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1 # SWIMMING PREVENTS MEMORY IMPAIRMENT BY
2 # INCREASING THE ANTIOXIDANT DEFENSE IN AN ANIMAL
3 # MODEL OF DUCHENNE MUSCULAR DYSTROPHY

4

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16 Duchenne muscular dystrophy (DMD) is a genetic disease which is associated to a
17 progressive skeletical muscle degeneration. Swimming is usually indicated for avoiding
18 impact and facilitating adherence because of a better adaptation to a warm water
19 invironment and also for its benefits on cognition, and modulating memory and learning
20 processes and for increasing antioxidant defenses in oxidative stress. The objective of this
21 study was to evaluate the effects of a swimming protocol on memory and oxidative stress
22 in an animal model of Duchenne muscular dystrophy. Methods: male mdx and wild type
23 mice within 28 days were used in this study. The animals were trained in an stepped
24 swimming protocol for four consecutive weeks. Twenty four hours after the last exercise
25 day, aversive memory and habituation memory tests were performed and removed the
26 encephalic structures of striatus, pre frontal cortex, hippocampus, and cortex and
27 gastrocnemius and diafragma muscles to evaluate protein carbonilation and lipid

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28 peroxidation and free thiols. Results: it was verified that swimming was able to reduce
29 significantly the levels of lipid peroxidation and protein carbonilation in gastrocnemius
30 and hippocampus and striatus in exercised animals. Swimming has also prevented lipid
31 peroxidation in diafragma. Besides, this swimming protocol was able to increase free
32 thiols in gastrocnemius, diafragma and in analysed SNC structures. These results showed
33 that swimming prevented aversive and habituation memory in mdx mice.
34 Keywords: Duchenne muscular dystrophy, Swimming , oxidative stress
35
36

37 Introduction

38 Duchenne muscular dystrophy (DMD) is one of the recessive X-linked PMS and
39 mainly affects males. DMD usually presents in early childhood, characterized by delays
40 in motor milestones. DMD leads to weakening of the skeletal muscles and is known to be
41 the most common and severe type, due to its early onset and the rapid evolving of
42 symptoms [1-3].
43 Treatment of patients diagnosed with Duchenne muscular dystrophy (DMD) needs
44 to be multidisciplinary, careful, and always focused on the well-being of the patient.
45 Given this, the role of physical therapy becomes crucial to the success of treatment, as it
46 has achieved good results in the short term, such as maintenance of the autonomy of these
47 individuals [4,5]. Another method that has been used to treat DMD is physical exercise
48 [6,7]. Studies have been using physical exercise to decrease muscle deterioration, muscle
49 contractures, bone fractures, and increase the time of functional independence, in
50 individuals with DMD [8,9].
51 Swimming is one of the aerobic exercise modalities that has become very popular.
52 Aerobic exercise in water is a viable alternative to exercise on land, because water,

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53 through its physical properties such as thrust, hydrostatic pressure, and viscosity, among
54 others, brings increased well-being and quality of life [10]. Swimming, like walking and
55 running, has health benefits compared to a sedentary lifestyle [11]. Swimming is
56 generally indicated because with heated water it avoids impact and facilitates adherence
57 by better adaptation to the environment [11]. Swimming is further described in the
58 literature as having beneficial effects on patients: on cognition by modulating memory
59 and learning processes; and in oxidative stress, by increasing antioxidant defenses [11].
60 Thus, the present study aims to evaluate the effects of swimming on memory and
61 oxidative stress in an animal model of DMD.
62

63 # Materials and methods

64 ## Animals

65 Male mdx and wild-type (WT) mice of the C57BL / 6 strain, 28 days old, weighing
66 between 18 and 23 g from USP - São Paulo, were used. The animals were kept in the
67 LANEX Vivarium and per box five animals were packaged, in a 12-h light/dark cycle
68 (06:00 to 18:00), with food and water ad libitum. The environment was maintained at a
69 temperature of 22 ± 2 °C. The study was carried out at LANEX and the Laboratory of
70 Biochemistry and Molecular Biology during 2016 and 2017. This project was approved
71 by the Animal Use Ethics Committee - CEUA of UNISUL under the number
72 16.003.4.01.IV.
73

74 ## Experimental draw

75 The animals were divided into four groups of eight animals each: (1) unexercized
76 WT; (2) exercised WT; (3) mdx not exercised; and (4) mdx exercised. Groups 2 and 4

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77 were submitted to the swimming type aerobic exercise protocol for four weeks. Twenty
78 four hours after the last day of training, aversive memory and habituation tests were
79 performed. Afterward, the animals were sedated and submitted to assisted painless death
80 procedure and the following structures were removed: gastrocnemius; quadriceps;
81 diaphragm; prefrontal cortex; cerebellum; hippocampus; striatum; and cerebral cortex,
82 for determination of lipid peroxidation, protein carbonylation, and thiol grouping.
83

84 ## Swimming type aerobic exercise protocol

85 The groups of exercised animals were submitted to an aerobic swimming exercise
86 protocol in a plastic container adapted for this purpose (170 x 100 mm), with 35 liters of
87 water at 28 to 30 ºC, divided into eight lanes. There were four consecutive weeks of
88 exercise, four times a week, with daily sessions of 15 minutes in the first week, 20 minutes
89 in the second week, and 30 minutes in the third and fourth week [12]. Of baby shampoo,
90 1 ml was used throughout the container to decrease surface tension of water [13]. After
91 the protocol, the animals were gently dried. The groups of non-exercised animals did not
92 perform any type of physical exercise, remaining in their housing boxes during the entire
93 study period.

94 Exercise intensity was determined in the fourth week of protocol in WT and mdx
95 animals. Blood samples were collected before the test and at the 10th and 30th minutes of
96 exercise for subsequent analysis of lactate concentration. The criterion for considering
97 intensity was the increase in concentration of no more than 1 mmol / L between the 10th
98 and 30th minute of physical exercise. Hygienization of the blood collection site was
99 performed with alcohol (70%). After this procedure, the distal portion of the tail of the
100 animal was slightly sectioned with surgical scissors and 25 μl and a drop of blood was
101 inserted in the lactate collection tape, and then, by means of a portable lactimeter, the

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102 blood level was measured. Before each test, the equipment was calibrated according to
103 the manufacturer's instructions [14].
104

105 Aversive Memory Test

106 This test consisted of an acrylic box in which the floor was formed by parallel
107 metal bars. A platform 7 cm wide and 2.5 cm long was placed near the left wall of the
108 appliance. In the training session, the animals were placed on the platform and the time
109 in seconds was measured for the animal to descend with all four legs from the platform.
110 This time is called latency. Immediately after descending from the platform, the animal
111 received a 0.2 mA shock for 2 s. In the test session, the animal was again placed on the
112 platform and the time it took to descend (latency) the platform was measured, but no
113 shock was given. The test was also terminated if the animal did not descend within a
114 maximum of three minutes [15].
115

116 Habituation memory test

117 This test was performed in a 40 x 60 cm open field, with four 50 cm high walls
118 delimiting the area; three of them wood and one of transparent glass. The open field floor
119 was divided into 16 equal squares, marked by black lines. In the training session, the
120 animals were carefully placed in the square of the back left corner of the apparatus, from
121 which they freely explored the environment for five minutes. Immediately afterward, the
122 animals were returned to the box. The test session was held 24 hours after training, and
123 the training procedure was then repeated. The number of four-legged crossings
124 (crossings: motor activity) across the black lines and the number of times animals reared
125 on their hind legs (rearings: exploratory activity) were evaluated in both sessions [16].
126

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127 # Oxidative stress measures

128 Measurement of thiobarbituric acid reactive substances (TBARS)
129 This method is used to evaluate the oxidation state of hydroperoxides in biological
130 systems. Membrane lipid damage is determined by the formation of lipoperoxidation
131 byproducts (such as MDA or malondialdehyde), which are substances reactive to
132 thiobarbituric acid heating that are formed during peroxidation in membrane systems.
133 MDA reacts with thiobarbituric acid (TBA), generating a pinkish-colored product read in
134 a 535 nm microplate reader. The technique consists of the following form: firstly, the
135 dilution value was calculated so that the TBARS reaction tube has 100 µl of tissue protein
136 in 500 µl of BHT buffer. Thereafter, 500 µl of the 0.67% TBA solution was added. The
137 tubes were placed in a dry bath at 96 °C for 30 minutes. To stop the reaction, the samples
138 were placed on ice for 5 minutes. Finally, 200 µl of the reaction mixture was placed in
139 96-well microplates and read in the 535 nm microplate reader.
140

141 # Measurement of oxidative damage in proteins

142 This method was used for protein oxidation dosing. It is based on the principle
143 that several ROS attack protein residues, such as amino acids, produce products with the
144 carbonyl group, which can be measured by reaction with 2,4-dinitrophenylhydrazine. The
145 carbonyl content is determined by a 370 nm microplate reader as described in the study
146 by Levine et al. (1990). Firstly, the tissue was homogenized in 1 ml BHT buffer. Samples
147 were centrifuged for 15 min at 4 °C at 14,000 rpm. Of the sample, 200 µl was separated
148 to blank and 200 µl was separated for the test. Of 20% trichloro acetic acid (TCA), 100
149 µl was placed in all eppendorfs. It was centrifuged for 5 min at 14,000 rpm. The
150 supernatant was discarded. The pellet was redissolved in 100 µl of 0.2 molar NaOH. Of
151 dinitrophenylhydrazine 2.4 (DNTP), 100 µl was placed in the sample and allowed to stand

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152 for 1 h. Of 20% tTCA, 100 µl was placed in all eppendorfs, which were centrifuged for 3
153 min. The supernatant was then discarded. The pellet was washed 3 times with 500 µl
154 ethanol and ethyl acetate (1: 1). For each wash, it was centrifuged for 3 min at 14,000
155 rpm and the supernatant discarded. After discarding the last wash, 1 ml of 3% sodium
156 hydroxide (NaOH) was placed in all eppendorfs. The samples were taken to the bath at
157 60 °C for 30 minutes and read on the microplate reader at 370 nm.
158

159 Thiol groupings

160 Sulfhydryl radicals represent all groups of thiols found in proteins such as albumin
161 and in low molecular weight compounds such as glutathione. These groups may be
162 oxidized when the oxidative stress is elevated. Determination of total sulfhydryl groups,
163 protein-bound sulfhydryl groups, and sulfhydryl groups in low molecular weight
164 compounds (free sulfhydryls) can be carried out using Ellman's reagent (2,2-dinitro-5,5-
165 dithiobenzoic acid – DTNB). The thiol groups react with DTNB to form a light-absorbing
166 complex at 412 nm. The technique consists of adding 10% TCA to the same sample
167 volume (1: 1 dilution). Prepared blank containing 100 µl TCA was added to 100 µl PBS.
168 It was centrifuged for 15 minutes at 3,000 rpm (temperature 4 °C); the supernatant was
169 collected and at 75 µl of this, 30 µl DTNB (1.7 mM) and 300 µl hydrochloric acid (TRIS-
170 HCl) were added. It was allowed to react for 30 minutes and transferred to a 96-well plate.
171 The samples were read in a 412 nm microplate reader.
172

173 Protein Dosages

174 Proteins were determined by the BCA method and bovine serum albumin was
175 used as standard. The method was based on the reaction of copper with proteins in basic
176 medium. Samples were analyzed by a 562 nm plate reader.

