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MICROBIOLOGY

Physical activity reduces intradermal bacterial load in a murine model submitted to forced swim training – a pilot study

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Abstract: Regular exercise is beneficial to health. This study evaluated the effects of moderate and intense physical exercise modalities on intradermal infection by *Staphylococcus aureus* in a murine model. Mice that practiced moderate exercise had lower bacterial load on lymph nodes and less inflammatory infiltrate in dermis. They presented greater weight, however, less amount of epididymal fat: the weight was increased while they had fat diminished. A positive correlation was observed between lipid content and bacterial load in mice trained at moderate intensity. Animals that were under high intensity exercises presented superior bacterial load on the lymph nodes, increased neutrophil count and circulating lymphocytes, and had leukocyte recruitment to the dermis augmented, when compared to the ones in moderate exercise. These findings suggest that moderate physical activity modulates the immune response in dermal infection caused by *S. aureus* in a murine model.

Key words: infection, mice, physical exercise, *Staphylococcus aureus*.

INTRODUCTION

Moderate physical exercise represents a resistance factor for the onset of infections by stimulating the immune system protective response (Viana et al. 2014). However, high intensity exercises may lead to increased susceptibility to infectious conditions (Nieman 2007, Priebe et al. 2009). The duration, intensity, and frequency of exercise can directly influence the formulation of a protective response by modulating the number of circulating or recruited leukocytes to the infectious site (Nieman & Nehlsen-Cannarella 1994, Matthews et al. 2002, Escribano et al. 2005, Nieman et al. 2011, Araneda et al. 2016, Lu et al. 2017).

The fluctuation in leukocyte concentration in different body regions is determined by pathophysiological reactions (Natale et al. 2003). Chronic moderate physical exercise has been associated with an increment in the proliferative response of T lymphocytes and in the number of neutrophils, thus, intensifying antimicrobial and phagocytosis functions (Malm 2004, Yeh et al. 2014, Gomes et al. 2016) .

Neutrophils and lymphocytes are key cells in control of infections caused by *Staphylococcus aureus* (Harrington et al. 2005, Sladek & Rysanek 2006, Hill & Imai 2016). Neutrophil recruitment and abscess formation are characteristic of *S. aureus* infections and are required for the pathogen elimination (Kobayashi et al. 2015).

S. aureus is a gram-positive bacteria and catalase-positive cocci. This pathogen generally is an unencapsulated bacteria, has approximately 0.5 to 1.5 µm in diameter, is immobile, and

non-sporulated (Kloos & Wolfshohl 1982). Among bacterial microorganisms, *S. aureus* has extreme importance nowadays (Lowy 1998, Wertheim et al. 2004, Daum & Spellberg 2014).

Besides its high prevalence, *S. aureus* is well known for its ability to acquire resistance to antibiotics (Kobayashi et al. 2015, Boswihi & Udo 2018). The methicillin-resistant *S. aureus* (MRSA) was reported in the early 1960s and then spread throughout the world, being currently endemic in hospitals and communities (CA-MRSA) (Rigby & DeLeo 2012).

This pathogen is associated with a significant diversity of diseases and syndromes, including bacteremia, pneumonia, and osteomyelitis; most of the infections occurs in skin and soft tissues (Malachowa et al. 2016). In order to develop more effective strategies on prevent or treat the infections, it is crucial to understand why the immune response is unable to eradicate this bacterium.

Intradermal infection by *S. aureus* in rodents display of many inflammatory features like those observed in humans (Asai et al. 2010, Alabi et al. 2013). Dermis and epidermis are regions rich in antigen presenting cells. This factor guarantees to the intradermal infection model a more efficient antigen delivery, even though using a smaller dose (Miller et al. 2007, Miller & Cho 2016, Santana et al. 2016).

Little is known about the influence of exercise and its execution in different intensities on experimental intradermal infection in rodents. In this regard, the objective of this study was to evaluate the effects of moderate and high intensity physical exercises on intradermal infection by *Staphylococcus aureus* in a murine model.

MATERIALS AND METHODS

Animals

Male mice, aged 6 to 8 weeks old, were obtained from the laboratory of Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira (UFBA/IMS-CAT), and maintained in a controlled temperature room, 12-hours photoperiod, and free access to water and food. This project was submitted and approved by the Ethics Committee on Animal Use (Comitê de Ética no Uso de Animais - CEUA) of IMS-CAT (Protocol No. 031/2015).

