Eccentric exercise 48 hours prior to simulated diving has no effect on vascular bubble formation in rats

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Abstract

Purpose Decompression sickness caused by vascular bubble formation is a major risk of injury when diving. Prior studies have shown that physical exercise has a significant impact in both reducing and increasing bubble formation. There is limited knowledge about the mechanisms, but there are indications that exercise-induced muscle injury prior to diving may increase bubble formation. The purpose of this study was to investigate the role of exercise-induced muscle injury as a possible mechanism of bubble formation during diving.

Methods Muscle injury was induced by exposing female Sprague-Dawley rats (n=30) to a single bout of eccentric exercise; 100 min intermittent downhill (-16°) treadmill running. Forty-eight hours later, the animals were exposed to a 50 min simulated saturation dive (709 kPa) in a pressure chamber, when the degree of muscle injury and inflammation would be the most pronounced. Bubble formation after the dive was observed by ultrasonic imaging for four hours.

Results No difference in bubble loads were found between the groups at any time despite evident muscle injury. Maximum bubble loads (bubbles·cm^{-2}·heart cycle^{-1}) were not different, exercise: 1.6 ± 3.5SD *vs.* control: 2.2 ± 4.1SD, P=0.90, n=15 in each group.

Conclusions Eccentric exercise performed 48 hours prior to diving causes skeletal muscle injury but does not increase the amount of vascular bubbles in rats. The prevailing recommendation is that physical activity prior to diving is a risk factor of DCS. However, present and previous studies implicate that pre dive physical activity does not increase the DCS risk.

Keywords Bubble formation · Decompression sickness · Diving · Eccentric exercise

Abbreviations

DCS	Decompression sickness
IHC	Immunohistochemistry

Introduction

During diving, intra- and extravascular gas bubbles may form as a result of ambient pressure reduction (decompression) on ascent to the surface. The general opinion is that these bubbles are the cause of the clinical manifestations termed decompression sickness (DCS), which is a major health risk to divers (Sellers 2005). The traditional view is that physical activity prior to diving is a risk factor for DCS (Claybaugh and Lin 2004), but there is no firm evidence to support this. However, physical activity prior to altitude decompression has been shown to increase the risk of both decompression-induced bubble formation and DCS (Harvey 1951; Dervay et al. 2002; Foster and Butler 2009). The understanding of the mechanisms involved is limited. It has been hypothesized that physical activity with eccentric muscle contractions may be involved (Foster and Butler 2009).

As early as 1912, Hill (1912) discovered that gas bubbles will not form in solutions exposed to decompression, unless "points" were given for the bubbles to form on. In the work by Harvey and colleagues (1951) it is proposed that small gas bubble precursors called micronuclei could form in liquids, unexposed to decompression, inside hydrophobic surface cracks or acute angled cavities. In these hydrophobic cracks, micronuclei could probably remain stable for indefinite periods of time. They further suggested that micronuclei could be formed at sites with high negative mechanical pressures as a result of physical activity or movements. As suggested by Ikels (1970), the most obvious sites would be the articulating surfaces of joints, muscle tendon insertions and the inside of blood vessels. In a decompression model by Chappell and Payne (2006), it is proposed that gas bubbles may originate from hydrophobic regions in the vasculature. These regions could be seed-sites of bubble formation, where bubbles grow during decompression. McDonough and Hemmingsen (1984) suggested that physical movement or activity is necessary for bubbles to form. Furthermore, Wilbur et al. (2010) have provided direct evidence that physical exercise can cause micronuclei formation even without any exposure to decompression. They reported that the lifetime of exercise-induced micronuclei was in the order of minutes. However, in an experiment by Dervay et al. (2002) the lifetime of exercise-induced gas bubbles was approximately one hour. The experimental interventions performed in these two studies were different: whereas Wilbur et al. exposed their study subjects to bicycling which consists of concentric muscle movements, the study by Dervay et al. used deep knee squats which have an important eccentric component. Concentric exercise causes little muscle injury (Gibala et al. 1995; Newham et al. 1983), whereas eccentric exercise is known to result in a high degree of muscle injury (Armstrong et al. 1983; Proske and Morgan 2001). In addition, animal experiments have shown that trauma-induced muscle injury (Harvey 1951) and