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177

178 ## Statistical analysis

179 Data were entered into an electronic database in the IBM SPSS Statistics 24.0
180 software (@copyright IBM corporations and its licensors 1989, 2016). The Shapiro-Wilk
181 normality test was applied to verify the behavior of the data. The data related to the open
182 field habituation test were expressed as mean and standard deviation because they are
183 parametric data. For differences between groups, a two-way analysis of variance
184 (ANOVA) with Bonferroni post-hoc test was used. For differences between training and
185 test in the same group, we used Student's *t*-test for paired samples. The inhibitory
186 avoidance test data were expressed as median and interquartile range because they are
187 nonparametric data. Wilcoxon test was used for analysis between training and test in the
188 same group. Biochemical test data were expressed as mean and standard deviation
189 because they are parametric data. The two-way ANOVA with Bonferroni post-hoc test
190 was used for analysis between groups. Data were considered statistically significant when
191 $p < 0.05$.

192

193 # Results

194 ## Lactate measure

195 Figure 3 shows the results obtained after the measurement of lactate in the blood
196 taken from the animals during the swimming protocol.

197 Figure 3 shows that in both mdx and WT animals submitted to the swimming
198 protocol, there was no change between the 10th and 30th minutes of exercise in blood
199 lactate above 1 mmol / L in relation to rest values, being mean lactate values of 2.31 nmol
200 / l for WT animals and 2.21 for mdx animals (i.e., remained below the lactate threshold).

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201 Thus, the swimming protocol used in this study can be considered to be of moderate
202 intensity.
203

204 Learning and memory evaluation

205 Figure 4 shows the results obtained after the application of the swimming protocol
206 on aversive memory (Fig. 4A) and habituation (Fig. 4B).
207 Figure 4A shows the results of habituation memory evaluation through the open field test.
208 It can be observed that there was no difference in the number of crossings and rearings (p
209 > 0.05) between groups during the training phase, demonstrating that there was no
210 difference in locomotor activity between the groups. The non-exercised and exercised
211 WT animals showed significant changes between training and testing, both in the number
212 of crossings and the number of rearings ($p < 0.05$), that is, there was no impairment of the
213 evaluated memory. The group of non-exercised mdx animals (DMD) did not show
214 significant changes between training and tests in the number of crossings and rearings (p
215 > 0.95), evidencing a memory impairment. In contrast, the group of mdx animals that
216 underwent the protocol showed a decrease in the number of crossings and rearings
217 between training and testing ($p < 0.05$), suggesting a possible prevention of aversive
218 memory impairment in mdx mice.
219 The results of aversive memory assessment by the inhibitory avoidance test are
220 shown in Fig. 4B. In the group of non-exercised and exercised WT animals, there was a
221 statistically significant difference in the latency time between training and testing,
222 showing no impairment in aversive memory ($p < 0.05$). In the group of non-exercised
223 mdx animals, there was no statistically significant difference between training and testing,
224 evidencing an impairment of aversive memory ($p > 0.05$). The mdx animals submitted to

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225 the experimental protocol showed a statistically significant difference between training
226 and testing, i.e., there was no impairment of aversive memory in these animals ($p < 0.05$).
227

228 Evaluation of oxidative stress in the gastrocnemium muscle

229 Figure 5 shows the results of using a swimming protocol on lipid peroxidation
230 (Figure 5A), protein carbonylation (Figure 5B), and free thiols (Figure 5C) in the
231 gastrocnemius muscle.

232 Figure 5A shows the result of the assessment of lipid peroxidation in
233 gastrocnemius. Unexercised mdx animals showed significantly higher levels of lipid
234 peroxidation in gastrocnemius when compared to untrained wild animals ($p < 0.05$). The
235 mdx animals that underwent the experimental protocol presented a significant reduction
236 of these levels when compared to the non-exercised mdx animals ($p < 0.05$), showing that
237 the experimental protocol used in this study protected against the increase of lipid
238 peroxidation in gastrocnemius in mdx animals. It was observed that the non-exercised
239 mdx animals showed a significant increase in protein carbonylation in gastrocnemius
240 when compared to the non-exercised wild animals group ($p < 0.05$). After the
241 experimental protocol, the mdx animals showed significantly lower protein carbonylation
242 levels when compared to the non-exercised mdx animals ($p < 0.05$), demonstrating that
243 the four-week swimming protocol was able to prevent the carbonylation increase. of
244 protein observed in the gastrocnemius muscle of mdx animals (Figure 5B). The
245 quantification of free thiols in gastrocnemius is shown in Figure 5C. It can be observed
246 that there was a significant increase in the amount of free thiols in gastrocnemius of the
247 group of wild animals submitted to the experimental protocol, when compared to the non-
248 exercised wild animals ($p < 0.05$). There was also a significant decrease in non-exercised
249 mdx animals as compared to non-exercised wild animals ($p < 0.05$). When the mdx

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250 animals underwent the experimental protocol, there was an increase in the amount of free
251 thiols when compared to the unexercised mdx animals.

252 Assessment of oxidative stress in diaphragm muscle

253 Figure 6 shows the results of a training protocol on lipid peroxidation (Figure 6A),
254 protein carbonylation (Figure 6B), and several pounds (Figure 6C) in the diaphragm
255 muscle.

256 The non-exercised mdx animals showed a significant increase in diaphragm lipid
257 peroxidation when compared to the non-exercised wild animals group (p <0.05). After
258 the experimental protocol, the mdx animals showed significantly lower lipid peroxidation
259 levels when compared to the non-exercised mdx animals (p <0.05), demonstrating that
260 the four-week swimming protocol was able to prevent increased peroxidation. observed
261 in the diaphragm muscle of mdx animals (Figure 6A). Figure 6B shows the result of the
262 evaluation of protein carbonylation in diaphragm. The non-exercised mdx animals
263 showed significantly higher levels of protein carbonation in diaphragm when compared
264 to the non-exercised wild animals (p <0.05). However, the mdx animals that underwent
265 the experimental protocol did not show a significant reduction of these levels when
266 compared to the non-exercised mdx animals, showing that the experimental protocol used
267 in this study did not protect against the increase of diaphragm protein carbonylation in
268 mdx animals. The quantitation of free diaphragm thiols is shown in Figure 6C. It can be
269 observed that there was a significant increase in the amount of diaphragm free thiols in
270 the group of wild animals submitted to the experimental protocol, when compared to the
271 non-exercised wild animals (p <0.05). There was also a significant decrease in non-
272 exercised mdx animals when compared to non-exercised wild animals (p <0.05). When
273 the mdx animals underwent the experimental protocol, there was an increase in the

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274 amount of free thiols when compared to the non-exercised mdx animals, demonstrating
275 that swimming was able to protect this change.
276

277 Assessment of oxidative stress in central nervous system 278 structures

279 Figure 7 shows the results of using a swimming protocol on lipid peroxidation in
280 the prefrontal cortex (Figure 7A), hippocampus (Figure 7B), striated (Figure 7C), and
281 cortex (Figure 7D).

282 The non-exercised mdx animals showed a significant increase in hippocampal and
283 striatum lipid peroxidation when compared to the non-exercised wild animals group (p
284 <0.05). After the experimental protocol, the mdx animals presented significantly lower
285 lipid peroxidation levels in the hippocampus and striatum when compared to the untrained
286 mdx animals ($p <0.05$), demonstrating that the four-week swimming protocol was able to
287 prevent the increase in lipid peroxidation observed in the hippocampus and striatum
288 structures of mdx animals (Figures 7B and 7C). Figures 7A and 7D show that in the
289 prefrontal cortex and cortex structures there were no significant changes between the
290 analyzed groups. Figure 8 shows the results of using a swimming protocol on protein
291 carbonylation in the prefrontal cortex (Figure 8A), hippocampus (Figure 8B), striated
292 (Figure 8C) and cortex (Figure 8D).

293 It can be observed that the non-exercised mdx animals showed a significant
294 increase in protein carbonylation in prefrontal, hippocampal and striated cortex when
295 compared to the non-exercised wild animals group ($p <0.05$). After the experimental
296 protocol, the mdx animals showed significantly lower protein carbonylation levels in the
297 prefrontal cortex when compared to the untrained mdx animals ($p <0.05$), demonstrating
298 that the four-week swimming protocol was able to prevent increased protein

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299 carbonylation, observed only in the prefrontal cortex structure of mdx animals (Figure
300 8A). Figures 8B and 8C show that the four-week swimming protocol was unable to
301 prevent increased protein carbonation in the hippocampus and striatum. Figure 8D shows
302 that in the cortex structure there was no significant change between the analyzed groups.

303 Figure 9 shows the results of using a swimming protocol on free thiols in the
304 prefrontal cortex (Figure 9A), hippocampus (Figure 9B), striated (Figure 9C) and cortex
305 (Figure 9D).

306 It can be observed that the non-exercised mdx animals showed a significant
307 decrease in free thiols in prefrontal cortex, hippocampus, striatum and cortex when
308 compared to the group of non-exercised wild animals (p <0.05). After the experimental
309 protocol, the mdx animals showed significantly higher free thiol levels in prefrontal
310 cortex, hippocampus, striatum and cortex when compared to untrained mdx animals (p
311 <0.05), demonstrating that the swimming protocol, by four weeks, it was able to increase
312 antioxidant defenses in all CNS structures of mdx animals. (Figures 9A, 9B, 9C and 9D).

313

314 Discussion

315 This study aimed to evaluate the effects of swimming on memory and oxidative
316 stress in skeletal muscle and brain tissue in an animal model of Duchenne muscular
317 dystrophy. For this, we used a moderate intensity swimming protocol performed four
318 times a week for four consecutive weeks. The results showed that swimming prevented
319 the aversive memory and habituation impairment in mdx mice. Parallel to this effect, an
320 increase of protein carbonylation in the prefrontal cortex, hippocampus, striatum,
321 diaphragm, and gastrocnemius, and increased lipid peroxidation in the hippocampus,
322 striatum, diaphragm, and gastrocnemius were also observed by the analysis of the
323 parameters related to the oxidative damage, concomitant with the decrease in free thiols

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324 in non-exercised mdx animals, evidencing oxidative stress. Interestingly, low intensity
325 swimming was able to prevent oxidative stress in the gastrocnemius and hippocampal and
326 striated structures of these animals. This same protocol increased free thiols in the
327 gastrocnemius, diaphragm, and CNS structures analyzed.

328 The exercise of swimming (aerobic exercise) used in this study was of moderate
329 intensity, according to the lactate measurements made in the animals. For this, one ml of
330 shampoo was used in the whole container adapted for swimming. Regarding exercise
331 intensity, in addition to the classification based on VO2max and maximum heart rate
332 measurement, which classifies exercise as mild/ low intensity, moderate intensity, or high
333 intensity [17], there is also the model proposed by Gaesser and Poole (1996) proposing
334 three domains relative to the intensity of effort: moderate; heavy; and severe. The
335 moderate domain comprises all the exertion intensities that can be performed without
336 blood lactate modification in relation to the resting values, that is, below the lactate
337 threshold (LL). The heavy domain starts from the lowest effort intensity where lactate
338 rises, and has as its upper limit an intensity corresponding to an average of 4 mM lactate.
339 In the severe domain, there is no stable phase of blood lactate, with blood lactate rising
340 throughout the exercise time, until the point of individual exhaustion [18].

341 DMD is characterized by an absence of dystrophin protein in skeletal muscle5.
342 However, the literature also shows that dystrophin is absent in brain tissue, and this
343 change is associated with other changes such as oxidative stress [19]. The absence of
344 dystrophin and oxidative stress in the CNS makes cognitive impairment part of the
345 pathophysiology of this disease. In this study, it was evidenced that the swimming
346 protocol was able to protect the aversive memory and habituation memory impairment in
347 the mdx mice submitted to the protocol.

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348 The protocol of the present study was started with 28-day-old animals and ended
349 with animals at 56 days old. At this age, these animals would be expected to have
350 impaired memory and learning, which does not occur in animals submitted to swimming.
351 Thus, it can be said that exercise prevents memory and learning deficits. Although there
352 are no studies relating swimming directly to the prevention of memory impairment and
353 DMD-related learning, there are reports of benefits of swimming practice in cognitive
354 aging [20]. Another study, still with middle-aged animals, showed the benefits of
355 swimming in object recognition memory tests, when combined with dietary
356 supplementation, demonstrating improvement in short and long-term memory [21].