Experimental design

Animals were randomly divided into 6 groups with 8 to 10 animals per group. Two sedentary groups (SC- sedentary control, SI- sedentary infected by *S. aureus*) and four exercise groups (MIC- moderate intensity control, MII- moderate intensity infected, HIC - high intensity control, HII- high intensity infected) were submitted to 2 weeks of pre-training and 8 weeks of swimming training. Both sedentary and exercise groups were considered able to swim, however, only the exercise groups performed the swimming training.

Training protocol

Animals submitted to the exercises performed swimming sessions for adaptation, for 2 weeks long. These training sessions lasted 5 minutes each one without adding a load into animal's back. In order to carry out high intensity exercises, additional load weighing 5% of the animal body mass was coupled in its dorsal region; for moderate intensity exercise the load was of 2.5% of body mass. After adaptation period, mice started swimming five times a week with a minimum interval of 24 hours between sessions for eight weeks, with a training session time of 10 minutes for the animals in moderate

intensity group and 15 minutes for the ones in the high intensity group. The weight attached to the animal's back was a pre-weighed heavy ball. The application of mass attached to animal body was used so that the level of effort was greater and proportional to the intensity intended to reach. The swimming protocol utilized was adapted from Gobatto et al. (2001).

Bacterial culture

Staphylococcus aureus ATCC 43300 (MRSA) strain obtained from Laboratório de Histopatologia at Universidade Federal da Bahia, Instituto Multidisciplinar em Saúde, Campus Anísio Teixeira (UFBA/IMS-CAT), were reactivated and cultured in brain-heart infusion agar (BHI) for 24 hours at 37°C. After growth, colonies of bacteria were collected, diluted in sterile saline, and the number of colony forming unit (CFU) was determined based on analysis of spectrophotometric absorbance at 660nm (value equivalent to 10^8 CFUs in McFarland scale).

Preparation of anesthetic, challenge, and euthanasia

After the training period, animals were anesthetized with 50 mg/kg of ketamine and 10 mg/kg of xylazine, intraperitoneally. Later, they were challenged intradermally (right ear) with 10 µL of saline solution or *S. aureus,* according to the experimental design. Mice were euthanized 72 hours after challenge by intraperitoneal injection with lethal dose of xylazine and ketamine (40 mg/kg and 400 mg/kg, respectively).

Hematological analysis

Blood Samples were collected by opening peritoneal cavity and through incision of a large-caliber blood vessel. Total and differential numbers of leukocytes were determined. Twenty microliters of blood were solubilized in 380 µL of Turk blue for total cell count in Neubauer's chamber.

Blood smears were made with 10 µL of blood and differential leukocyte count were carried out. Serum was obtained after centrifugation of total blood for 10 min at 300g.

Histopathological analysis

Following euthanasia, a fragment of the infected ear region was cut off for fixation in 10% formalin. After dehydration in alcohol solution and diaphanization in xylol (60°C in an oven), tissue was included in histological paraffin, and sections of 4 µm were done by microtomy. Sections were stained with hematoxylin-eosin. Inflammatory infiltrate cells and epithelial architecture were analyzed. Photomicrographs were acquired and evaluated by two pathologists who were blinded to the experiment, through Image J-SisGET IT Image capture system (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Total count of tissue infiltrated cells was performed with the mean counts of twenty fields in two magnification levels: 4X and 20X.

Determination of bacterial load

Retromaxillary lymph nodes were removed 72 hours post-infection and macerated in a sterile Petri dish in 500 µL saline solution. Fifty microliters of the supernatant were collected and spread in Petri dish with BHI medium by the pour plate method and conditioned in a bacteriological oven at 37°C for 24 hours, and after that, the colony forming units (CFUs) were quantified.

Epididymal adipose tissue

Throughout the project, the animal weight was weekly monitored. After euthanasia, epididymal adipose tissue was removed and immediately weighed to avoid dehydration. This tissue was applied as an indicator of lipid content in

these animals. The epididymal fat weight was divided by animal body weight, obtained before euthanasia, and multiplied by 100 to provide the relative value and relative percentage of this tissue in the assessed animals (Bueno et al. 2005).

Statistical analysis

In order to evaluate statistical differences among the groups, Kruskal Wallis test was used with Duns' test for non-parametric samples. ANOVA test was applied for parametric samples. Pearson's test was utilized for the non-parametric correlations, being considered a significant difference when $p<0.05$, with a 95% confidence interval.

