EXERCISE TRAINING AND LIVER CELLULAR TURNOVER

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Keywords: EXERCISE TRAINING, HEPATOCYTE, CELLULAR TURNOVER, LIVER ADAPTATION, LOBULES ZONES.

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To my parents

“Patience et longueur de temps. Font plus que force ni que rage.”

Jean de La Fontaine

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Abstract

During the practice of physical exercise, there are numerous structural, functional and metabolic changes in the body. Some of these changes, such as increased oxidative stress, changes in blood flow or nutrient and oxygen supply, associated with increased functional requirement imposed by neuro-hormonal stimuli, can damage some organs / tissues with consequent dysfunction of same, especially if the exercise in question is exhaustive. However, there are evidences that repeated acute organic attacks of, promoted by a regular exercise practice, can bring organic benefit, inducing chronic adaptations with reduced organic sensitivity to future aggressions.

Due to its functions, its location and its blood supply, the liver seems to be directly exposed to damaging factors induced by acute exercise, however it high regenerative capacity after assault leaves assume a distinct adaptive capacity of this organ.

In this sense, the main objectives of this study were, initially, to make a state of play about the state of knowledge existing in the literature on this subject (review article) and, secondly, to evaluate the effect of repeated exercise of moderate intensity in the mice hepatocyte turnover (experimental study). For this purpose we evaluated the frequency of death and hepatocyte proliferation by immunohistochemistry in two distinct zones of the hepatic lobule, in the livers of animals subjected to seven weeks of daily exercise on treadmill.

The results show no increase in the number of apoptotic cells in the livers of animals trained when compared to the control group. Regarding indicators of cell proliferation, data suggest that repeated exercise time reduces the rate of cellular division, especially in the periportal region.

Depending on the results, we conclude that moderate physical exercise, repeated over seven weeks, in chronic terms, attenuate the rate of hepatocyte proliferation without apparent influence on the rate of cell death, suggesting a decrease in hepatocyte turnover.

Keywords: EXERCISE TRAINING, HEPATOCYTE, CELLULAR TURNOVER, LIVER ADAPTATION, LOBULES ZONES.

Resumo

Durante a prática de um exercício físico, ocorrem inúmeras alterações estruturais, funcionais e metabólicas no organismo. Algumas destas mudanças, tais como o aumento do stress oxidativo, as alterações do fluxo sanguíneo ou o aporte em nutrientes e oxigénio, associados com o aumento da exigência funcional imposta por estímulos neuro-hormonais, podem danificar alguns órgãos/tecidos com a consequente disfunção dos mesmos, especialmente se o exercício em causa for exaustivo. No entanto, existem evidências de que a repetição das agressões orgânicas agudas, promovidas por uma prática regular do exercício, podem trazer benefícios orgânicos, induzindo adaptações crónicas com redução da sensibilidade orgânica a futuras agressões.

Devido às suas funções, à sua localização e ao seu aporte sanguíneo, o fígado parece estar diretamente exposto aos fatores danosos induzidos pelo exercício agudo, contudo a sua elevada capacidade regenerativa após agressão, deixa supor uma distinta capacidade adaptativa deste órgão.

Neste sentido, os objetivos principais deste trabalho foram, numa primeira fase, fazer um ponto da situação acerca dos conhecimentos já existentes na literatura sob este tema (artigo de revisão) e, numa segunda fase, avaliar o efeito do exercício repetido, de moderada intensidade, no turnover hepatocitário do rato (estudo experimental). Com esse propósito foram avaliadas a frequência de morte e de proliferação de hepatócitos por imunohistoquímica, em duas zonas distintas do lóbulo hepático, nos fígados de animais sujeitos a sete semanas de exercício diário em tapete rolante.

Os resultados obtidos mostram não haver aumento do número de células apoptóticas nos fígados dos animais treinados quando comparados aos do grupo de controlo. Relativamente aos indicadores de proliferação celular, os dados sugerem que o exercício repetido no tempo reduz a taxa de divisão celular, especialmente na região periportal.

Em função dos resultados obtidos, é possível concluir que o exercício físico moderado, repetido ao longo de sete semanas, em termos crónicos, atenua a

taxa de proliferação hepatocitária sem aparente influência na sua taxa de morte celular, sugerindo uma diminuição do turnover hepatocitário.

Palavras-chave: EXERCÍCIO CRÓNICO, HEPATÓCITO, TURNOVER CELULAR, ADAPTAÇÕES HEPÁTICAS, ZONAS LOBULARES.

List of Abbreviations

.OH: hydroxyl radical

ALT: alanine transaminase

AST: aspartate transaminase

ATP: adenosine-5'-triphosphate

BrdU: 5-bromo-2'-deoxyuridine

BSA: bovine serum albumin

Ca^{2+}: calcium

CAT: catalase

CL: centrilobular

CNS: central nervous system

CONT: control group

CYP: enzymes of the cytochrome P450 complex

DNA: deoxyribonucleic acid

EHS: exertional heat stroke

EIAH: exercise-induced arterial hypoxemia

ET: exercise training

ɣ-GST: lambda class glutathione transferase

ɣGT: bilirubin and gamma glutamyl transpeptidase

GPx: glutathione peroxidises

GSH: glutathione

H**&**E: hematoxylin/eosin

H_2O_2: hydrogen peroxide

HCL: hydrochloric acid

I/R: ischemia/reperfusion

IL-1β, IL-6 e IL-10: Interleukines

K^+: potassium

L-FABP: liver-type fatty acid-binding protein

M_{ed}: median

Na^{2+}: sodium

NAD+: nicotinamide adenine dinucleotide

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

O^{2-}: superoxide anion

PaO_2: arterial oxygen partial pressure

PBS: phosphate buffered saline

PP: periportal

ROS: reactive oxygen species

SaO_2: haemoglobin oxygen saturation capacity

SOD: superoxide dismutases

TNF-α: tumor necrosis factor-alpha

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

VO_{2max}: maximal oxygen uptake

XO: xanthine oxidase

1. General Introduction

Exercise is widely recommended as a healthy lifestyle promoter (16, 33) however the acute metabolic, functional and structural organic changes that occur during it practice, represent a real challenge to the body's ability to maintain homeostasis (26, 34). Evidences exist in the literature that exercise induces a set of physiological alterations in the body. Depending of it characteristics (intensity, duration and frequency), the acute exercise seem to have different repercussions to the body structures (3, 7). Data have demonstrated that exercises of high intensity and/or long duration can promote damage in several tissues and may lead the cells death (20, 22, 31, 38). This fact can result from homeostatic changes such as, for example, hyperthermia (10, 15), increase of oxidative stress, and redistribution of blood flow (35-37) with alterations on the supply of nutrients and oxygen (14, 17). Nevertheless the exercise training seems to encourage positive adjustments at least on the cardiovascular system and in the skeletal muscle, increasing it tolerance to acute exercise and resistance to different pathologies (6, 21, 25, 32). Beyond the chronic adaptations within each cell due to exercise training-induced alterations in gene expression, it is speculated that this enhanced tolerance to acute exercise and other kind of harmful stimuli might also be explained by the progressive elimination of more susceptible and dysfunctional cells during the repetition of exercise, with the simultaneous proliferation of more capable cells, potentiating in this sense the organ's resistance to injury.

The acute and chronic repercussions of exercise and exercise training on skeletal muscle and cardiovascular system are relatively well known. However, other fundamental organs, such as the liver, have received little attention on this subject. Liver is considered a vital organ (13) and it complexity and multifunctionality in the metabolic tasks make as that no other tissue can replace it (18, 19). Histologically, it is constituted by a large phonotypical type of cells, each one with singular characteristics and functions (1, 2, 4, 8), yet due

to its presence in large numbers and its active functional activity, hepatocytes are consider the main important structures of the organ (30). Liver functions, localization and blood supply might expose it to adverse environments (18),furthermore its important role in the xenobiotic depuration have demonstrate that hepatocytes are very susceptible to injury induced by toxins (e.g. alcohol and tetracycline), drugs (e.g. acetaminophen and halothane) and pathologies (e.g. viral hepatitis) jeopardize the cell viability (18, 19, 28, 39). Fortunately, the liver seems to have compensatory mechanisms, such as its great regenerative capacity and cells aneuploidy, which permit it to counteract the injuries induced, maintaining it functional (5, 29).

Considering the evidences that exhaustive acute physical exercise may induce liver damage (12, 16, 24) and that exercise training, similarly to what is already described to skeletal muscle and cardiac muscle (9, 21, 25, 34) , may promote protective compensatory liver adaptations (11, 23, 27), it might be hypothesized that exercise training would endorse a progressive process of cell selection, to get an organ more fit to tolerate future demanding environments.

This dissertation arose due to the insufficient data in literature about this issue. Aiming to add some knowledge to the one currently existing, this dissertation includes: i) a literature review about the acute and chronic effects of physical exercise and exercise training on liver, exploring hypothetical mechanisms of exercise-induced cell damage and of training-induced cell tolerance, and ii) an experimental study aimed to determine, in male rats, the effects of moderate exercise training on the hepatocytes turnover, according to their location in centrilobular and periportal zones.

2. Review Article

Underlying mechanisms of exercise-induced liver damage and possible benefits of exercise training.

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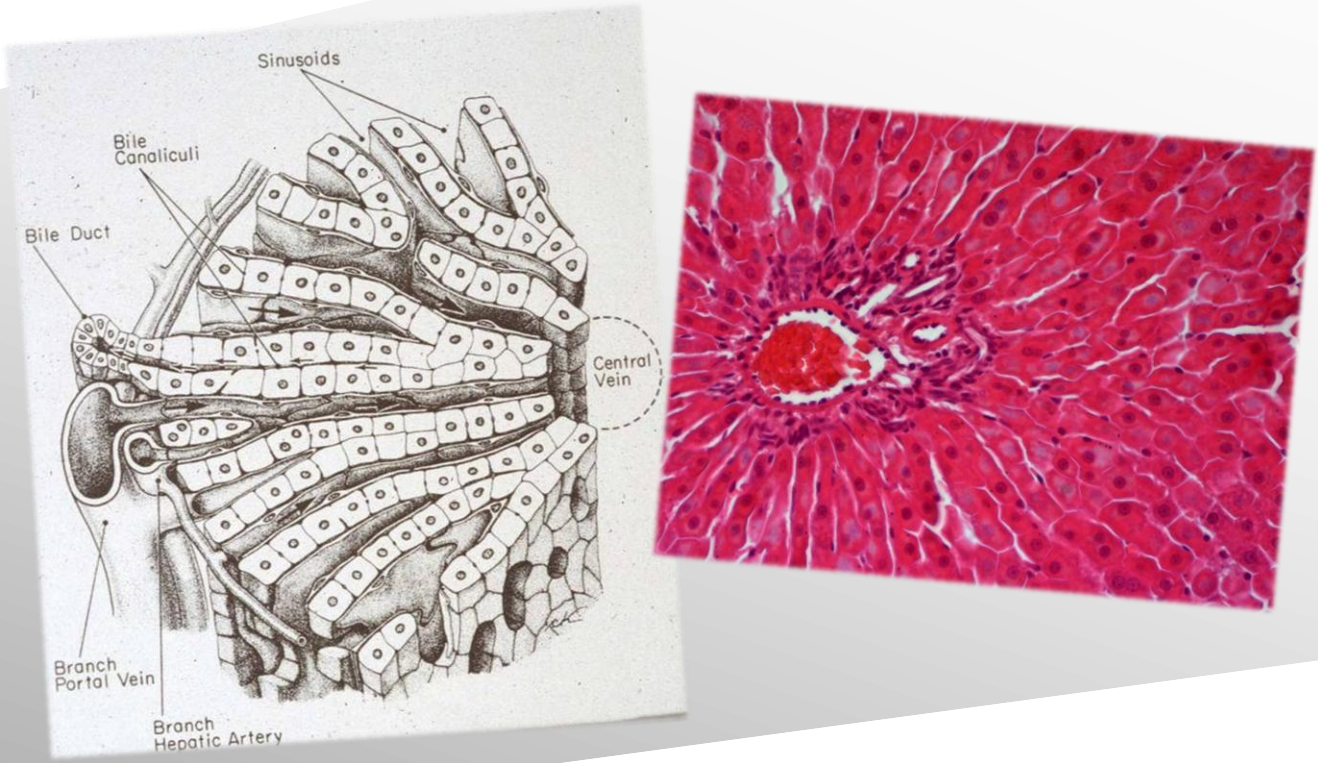