357 Besides cognitive impairment, another objective of this study was to verify if
358 swimming can alter oxidative stress in neuronal and skeletal muscle tissue. Studies show
359 that oxidative stress is present in the pathophysiological process of DMD, as there is an
360 imbalance between the formation of oxidizing agents and antioxidant activity [22-24].
361 Oxidative stress is present in DMD, skeletal muscle, and also in the CNS [22]. One of the
362 effects of exercise is increased antioxidant activity. Exercise produces ROS, which act as
363 signals of molecular events, which regulate adaptations in muscle cells, such as the
364 regulation of antioxidant enzymes [25]. In this study, it was observed that swimming
365 increased free thiols, demonstrating that physical exercise is able to increase antioxidant
366 glutathione levels, in agreement with previous studies that demonstrated the protective
367 role of exercise [26,27]. Free thiols are an indirect measure of glutathione activity, an
368 antioxidant present in greater numbers in the CNS [28].

369 In addition to verifying the influence of swimming on brain impairment, the
370 evaluation of some skeletal muscle tissues is necessary. Since DMD is an essentially
371 neuromuscular disease, it is necessary to include skeletal muscle assessments
372 (gastrocnemius and diaphragm), since the main characteristic of this disease is related to

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373 the involvement of these structures with calf pseudohypertrophy, falls, frequent gait loss,
374 and cardiorespiratory dysfunction [29]. Evidence suggests that oxidative stress is
375 associated with aggravation of both respiratory and muscular pathology in these patients
376 [22]. In addition, there are studies showing that exercise can protect against oxidative
377 stress in skeletal muscle in mdx [30,12] mice. The protocol used in this study protected
378 against increased lipid peroxidation in the gastrocnemius in mdx animals. Swimming for
379 four weeks was able to prevent the increased protein carbonylation observed in the
380 gastrocnemius muscle of mdx animals. This finding is in agreement with a study
381 conducted in 2015 which used a swimming protocol and found a decrease in protein
382 carbonylation [12].

383 The same occurred with the diaphragm muscle, in which the mdx animals had
384 significantly lower lipid peroxidation levels when compared to the non-exercised mdx
385 animals, demonstrating that the four-week exercise protocol was able to prevent increased
386 lipid peroxidation. However, swimming was not able to reverse the increased protein
387 carbonation in the diaphragm. Diaphragm degeneration is a major contributor to
388 dystrophic right ventricular pathology. One study has concluded that although swimming
389 is beneficial for skeletal muscle and increases cardiac function, a 60-min protocol, six
390 days a week, for two months, exacerbates diaphragm degeneration and increases
391 dystrophic phenotype, and causes pulmonary hypertension [30].

392 Considering the above, it can be suggested, having seen the increase in free thiol
393 formation shown in the results of this study, that the moderate intensity aerobic exercise
394 of swimming may reduce oxidative stress by increasing antioxidant defenses, such as
395 glutathione. However, as there is no consensus in the literature on exercise volume,
396 frequency, and intensity in the treatment of DMD, this study may help to propose new
397 perspectives on the therapeutic use of exercise in the treatment of this disease.

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398

399 # Conclusion

400 A swimming protocol applied in mdx mice was able to prevent memory damage
401 and oxidative stress in gastrocnemius and in most of analysed SNC structures with a
402 significant increase in antioxidant activity in all analysed structures.

403

404 # Acknowledgments

405 To research group for their commitment and dedication.

406

407

408 # References

409

410 1. Matthew PW. The muscular dystrophies. Continuum Journ 2013;19(6):1535-70.
411 2. Ljubicic V, Burt M, Jasmin BJ. The therapeutic potential of skeletal muscle
412 plasticity in Duchenne muscular dystrophy: phenotypic modifiers as
413 pharmacologic targets. The FASEB Journ 2014;28:548-68.
414 3. Savino W, Pinto-Mariz F, Mouly V. Flow Cytometry-Defined CD49d
415 Expression in Circulating T-Lymphocytes Is a Biomarker for Disease
416 Progression in Duchenne Muscular Dystrophy. In Duchenne Muscular
417 Dystrophy. New York, NY: Humana Press; 2018. p. 219-27.
418 4. Gevaerd MS, Domenech SC, Junior NGB, Higa DF, Lima-Silva AE. Alterações
419 fisiológicas e metabólicas em indivíduo com distrofia muscular de Duchenne
420 durante tratamento fisioterapêutico: um estudo de caso. Fisiot Mov
421 2010;23(1):93-103.

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422 5. Le Rumeur E. Dystrophin and the two related genetic diseases, Duchenne and
423 Becker muscular dystrophies. Bosn J Basic Med Sci 2015;15(3):14-20.
424 6. Alemdaroğlu Đ, Karaduman A, Yilmaz ÖT, Topaloğlu H. Different types of
425 upper extremity exercise training in duchenne muscular dystrophy: effects on
426 functional performance, strength, endurance, and ambulation. Muscle & Nerve
427 2015;51(5):697-705.
428 7. Hyzewicz J, Ruegg UT, Takeda S. Comparison of experimental protocols of
429 physical exercise for mdx mice and duchenne muscular dystrophy patients. J
430 Neuromuscul Dis 2015;2:325-42.
431 8. Jansen M, Alfen NV, Geurts ACH, Groot IJM. Assisted bicycle training delays
432 functional deterioration in boys with duchenne muscular dystrophy: the
433 randomized controlled trial "No Use Is Disuse". Neurorehabilit and Neural
434 Repair 2013;27(9):816-27.
435 9. Mankodi A, Azzabou N, Bulea T, Reyngoudt H, Shimellis H, Ren Y, et al.
436 Concentric exercise effects on skeletal muscle water T2 in Duchenne muscular
437 dystrophy (P2. 131). Neurology 2017; 88(16 Supplement):P2-131.
438 10. Nikolai, A. L., Novotny, B. A., Bohnen, C. L., Schleis, K. M. & Dalleck, L. C.
439 Cardiovascular and Metabolic Responses to Water Aerobics Exercise in Middle-
440 Aged and Older Adults. J of Phys Act & Health. 2009; 6,(3): 333–338.
441 11. Chase, N. L., Sui, X. & Blair, S. N. Comparison of the Health Aspects of
442 Swimming With Other Types of Physical Activity and Sedentary Lifestyle
443 Habits.. Int. J. of Aquatic Res & Educ. 2008; 2, (2): 151-161.
444 12. Hyzewicz, J. Tanihata J, Kuraoka M, Ito N, Miyagoe-Suzuki Y, Takeda S. Low
445 intensity training of mdx mice reduces carbonylation and increases expression
446 levels of proteins involved in energy metabolism and muscle contraction. Free

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447 Radic. Biol. Med. (2015); 82, 122–36.

448 13. Mazzardo-Martins, L. Martins DF, Marcon R, Dos Santos UD, Speckhann B,
449 Gadotti VM, Sigwalt AR, Guglielmo LG, Santos AR. High-intensity extended
450 swimming exercise reduces pain-related behavior in mice: involvement of
451 endogenous opioids and the serotonergic system. J. Pain.2010; 11, 1384–93.

452 14. Ishii H, N. Y. Effect of lactate accumulation during exercise-induced muscle
453 fatigue on the sensorimotor cortex. J Phys Ther Sci. 2013; 25, 1637–42.

454 15. Leussis, M. P. & Bolivar, V. J. Habituation in rodents: A review of behavior ,
455 neurobiology , and genetics. Neurosc and Biobehav Rev. 2006; 30, 1045– 1064.

456 16. Ogihara, C. A. Schoorlemmer GH, Lazari Mde F, Giannocco G, Lopes OU,
457 Colombari E, Sato MA. Swimming Exercise Changes Hemodynamic Responses
458 Evoked by Blockade of Excitatory Amino Receptors in the Rostral Ventrolateral
459 Medulla in Spontaneously Hypertensive Rats. BioMed Research International.
460 2014; 2014.

461 17. Schubert MM, Washburn RA, Honas JJ, Lee J, D. J. Exercise volume and
462 aerobic fitness in young adults: the Midwest Exercise Trial-2. Springerplus.
463 2016; 5, (1): 183.

464 18. Gaesser GA, P. D. The slow component of oxygen uptake kinetics in humans.
465 Exerc Sport Sci Rev. 1996; 24, 35–71.

466 19. Comim, C. M. Tuon L, Stertz L, Vainzof M, Kapczinski F, Quevedo J. Striatum
467 brain-derived neurotrophic factor levels are decreased in dystrophin-deficient
468 mice. Neuroscience Letters. 2009; 459, (2) 66–68.

469 20. De Araujo, G. G. Papoti M, Dos Reis IG, de Mello MA, Gobatto CA.
470 Physiological responses during linear periodized training in rats. Eur. J. Appl.
471 Physiol. 2012; 112, (13): 839-852.

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472 21. Cechella, J. L., Leite, M. R., Gai, R. M. & Zeni, G. The impact of a diphenyl
473 diselenide-supplemented diet and aerobic exercise on memory of middle-aged
474 rats. Physiol Behav. 2014; 135, 125-129.
475 22. Lawler, J. M. Exacerbation of pathology by oxidative stress in respiratory and
476 locomotor muscles with Duchenne muscular dystrophy. J Physiol. 2011; 589, (9):
477 2161–2170.
478 23. LH, M. Bollineli RC, Mizobuti DS, Silveira Ldos R, Marques MJ, Minatel E.
479 Effect of N-acetylcysteine plus deferoxamine on oxidative stress and
480 inflammation in dystrophic muscle cells. Redox Report. 2015; 20, (3): 109115.
481 24. Kozakowska, M., Pietraszek-Gremplewicz, K., Jozkowicz, A. & Dulak, J. The
482 role of oxidative stress in skeletal muscle injury and regeneration: focus on
483 antioxidant enzymes. J. Muscle Res. Cell Motil. 2015; 36, 377–393.
484 25. Gomez-Cabrera, M. C., Domenech, E. & Viña, J. Moderate exercise is an
485 antioxidant: Upregulation of antioxidant genes by training. Free Radic. Biol.
486 Med. 2008; 44, 126–131.
487 26. Antoncic-Svetina, M. et al. Ergometry induces systemic oxidative stress in
488 healthy human subjects. Tohoku University Medical Library. 2010; 221, 43-48.
489 27. 11. Jia, B. et al. The effects of long term aerobic exercise. Clin. Hemorheol.
490 Microcirc. 2012; 51, 117–127.
491 28. Halliwell, B. Reactive oxygen species and the central nervous system. J
492 Neurochem. 1992; 59, 609–23.
493 29. Okama, L. O. et al. Functional and postural evaluation in duchenne and becker
494 muscular dystrophies. Cons e Saúde. 2010; 9,(4) 649–658.
495 30. Barbin, I. C. C. Pereira JA, Bersan Rovere M, de Oliveira Moreira D, Marques
496 MJ, Santo Neto H. Diaphragm degeneration and cardiac structure in mdx mouse:

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497 Potential clinical implications for Duchenne muscular dystrophy. J. Anat. 2016;
498 228 (5): 784–791.

499

500 # Supporting information

501

502

503 Figure 3 - Lactate measurement of mdx and wt animals submitted to the swimming
504 protocol.

505

506 Figure 4 - Effect of a swimming protocol on aversive and habituation memory of mice.

507

508

509 Figure 5 - Effect of a swimming protocol on oxidative stress in mouse gastrocnemius
510 muscle. (* when compared to the non-exercised wt group; ** when compared to the non-
511 exercised mdx group).