violent exercise (Whitaker et al. 1945) immediately before altitude decompression increases bubble formation. On such injury, there may be a momentary exposure of hydrophobic sites, favoring the formation of micronuclei (Dervay et al. 2002). Thus, it is reasonable to assume that eccentric exercise-induced disruptions or damage to muscle tissues could lead to exposure of hydrophobic surfaces that enhances the formation and stabilization of gas micronuclei, leading to enhanced bubble growth on subsequent decompression (Foster and Butler 2009). In a previous study, we investigated whether eccentric exercise immediately prior to diving would increase the formation of vascular bubbles after diving (Jorgensen et al. 2013b), and we found no such effect despite evident muscle injury at the time of decompression. In the present study we aimed to further investigate the effect of eccentric exercise on bubble formation after diving, by introducing a 48 hour delay between eccentric exercise and diving. Decompression then occurs at a time when the degree of muscle injury after exercise would be most pronounced (Armstrong et al. 1983). We hypothesized that a single bout of eccentric exercise resulting in skeletal muscle injury would cause enhanced vascular bubble formation after a simulated saturation dive performed 48 hours later.

Methods

Ethical approval

The experimental protocols were reviewed and approved prior to the study by the Norwegian Committee for Animal Experiments, and conform to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes.

Diving animals

Thirty previously untrained adult female Sprague-Dawley rats (Taconic, Denmark), 278 ± 10 g SD, were randomly assigned to two groups. Group 1 (n = 15) was the exercise group. These animals performed eccentric exercise prior to diving. Group 2 (n = 15) was the control group. These animals did not exercise prior to diving. Before experiments and between the exercise and diving, the animals were housed in groups of three per cage in an animal facility. Illumination was controlled on a 12:12-hour light-dark cycle at room temperature 21.1 ± 0.6°C SD and humidity 28 ± 5% SD, and the animals had free access to water and a pellet rodent diet. Animals from both the exercise and the control group were included simultaneously on each day of experiments.

Exercise protocol

The eccentric exercise protocol has been described previously in detail by Jørgensen et al. (2013b). In summary, the rats were exposed to intermittent, downhill (16° decline) treadmill running for 100 min to exhaustion. This protocol has been shown to result in significant muscle injury in the fore- and hind-limbs, consistent with similar eccentric exercise protocols (Armstrong et al. 1983; Takekura et al. 2001). The control animals were placed on the treadmill, but did not run. All animals were allowed to rest in their cages for 48 hours after the exercise bout prior to diving.

Diving protocol

Forty-eight hours after completion of the exercise, the animals were exposed to a simulated saturation dive (Lillo and Parker 2000) in a pressure chamber while breathing compressed air, with a compression rate of 200 kPa·min^{-1} to a depth of 709 kPa for 50 min, and a linear decompression rate of 50 kPa·min^{-1}.

Anaesthetics

Immediately after decompression from diving, the animals were anesthetized with a one bolus subcutaneous injection of; midazolam 0.5 mg·100 g^{-1}, fentanyl 5 µg·100 g^{-1} and haldol 0.33 mg·100 g^{-1}, followed by regular booster doses of; midazolam 0.3 mg·100 g^{-1}·h^{-1}, fentanyl 3 µg·100 g^{-1}·h^{-1} and haldol 0.2 mg·100 g^{-1}·h^{-1}.