Underlying mechanisms of exercise-induced liver damage and possible benefits of exercise training

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Objective: To summarize the scattered existing literature about the exercise-induced liver damage mechanisms as well as the possible benefits of exercise training on liver structure. ***Methods:*** Major databases, such as EBSCO and PubMed, were searched for relevant terms associated with the subject of this review. In addition to articles published in peer-review journals, book chapters were also analysed for this review. ***Results:*** Several studies were found showing evidences that exhaustive exercise can induce liver damage. This damage is reflected by an elevation of serum transaminases following, for instance, ultra-marathon races. Several ethological factors are pointed as triggers for this damage. Changes in the blood flow, oxygen supply, accumulation of metabolic waste products and the increases in temperature are some of the factors that have been described in the literature as potential inducers of hepatic lesion during strenuous exercise training. Evidences regarding the beneficial effects of exercise on hepatic function however are almost inexistent. ***Conclusion:*** Due to the physiological alterations that occur during exercise in the liver metabolic environment it is possible to predict the injurious effects of exhaustive exercise on liver structure. However, further research is needed to investigate if this exercise-induced aggression is able to trigger compensatory beneficial adaptations at the resemblance of what happens in other organs.

Key Words: Exhaustive exercise, Hepatocytes, Physical training, Liver adaptation, Damage

1. INTRODUCTION

Similarly to the heart, lungs, kidneys and brain, the liver is consider a vital organ (2). Due to its strategic location (57, 125), it has a dual blood supply in venous and arterial blood via the portal vein and the hepatic artery, respectively (5, 57, 125), as well as a dynamic interaction between them to ensure a standard blood flow (62). The liver plays important roles in several organic functions such as in the energetic metabolism (67), plasmatic proteins synthesis (55), hormone secretion (68, 73), vitamins and minerals storage (5, 27) and it also has a detoxification function, metabolizing and removing several noxious substances (137).

The hepatocyte is the cell with the highest metabolic activity in liver (85). Despite the existence of other cells such as Kupffer cells, Stellate cells and stem-cells (6, 46, 122), hepatocytes represent 80% of the total cellular mass of the organ and are responsible for the majority of the liver functions (126). Hepatocytes have an estimated lifespan ranging between 5 months and a little more than 1 year (13). During this period, and in part as a consequence of its functional demands, the hepatocyte progressively loses efficiency and eventually dies triggering a normal clearance process being later replaced by new and more functional cells, which allows the organ to maintain a stable mass and to fulfil its function (18, 75, 83).

Several endogenous and exogenous substances such as toxins (e.g. alcohol, tetracycline) (99, 102), drugs (e.g. acetaminophen, halothane) (28, 99, 128), hormones (e.g. thyroid hormone, sex steroids) (140) as well as some pathologies (e.g. viral hepatitis, alcoholic hepatitis) (103) can jeopardize the hepatocytes viability, altering the normal cellular turnover and promoting an imbalance in the hepatocyte population that can compromise the liver functions (18, 84, 140).

The literature globally supports the beneficial effects of exercise in the organism and its role in the promotion of a healthy lifestyle (108). Nevertheless, the functional demands and metabolic environments induced by exercise can impose a real challenge to the organism homeostasis (19, 116). Depending on the intensity (high, moderate or low), the duration (long, moderate or short) and the frequency (acute or chronic), exercise induces a set of physiological adaptations, such as the alteration of the circulatory

and respiratory systems (19, 80, 154), changes in energetic pathways and on the utilization of substrates (15, 19, 66), changes in the body temperature (79) and hormonal adaptations (71, 112). However, when compared, for example, with skeletal or cardiac muscle for which the exercise-induced acute and chronic adaptations have been thoroughly investigated, there are only few and disperse evidences in the literature suggesting that exercise induces hepatic alterations. The aim of this review is therefore to revisit and assemble the several literature evidences about the hepatic repercussions of acute bouts of exercise and of exercise training, the underlying mechanisms that might be on the origin of exercise-induced liver damage, as well as the potential compensatory liver adaptations associated with exercise training.

2. Overview

Exercise is often associated with a healthy lifestyle (12, 143) and is a helpful therapeutic aid in the management of several pathologies (10, 23, 38) due to its ability to induce favourable metabolic and structural adaptations to several organic systems (54, 74). However, exercise is also a very demanding metabolic stimulus, representing a real challenge for the organs ability to maintain their function and homeostatic equilibrium (144). The heart is a good example of this dual effect of exercise. Exercise training is nowadays an effective strategy in the prevention of cardiovascular diseases (139), as it induces beneficial cardiac adaptations such as a decrease in resting heart rate and an increase in stroke volume (112). Furthermore, Moreira-Gonçalves et al. (96) have demonstrated that moderate exercise training enables cardioprotection against several deleterious stimuli. According with the authors of this study, exercise training induces remodelling of the heart, in which cells that are more fit to handle aggression are selected while cells that are less fit tend to die, decreasing thereby the vulnerability of the heart to stresses such as acute pressure overload.

However, several studies have also shown data suggesting that exercise can cause damage to the heart, which can be appreciated for instance by increases in cardiac troponin in healthy individuals following exercise bouts (37, 43, 97, 127).

Also in skeletal muscle, the increased contractile activity associated with exercise is paralleled by an increase in mitochondrial respiration, triggering an increase in the production of reactive oxygen species (30, 88, 89). In spite the fact that an increase in ROS might damage several cellular structures such as proteins, lipids and nucleic acids (53, 98) being for that reason responsible for a number of disorders (53), it is also noteworthy that the aggression induced by ROS can trigger some adaptations in skeletal muscle, conferring thereby muscle fibres a greater resistance to handle future aggressions (119, 136).

3. Exercise-induced liver damage

There are several evidences in the literature suggesting that physical exercise can induce liver damage. Fallon et al. (39) for instance, analyzed the effect of an ultra marathon race competition on several biochemical markers of hepatic damage, including hepatic enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and gamma glutamyl transpeptidase (γGT). It was observed that the serum concentration of these markers increased immediately after the race, and that remained elevated from four to eleven days after the race. A study performed by Kinoshita et al. (72), using animal models, was also able to demonstrate that rodents exercised at high intensities had an increase in hepatic cells damage, particularly in the centrilobular region, being that damage more relevant only some hours after the exercise bout. Other investigations performed with ultra marathon racers, cyclists as well as in athletes performing other kinds of strenuous exercise have also been able to demonstrate increases in plasma biomarkers of hepatic lesion induced by exercise (81, 110). Collectively, these evidences clearly suggest that, at least more strenuous exercise is able to induce some liver damage. However there is limited information about how much is too much in terms of the exercise intensity/duration necessary to induce liver damage as well as the extent of this exercise-induced damage.

The possible mechanisms underlying the exercise-induced hepatic damage will now be addressed.

3.1. Ischemia and Hypoxia

During acute exercise, the increased needs of oxygen and nutrients associated with an increase in metabolites production, leads to an increase in blood flow demands towards the actively contracting tissues (112) so that the cardiovascular system needs to perform a series of adaptations to ensure the increasing needs of muscle blood supply (32). One of these adaptations is a redistribution of blood flow according to the tissue necessities (32, 112).

The splanchnic circulation is constituted by several small circulatory routs (gastric, small intestinal, colonic, pancreatic, hepatic and splenic) and these routes are responsible for the transport of blood within those regions (107). The amount of blood involved in the splanchnic circulation represents an important fraction of the total organism blood and numerous extrinsic and intrinsic factors can affect its flux (107).

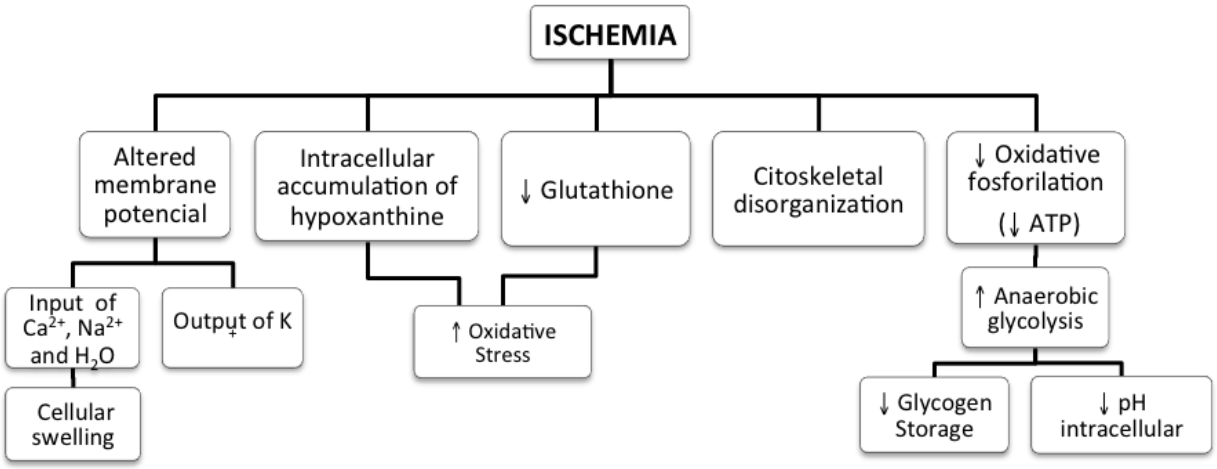

Fig. 1: Principal cellular repercussions induced by ischemia. Adapted from Collard and Gelman (25) and from Vinay Kumar et al. (151)

At rest, approximately 20% to 25% of the blood the expelled by the left ventricle is destined to the splanchnic region (124). However, during exercise, blood flow is mainly directed towards the recruited skeletal muscle and the skin (112) causing a hypoperfusion of the splanchnic region as shown by several studies (148, 149). Qamar and Read (114) for instance, have found a decrease of 43% in the superior mesenteric artery blood flow immediately after treadmill exercise (5km/h, 15min) and significant differences were observed when compared to baseline until 10 minutes after the exercise. A similar study performed by Perko et al. (109) with an exercise intensity of 70% of VO_{2max} also showed a decrease of 50% and 25% in the blood flow of the hepato-splenic and mesenteric arteries, respectively. A study with healthy volunteers also showed that high intensity exercise may induce gastro-mucosal ischemia and that this is directly related with the intensity of the exercise (42) . Moreover, Flamm et al. (42) described the blood flow volume changes of several organs of the abdominal region as a function of exercise intensity, namely between resting and 50%, 75% and 100% of VO_{2max} and their results showed that the increase in exercise intensity was paralleled by a reduction in the liver blood flow, with an approximately 18% decrease between maximal intensity exercise and resting conditions. Recently, a study performed by Van Wijck et al. (149) studied the liver repercussions of splanchnic hypoperfusion induced by exercise (cycling) using four plasmatic indicators of liver injury (L-FABP, ALT, AST and ϒ-GST). All biomarkers were shown to increase with exercise, supporting therefore the idea that hypoperfusion during exercise may cause liver damage.