512

513

514 Figure 6 - Effect of a swimming protocol on oxidative stress on mouse diaphragm muscle.
515 (* when compared to the non-exercised wt group; ** when compared to the non-exercised
516 mdx group).

517

518

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519 Figure 7 - Effect of a swimming protocol on lipid peroxidation in mouse CNS structures.
520 (* when compared to the non-exercised wt group; ** when compared to the non-exercised
521 mdx group).
522
523
524 Figure 8 - Effect of a swimming protocol on protein carbonylation in mouse CNS
525 structures. (* when compared to the non-exercised wt group; ** when compared to the
526 non-exercised mdx group).
527
528
529 Figure 9 - Effect of a swimming protocol on free thiols in mouse CNS structures. (* when
530 compared to the non-exercised wt group; ** when compared to the non-exercised mdx
531 group).
532
533
534
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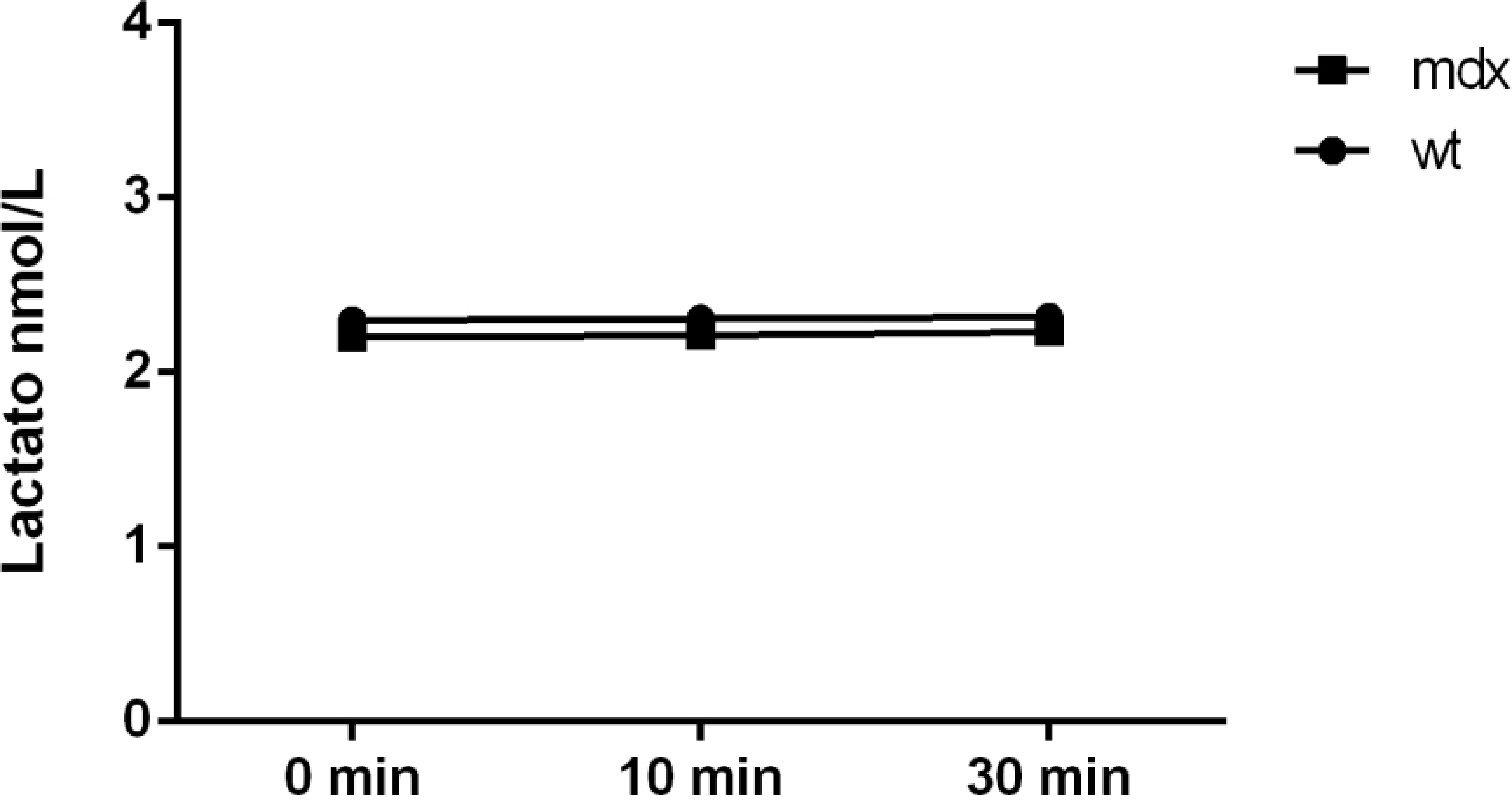

Figure 3

A)

Treino - *Crossing*
Teste - *Crossing*
Treino - *Rearing*
Teste - *Rearing*

Número
crossings e rearings
150
100
50
0
*
*
*
*
*
*
não exercitado
exercitado
não exercitado
exercitado
wt
mdx

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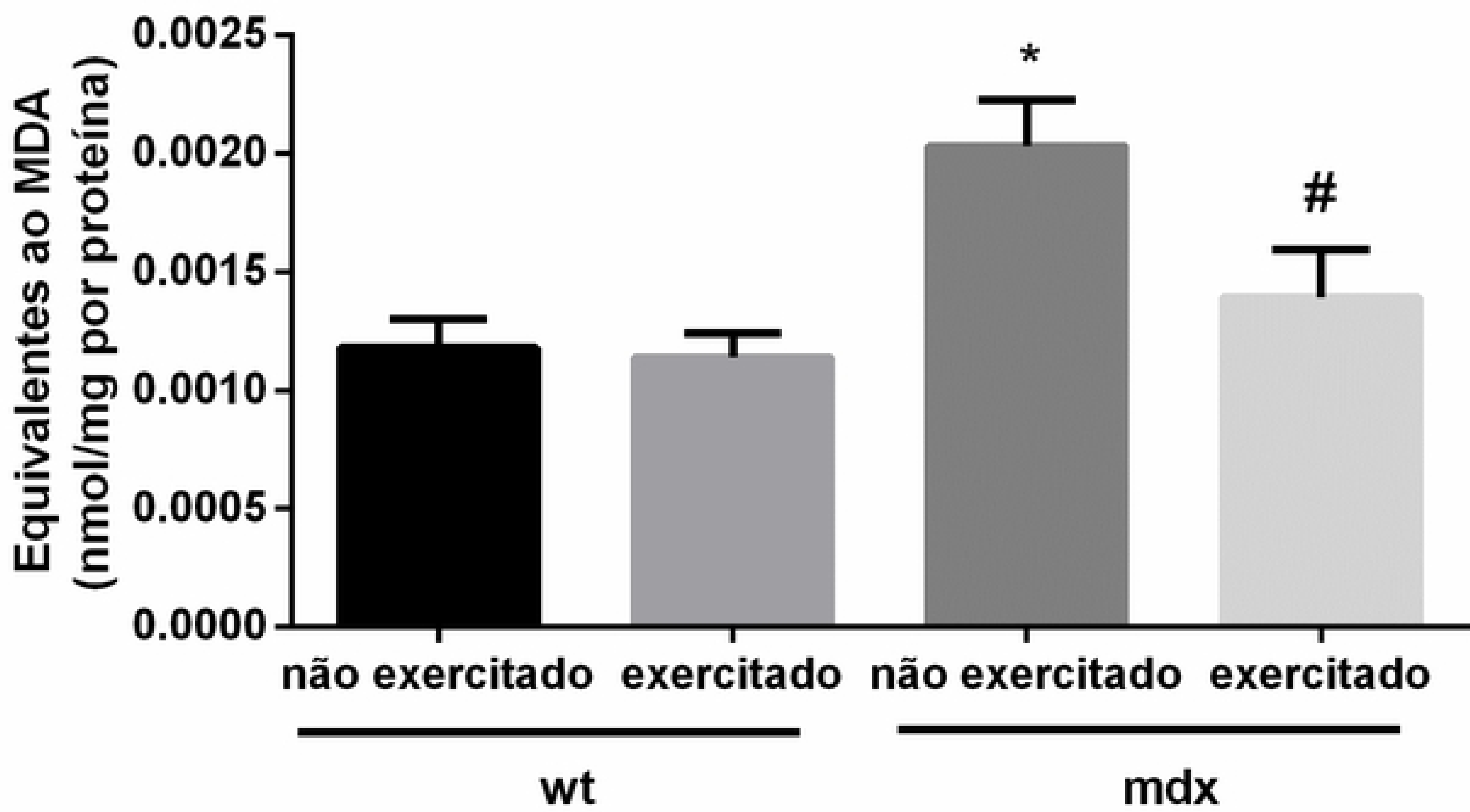
B)

Treino
Teste

Tempo de latencia (seg)
400
300
200
100
0
*
*
*
não exercitado
exercitado
não exercitado
exercitado
wt
mdx

Figure 4

A)



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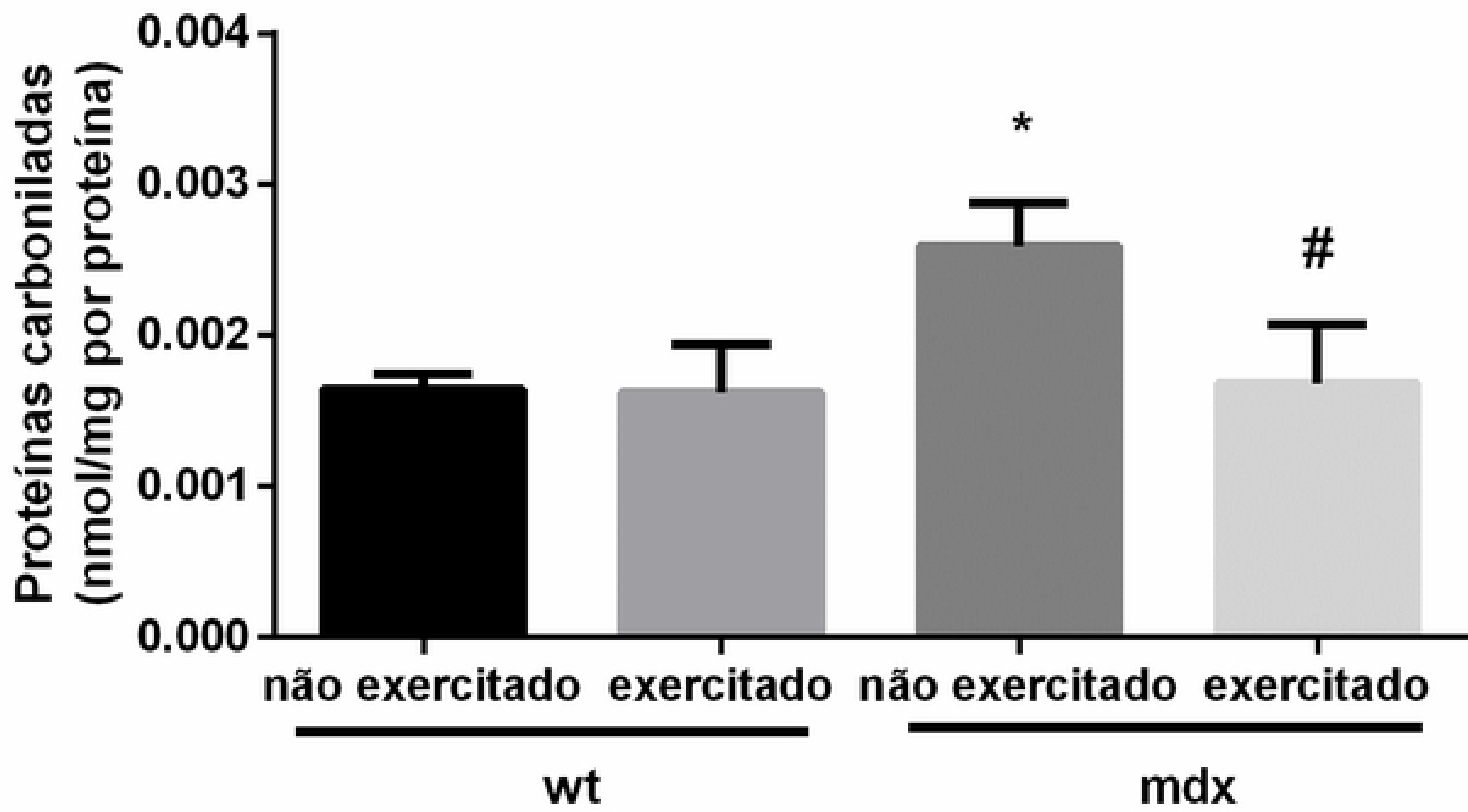
B)