Muscle injury detection

An additional 18 Sprague-Dawley rats, similar to those in the diving groups, were used to assess the extent of muscle damage after eccentric exercise. Muscle injury was evaluated by the presence of leukocyte infiltration inside muscle cells, and a marker of muscle injury, αB-crystallin, was detected by the use of immunohistochemistry (IHC). Nine rats were exposed to the exercise protocol, whereas nine control rats were placed on the treadmill, but did not run. The animals were euthanized 48 hours after the completion of exercise, and vastus intermedius and soleus muscles were dissected out from each rat and immediately transferred to liquid nitrogen for IHC or to formalin for histology. Frozen biopsies were cut into 10 µM cross- and longitudinal sections using a cryostat microtome set to -22°C, mounted on glass slides and double stained with antibodies against total αB-crystallin (SPA-222, diluted 1:400, Enzo Life Sciences AG, Lausen, Switzerland) and dystrophin (ab15277 diluted 1:400, AbCam, Cambrinde, UK) to visualize the muscle cell membrane. The cross sections were prepared for fluorescence microscopy using a previously described protocol (Jorgensen et al. 2013b). Protein distribution of αB-crystallin and dystrophin were detected on an Olympus BX 41 light microscope with fluorescence, captured with an Olympus DP25 camera and images for both proteins were superimposed on each other using the Olympus Cell B analysis software, v. 3.3. The formalin fixated biopsies were paraffin embedded and cut into 4 µM cross- and longitudinal sections that were

mounted on glass sides and stained with haematoxylin, eosin and saffron (HES) for histological assessment of leukocyte infiltration in muscle cells. The stained sections were photographed with 20x objectives.

For semi-quantitative analysis of αB-crystallin staining, three randomly chosen areas (20x objective) in each muscle cross-section from exercised (n=9) and non-exercised (n=8) animals were captured under equal conditions. All adjustments and settings to the microscope and the image software were kept the identical in all images. Images of αB-crystallin were equally processed by computer software (Cell B) to remove background staining so that only clusters of αB-crystallin staining remained in the image. αB-crystallin clusters were automatically counted and the total immunostained area measured by the use of computer software (ImageJ 1.48, National Institutes of Health, USA). The average value of the three randomly chosen areas from each cover slide was used for statistical calculations.

Vascular bubble detection

The pulmonary artery and ascending aorta were insonated for 4 hours using a GE Vingmed Vivid 5 scanner, with a 10 MHz transducer. The amount of bubbles in the venous (pulmonary artery) and arterial circulation (ascending aorta) was detected for one min at discrete time points (15, 30, 60, 90, 150, 180, 210 and 240 min) after decompression. Data were stored and played back in slow motion for analysis. Images were graded according to a previously described method with the observer blinded to the group allocation of the rat (Eftedal and Brubakk 1997). The scoring system is composed of the following grades: 0, no bubbles; 1, occasional bubbles; 2, at least one bubble per fourth heart cycle; 3, at least one bubble per heart cycle; 4, at least ten bubbles per heart cycle; and 5, "signal whiteout" where individual bubbles cannot be separated. Bubble grades were converted to the number of bubbles$\cdot$cm$^{-2}\cdot$heart cycle^{-1} as previously described by Nishi et al. (2003). After the 4 hour observation period, the animals were euthanized by thoracotomy and excision of the heart.

Statistics

Data are expressed as median and range or mean ± SD. A Mann–Whitney *U* test was used to evaluate differences in detected bubble amounts, number of αB-crystallin clusters and total area immunostained by αB-crystallin. *P* values < 0.05 were considered significant.

Results

Evidence of muscle injury

Histology and IHC of muscle tissues verified that the eccentric exercise protocol generated skeletal muscle injury that was manifest 48 hours later. Immunostained slices (20x objective) of exercised muscles had a significantly higher number of clusters of αB-crystallin ($P<0.0001$) and the total area stained with αB-crystallin was larger ($P<0.0001$), Fig. 1a-d. In the light micrographs of HES stained tissue sections, there was evident leukocyte infiltration in exercised muscle cells but not in non-exercised animals (Fig. 2a-d), indicating an inflammatory response in injured muscle tissue from exercised animals.

Vascular bubble loads

In both groups, 7 out of 15 animals (47%) had detectable bubbles in the pulmonary artery after the dive. One animal in the exercise group, and three animals in the control group, died during the first hour after diving (13% mortality rate). These animals are included in the data in Fig. 3a. All animals that died had massive venous bubble loads (grade 5) at the time of death. For the surviving animals, there was no significant difference in median or maximum bubble loads (bubbles·cm^{-2}·heart cycle^{-1}) between the groups at any time point (Exercise: 1.6 ± 3.5 SD *vs.* Control: 2.2 ± 4.1 SD, $P = 0.90$, n = 15 in each group). The distribution of venous bubble grades and the mean bubble loads throughout the observation period are shown in Fig. 3a and b respectively. No gas bubbles were detected in the ascending aorta of any of the animals that survived the observation period.