Together, these findings clearly show that exercise is associated with major changes in the redistribution of the blood flow, and that the increase in blood supply mostly to the skin and skeletal muscle occur at the expense of a significant decrease in blood supply towards the splanchnic region, namely to the liver. This reduction of blood flood might precipitate an ischemic situation due to the lack of an adequate supply of oxygen and nutrients (93) as well as due to the accumulation of toxic metabolites resulting from cellular activity (56) ultimately compromising the liver cells enzymatic activity and biochemical pathways (93).

Depending on the ischemia duration, the damage caused to the cells can be reversible, when the reperfusion of the tissue is sufficient to resolve the situation or it can be irreversible when the cellular damages become permanent independently of the reperfusion. In this last case, the reperfusion of the tissue may also enhance the damage (21). Some of the cell alterations induced by ischemia are summarized in figure 1.

Although the decrease of ATP has a central role in ischemia-induced damage, affecting all ATP-dependent processes (e.g. sodium-potassium pump), ischemia also can damage directly the cellular and mitochondria membrane (93). It is important to highlight that mitochondria are the cell powerhouses and that their dysfunction inevitably promotes cellular damage and eventually cell death (151). One of the possible indirect membrane injurious agents is calcium (Ca^{2+}). As we can appreciate in figure 1, ischemia induces cytosolic calcium accumulation coming from intracellular reserves as well as from the extracellular medium. This increase in Ca^{2+} concentration leads to the activation of several enzymatic pathways (150). The activation of phospholipases for instance, leads to the degradation of the phospholipid bilayer (121, 151), while the activation of proteases disrupts the proteins in the membrane and in the cytoskeleton inducing thereby widespread damage (151, 156).

Ischemia (reduced blood flow to the tissues) however has a greater potential to produce cell damage than hypoxia (decrease of oxygen supply) (151) because of the ability of the cell to produce ATP in the absence of oxygen by anaerobic glycolysis (132). During an ischemic situation however the insufficient supply of substrates entirely disables the cell capacity to produce energy (151). However, these two situations are directly linked as ischemia inevitably induces hypoxia (91). In the liver, the inadequate deliver of oxygen induces an injury designated by hypoxic hepatitis (also called ischemic hepatitis or shock liver) (36, 45). Some of the signs of hypoxic injury are the formation of blebs (protuberances in the plasmatic membrane) endoplasmic reticulum and mitochondrial swelling as well as a global increase in the cell volume (60). The cell damage, depending on the time without oxygen, may be reversed when early reoxygenation occurs, however a prolongation of the hypoxia can lead the blebs rupture (77, 104), emptying the contents of the cell into the extra cellular space which results in cell necrosis (59). Hepatic damage can be readily identified by a rapid increase of the serum aminotransferases concentration as seen for instance in patients with ischemic hepatitis (48).

Several factors have been considered as the cause for the limited availability of oxygen to the liver, namely an increase in venous constriction, metabolic hypoxemia, incapacity of the liver to capture the blood oxygen and arterial ischemia (36, 45, 59, 147). Hypoxemia is the term used to define the low oxygen concentration in the blood and it may be responsible for tissue hypoxia (hypoxemic hypoxia) (76). Exercise-induced arterial hypoxemia (EIAH), is characterized by a reduction in the haemoglobin oxygen saturation capacity (SaO_2) and by a decrease in arterial oxygen partial pressure (PaO_2)(34). EIAH has been mainly associated in the literature with high or sub-maximal exercise intensities in athletes with high oxygen consumption (VO_{2max}) (33, 47, 58, 94, 111). Although hypoxia may be responsible for damage in several tissues (4, 138), there are no experimental evidences showing that hepatic hypoxia induced by exercise has a possible role in liver damage and therefore this mechanism remains largely speculative.

3.2 Reactive Oxygen Species (ROS), Oxidative Stress and Hyperthermia

Reactive oxygen species (ROS), such as superoxide anion (O_2-), hydroxyl radical (.OH) and hydrogen peroxide (H_2O_2), are physiological products (63, 98) mediating several important physiological tasks as for example signalling pathways, induction of apoptosis, or stimulation of antioxidant synthesis (106). On the other hand, ROS might also have cellular detrimental effects especially when produced in large amounts or during prolonged periods (20, 142, 146). Due to their instability, ROS easily react with macromolecules such as DNA, lipids and proteins causing their oxidation (53, 98). It is widely accepted that acute exercise increases the production of ROS leading to a situation of oxidative stress (30, 63, 69, 95, 118), which paradoxically also seems to be favoured by hypoxia (24, 61, 78, 82).

In normal conditions, the organism has mechanisms of protection against ROS injures. The cells anti-oxidant capacity is comprised by a series of enzymatic and non-enzymatic defences such as glutathione (GSH), vitamins C and E, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidises (GPx) (51, 63). Each antioxidant defense has its distinct main cellular storage site. For example, enzymatic antioxidant defenses (SOD, CAT, GPx) were mainly found in the cytosol and mitochondria of skeletal muscle (51) but were also shown to have an antioxidant protective role in the liver (64, 65) and heart (63). Other example is the GSH, one of most important endogenous antioxidants.

When the increase of ROS production is not compensated by an increment of the antioxidant mechanisms, it creates a prejudicial redox imbalance (oxidative stress) with harmful consequences to cellular components (53).

Initially it was thought that ROS resulted exclusively from the mitochondrial respiratory chain, however it is now well accepted that other cellular organelles such as the endoplasmic reticulum and peroxisomes (16) as well as some immune cells like Kupffer cells, neutrophils, eosinophils and macrophages also have the capacity to produce ROS (7, 78). Despite the several sources of ROS, the mitochondrial respiratory chain continues to be regarded in the literature as one of main sources of ROS production within the cell (3, 31, 145).

It is well established that exercise increases ROS production (63, 113). The increases in the cell metabolic activity and mitochondrial oxygen consumption during exercise can be responsible to this increase (30, 52). Evidences have demonstrated that mitochondrial oxidative stress increase during the endurance exercise, however this increase seem to balanced by an up-regulation of the oxidative mechanisms (135).

Other hypothesis are associated with the enzymatic activity of xanthine oxidase (XO) (63). According with Radák et al. (115), exercise promotes the conversion of xanthine dehydrogenase (XD), that exists in great amounts in the liver, to XO, which utilizes the oxygen, as an alternative to NAD+, as an electron receptor, promoting the synthesis of superoxide anion and other ROS such as H_2O_2. These substances after may cause cell damage, as previously mentioned.

Hyperthermia also seems to be associated with oxidative stress (31, 86, 129). The idea is that hyperthermia may precipitate hepatic damage associated with oxidative stress, namely by promoting the formation of superoxide by XO and by decreasing the hepatic level of glutathione (GSH) (130, 131) promoting thereby hepatic injury (155).

During muscular contraction the bioenergetics mechanisms convert the energy contained within the several energetic substrates into adenosine-5'-triphosphate (ATP) which can be later used in the metabolism, namely for muscle contraction (57). However, the process of converting chemical energy into mechanical work is not entirely efficient, since only 20%-25% of the energetic potential is used for muscle contraction, being the remaining energy released in the form of heat (19, 57, 112).

The activation of the skeletal muscle induced by exercise promotes an increase in ATP degradation to ensure the higher energetic demands of the skeletal muscle fibres but concomitantly also increases the heat release from the organism (50, 57). In normal conditions, the thermogenesis is compensated by an up-regulation of the heat loss mechanism, coordinated by the hypothalamus and changes in the local microcirculation (44, 50). This balance between the gains and losses of heat allow maintaining the temperature within the reference values (152). In normal conditions the body core temperature is relatively stable (variation ±0,6°C), however, during exercise the central temperature may reach values higher than 40 degrees (57).

The practice of acute exercise in extreme environment conditions (e.g. hot, humid, unvented, clothes), with the prior existence of some pathologies (e.g. obesity, diabetes), or even with the intake of certain drugs (e.g. alcohol, amphetamines, and neuroleptics) can promote a heat stroke, which is usually designated by exertional heat stroke (EHS) (123, 141, 152). McAnulty et al. (87) have studied the repercussions of hyperthermia and the oxidative stress. Using two different ambient temperatures they observed that hot environment promoted a rise of the oxidative stress and an increase in the body core temperature.

The EHS is characterized by an increase in the body core temperature above 40°C (1, 11, 105, 141) promoting perturbation in the central nervous system (CNS) expressed by dizziness, nausea, seizures, malaise and coma (1, 11, 49) and can induce hepatic failure (1, 14). Independently of the intensity and duration of the exercise, EHS always causes liver damage which is believed to be associated with protein and membrane phospholipid denaturation caused by heat (141, 152) promoting hepatocyte necrosis, mainly in centrilobular region (22, 153). Furthermore, alterations of the membrane permeability of the enterocytes facilitate the entry of bacteria from the digestive tract that when phagocytised by Kupffer cells, induce the secretion of cytokines (e.g. TNF-α, IL-1β, IL-6 e IL-10) promoting an inflammatory response (11, 152). The hepatic damage caused by EHS is generally considered moderate, asymptomatic and transitory. However, estimates suggest that 10% of EHS can induce acute liver failure (141, 152).

In summary, moderate increases in body temperature associated with exercise seem to potentiate the injurious effects of oxidative stress on hepatic structure and function while dramatic increases in body temperature are shown to trigger damage to enzymes and membranes due to denaturation, causing thereby hepatic injury.

4. Exercise training and liver adaptations

After having analysed the adverse effects of exercise on liver structure and the mechanisms of liver injury we will revise the findings suggesting possible positive effects of exercise on hepatic function.