C)

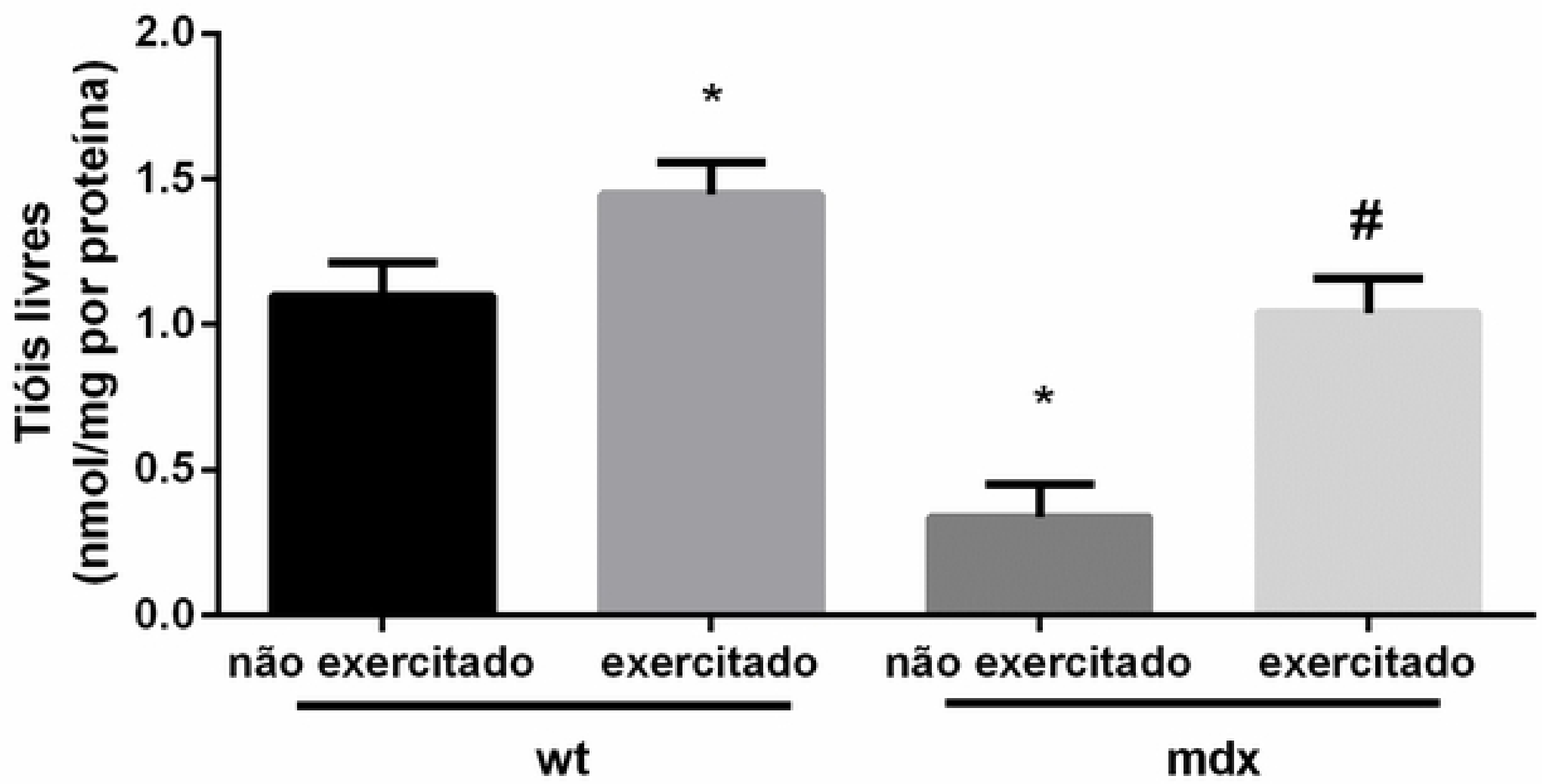
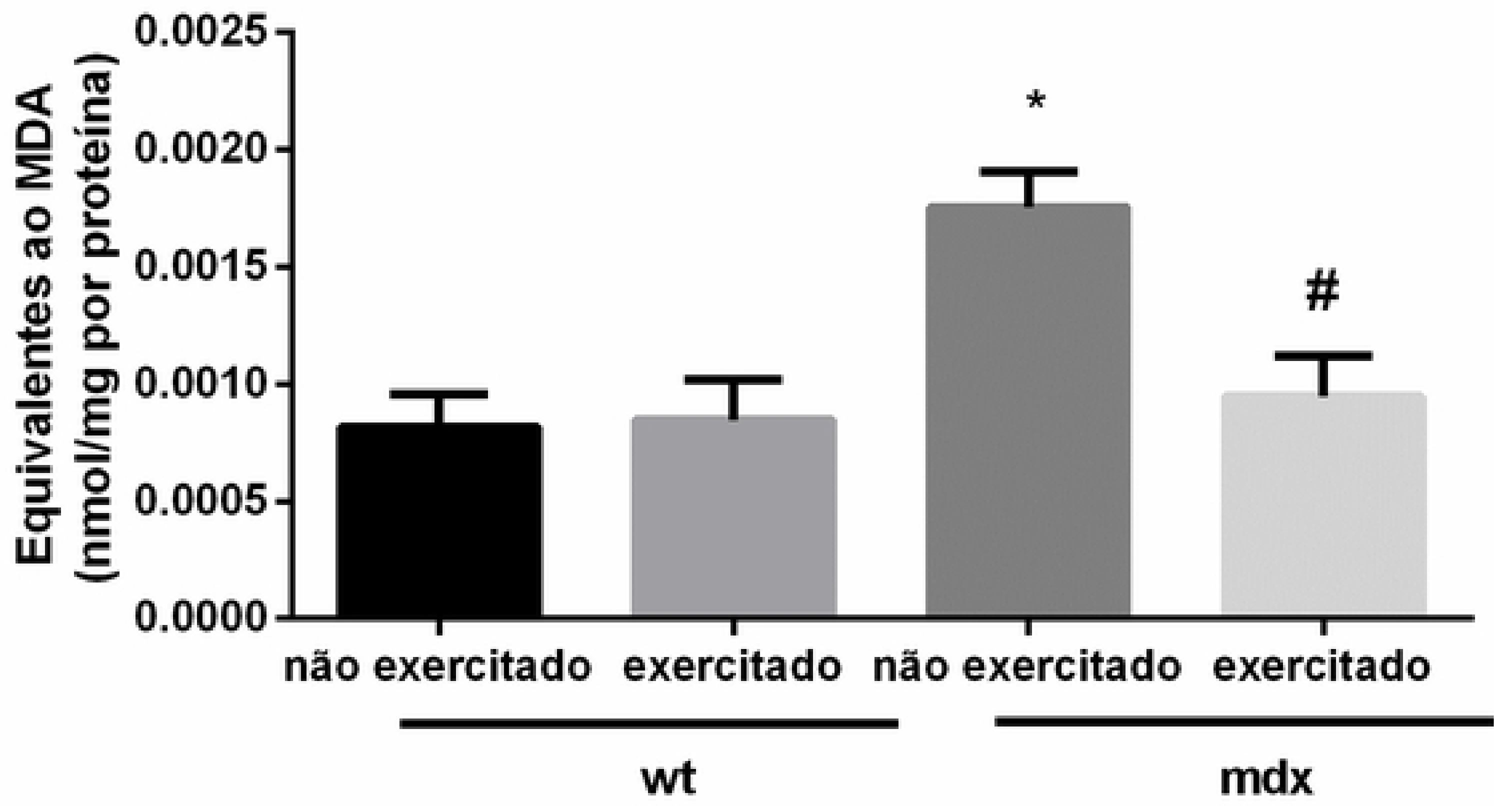


Figure 5

A)



B)

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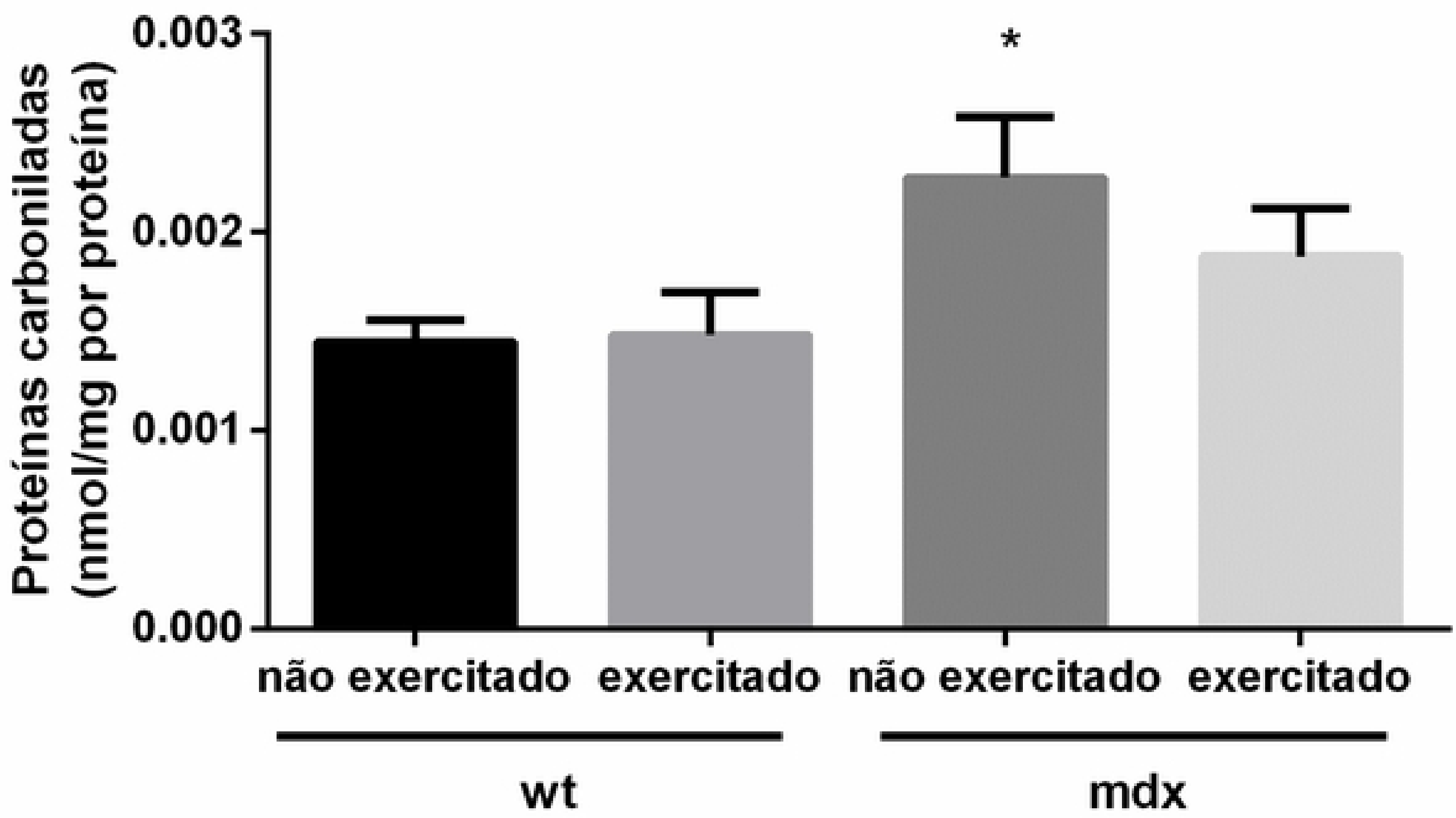