Discussion

In this study we found that muscle-injuring eccentric exercise performed 48 hours prior to a saturation dive did not cause increased vascular bubble formation after diving. This is in agreement with our previous results when the same exercise protocol was performed 90 min prior to diving (Jorgensen et al. 2013b). Our results do thus not endorse a link between exercise-induced muscle injury and vascular bubble formation after diving.

Eccentric exercise in individuals unaccustomed to such exercise, especially downhill running, induces widespread muscle damage and systemic inflammatory responses (Peake et al. 2005). Initially muscle fibres are damaged by direct mechanical forces during eccentric contractions, and secondly the muscle cells are further damaged by increased proteolytic activity and inflammatory processes (Proske and Morgan 2001; Smith et al. 2008). It has been shown that both muscle injury and maximum muscle soreness is most prominent around 48 hours after eccentric exercise in both rats and humans (Proske and Morgan 2001; Armstrong et al. 1983; Morton et al. 2009). We have previously shown that the eccentric exercise protocol used in the present study causes immediate muscle injury, with increased levels of the small heat shock protein αB-crystallin and of the key pro-inflammatory mediators NF-κB and TNF-α (Jorgensen et al. 2013b). Protein accumulation and granular appearance of αB-crystallin in skeletal muscle tissue is a valid and reliable marker of muscle injury as a response to eccentric exercise (Koh and Escobedo 2004; Paulsen et al. 2009). As in our preceding study, the same exercise protocol in the present study resulted in accumulation of αB-crystallin in skeletal muscles when muscle tissues were examined 48 hours later (fig. 1). Paulsen et al. (2009) have demonstrated that αB-crystallin accumulates in muscle tissues immediately after eccentric exercise, and that αB-crystallin continues to accumulate with levels peaking at 48 hours after the exercise bout. The design of the present study does not allow for comparison of αB-crystallin levels in the exercised muscles 48 hours after compared to immediately after exercise. In contrast to the findings immediately after eccentric exercise (Jorgensen et al. 2013b), there was an accumulation of leukocytes inside exercised muscle cells after 48 hours (fig. 2). During the first hours after eccentric exercise, leukocytes start to accumulate in the exercised muscles, and migrate into the damaged muscle cells. The number of cells showing leukocyte infiltration has been reported to reach a peak 1-3 days after eccentric exercise (MacIntyre et al. 1995; Paulsen et al. 2010; Tsivitse et al. 2003), and verification of leukocyte accumulation in muscle tissues is a valid sign of muscle injury.

It has long been recognized that physical exercise prior to altitude exposure increases the risk of both bubble formation and DCS (Foster and Butler 2009). In contrast to what is observed in altitude experiments, physical

exercise prior to diving is reported to cause decreased bubble formation and DCS risk (Wisloff and Brubakk 2001; Dujic et al. 2004). Wisløff et al. (2004; 2001) demonstrated that treadmill running provided significant protection against bubble formation and DCS in rats if performed 20 hours prior to diving. The exercise protocol, diving protocol and animals were similar to the ones used here, but instead of eccentric exercise (downhill running), concentric exercise was applied (uphill running). Concentric exercise, in contrast to eccentric exercise, is known to provoke little muscle injury (Newham et al. 1983; Gibala et al. 1995). We might speculate that in these previous diving experiments involving concentric exercise, there may have been a low degree of muscle injury, with no exposure of hydrophobic regions and therefore no enhanced bubble formation. Thus, it is possible that the adverse effects of eccentric exercise-induced muscle injury may have been mitigated in the present study by a protective effect of exercise on bubble formation. However, it is less likely that eccentric exercise induces a protective effect 48 hours later, since Wisløff et al. (2004; 2001) did not find any protective effect if concentric exercise was performed 48 hours prior to the dive. In human diving experiments, level treadmill running performed 24 and 2 hours prior to diving resulted in reduced bubble formation (Dujic et al. 2004; Blatteau et al. 2007). Level running involves similar amounts of concentric and eccentric contractions. Therefore, it seems likely that eccentric muscle contractions can be introduced prior to diving without enhancing the formation of vascular bubbles.