Although there are few studies about hepatic function improvements induced by exercise, the adaptations caused by exercise training in other tissue as such the skeletal muscle, may suppose that similar beneficial adaptations may occur in the liver (152). Studies with skeletal muscle have demonstrated that acute bouts of exercise have structural repercussions in the tissue structure, causing cell damage and function impairment (29, 90). However, this cell damage induced by exercise seems to be an important stimulus towards beneficial structural, neural and physiological adaptations during the repair and regenerative process, endowing the cells of a better resistance capacity to future bouts (90, 113). McArdle et al. (89) have observed the adaptations in skeletal muscle induced by a previous muscular contraction and exposure to hydrogen peroxide (H_2O_2). Results showed that after the induced stress, the tissue developed protective adaptations such as changes in gene expression. The author reinforced the idea that ROS may be responsible to this genetic activation that induces a shielding against future adverse exposures. Indeed, the repetitive contact with ROS induced by exercise appears to promote systemic adaptations that induce a better resistance of the tissue against oxidative stress (52, 118). These adaptations can affect several mechanisms, namely by directly decreasing the ROS production or by raising the antioxidant defense mechanisms (116).

According to this perspective, Radák et al. (117) showed that regular exercise can induce a down-regulation of the ROS synthesis and potentiate the antioxidant defences, namely by increasing GSH, in

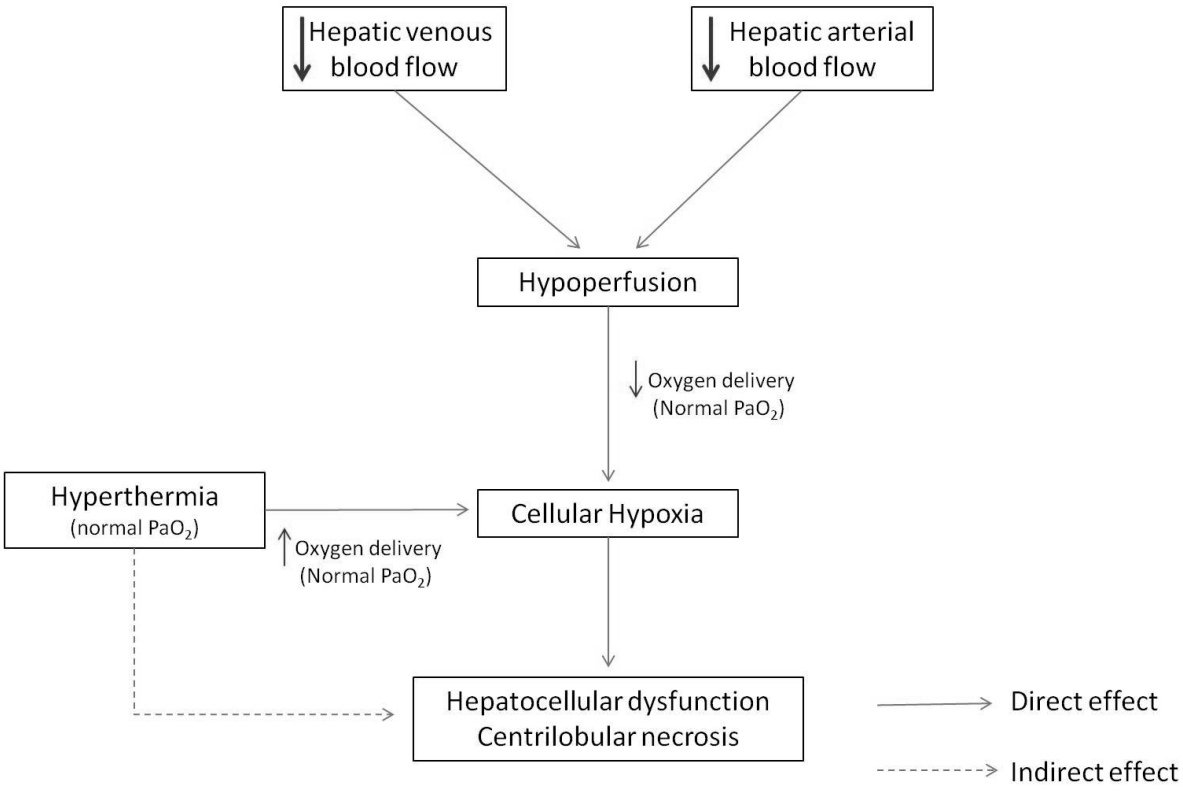

Fig. 2: Proposed interaction between hepatopathy factors. Adapted from Birrer et al. (9)

the liver of aged rats. Moreover, they also observed a decrease in hepatic NF-kB activity. Nakamoto et al. (100), have also observed liver beneficial adaptations induced by exercise. They demonstrated that after 8 weeks of treadmill training the oxidative stress in the liver of aged experimental animals, which usually increases due to age, was reduced to a level similar to that of young animals, causing a decrease in nucleus and mitochondria DNA injury. Based on these data, Radak et al. (116) concluded that exercise might have a beneficial role in the control of age related hepatic cells DNA mutations.

The liver functionality has also been investigated in response to exercise training. Bergman et al. (8) centred they investigations in the liver energetic metabolism changes in response to exercise, more precisely in gluconeogenesis. Concomitantly with the results obtained by other authors they concluded that exercise enhances this metabolic pathway. Burelle et al. (17) investigated the effects of endurance training on gluconeogenesis of isolated hepatocytes and observed that cells from trained rats had an increased gluconeogenesis as well as a significantly higher glucose accumulation. Sumida et al. (134) also observed increases in several hepatic indicators such as maximal rate for hepatic glucose production and lactate uptake as well as in oxygen consumption in livers from endurance trained rats. Several characteristics of the liver improve its ability to resist injury. Duncan et al. (35) showed that over 50% of the mature hepatocytes present in the liver of healthy young men have an abnormal number of chromosomes. Although this aneuploidy has been associated with hepatic pathologies, as for example, hepatocellular carcinoma (70, 101), they also seem to be responsible, when specific chromosomes are added or depleted, by increases in hepatocyte resistance to injury.

Other important liver characteristic is the fast and efficient (92, 133) regenerative capacity after injuries or cell mass losses (40, 41, 120). This process, which is based in rapid hepatocyte proliferation allows the liver to rapidly recover its functional cell mass following injury (26).

5. Conclusion

The objective of this work was to review the state of the art regarding the possible interactions between exercise training and liver. Based on the data of the several studies analyzed it was possible to observe that the liver structure and function are potentially jeopardized by a set of factors. Some of these factors are induced by exercise, namely alterations in blood

flood, oxygen supply, accumulation of metabolites and increases in temperature. These alterations are associated with losses of homeostasis and contribute to the creation of an injurious liver environment that promotes cellular damage and ultimately organ dysfunction. We also observed that although each factor can act as a damaging stimulus, exercise can trigger several injurious stimuli simultaneously and they can potentiate the injurious effects of each other (as demonstrated in figure 2).

We have also found evidences suggesting that the harsh environment originated by exercise can, when repeated over time, become an important mechanism inducing resistance, possibly by potentiating the selection of the more efficient cells and ultimately lowering the liver susceptibility to future aggression. Nevertheless, these conclusions are mainly speculative and the few literature that exists about this subject does not provide evidence about how much exercise is sufficient to promote favorable liver adaptations and how much will induce damage. Clearly more investigation is needed to better understand the possible effects of exercise on liver function.

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3. Experimental Study

Moderate exercise training attenuates the rate of hepatocytes turnover in rats.

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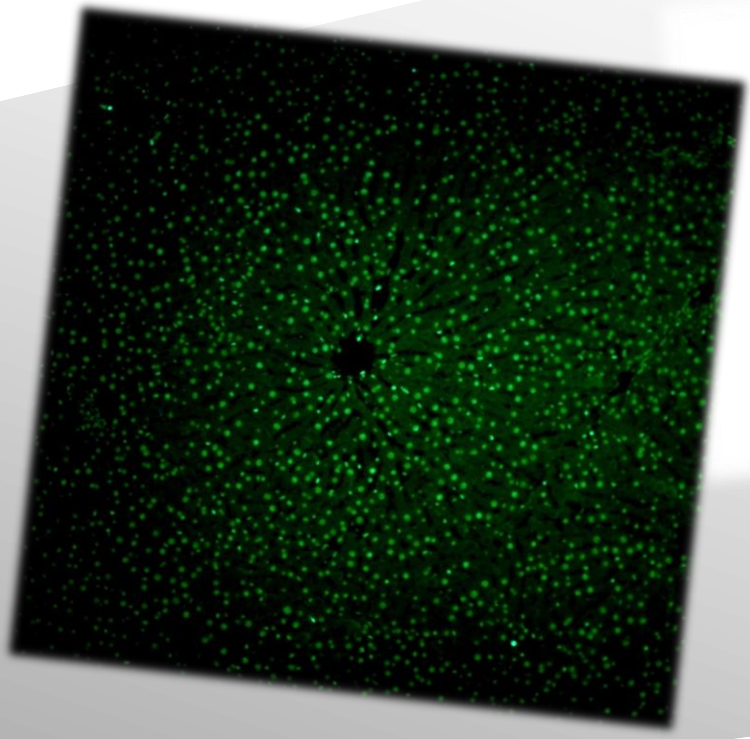

Moderate exercise training attenuates the rate of hepatocytes turnover in rats

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Objective: To evaluate the effect of moderate exercise training on the rate of hepatocyte turnover in male Wistar rats. ***Methods:*** Eleven male Wistar rats were randomly assigned to either a control (CONT, n=6) or exercise training group (ET, n=5). Animals from the ET group were submitted to a daily treadmill exercise training protocol (20m/min; 60min/day; 5days/week) for a period of 7 weeks. Seven days prior to their sacrifice, 5-bromo-2'-deoxyuridine (BrdU) was administered to all animals through their drinking water (1mg/ml). At the end of the 7th week all animals were sacrificed and samples from the different liver lobes were collected for histological (H&E staining) and immunohistochemical analysis (TUNEL and BrdU). ***Results:*** Our results show that moderate exercise training failed to modify the percentage of hepatocyte apoptosis ($p>0.05$) observed in CONT animals. However differences in the percentage of TUNEL stained cells ($p=0.049$) were observed between lobule regions in ET animals (Median of centrilobular and periportal zones were 33.80 and 28.13, respectively). Simultaneously, the moderate exercise training promoted a significant decrease in hepatocyte proliferation (CONT=3.53 vs. ET=1.86; $p=0.000$), mainly in the periportal lobule region (CONT=4.00 vs. ET=1.94; $p=0.003$). ***Conclusion:*** After 7 weeks of moderate exercise training, the daily repetition of physical exercise does not seem to induce liver damage but apparently it reduces cell proliferation affecting thereby hepatocyte turnover in rat liver.