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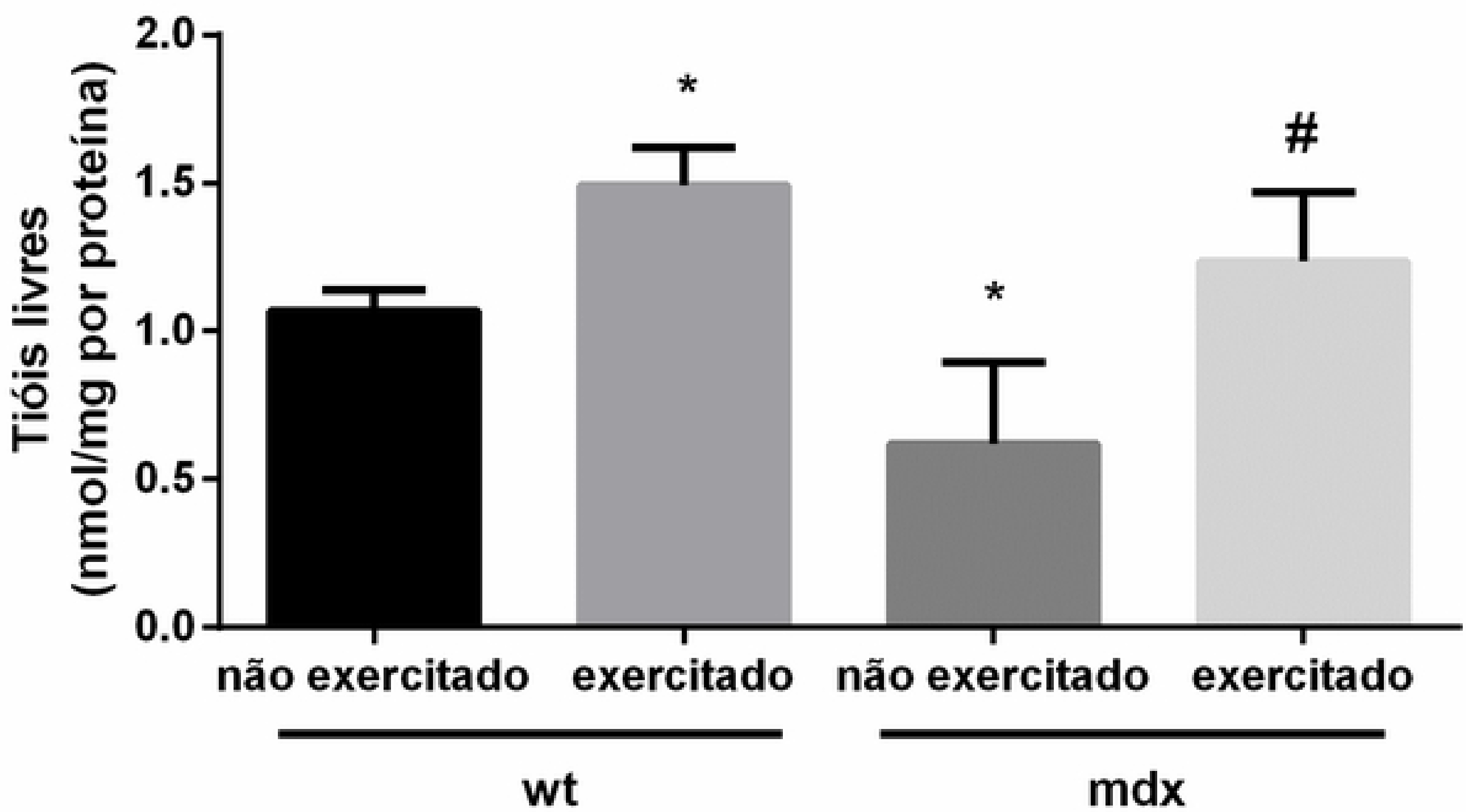
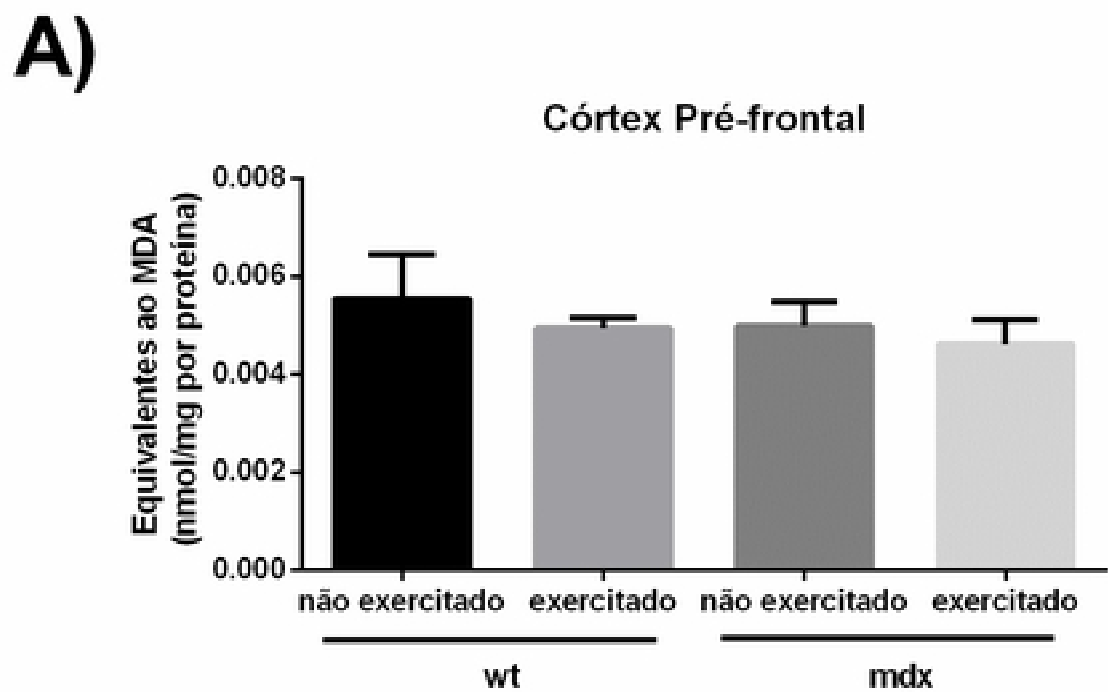
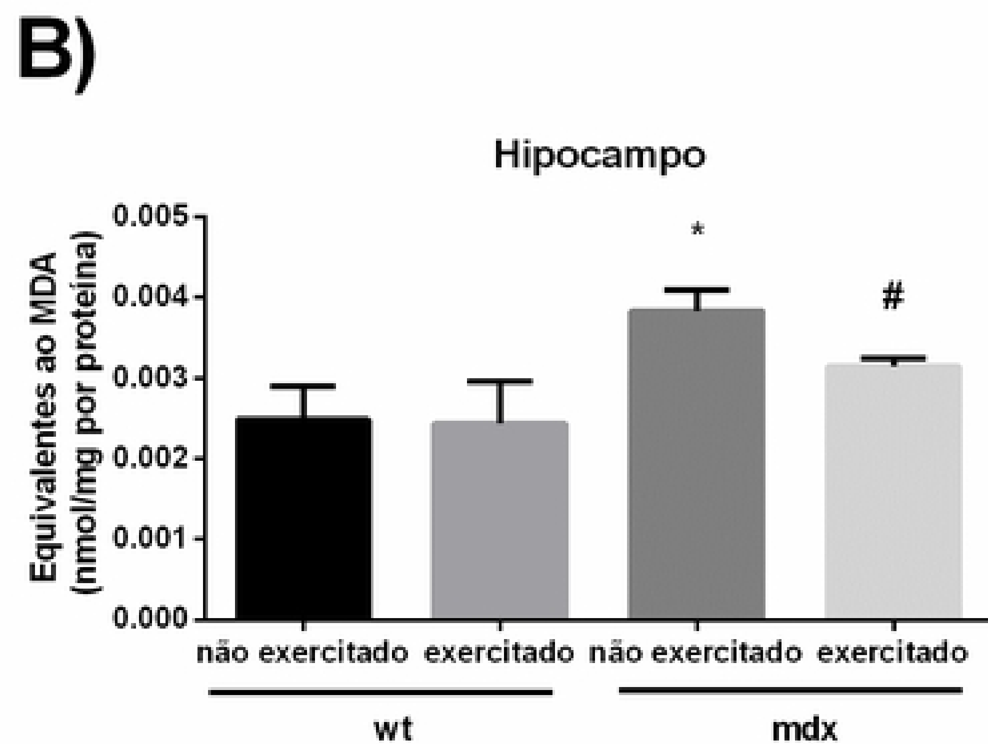
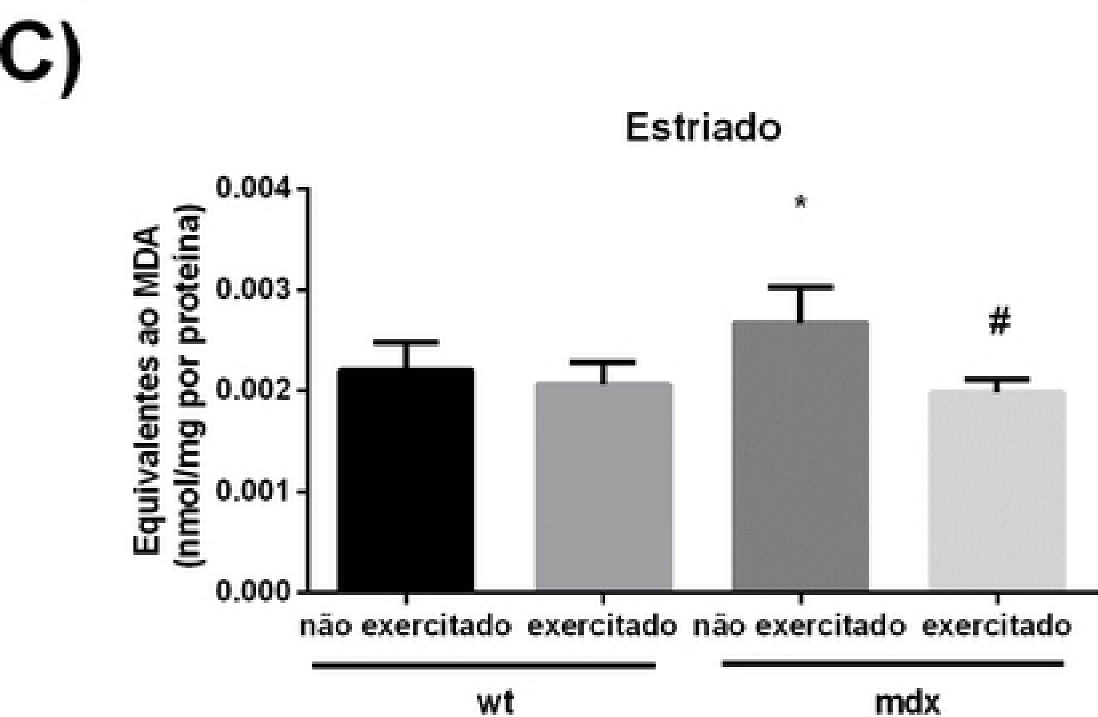