In the present study, decompression-induced bubbles were used as a quantifiable indicator of decompression stress. Bubble grades are a common surrogate marker for DCS manifestations in studies on anesthetized animals or in human studies with low risk of DCS (Doolette et al. 2014). The intra-individual variability in bubble grades in this study was high, ranging from no detectable bubbles to maximum bubble grade with lethal outcome within both the exercise and control group. This variation between individuals who perform identical dives is well known from previous studies on animals and humans (Doolette et al. 2014; Jorgensen et al. 2013a; Berge et al. 2005). Endogenous factors, such as differences in fat distribution, genetic composition and stress responses to handling and diving are likely causes (Francis and Mitchell 2003). In order to detect differences between groups of individuals that differ significantly in their responses, large sample sizes are required. In this study we included 15 animals in each group, which in similar studies have been shown to provide sufficient power for detection of differences between the groups (Jorgensen et al. 2013b; Wisloff and Brubakk 2001; Wisloff et al. 2003, 2004).

The interpretation of the results from the present study may be limited by the inability to detect the true maximum bubble loads in the pulmonary artery due to the timing of measurements. In our data, the mean bubble loads decline uniformly from the first recording at 15 min post decompression (Fig. 3b). In only three out of 14 rats with bubbles, peak bubble amounts were recorded later than 15 min. The timing of the first measurement was restricted by the time required for all animals to achieve sufficient depth of anesthesia. All animals were in general anesthesia at 15 min post decompression. However, the results indicate that in future studies bubble measurements should commence sooner. Another possible limitation of this study may be the inability to detect the total amount of bubbles produced during and after decompression. The amount of circulating bubbles that passes through the pulmonary artery are assumed to reflect the amount of bubbles that is produced due to decompression stress. However, it is still possible that at sites of injury, eccentric exercise may still lead to enhanced formation gas bubbles that are not dislodged into the circulation. To elucidate this further, bubble formation will have to be studied in situ at the site of injury.

This study has investigated whether exercise-induced muscle injury will increase vascular bubble formation after diving. However, results from the present and previous studies indicate that several factors are critical in determining whether the effects of exercise are harmful or beneficial, i.e, the choice of diving protocols, exercise modalities and intensity, the timing of exercise bouts, and the animal species. It also appears crucial for the outcome on bubble formation whether the exercise is performed in relation to altitude or diving exposures.

Conclusions

In partial answer to the question of how physical exercise affects the formation of decompression-induced bubbles, the present study leads us to conclude that eccentric exercise with skeletal muscle injury performed 48 hours prior to diving does not increase the amount of vascular bubbles in rats. The prevailing recommendation is that physical exercise prior to diving is a risk factor for DCS. However, the physiological implication of the present and previous studies is that pre dive physical exercise does not seem to increase the DCS risk, but may rather be beneficial if performed with the appropriate timing and intensity. The mechanisms behind the effects of pre dive physical exercise on decompression-induced bubbles and DCS manifestations are still unknown. Further studies are needed in animals

and humans to elucidate the mechanisms of how exercise modality and timing may be optimized for protection against DCS development.

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Conflict of interest: The authors declare that they have no conflict of interest.

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Legends

Fig. 1 Exercised skeletal muscles (**a**) were more immunostained for αB-crystallin (green) compared to non-exercised muscles (**b**). The cross sectional light micrographs were immunostained for αB-crystallin as a valid sign of muscle injury, dystrophin (red) to visualize the cell membranes, and with DAPI to visualize cell nuclei (purple) as a sign of leukocyte infiltration. Note the increased number of cell nuclei in the exercised muscle. The number of αB-crystallin clusters (**c**) and total immunostained area (**d**) were increased in exercised rats (gray boxes) compared to non-exercised rats (white boxes). Values are presented on a logarithmic scale. In the box plots, bands are drawn at the median values, and bars and whiskers indicate lower and upper quartiles and min/max values, respectively. Singular dots are outliers that deviate >1.5x from interquartile range, n=9 in the exercise and n=8 in the control group. **P*<0.0001.