Key Words: Moderate exercise training; Hepatocyte; Apoptosis; Cell proliferation; Lobule zones

INTRODUCTION

The liver is constituted by different cells, each one with singular characteristics and functions. This whole array of cells is responsible for the huge diversity of liver roles in the organism (2, 10, 41). From all liver cells, the hepatocyte represents about 80% of the organ mass and it is its principal functional structure (44). Similarly with what happens with others cells in the body, the human hepatocytes have a functional lifespan, which is estimated to range between 150 to 400 days (1, 19). To ensure the efficiency of liver, the old and non functional hepatocytes are naturally eliminated by programmed cell death and replaced by new and more functional cells (54). It is this capacity to regulate the balance between cell death and proliferation that ensures the maintenance of the hepatic mass and ultimately the liver vital functions (19). In normal conditions, hepatocytes turnover is considered to be relatively low. In normal steady-state condition, only 0.025% of hepatocyte population is estimated to be engaged with the DNA synthesis (1, 28). However, the hepatocyte turnover rate can be affected by diverse endogenous or exogenous factors. Some toxins (e.g. alcohol, tetracycline present in some mushrooms), drugs (e.g. acetaminophen, halothane), hormones (e.g. thyroid hormone, sex steroids) and pathologies (e.g. viral hepatitis) can alter the stimulus for hepatocyte death or proliferation, causing a temporarily or permanently imbalance in hepatocyte mass (30, 49).

The practice of physical exercise has been considered as having a beneficial role to the human body, but it also represents a real challenge to the ability to maintain the organ/tissue homeostasis (50). Indeed, during acute exercise, the enhancement of functional, metabolic and mechanical demands tends to create hostile tissue conditions prone to increase the rate of cell damage/death, not only affecting the recruited skeletal muscles but also other organs and tissues deeply stimulated by the neuroendocrine alterations induced by exercise (22, 26, 52).

Indeed, several studies have demonstrated that exhaustive exercise may induce liver damage. Sun et al. (47) using an animal protocol, established that long-term endurance exercise promoted an increase in oxidative stress and mitochondrial damage in liver cells. Others authors have also shown in plasma an

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increase of indirect markers of hepatic injury following acute demanding exercise. For instance, plasmatic biomarkers such as aspartate transaminase (AST), bilirubin and gamma glutamyl transpeptidase (γGT) are shown to increase after exhaustive exercise (5, 24). Together, these evidences suggest a possible negative effect of acute exercise in the liver. Due to the homeostatic imbalances originated by exercise and because of the important function of the liver in supplying energetic substrates as well as depurating several metabolites, the acute exercise constitutes a real test to the efficiency and efficacy of liver cells (15, 25).

On the other hand, beyond the negative repercussions of a single bout of exercise, there is also evidences of an increased gene transcription in liver cells following exercise training (14, 15), suggesting that beneficial compensatory mechanism may be activated in hepatocytes following repeated exercise bouts. Interestingly, there is also evidence that the practice of regular physical exercise is able to reduce hepatocyte apoptosis markers in obese patients with nonalcoholic fatty liver disease, which suggests that exercise training may induce favorable liver adaptations that are benefic for the management of the disease (8, 13).

Considering the evidences of liver damage following exhaustive physical exercise and that exercise training may promote protective compensatory liver adaptations, it might be hypothesized that the repeated cellular aggression of exercise training would modify the rate of hepatocyte turnover due to a progressive elimination of the more susceptible and dysfunctional cells with the survival of the higher efficient ones, leading to the formation of an organ more fit to overcome future demanding environments. Regarding the absence of data in literature about this issue, the objective of this study was to determinate, in male rats, the effects of moderate exercise training on histological markers of hepatocytes apoptosis and proliferation, according to their location in centrilobular and periportal zones.

MATERIALS AND METHODS

Study design

Following one week of quarantine, 11 male Wistar rats aged 8 weeks (purchased from Charles River laboratories, Barcelona) were randomly assigned to either exercise training (ET; n=5) or sedentary control (CONT; n=6) groups. All animals were individually housed in standard cages (floor area of 800 cm2; Tecniplast, Italy) in a controlled environment (i.e., constant humidity of 50%, and temperature of 21±1º C) with a 12h inverted light/dark cycle. Standard rat chow and water were provided *ad libitum* throughout the experimental period to all animals.

Seven days prior to their sacrifice, 5-bromo-2'-deoxyuridine (BrdU; Sigma, USA) was administered to all animals through their drinking water (1mg/ml). Fresh solution of BrdU was added daily in bottles covered with aluminum foil to prevent the loss of BrdU reactivity due to light exposure.

Animals of the ET group were submitted to a daily treadmill exercise training protocol for a period of 7 weeks. At the end of the 7th weeks animals were sacrificed. Animal handling and experiments were conducted according to the guidelines of the European Communities Council Directive (86/609/EEC).

Exercise Training protocol

The first two weeks of the exercise training protocol (5 days/wk) aimed to adapt the animals to the treadmill exercise. This adaptation period included a gradual 10 min/day increase in running time, beginning with 10 min in the first day and ending with 50 min duration at the end of the 1st week, maintaining a constant speed of 15m/min and 0% grade. During the second week, the running speed and the exercise duration were progressively increased, to 20m/min (0% grade) and to 60min, respectively. Following this adaptation period, exercise training during the additional 5 weeks consisted of daily sessions (5 days/wk) of 60 min duration; 20m/min and 0% grade (Fig. 1). A similar exercise training program to the one used in this study was previously shown to attenuate mice hepatic inflammation, fibrosis and macrophage infiltration during diet induced-obesity (17). All animals from the exercise training group completed the training protocol.

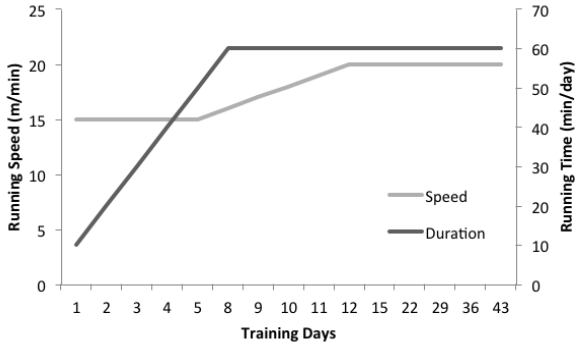

Fig. 1: Temporal variations in the training protocol, considering the changes of duration and running speed of exercise bouts. Running speed was initiated at 15 m/min and was increased until 20 m/min (end of 2nd week). Running duration started at 10 min/day and increased until reaching 60 min/day (beginning of the 2nd week).

Animal sacrifice and organ collection

After the completion of exercise training protocol, ET animals were weighted and anesthetized by an

intraperitoneal injection of ketamin (90mg/kg, Imalgene 1000, France) and xylazine (10mg/kg, Rompun, Brazil). After assessing the anesthetic depth, the animal's anterior abdominal wall was opened with a scalpel, with the exposure of the abdominal cavity from the xiphoid appendix to the pubic symphysis. The animals were then sacrificed by exsanguination by drawing approximately 6 ml of blood from the ascending *vena cava*. Afterwards, liver was exposed and carefully dissected. Immediately after clearing the surrounding adipose and connective tissue, a sample of liver tissue (approximately 3x3x3mm) was collected from the four liver lobes (right, left, median and caudate) and immediately immersed in cold fixative solution containing 4% paraformaldehyde, 2.5% sucrose (Sigma, Saint Louis) and 0.1% gluteraldehyde (TAAB) in phosphate buffered saline buffer (PBS; pH 7.2). Sedentary control animals were sacrificed according to the same procedure.

Histology

Tissue processing for light microscopy: Following 24h of fixation at 4°C, liver samples were rinsed in PBS (pH 7.2) for 30 min, dehydrated through graded ethanol solutions (70%, 80%, 95% and 2x100%) for 60 min each at 4°C, cleared in graded xylene and paraffin solutions (3:1, 1:1 and 1:3) for 60 min each at 56°C in an oven, and mounted in paraffin blocks (Merk). Five µm thick sections were cut from each liver sample block with a Leica 2125 rotary microtome (Leica Microsystems Inc) and individually collected to silane-prep slides. From each lobe, one slide was stained with hematoxylin and eosin for gross morphological analysis of the liver structure; another slide was used for immunohistochemical detection of cells displaying apoptotic nuclei (TUNEL) and a third slide was used for immunohistochemical detection of cellular proliferation (BrdU).

Liver gross structure: Morphological analysis to the liver tissue was made in eosin & hematoxylin stained sections according to standard procedures. Briefly, following deparaffinization with xylene, sections were rehydrated by successive immersions in graded ethanol solutions (100%, 95%, 80% and 75%) and water and then were stained with hematoxylin and, after rinsing with water, with eosin. Afterwards, slides were dehydrated in ethanol, cleared with xylene and finally mounted with DPX (dibutyl phthalate xylene; Shandon EZ-Mount, Thermo Electron Corporation, USA) and glass covers lip. After staining, all slides were observed with light microscope (Axio Imager A1, Carl Zeiss) and a qualitative analysis of global tissue structure organization, including the sinusoidal dimensions, the hepatocyte morphology and arrangement, as well as the presence of necrosis or cellular infiltration was made.

Detection of cell death by TUNEL: The presence of apoptotic nuclei in liver tissue sections was assayed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using a commercially available kit (In situ cell death detection kit AP, Roche) according to the manufacturer instructions. Briefly, after deparaffinization, sections were immersed in 0.1M citrate buffer (pH 6.0) and microwave irradiated for 1 min (750W). After rinsing in could PBS buffer, sections were first blocked with 3% BSA in 0.1M Tris-HCl (pH 7.5) for 30min at 20°C and then incubated in freshly prepared TUNEL reaction solution (nucleotide mixture + terminal deoxynucleotidyl transferase) in a humidified chamber at 37°C for 60 min in the dark. Negative and positive controls were simultaneously prepared by incubation with label solution only (nucleotide mixture) or by incubation with DNase (Sigma) prior to the labeling procedure, respectively. Sections were analyzed with a fluorescent microscope coupled to a digital camera (Axio Imager A1, Carl Zeiss) and apoptotic cells were identified as brightly fluorescent in opposition to the pale green background staining. Since the fluorescence staining is highly labile and disappears following a few instances of exposure to light, to ensure permanent detection, slides were incubated with converter-AP (anti-fluorescein antibody, fap fragment from sheep, conjugated with alkaline phosphatase (AP)) in a humidified chamber for 30 min at 37°C. After rinsing 3x with PBS, slides were incubated with Fast Red TR/Naphthol AS-MX tablets (Fast Red, Sigma) until an obvious red staining was detectable. Sections were then immediately rinsed in PBS, counterstained with diluted aqueous hematoxylin solution (1:3) for 1 min and mounted with aqueous mounting medium (Cristal Mount, Sigma) and glass cover slip.

Counting of apoptotic cells was only performed on images taken from the tissue sections following incubation with fast-red and assuring permanent detection. Each tissue section was thoroughly analyzed with a 40x objective and 7 to 10 (according to the section dimension) non-overlapping fields were randomly photographed. Quantification of the TUNEL positive ($TUNEL^+$) and TUNEL negative ($TUNEL^-$) hepatocytes was performed with ImageJ software (NIH, Bethesda, MD) and a percentage of labeled hepatocytes was calculated regarding the total number of hepatocytes observed in each field. To determine the intra-observer hepatocyte classification, a randomly selected sub-sample comprising about 30% of the original sample was re-analyzed. Statistical significant differences were absent between the initial and re-counting ($p=0.238$) and correlation coefficient was $r=0.891$ ($p=0.000$).