RESULTS

Physical exercise promotes bacterial load reduction in lymph nodes

In order to analyze if different intensity exercises change bacterial clearance pattern after infection, colony forming units (CFU) were quantified in lymph node macerates. CFUs count are shown in Figure 1. In infected exercised mice, there was lower growth in number of CFUs when compared to the infected sedentary group. No bacterial growth was observed in control groups.

Trained animals have lower lipid content ratio by animal weight

Sedentary groups animals showed lower average of body weight when compared to exercised groups (Figure 2). In contrast, Figures 3a and 3b brings that, despite having a lower gross weight, when the epididymal fat weight and the epididymal fat/weight ratio of the mice were evaluated, sedentary groups presented higher epididymal fat weight and greater epididymal/weight of the mice, for both the sedentary control group (SC) and for the sedentary infected group

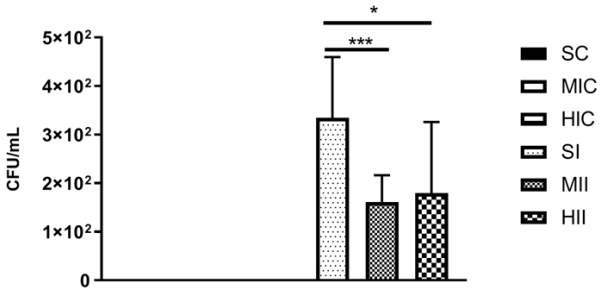

Figure 1. Bacterial load on exercised and sedentary animals. A / J mice were challenged with the number of 10^8 CFU of MRSA strains. Animals were euthanized 72 hours post-infection. Animals had the lymph nodes macerated and cultured in BHI medium (Brain Heart Infusion). CFUs were quantified after 24 hours of culture; (MRSA: Methicillin-resistant *Staphylococcus aureus*). CFU- colony forming unit; SC- sedentary control; MIC- moderate intensity control; HIC- high intensity control; SI- sedentary infected; MII- moderate intensity infected; HII- high intensity infected. n = 8 to 10 / group. *p<0.05; *** p<0.001.

(SI). The groups in moderate intensity exercise, controls and infected, despite presenting the highest gross weight, exhibited lower epididymal fat weight and epididymal fat/weight ratio when compared to the other groups.

Animals exercised in high intensity present neutrophilia and lymphocytosis

Aiming to assess the absolute number of neutrophils and lymphocytes in peripheral blood, a differential count of leukocytes was performed through light microscopy. Figure 4 illustrates the main cell types observed. It is possible to notice that there were quantitative differences in the number of neutrophils and lymphocytes in *S. aureus* infected animals belonging to the HIC and HII groups.

Animals infected with *S. aureus* present inflammatory infiltrate in the ear

The number of inflammatory cells was determined by histopathological evaluation of the ears where the intradermal challenge was performed. In this model, cellular populations