Fig. 2 HES stained sections of eccentric exercised muscle fibers (**a** longitudial, **c** transversal) shows high degree of leukocyte infiltration (arrows) compared to non-exercised muscle fibers (**b** longitudinal and **d** transveral).

Fig. 3 No difference in the amount of venous gas bubbles between exercised and control animals after diving. Bubbles were detected by ultrasonic imaging in the pulmonary artery. **Panel a** shows the distribution of bubble grades (from 0 to 5) in the venous circulation post dive observation for exercised and control animals (n = 15, in each group). ***** marks the time of death of individual rats. In **Panel b** the mean bubble loads (bubbles·cm^{-2}·heart cycle^{-1}) detected during the post dive observation are graphed. Maximum mean loads were observed in the first post dive measurement (at 15 min) for both groups, with 1.2 bubbles·cm^{-2}·heart cycle^{-1} for the exercise group and 1.8 bubbles·cm^{-2}·heart cycle^{-1} for the control group respectively. The differences in bubble loads between the two groups are not significant at any time point.

Illustrations

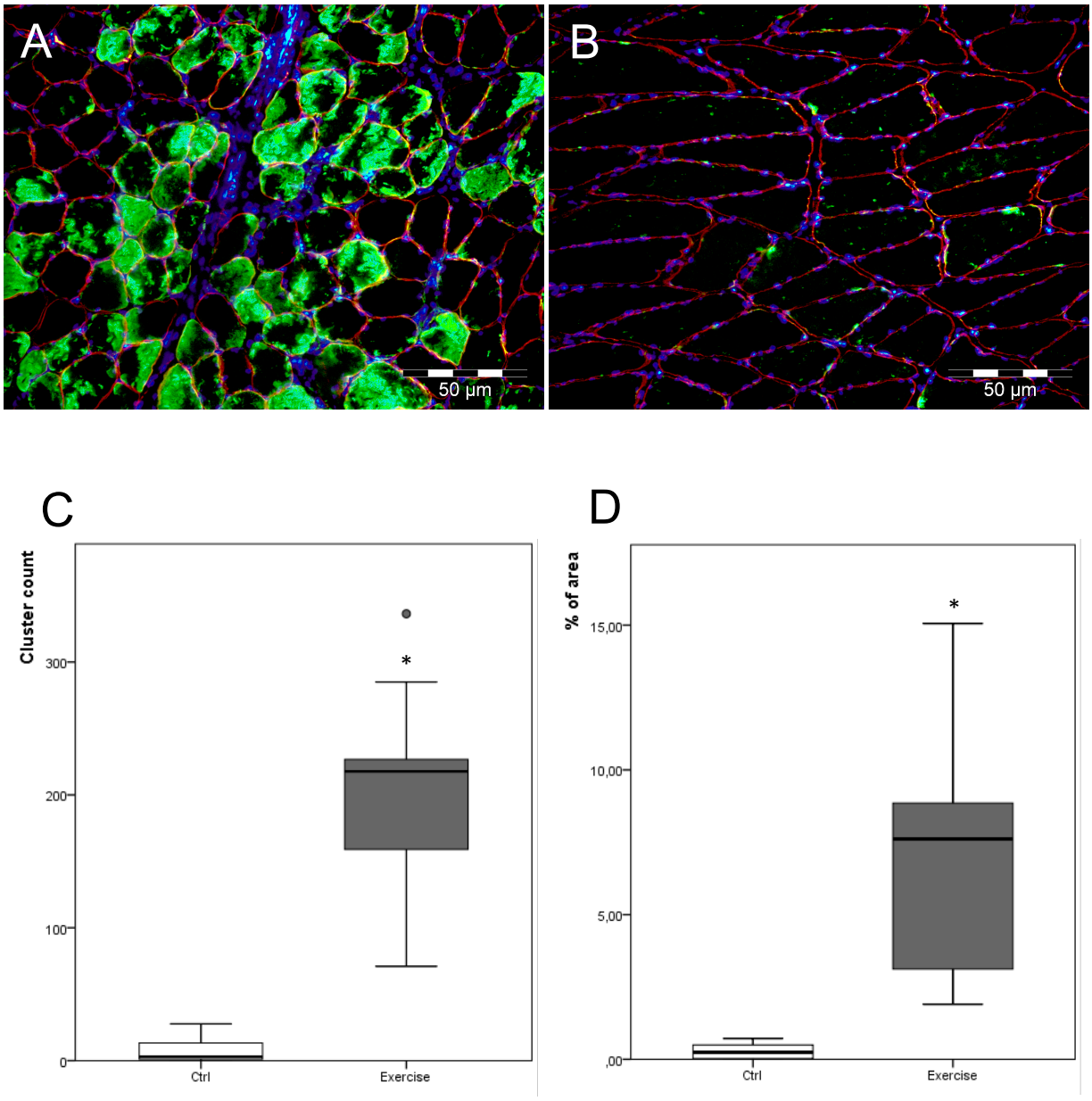
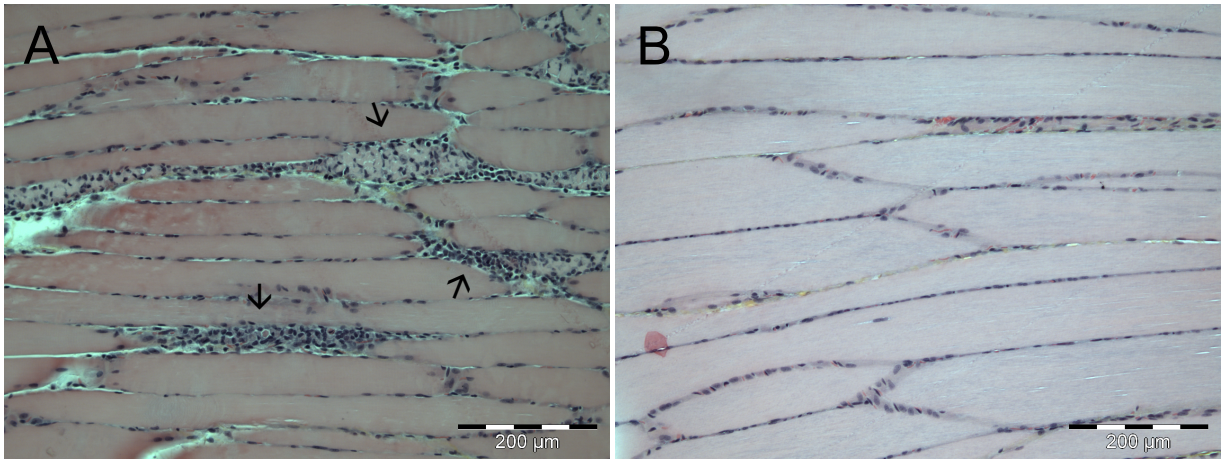
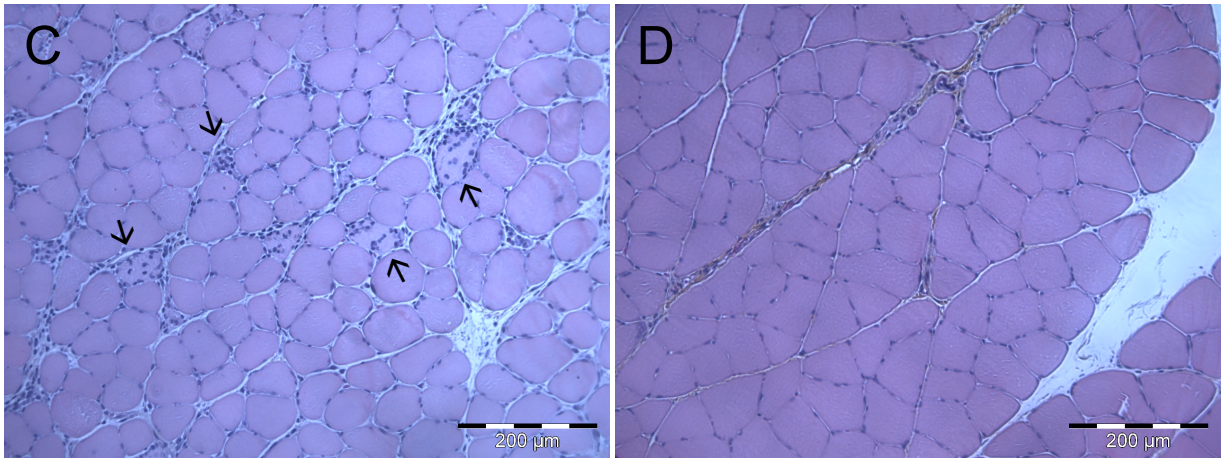