Detection of cellular proliferation by BrdU: For the assessment of cellular proliferation, liver tissue

sections were stained according to the antibody manufacturer instructions (Abcam) for detection of BrdU, which was previous administered to all animals along the 7 days that preceded sacrifice. Briefly, after deparaffinization and rehydration, liver sections were rinsed in PBS for 10 min and microwave irradiated for 15 min at 100W while immersed in sodium citrate buffer (10 mM, 0.05% Tween 20, pH 6.0). Slides were then cooled in citrate buffer, washed in PBS for 5 min and immersed in 1N HCl for 10 min while resting on ice. After rinsing with PBS, slides were immersed in 2N HCl for 10 min at room temperature and for another 20 min at 37°C in a humidified chamber. After rinsing with PBS, sections were neutralized with 0.1M borate buffer (pH 8.4) for 2x 5 min. Sections were again rinsed with PBS and incubated for 1h at room temperature in a solution composed by 160 ml of PBS, 20 ml of methanol and 20 µl of H_2O_2 at 30% and 0.05% tween for endogenous peroxidase blocking. After rinsing with PBS, non-specific binding was blocked by incubation with 3% BSA in 0.1% PBS-T for 1h at room temperature. Sections were then incubated with sheep polyclonal anti-BrdU primary antibody (ab1893, Abcam, UK) diluted 1:100 in 0.05% PBS-T overnight in a humidified chamber at 4°C. After rinsing 3x 5min in PBS, sections were incubated with horseradish peroxidase donkey conjugated polyclonal secondary antibody to Goat IgG (ab7125, Abcam, UK) diluted 1:100 in 0.05% PBS-T for 2h at 37°C in a humidified chamber. Detection was performed by incubation with Fast Dab Tablets for approximately 2 min (Sigma). Negative controls were performed for each section by omission of the primary antibody. Counterstaining was performed with hematoxylin diluted in water at 50%. A similar procedure to that used to determine the percentage of apoptotic hepatocytes in each photographic field was used to assess the number BrdU positively stained hepatocytes ($BrdU^+$) (recounting p=0.205, correlation coefficient r=0.933, p=0.000).

Statistic analysis

Results were expressed as median (M_{ed}) and interquartile range (25^{th}-75^{th} percentiles). Comparisons regarding percentage of hepatocytes labeled with BrdU ($BrdU^+$) and TUNEL ($TUNEL^+$) between animals from the control group and animals from the exercise group were performed with Mann-Whitney U nonparametric test. The Kruskal-Wallis test was used to analyze the intra and inter group differences of labeled hepatocytes located in centrilobular (CL) and periportal (PP) zones. Differences were considered statistically significant when $p<0.05$. All statistical procedures were performed with SPSS software (SPSS v. 18, Chicago).

RESULTS

Liver gross structure

The liver gross structure was analysed in sections of the hepatic lobules stained by H&E. Overall, the histological features observed in several random fields failed to evidence sings of cell damage both in CONT as well as in ET animals, with all animals evidencing a normal liver structure. The liver of both groups showed a normal lobule organization without signs of tissue deformation, dilation of the sinusoidal spaces or appearance of fatty droplets (Fig. 2a). There were also no signs of neutrophil invasion or macrophage activation. All hepatocytes observed showed normal regular borders and no evidences of swelling, eosinophilia or vacuolization (Fig. 2b). There were also no signs of distinct nuclear changes, with integrity of the nuclear membrane, normal nuclear size and basophilia (Fig. 2b). No signs of increased fibrous tissue were also detected (40).

TUNEL labeled hepatocytes

To investigate the chronic effects of exercise training on hepatocyte death, the percentage of TUNEL labeled hepatocytes between CONT and ET animals was compared (Fig. 3). The results demonstrate that livers collected from trained animals do not have significant differences (p=0.387) in the ratio of $TUNEL^+$ hepatocytes when compared to animals of the control group (Fig. 3b).

Likewise, when the differences between these two groups were analyzed according to the lobule zone, no significant changes were observed in the CL or in the PP (p= 0.699 and p=0.210, respectively) (Fig. 4).

Interestingly, in ET group, the analysis of hepatocytes among zones showed significant differences between lobule zones in it was the CL region rather than the PP region to evidence a higher number of apoptotic hepatocytes (p=0.049) (Fig. 4b). Animals from the control group however showed no significant differences (p=0.796) in the number of $TUNEL^+$ hepatocytes among lobule regions (Fig. 4b).

Hepatocyte proliferation

The results of BrdU labeled hepatocytes obtained in the animals from the CONT and ET groups are depicted in Figure 5. Data revealed that animals from ET group had approximately 48% (p=0.000) less number of hepatocytes positively stained with BrdU when compared to control animals, suggesting therefore a significantly lower hepatocyte proliferation (Fig. 5).

In the zone analysis of animals from ET and Cont groups there were no significant differences in the percentage of BrdU+ stained nuclei between CL and PP zone in both groups p= 0.289 and p=0.443, respectively) (Fig. 6).

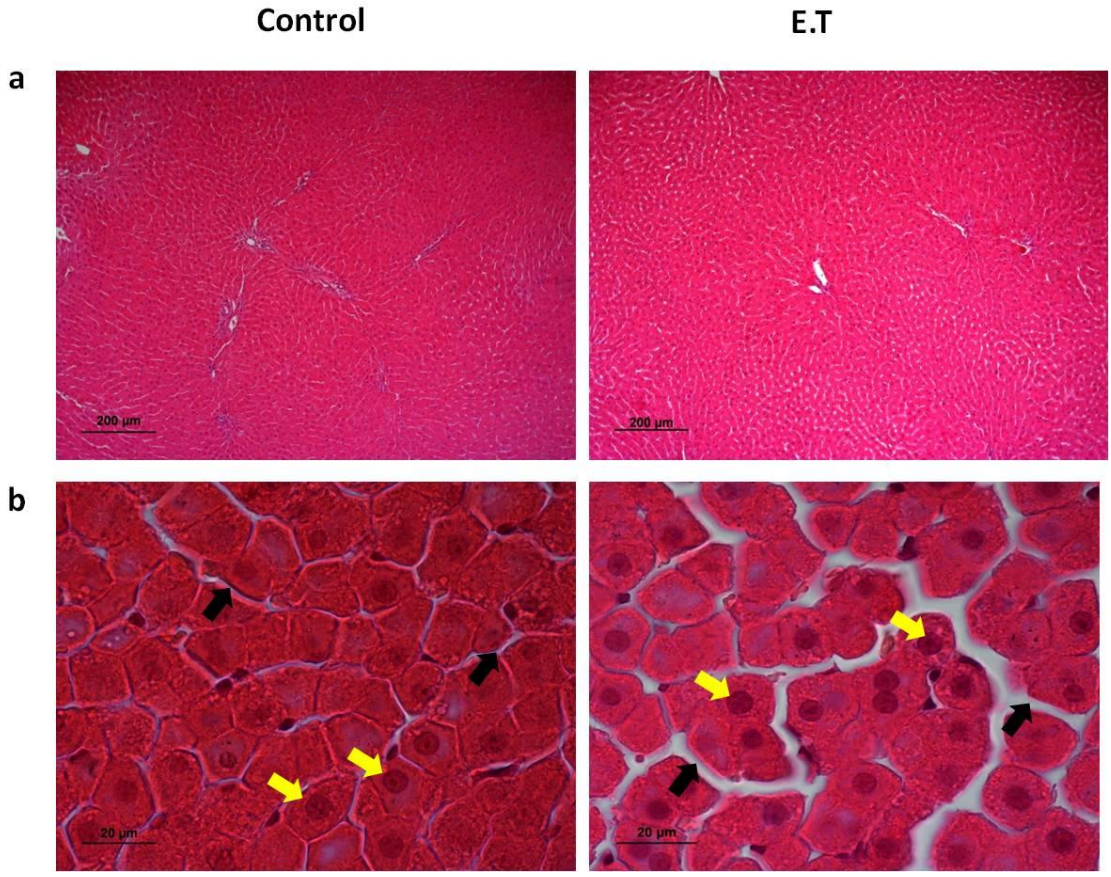

Fig. 2: Photomicrographs of liver sections from control (CONT) and exercise trained (ET) animals stained with hematoxylin & eosin (H&E). a) A normal gross liver lobule structure was identified in both groups (magnification 10×) b) Higher magnification (×100) micrographs evidence the normal organization of the lobule structures with hepatocytes (yellow arrows) arranged as single-cell thick plates separated from the vascular sinusoids (black arrows). All cells show normal nuclear morphology and plasma membrane integrity.

When we examined the results per lobule zone, it was observed a significant difference between the percentage of BrdU labeled hepatocytes in the PP region of the control group when compared with the same lobule region in animals from the ET group ($p=0.003$), with control animals having a significantly higher number of labeled cells. In the CL region, despite the differences did not reached statistical significance ($p=0.051$), a trend for a higher number of BrdU labeled hepatocytes was also found in control animals (Fig. 6b).

DISCUSSION

The obtained results demonstrate that exercise training does not lead to an increase in hepatocyte apoptosis, as there were no differences between exercise trained animals and sedentary controls. Nevertheless, exercise training still seems to induce some alterations in hepatocyte viability as we found differences in the number of apoptotic hepatocytes between hepatic lobule zones in trained animals but not in sedentary controls. Our results also suggest that exercise training decreases hepatocyte proliferation, mostly in the periportal zone.