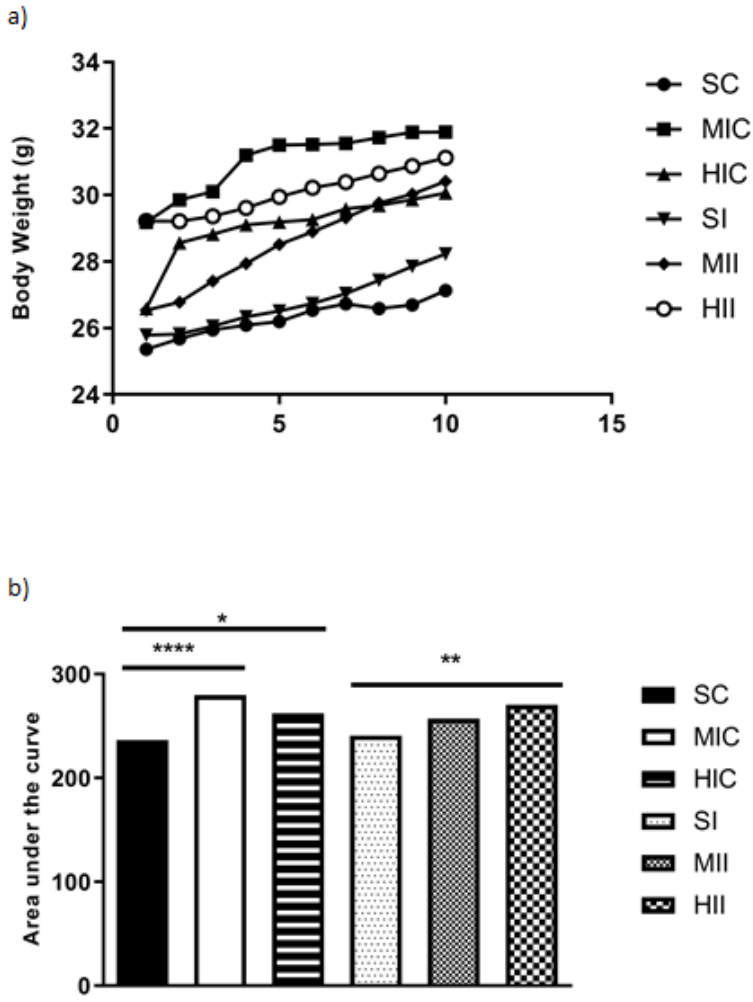

Figure 2. Determination of mice weight variation during training period. a) Weight mean of all animals in the group. b) Area under the curve corresponding to the Figure 2a. SC- sedentary control; MIC- moderate intensity control; HIC- high intensity control; SI- sedentary infected; MII- moderate intensity infected; HII- high intensity infected. n = 8 to 10 / group. * p<0.05; ** p<0.01; **** p<0.0001.

were observed and quantified through total cell count. Figure 5 outlines the six groups analyzed, revealing superior cellular infiltrate in infected sedentary group, followed by the infected group in high intensity exercise.

Sedentary animals have more intense inflammatory infiltrate than those exercised

Figure 6 indicates that animals infected with *S. aureus* had intense infiltration in the ear, however, in the previously exercised animals, this infiltrate was smaller than the number of cells presented in the sedentary group. Thus, it was also possible to observe that the animals that exercised in high intensity exhibited superior infiltration than those from the group that were in moderate intensity.

Lipid content positively correlated with the bacterial load in moderate exercise

Using the correlations, it could be observed that the higher the lipid content in animals that practiced moderate intensity exercises the greater the bacterial load presented by them. A positive correlation was also observed between the number of total leukocyte counts of the infected group in moderate exercise (MII) with its number of neutrophils. The same occurred between the total count of leukocytes and the number of neutrophils in the sedentary control group (Table I).

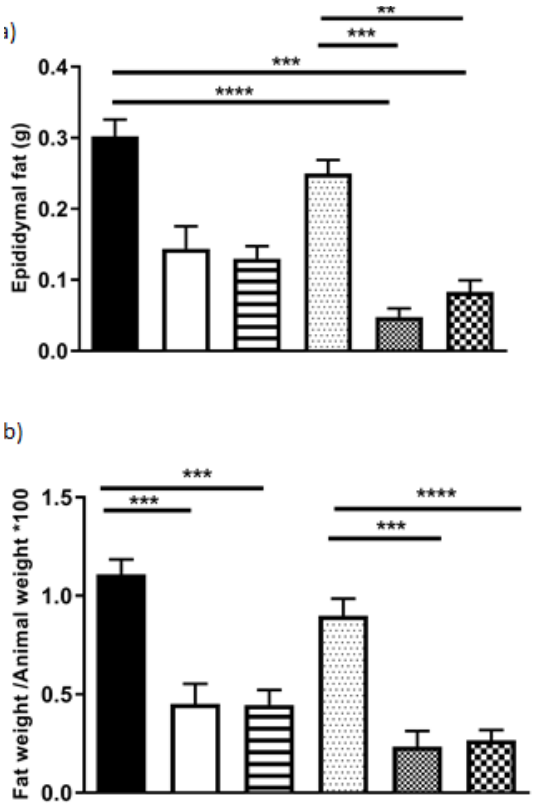

Figure 3. a) Determination of epididymal fat rate exhibited by the training group. This tissue is used as an indicator of animals' lipid content. Epididymal fat was removed and immediately weighed to avoid dehydration. **b)** Determination of the animals' lipid content. Epididymal fat weight was divided by the animal body weight, obtained before the sacrifice, and multiplied by 100 to provide the relative value and percentage. SC- sedentary control; MIC- moderate intensity control; HIC- high intensity control; SI- sedentary infected; MII- moderate intensity infected; HII- high intensity infected n = 8 to 10 / group. ** p<0.01; *** p<0.001; **** p<0.0001.