Figure 6







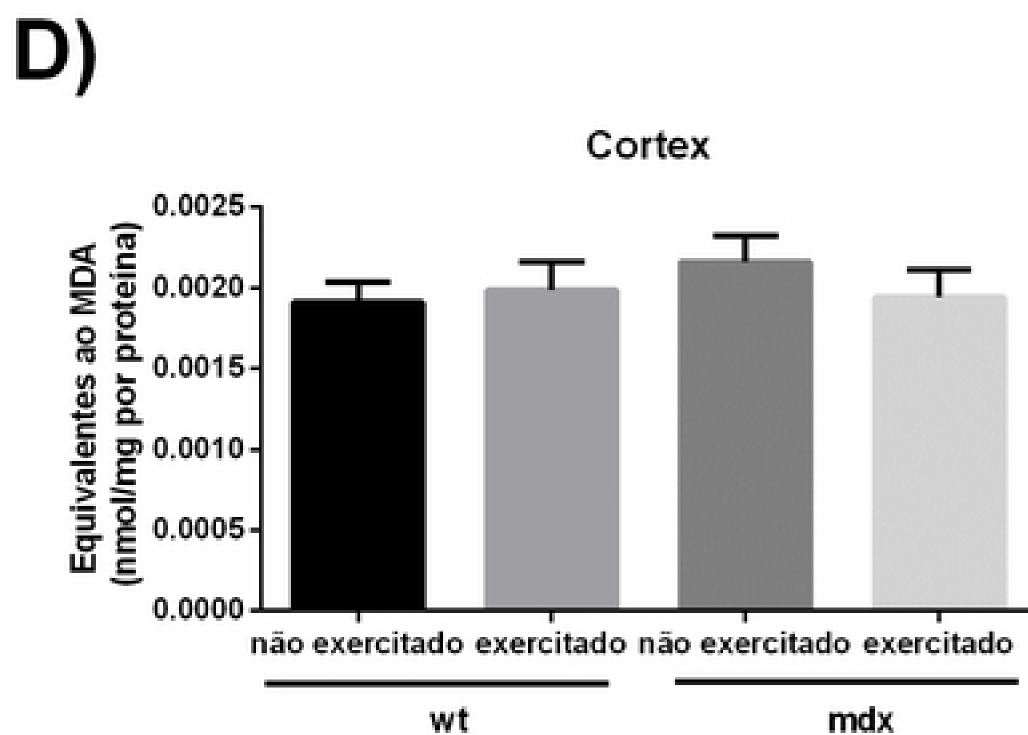
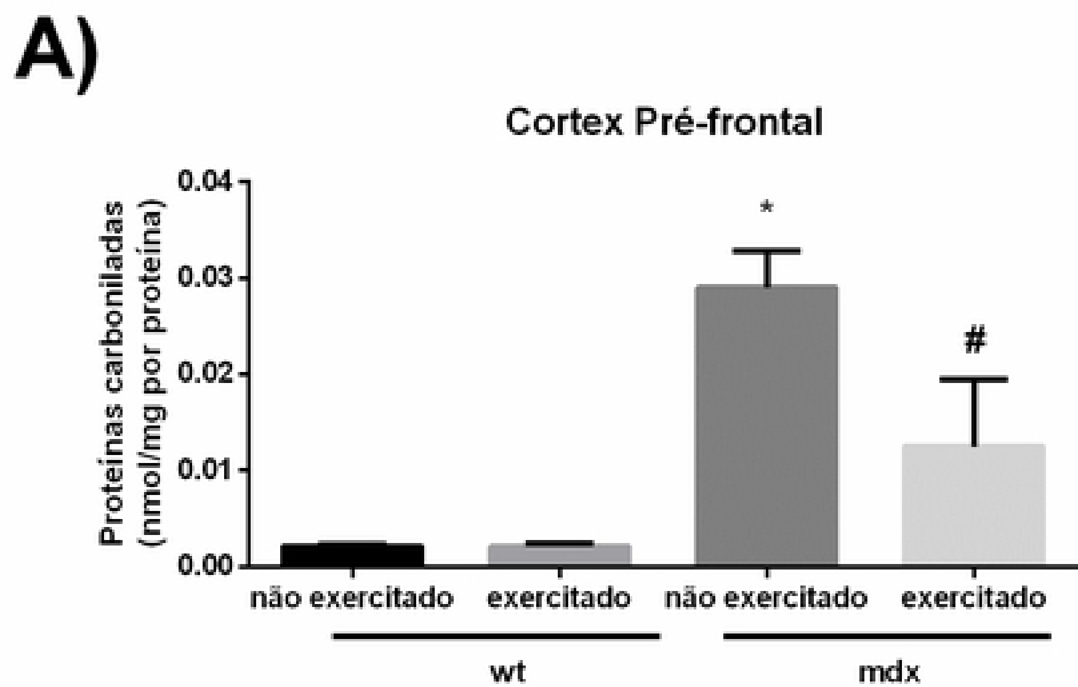


Figure 7

A)



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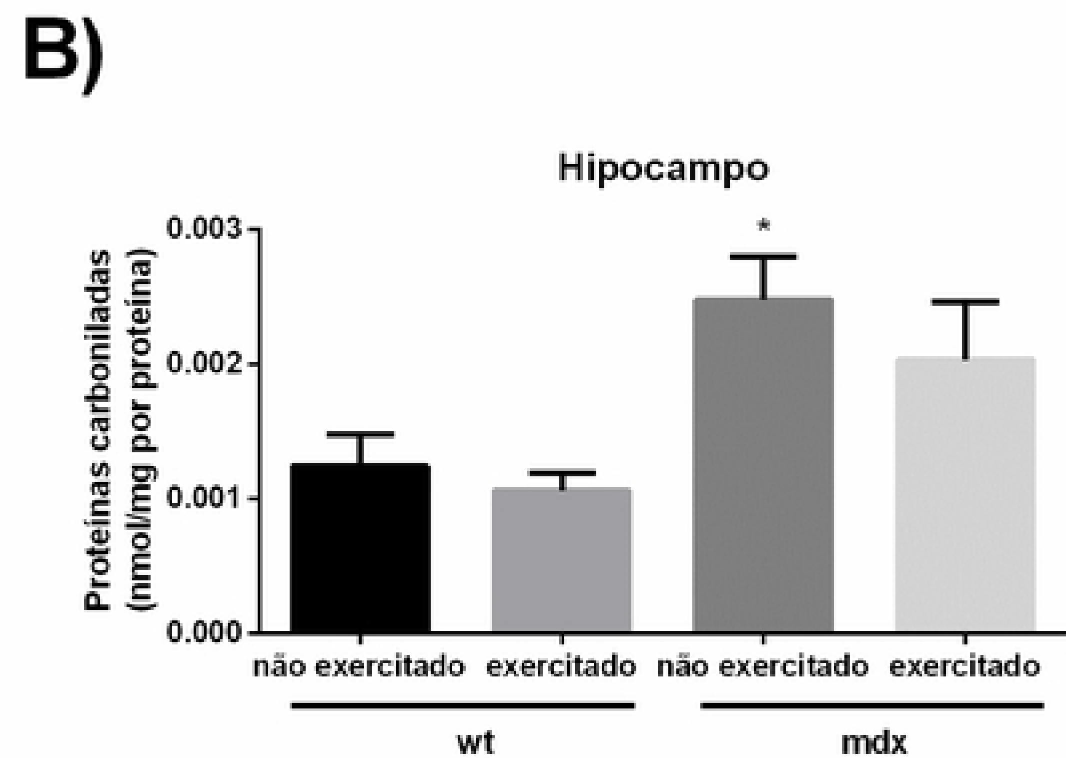

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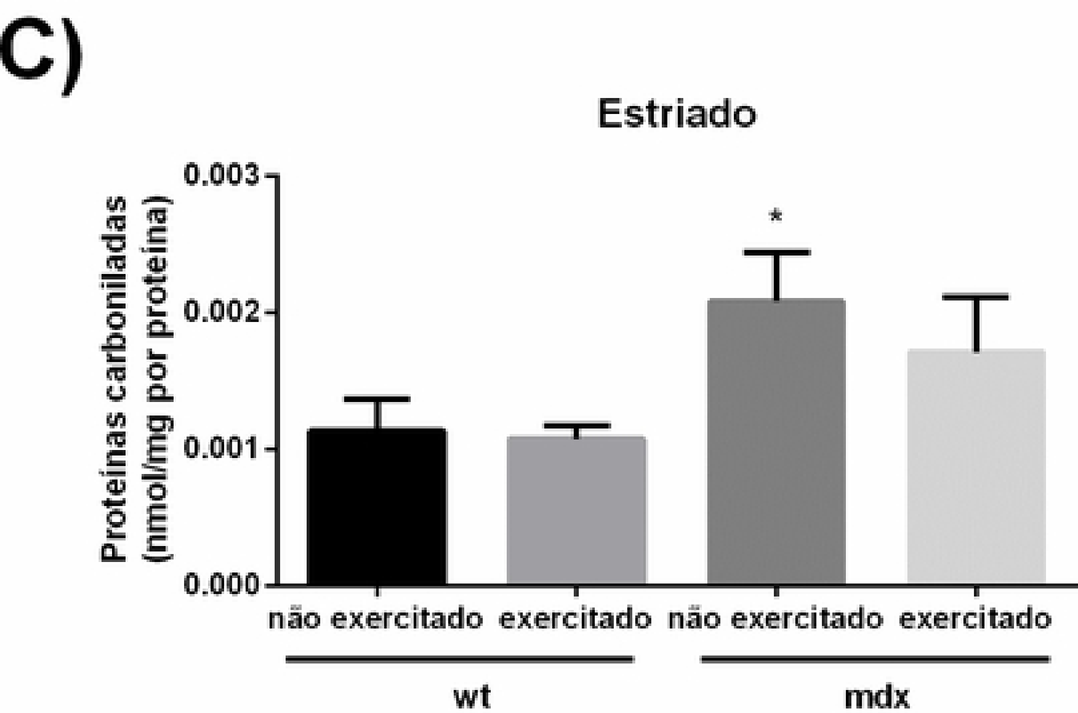


D)