Fig. 1a-d

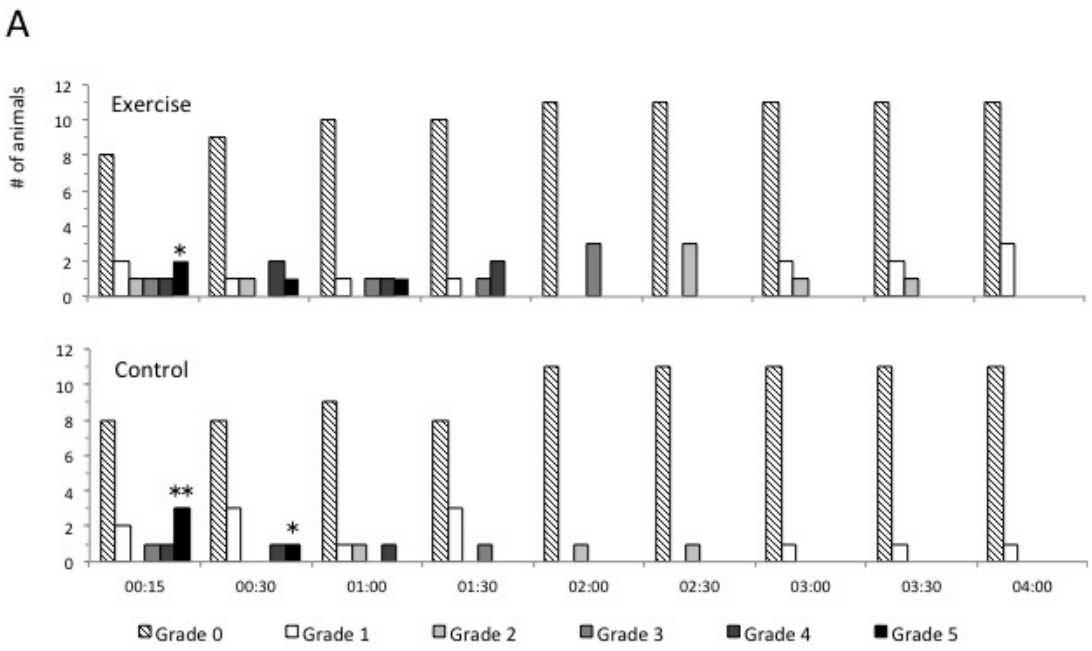
384



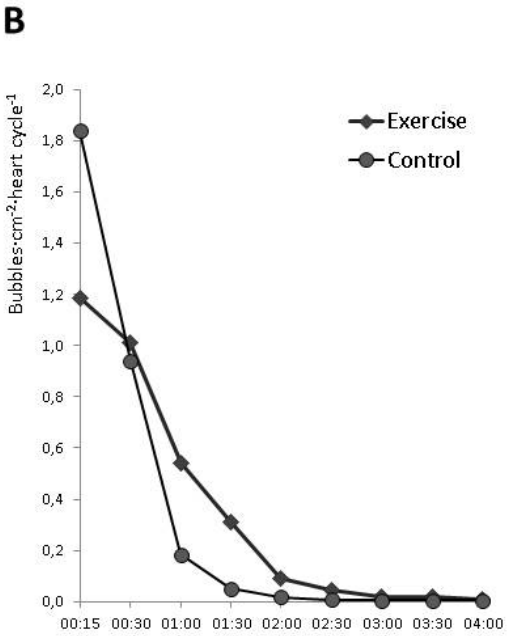
385



386 **Fig. 2 a-d**



387
388 **Fig. 3a**



389
390 **Fig. 3b**