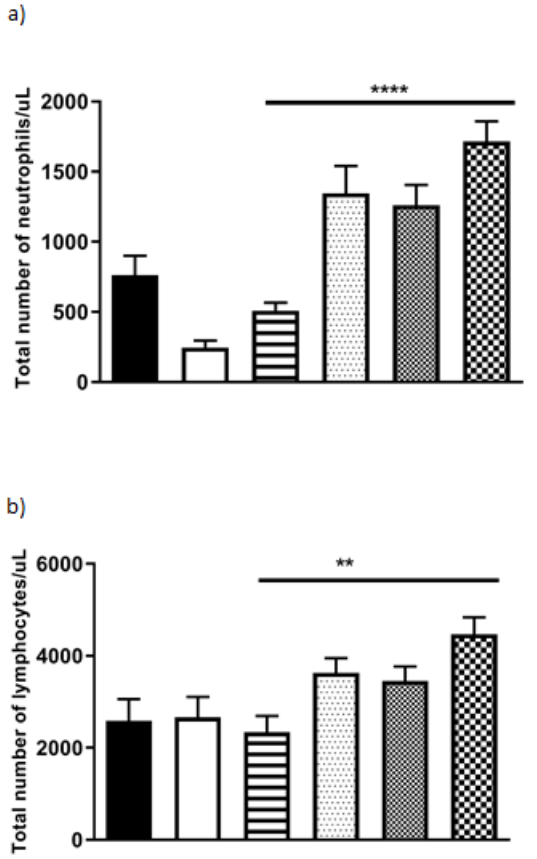

Figure 4. Neutrophil and Lymphocyte Differential Cell Counts - A / J mice were challenged with the amount of 10^8 CFU of MRSA strains. Following challenge, the animals were euthanized after 72 hours. After sacrifice, blood samples were collected for the differential count performed through light microscopy. **a)** Number of neutrophils; **b)** Number of lymphocytes. SC- sedentary control; MIC- moderate intensity control; HIC- high intensity control; SI- sedentary infected; MII- moderate intensity infected; HII- high intensity infected n = 8 to 10 / group. ** p<0.01; **** p<0.0001.

