the skeletal muscles directly engaged in treadmill running (Fig. 5A). In fact, IE is known to interfere with the cell redox status in skeletal muscle by altering the balance between oxidant production and antioxidant defense mechanisms (12, 28). Although a moderate exercise program can improve the organism redox status by considerably augmenting the defense mechanisms, thereby compensating for any simultaneous increase of oxidant prod-

Table 1. Effects of intense exercise on pharmacological parameters logEC50 (potency) and Emax (efficacy)

<table>
<thead>
<tr>
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<th>Control (n = 12)</th>
<th>EX.4D (n = 12)</th>
<th>EX.10D (n = 12)</th>
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<tr>
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<tr>
<td>KCl</td>
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<td>−1.80±0.03</td>
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<tr>
<td>BK</td>
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<td>−9.1±0.3</td>
<td>−8.1±0.2*</td>
</tr>
<tr>
<td>Emax, g</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1.3±0.2*</td>
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</tr>
<tr>
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</tr>
<tr>
<td>BK</td>
<td>2.1±0.2</td>
<td>0.9±0.1*</td>
<td>2.0±0.2</td>
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Values are means ± SE. Results were determined from the concentration-response curves of the ileum in response to KCl, carbachol (CCh), or bradykinin (BK) in control, 4-day exercise (EX.4D), and 10-day exercise (EX.10D) animal groups. *Significant difference in relation to control animals (P < 0.05).
current with an elevated concentration of blood lactate (Fig. 1, B and C). Despite this finding, there were no signs of malabsorption, since neither the ingestion of food by the animals nor body weight was affected by IE (data not shown). Together, these results demonstrated that 4 days of IE is a high-intensity stress stimulus to the whole body, which may certainly cause important physiological alterations in organs and systems not directly engaged in performing movements, as exemplified by the intestine in this study. It is clear that 4 days of IE was not enough to overwhelm the tissue antioxidant defense and to impose an oxidative stress condition because this short period did not cause significant changes in intestinal redox state, given that normal intestinal myocyte ultrastructure (Fig. 3; EX.4D), protein carbonyl content, and antioxidant capacity were observed (Fig. 5).

It is well known that ischemia-reperfusion events in intestinal muscularature are closely related to oxidative stress (23), and it has recently been reported that submaximal exercise causes intestinal ischemia in rats (20). Based on these facts, we initially hypothesized that intense and exhaustive exercise would lead to an increased level of intestine oxidative stress due to higher levels of tissue ischemia as a consequence of the drastic shift in cardiac debt to active muscle to the detriment of the splanchnic area. This seems to be the case of the mucosa layer, known to be very sensitive to oxidative stress (34), which was partially destroyed after 4 days of IE (Fig. 2B). However, this possibility was ruled out because the tissue lipid peroxidation was surprisingly lower and the antioxidant capacity was unchanged, rather than increased, in the ileum from the EX.4D animal group compared with results shown in the control group (Fig. 5).

Interestingly, exercise-induced morphological and functional effects were distinct after 10 days of IE. The mucosa layer (Fig. 2C), thickness of the muscular layer (Fig. 2D), and efficacy of both the electro- and pharmacomechanical couplings were almost restored to levels shown in control animals (Fig. 4, Table 1). Regarding potency, the sole significant influence of IE was on BK signaling, which was reduced after 10 days (Fig. 4C). This might be related to more complex responses because it is known that IE can trigger inflammatory and/or immunologic responses (24), although we did not further explore this point in this study. Moreover, in contrast to results shown in EX.4D tissues, EX.10D tissues presented considerable deterioration of intestinal mitochondria with distinct levels of damage to the cristae (Fig. 3) in association with significantly higher levels of oxidative stress, including decreased antioxidant capacity (Fig. 5). A plausible explanation for the above intriguing observations is that ileum is classified as a phasic smooth muscle, and its contraction development is more dependent on the ATP-creatine phosphate system and anaerobic metabolism than on mitochondrial oxidative metabolism (8).

These results strongly argue in favor of the distinct nature of the effects observed at the 4th and 10th day of IE, with distinct intestine adaptive responses under these two conditions. In fact, damage related to ischemia-reperfusion events were clearly established after 10 days of IE, at which point the antioxidant defense of the tissue was overwhelmed by oxidative production. In contrast, histological and contractile responsiveness disturbances seemed to be more related to animal physical performance and occurred within the intestine at a
faster pace than oxidative deleterious effects. Indeed, there was a high correlation ($r = 0.98$) between maximum running velocity and maximum ileum contractility. On the other hand, the low correlation between oxidative stress and contractile response ($r = 0.58$) is not a novel finding, since our group (27) has recently reported that aging-induced increases of ileum oxidative stress were also not related to impairment of tissue contractility responsiveness but related to muscular layer thickness, where the higher the hypertrophy the higher the lipid peroxidation and vice versa.

Finally, an alternative explanation for the partial loss of intestinal contractility after IE training might be related to exercise-induced inflammation and cytokine release (24). In fact, it has been demonstrated that exercise enhances IL-6 concentration in blood (19, 22) and that this cytokine impairs intestinal contraction (21). Therefore, it is quite possible that the exercise protocol designed in the present study might cause some degree of intestinal inflammation. Although some mild degree of mucosal cellular infiltration was perceived in histological analysis (data not shown), this requires further confirmation by investigating distinct inflammation markers. Obviously, this is merely an attractive speculation, and further studies concerning this possibility are needed to better clarify this point.

Thus the intestine should definitely be considered an important athletic organ, being an attractive exercise target. Health professionals who recommend exercise as a therapeutic tool against chronic diseases should be aware of the exercise-induced positive or negative influences on intestinal physiology. This point deserves closer attention mainly by considering the importance of the intestine as the organ responsible for organism homeostasis by controlling nutrient and liquid absorptions.

The potential value of these findings is evident, although we must be wary of directly extrapolating these results, observed in a rodent model, to humans. Although it is quite improbable that a subject could be voluntarily submitted to such a stressful exercise protocol as the one we studied, similar conditions could be attained by the so-called weekend athletes or even by professional and recreational athletes under special conditions. In fact, hard workout sessions in parks or street running by recreational athletes without any professional advice or professional athletes trying to win many competitive events in a row during a weekend could easily be associated with high-intensity and exhaustive exercise. Finally, the knowledge of the possible structural damage and functional impairments caused by IE protocols and inadequate rest conditions will certainly contribute toward improving strategies for employing exercise as a therapeutic tool against several chronic diseases (for example, Duchenne muscular dystrophy, hypertension, obesity, diabetes, Parkinson), where a moderate exercise protocol may represent an IE.

In summary, we demonstrate for the first time that an intense and exhaustive exercise program, causing the usual deleterious effects on physical performance, induced time-dependent structural and functional impairments of intestinal tissue in a murine animal model, which could not be directly attributed to exercise-induced ischemia-reperfusion events.

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