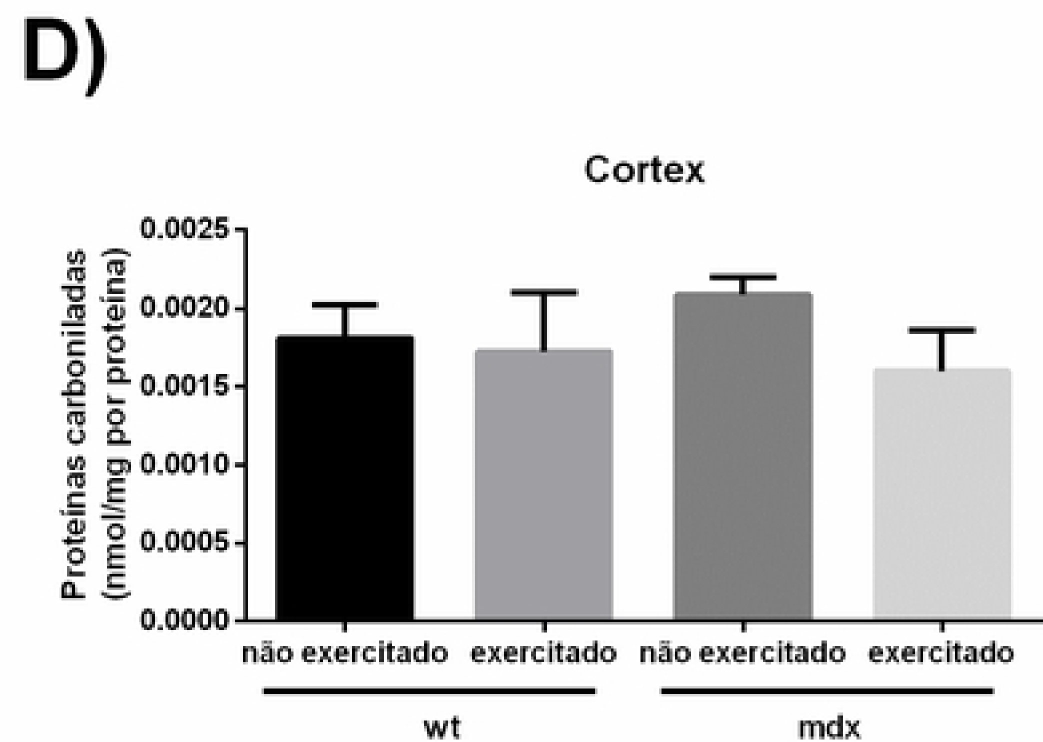
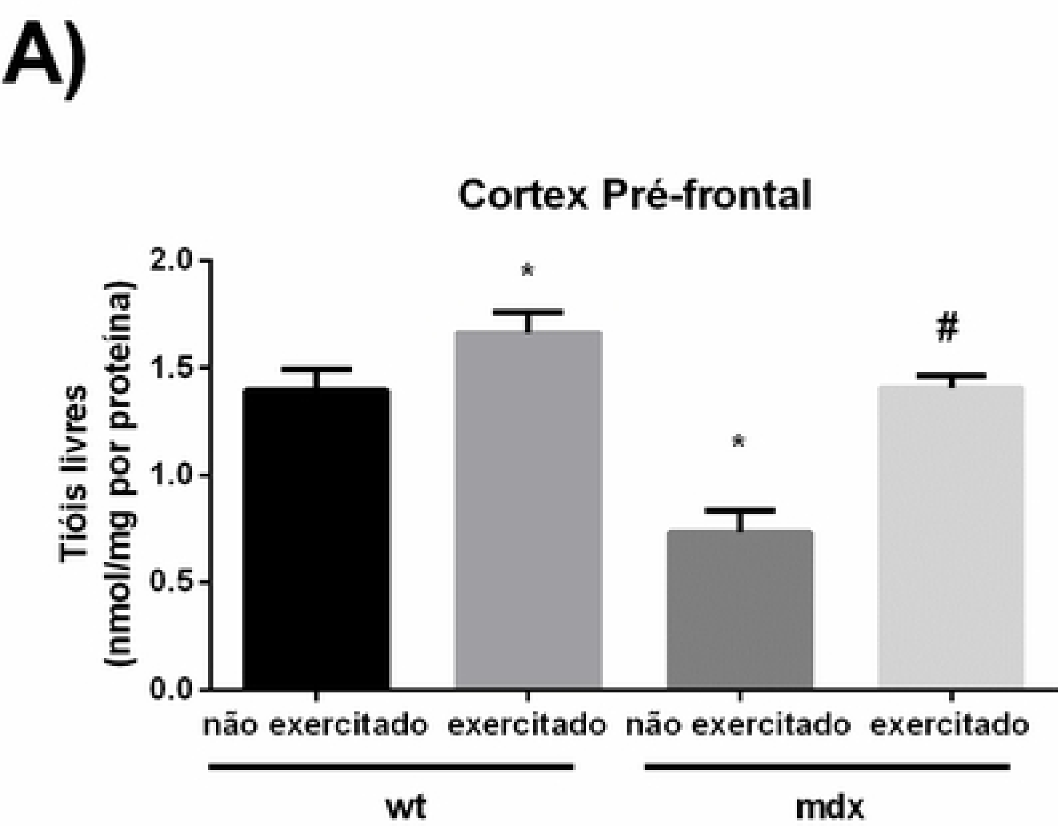
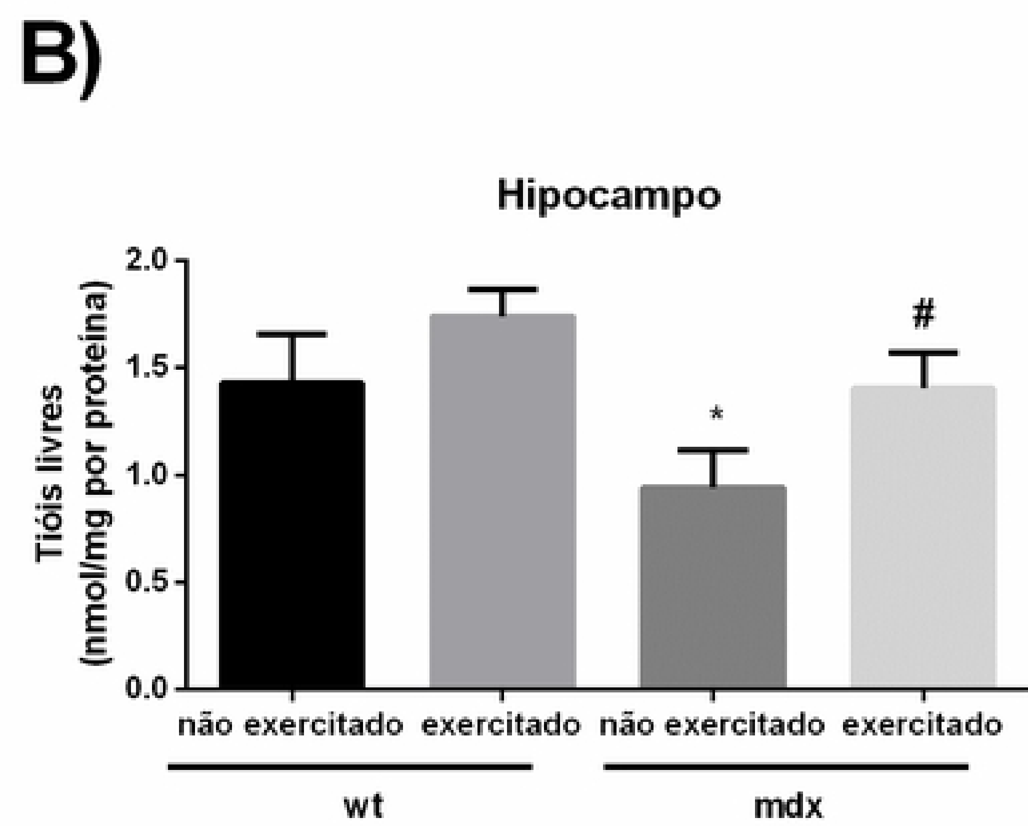
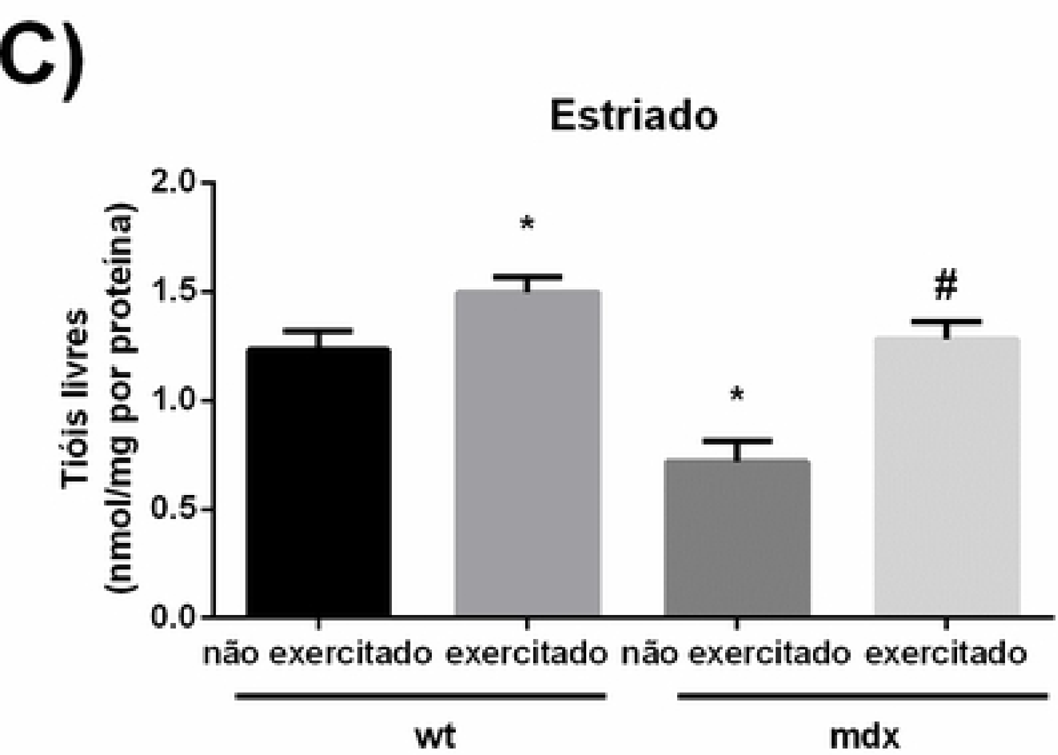


Figure 8







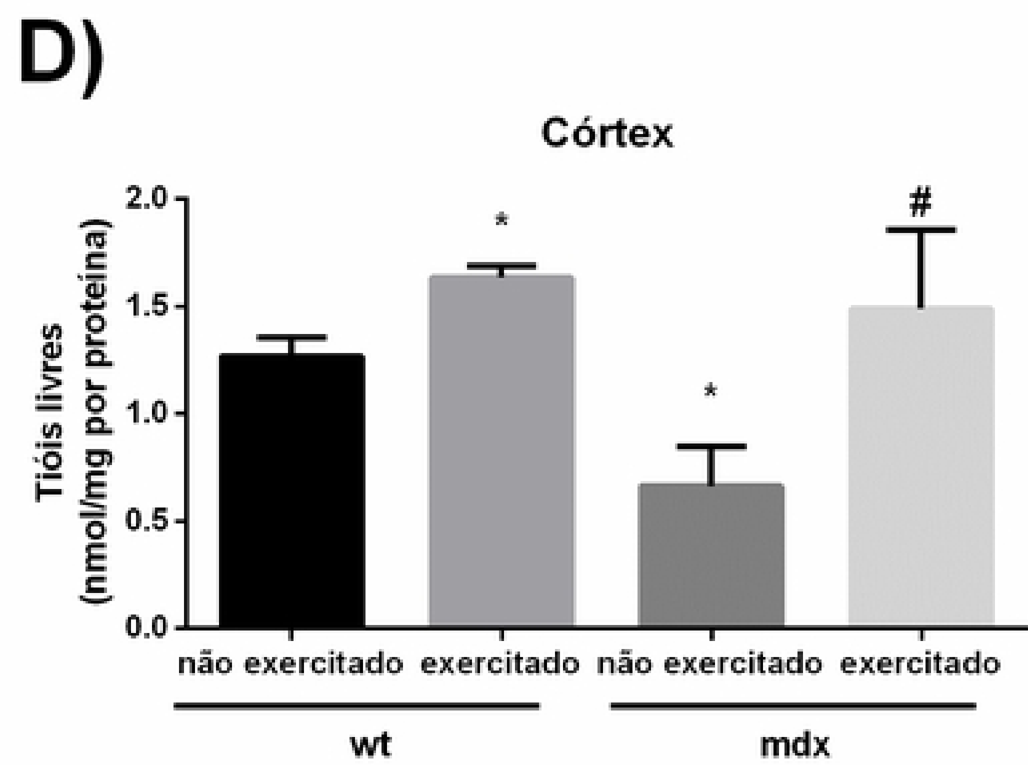


Figure 9