One of the main results of our study is that there were no differences in the number of hepatocytes undergoing cell death between control and exercised animals groups, which suggests that the repetitive metabolic stress to which the liver is submitted during moderate exercise training is not sufficiently injurious to induce cell damage. Notoriously, studies investigating the effects of more intense or more prolonged exercise training bouts show that those more demanding exercise stimuli are susceptible of inducing liver damage (37, 53). For instance, Kinoshita et al. (18), demonstrate that rats subjected to 2h of treadmill running at either 60% or 80% VO_{2max} have an increase in the number of damaged liver cells, assayed by trypan blue staining, especially in the centrilobular region. Furthermore, studies performed on athletes subjected to strenuous exercise

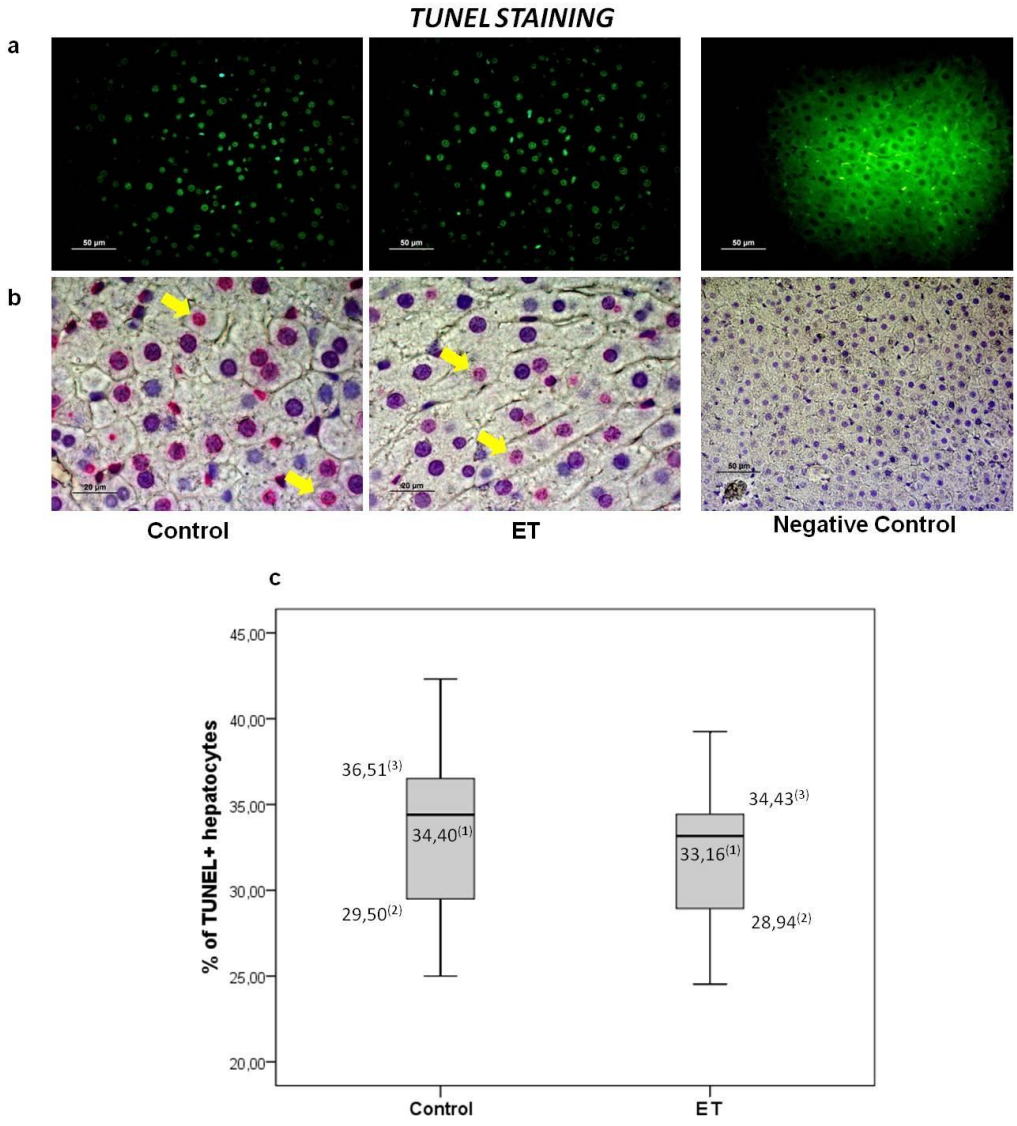

Fig. 3: Analysis of hepatocyte apoptosis performed by TUNEL staining in animals subjected to exercise training (ET) and controls (CONT). a) Images from fluorescence microscopy evidencing brightly fluorescent cells corresponding to cells in programmed cell death process in the livers of CONT and ET animals (×40). b) Following fluorescence analysis, sections were revealed with fast red and analyzed by light microscopy in order to determine the percentage of labeled hepatocytes. The arrows point to TUNEL stained cells in which a distinct red nucleus can be identified (×100) c) Box plot of the percentage of nuclei labeled with TUNEL in CONT and ET animals. Values are the median (1), 25th (2) and 75th (3) percentile in both groups. No significant differences were identified between groups regarding the median percentage of apoptotic nuclei in the micrographs analyzed ($p>0.05$).

competitions, such as ultramarathon races (53) or long distance kayaking (27) show increases in the serum concentration of biomarkers of hepatic lesion such as alanine transaminase (ALT), AST and $_{\gamma}$GT reinforcing therefore the notion that highly strenuous exercise conditions, but not moderate intensity or duration exercise, is able to induce substantial liver damage. In fact, evidences of exercise-induced liver damage described in the literature have been, to our knowledge, always associated with higher demanding exercise, while there is no evidence of moderate exercise training producing the same effects.

During exercise there is a redistribution of the blood flow between the actively contracting skeletal muscle and skin and the organs within the abdominal cavity (38), which under the condition of exercise receive less blood flow (9). There is evidence that even sub maximal exercise intensities (70% VO_{2max}) are able to induce a 50% reduction in the hepato-splenic blood flow (36). The hypoxia generated by the decline in

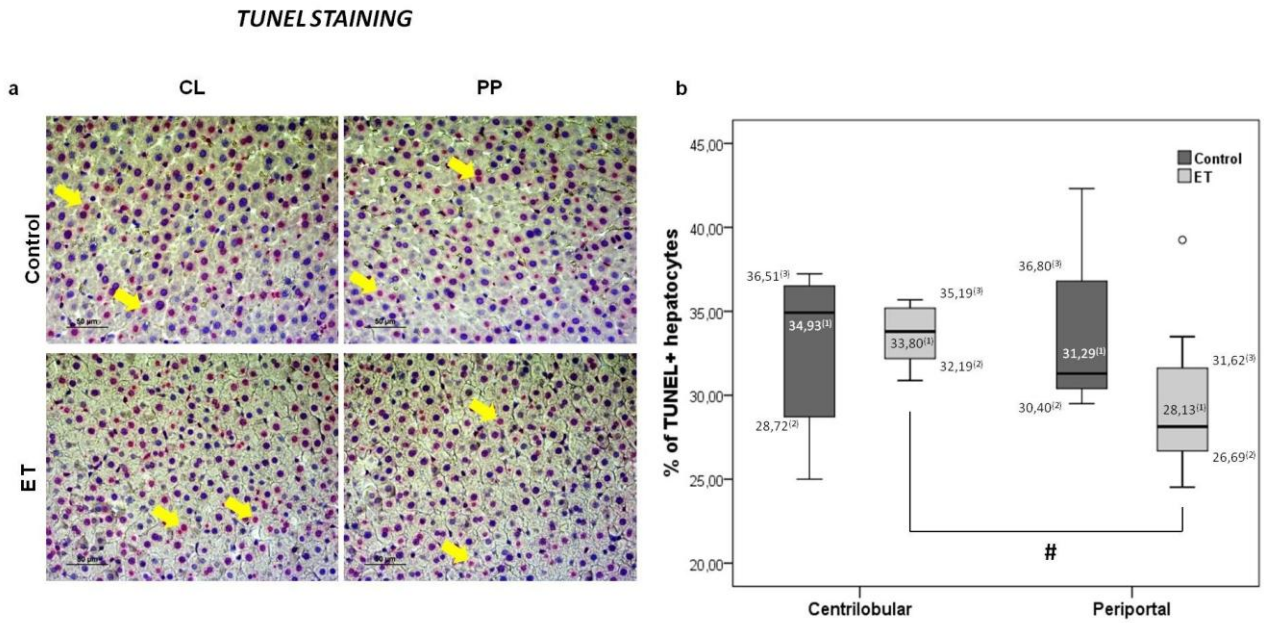

Fig. 4: Analysis of programmed cell death expression in control and exercise trained animals, according to the hepatic lobule region. a) Representative micrographs of TUNEL stained sections evidencing the centrilobular (CL) and periportal (PP) regions in control (CONT) and exercise trained (ET) animals (×40). b) Box plot with median (1), 25th (2) and 75th (3) percentile of TUNEL+ hepatocytes according to region in both groups. Significant differences in the number of apoptotic nuclei were only identified between the CL and PP regions in ET animals but not in CONT animals (# $p<0.05$). No significant differences were observed between equivalent hepatic lobule regions in control and ET groups ($p>0.05$) (° moderate outlier).

hepatic flow may eventually lead to gastrointestinal ischemia (34) resulting in damage of the hepatic cells, with primary incidence on hepatocytes and in sinusoidal endothelial cells (16). Moderate intensity exercise training however, seems to induce substantially lower changes in the splanchnic circulation (9). Therefore, in these exercise conditions hepatic cells might not be exposed to such hypoxic conditions, limiting increases in cell death.

Findings in the literature also suggest that the damage in liver cells induced by the regional redistribution of the blood flow might be a consequence of ischemia/reperfusion injury (45). Importantly, previous studies have also shown that only about 2% of ischemia/reperfusion (I/R) induced hepatic cell death occurs by apoptosis (30) and the majority of cell mass losses are a result of necrosis (12). Therefore, as in our study we aimed specifically for the detection of apoptotic cell death, it could be the case that increases in cell death by necrosis might have been undetected by our experimental approach. Therefore, we believe that further studies in this subject should also aim to detect other forms of cells death, as apoptosis might by insufficient to characterize exercise induced liver cell aggression.

Moreover, during the apoptotic process, a series of events occur that culminate in the formation of apoptotic bodies (Councilman bodies) which are later phagocytosed by immune cells (30). Studies using TUNEL staining (11, 48) and others markers of apoptotic cell death (48) were able to identify cellular changes corresponding to apoptotic cell death as soon as a few minutes following the initial liver injury (11, 48). These markers have also been shown to remain elevated for a few hours, declining thereafter (12). The quickness with which the apoptotic process and the apoptotic bodies removal process occur increases the difficulty in detecting with exactitude the exact peak of cell death (43).

Despite there were no signs of increased cell death in animals submitted to exercise training, our results show that the pattern of hepatocyte death was not identical throughout different lobule regions in exercised animals. Effectively, there is substantial evidence in literature showing metabolic differences between hepatocytes of the PP and CL regions (23, 29). These are related to differences in the expression of specific enzymes and organelles as well as are mainly a consequence of the proximity of the cell to the either the portal triad or to the central hepatic vein. As these cells are exposed to different metabolic environments, their metabolic profile and ultra structure are optimized to handle with different kinds of demands (23). This specialization of hepatocytes per zone is responsible, for example, for a different propensity to perform different functions in the energetic metabolism as well as a predisposition to utilize different energetic substrates (23). The cells