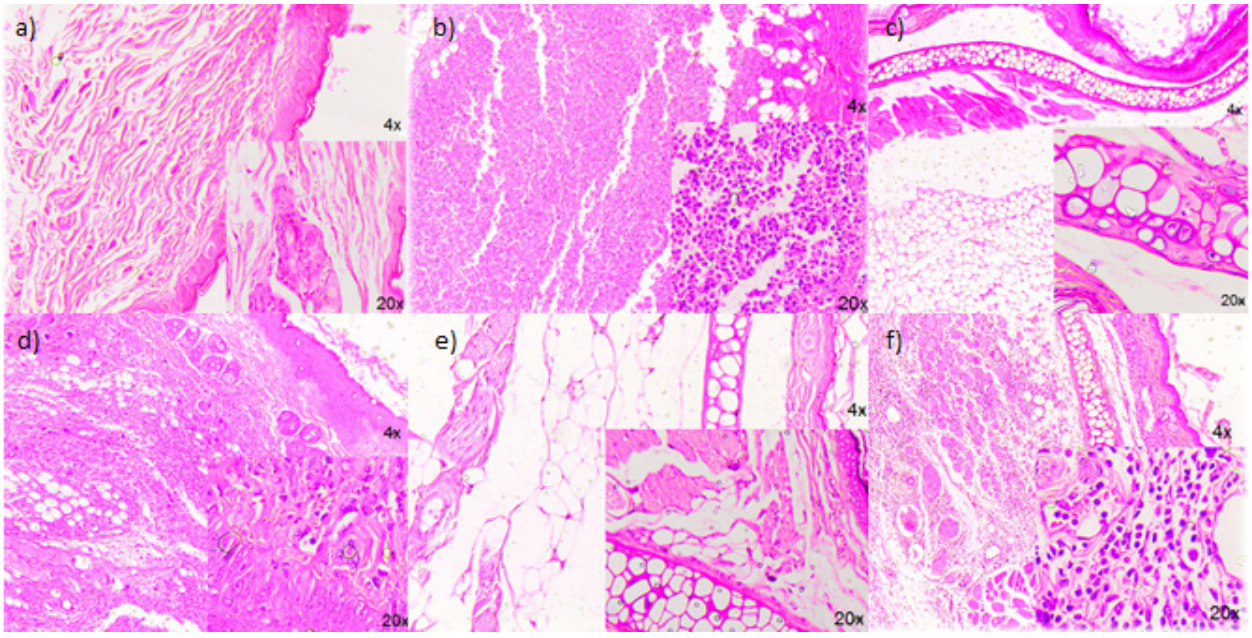

Figure 5. Leukocyte recruitment to the right ear dermis of A / J mice. Mice trained in moderate intensity, high intensity exercise, or the sedentaries, were infected with 10^8 methicillin resistant *Staphylococcus aureus* (MRSA). Animals were euthanized 72 hours after infection. Animals had excision of the right ear and histopathological slides were analyzed through light microscopy (MRSA: Methicillin-resistant *Staphylococcus aureus*) n = 8 to 10. Magnification 20X. a) SC (Sedentary control); b) MIC (Moderate intensity control); c) HIC (High intensity control); d) SI (Sedentary infected); e) MII (Moderate intensity infected); f) HII (High intensity infected).

DISCUSSION

S. aureus is responsible for several skin infections. Complications from these infections are an important and constant health problem. The present study investigated in an unprecedented way the effects of physical exercise in moderate and high intensities on intradermal infection by *Staphylococcus aureus* in a murine model. Here we demonstrate for the first time that exercise contributes not only to the bacterial load control, but also, to the reduction of dermis inflammation induced by this pathogen.

Skin is the body site most affected by *S. aureus* infections (Hill & Imai 2016). Thus, by intradermally infecting the mice, we can observe the cell migration to draining lymph nodes (Almeida et al. 2017). In our work, when quantifying the bacterial load in infected animals exercised or not, it was revealed that the exercise, regardless of the intensity, can decrease the bacterial load in mice dermis.

In *S. aureus* infections, the host initiates an innate immune response primarily mediated by neutrophils (Rigby & DeLeo 2012, dos Santos et al. 2018a, b), which is responsible for destroying and phagocytizing the bacteria, and migrating to the draining lymph node for antigen presentation (Hermida et al. 2014, Muniz et al. 2021). It is also known that the fluctuation of the number of circulating neutrophils in response to exercise depends on training intensity. Different reports have demonstrated that moderate exercise leads to an increase in the number of these cells, which is maintained even in rest (Nieman & Nehlsen-Cannarella 1994, Pereira et al. 1994, Mockinnon 1997, Rosa & Vaisberg 1998).

High intensity exercise has been correlated with intensified leukocytosis, sustained by the presence of neutrophils (Natale et al. 2003,

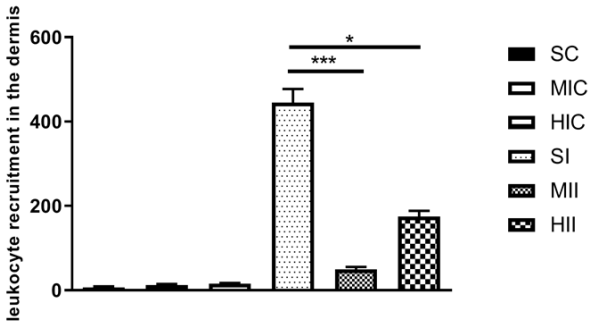

Figure 6. Leukocyte recruitment in the ear dermis (μm^2) – Sedentary A / J mice or previously trained in moderate intensity or high intensity exercise were infected with 10^8 strains of methicillin resistant *Staphylococcus aureus* (MRSA). Animals were euthanized 72 hours after infection. Animals had excision of the right ear and histopathological slides were prepared and analyzed through light microscopy. SC- sedentary control; MIC- moderate intensity control; HIC- high intensity control; SI- sedentary infected; MII- moderate intensity infected; HII- high intensity infected n = 8 to 10. *p<0,05; *** p<0.001.

Neves et al. 2015). We observed that animals trained in a high intensity level exhibited an increment in the number of leukocytes, corroborating with the previous studies. An endogenous inflammatory process is known to trigger the mobilization of mature neutrophils and more immature populations from bone marrow and blood to inflammatory sites, in this way, it is still necessary to analyze and characterize the different cell types present in the inflammatory site (Zhou et al. 2010, Cuenca et al. 2011, Nakamura & Ushigome 2018).