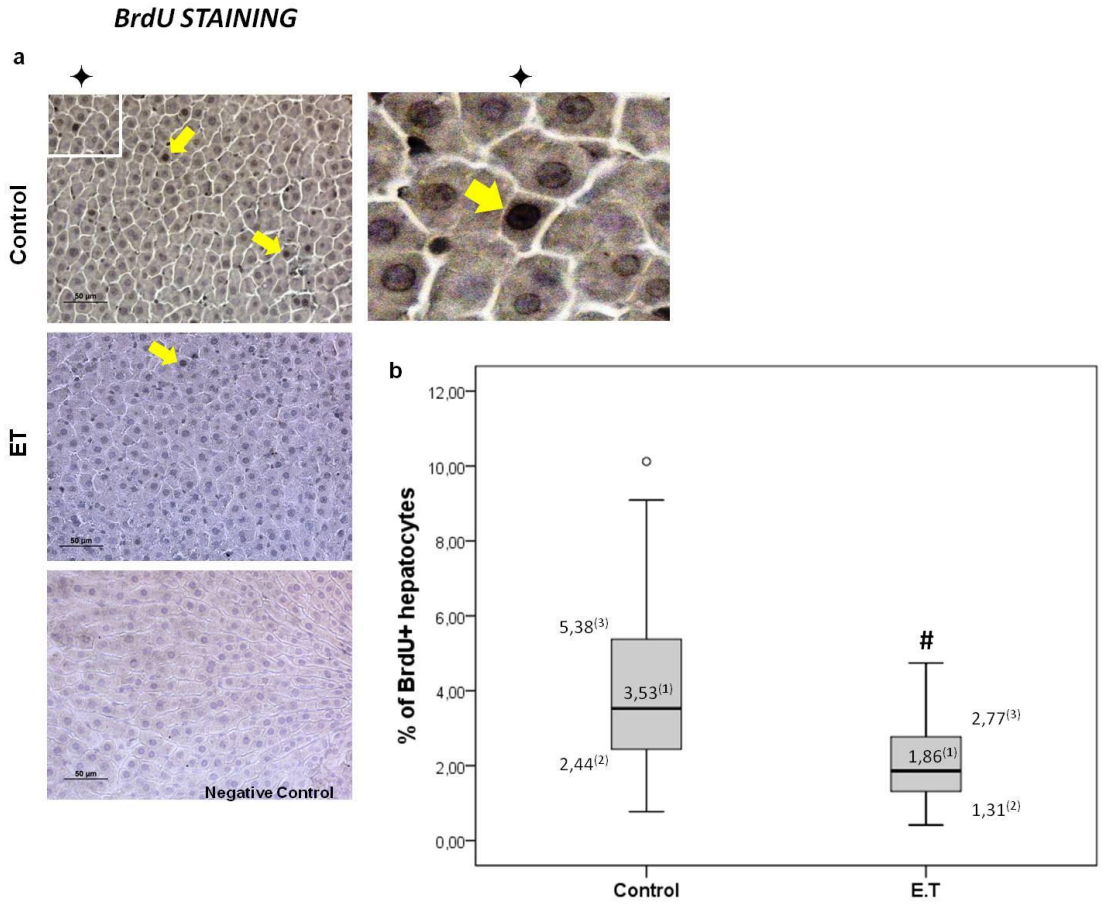

Fig. 5: Hepatocyte proliferation in control and exercise trained animals (ET). a) Micrographs of liver sections stained by immunohistochemistry for detection of BrdU positive cells. The presence of dark nuclei (arrows) reveals hepatocyte proliferation (×40). A higher magnification micrograph is provided highlighting the clear distinction between the darker nuclei detected by BrdU (arrow) and the more pale nuclei that were not (✦). b) Box plot representing the percentage of hepatocyte nuclei labeled with BrdU ($BrdU^+$) as well as the median $^{(1)}$, 25^{th} $^{(2)}$ and 75^{th} $^{(3)}$ percentile in both groups. A significantly lower number of labeled hepatocytes was observed in the ET group (# $p<0.05$) (° moderate outlier).

localization also seems to affect their susceptibility to the damage induced by a variety of factors (23, 29). This different susceptibility has been shown to be related for instance with differences in the expression of enzymes of the cytochrome P450 complex (CYP) throughout the different lobule region (3). These are a large and diverse group of enzymes that perform several functions in the organism (33), namely the bioactivation of several of drugs, toxins and carcinogens (35, 46). For instance, many hepatotoxins only acquire the capacity to cause damage after their activation by the CYP complex and due to the increased expression of the CYP complex in the CL region, this is the zone generally more injured by xenobiotics (23, 35). Moreover, and as it was previously referred, there are also significant differences between hepatic lobule zones in terms of the expression of enzymes related to different metabolic pathways (23, 29). In has also been suggested in the literature that another factor to induce mostly damage to the CL region is hypoxia (20). The CL zone is the area which is most distant from the portal triad, hence receiving less oxygenated blood supply (42). In cases of metabolic hypoxia or in which there are increased demands for oxygen such as intense exercise training, the supply of oxygen to the CL hepatocytes is further reduced. This situation may lead to local damage due to anoxia, while hepatocytes in the periportal zone are spared of this aggression due to their supply of more oxygenated blood (20, 21).

Considering therefore that liver cells are functionally distinct between regions and that they are exposed to different metabolic environments our results suggest that increased demands imposed by exercise training might have produced triggered different levels of stresses in different zones, which might explain our finding of different expression of apoptotic hepatocyte between lobule zones. Nevertheless, this hypothesis is.

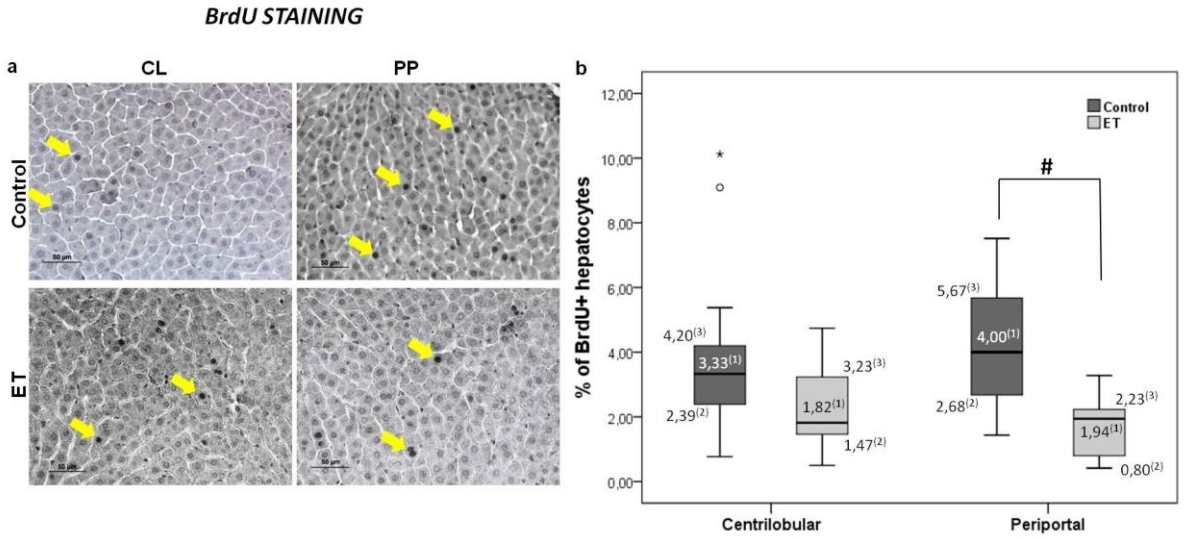

Fig. 6: BrdU staining of centrilobular (CL) and periportal (PP) liver lobule regions in control and exercise training groups (ET). a) Representative photomicrographs evidencing nuclei labeled by BrdU staining ($BrdU^+$) in both groups (control and exercise trained (ET)) according to the hepatic lobule region (CL or PP). Images from all sections evidence hepatocytes that actively incorporated BrdU into the DNA structure, suggesting therefore the occurrence of cell proliferation (arrows) (×40). b) Box plot representing median (1), 25th (2) and 75th (3) percentile of the percentage of hepatocytes labeled by BrdU in the CL and PP region in both groups. No differences in hepatocytes proliferation were observed between zones in the same group ($p>0.05$). However, a significantly higher number of $BrdU^+$ cells was detected in the PP zone of control group animals when compared to the same zone in ET animals (# $p<0.05$). (°moderate outlier,*severe outlier).

still highly speculative and further studies should be performed in order to investigate the effects of exercise training on different liver metabolic pathways.

Regarding our findings on hepatocyte proliferation, we found a decrease in proliferation in the livers of animals subjected to exercise training, which we believe might be an indicator of liver adaptation promoted by exercise training. For instance, some studies with skeletal and cardiac muscle demonstrate that one of beneficial effects of exercise training is to increase the tissue functional capacity as a result of favourable adaptations at the cellular and molecular levels (7, 32). In the specific case of skeletal muscle, for example, diverse results have positively connected the exercise-induced reactive oxygen species (ROS) with beneficial mitochondrial and gene expression (31, 39). Previous reports in the literature also suggest that a similar adaptive process might also occur in the liver (parenchyma hypertrophy), however this hypothesis remains to be confirmed (51). Nevertheless, there are some findings suggesting that exercise may optimize the hepatocyte functionality. Burelle et al. (4) investigated on isolated hepatocytes the possible effect of endurance training on the gluconeogenic process and observed an increase of gluconeogenesis as well as significantly higher glucose accumulation in trained vs. control animals. Importantly, there are also findings in the literature showing that the liver from trained animals has a significantly higher detoxification ability when exposed to chemical insults such as halothane, suggesting therefore that exercise training was able to improve hepatocyte function and its resistance against aggression (6).

These findings therefore put forward that exercise training may induce metabolic adaptations in the hepatocyte function which might suggest that the liver from trained animals has a lower need for cellular turnover in order to keep pace with its metabolic demands, since individual hepatocytes seem to have an enhanced metabolic function. Therefore, our results showing that exercise trained animals have lower proliferation rate might be a sign that the liver from these animals does not need to increase its cell population to handle with its metabolic demands. Nevertheless, these claims are largely speculative as there are very few findings in the literature suggesting exercise induced liver adaptation. More investigation will be needed to clarify the possible effects of exercise in the liver cells morphology.

In summary, our findings suggest that moderate exercise training does not increase global expression of apoptosis in liver. Rather, the moderate exercise training seems to amplify the cellular resistance, promoting a decrease of the hepatic cellular turnover suggested by the decreased percentage of BrdU stained hepatocytes. Thus, the livers of animals trained

appear to be more suitable than sedentary to tolerate the challenge of enhanced metabolic demands.

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4. General Conclusion

According with data collected in the literature we conclude that, likewise with the remaining organs and tissues, the liver is also confronted to adverse conditions during the acute exhaustive exercise. The consequent increase of metabolic demands, associated with disturbances in homeostatic environment promoted by the increased core temperature, accumulation of metabolites and reduction of hepatic blood flow (1, 2, 4), may explain the hepatocyte damage described after exhaustive exercises. However the literature also suggests that liver can chronically develop protective adaptations as a result of the exercise training, becoming, for example, more resistance to oxidative stress (3)

Many questions are still unclear, for example, no evidences were found in literature about how much intensity or duration of exercise or exercise training can be harmful or promote benefits to liver, respectively.

Regarding the experimental study we aimed to observe the influence of moderated exercise training on liver cellular turnover. The underlying hypothesis was that exercise training would promote a reduction in hepatic tissue turnover based on a progressive, elimination of dysfunctional hepatocytes with their substitution by more resistant and tolerant cells. After seven weeks of exercise training, livers did not present any alteration in the percentage of cells death; however, the rate of hepatocyte proliferation was significantly decreased in trained animals, especially in the periportal zone. These results support our hypothesis that exercise training attenuates the hepatocyte turnover.

Nevertheless, the possibility that this reduced liver cellular turnover may be associated with an increased cellular resistance to further acute exercises or other demanding conditions, needs to be confirmed in future studies.

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