Physical training may promote loss of body fat mass (Stanford et al. 2015), however, in our study, the groups exercised presented greater weight than the sedentary groups. On the other hand, it was observed that the exercised groups had lower epididymal fat weight as well as the weight/fat weight ratio. This finding suggests that physical exercise changed mice body composition, increasing the lean mass index and decreasing fat mass. This result can be correlated with previous studies reporting that, in general, physical exercises promote diminution of body fat, increase of muscle mass, and then, augment the strength, besides being important in muscle regeneration process (Foschini et al. 2004, Redman et al. 2007).

It was observed that, animals that were infected and were also practicing physical activity presented lower amount of adipose tissue than exercised control animals. Therefore, it is seen the positive correlation between the bacterial load and mouse weight/fat ratio, in both groups trained in moderated intensity. Different studies have shown that in situations of excessive fat accumulation, such as in obesity, the susceptibility to infections in animals and humans is increased (Ramírez-Orozco et al. 2018, Ramos-Muniz et al. 2018). The animals in this group presented lower weight/fat ratio and had lower bacterial loads than the other mice.

Studies have described the importance of the intradermal pathway for understanding the formulation of a protective immunity against infection by different pathogens (Lambert & Laurent 2008). In *S. aureus* infection, researches on cells recruited to the inflammatory site have collaborated to understand the inflammation associated with this microorganism (Santana et al. 2016). In our study it was possible to analyze that lesions in the dermis were characterized

Table I. Bacterial load correlate with lipid content in exercise mice.

CORRELATIONS BETWEEN GROUPS	p	r
Lipid content MII X Bacterial load MII	**0,0220**	0,7079
MII total count X Neutrophils MII	**0,0017**	0,8531
Total score SC X Neutrophils SC	**0,0004**	0,9453

SC - sedentary control; MII - moderate intensity infected; p – p-value (p<0.05); r – Pearson correlation coefficient; n = 8 to 10.

by intense inflammatory infiltrate. Although, in pre-exercised animals there was a smaller inflammatory infiltrate when compared to sedentary ones. Some studies have shown the regulatory role of Tregs in maintaining immune homeostasis, showing a cross-talk between Treg and neutrophils, limiting inflammation and inhibiting neutrophils accumulation (Richards et al. 2010, Okeke & Uzonna 2019). Understanding the association between Treg cells with neutrophils and macrophages can provide new insights into how the immune response is orchestrated in our model.

Several studies have reported that moderate intensity exercise is associated with decreased episodes of infection and improved immune response, probably associated with better neutrophil, macrophages, and T cell function (Matsudo & Matsudo 1992, Martin et al. 2009, Campos-Rodriguez et al. 2016, Lucchetti et al. 2017) . On the other hand, exercise, when practiced beyond a certain limit, is associated with an increase in cases of infectious diseases of the upper airways (Nieman 2007). These works convey that the number of cells at the inflammatory site was lower in the animals that practiced moderate intensity exercise. This lower infiltrate correlated with a lower bacterial load on the draining lymph node. These data allow us to infer that exercise may be interfering with the activation and the inflammatory profile of the cells recruited in the dermis.

This work corroborates to some existing studies that affirm that the moderate intensity exercise acts modulating a response against infections. Although, to date we are not aware of any other work that shows the influence of forced swimming training on *S. aureus* intradermal infections in mice. We have shown that both moderate and high intensity exercises may help reduce bacterial load on lymph nodes of animals intradermally infected with *S. aureus*. However, moderate exercise has a greater effect in reducing the infiltrate of cells at the infectious site.

The study is a pioneer in clarifying the cell recruitment and the reduction in intradermal bacterial load in a murine model submitted to forced swim. However, the characterization of the inflammatory cells influx and a more detailed evaluation of the influence of body composition on bacterial load in this model are still missing. In this regard, more studies are needed to bring new approaches to the different mechanisms that may be involved in this type of response.

Acknowledgments

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RAAS conceived the idea. MPLG, LSL, KBR, ISR, DPS, ISP and IPRM contributed with the interpretation of data, carried out the analysis of the data and the preparation of figures. LSL, KBR, CVG, SPMC contributed with the maintenance, weighing of the animals and assisted in swimming. MPLG, LSL anad IPRM contributed developing the discussion section and writing the manuscript. All authors are involved in interpreting the results and contributed reviewing the manuscript. RAAS, and MPLG supervised the final version. All authors read and approved the final manuscript.