Mariana Cervaens Costa Maia

HYPERBARIC OXYGEN THERAPY IN SPORTS MEDICINE

Universidade Fernando Pessoa
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Atesto a originalidade do trabalho

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"Tese apresentada à Universidade Fernando Pessoa como parte dos requisitos para obtenção do grau de Doutor em Biotecnologia e Saúde, sob a orientação do Professor Doutor Pedro Barata e co-orientação do Professor Doutor Franklim Marques"

RESUMO

As lesões desportivas são um grande problema no que diz respeito à sua rápida reabilitação. Inúmeros estudos tentam encontrar a técnica mais rápida capaz de acelerar o processo da recuperação. A oxigenoterapia hiperbárica (OTH) é a aplicação de 100% de oxigénio numa câmara hiperbárica a pressões mais elevadas do que o nível do mar. A inalação de OTH comporta-se como um fármaco multifacetado dotado de efeitos anti-isquémicos, anti-hipóxicos, anti-edematosos, pós-lesão e anti-infecciosos. Portanto, o objetivo desta tese foi analisar a influência da OTH na recuperação de lesões desportivas, como contusão muscular e do ligamento cruzado anterior (LCA) pós ruptura. Em primeiro lugar, verificou-se se a aplicação de OTH melhorou as propriedades biomecânicas, tais como rigidez, alongamento máximo e peso máximo, dos gastrocnémios de ratos após induzir contusão muscular. Em segundo lugar, era de nosso interesse analisar, após de se ter induzido uma contusão muscular nos gastrocnémios dos ratos, a influência da OTH na bioenergética mitocondrial avaliada através do consumo de oxigénio e potencial transmembranar e susceptibilidade à indução do poro de transição de permeabilidade mitocondrial, em mitocôndrias isoladas. Finalmente, no último trabalho experimental, tentou-se verificar se a aplicação de OTH tem a capacidade de melhorar a neovascularização, por meio da análise do factor de crescimento do endotélio vascular (VEGF), bem como analisar a proliferação e a produção de proteína, em coelhos com ruptura do LCA. A OTH parece desempenhar um papel importante na recuperação de lesões musculares, mais especificamente, na contusão muscular em ratos, melhorando as propriedades biomecânicas musculares, tais como a rigidez e peso máximo e na bioenergética mitocondrial, onde o tempo até que o inchaço na mitocôndria iniciasse em grande escala foi menor no grupo submetido a OTH, assim como a amplitude de inchaço foi maior, o que atrasou a apoptose mitocondrial. Contudo, em relação à ruptura do LCA, a OTH promoveu a neovascularização, activando VEGF, mas no entanto, contribuindo para o aumento da espessura da cápsula.

Palavras-chave: Oxigenoterapia hiperbárica, gastrocnémios, contusão muscular, propriedades biomecânicas, bioenergética mitocondrial, ligamento cruzado anterior, neovascularização, colagénio tipo I.

ABSTRACT

Sports injuries is a major problem what concerns to its rapid rehabilitation. There is innumerous attempting to find the faster technique to apply to the injured ones to accelerate its recovery. Hyperbaric oxygen therapy (HBO) is the application of 100% oxygen in a hyperbaric chamber at pressures higher than sea level. The inhalation of HBO has already shown that behaves like a multifaceted drug endowed with anti-ischemic, anti-hypoxic, anti-edematous, pro-healing and anti-infective effects. Therefore, the objective of this thesis was to analyze the influence of HBO in the recovery from sports injuries such as muscle contusion and anterior cruciate ligament (ACL) rupture. Firstly, it was verified if HBO improved the biomechanical properties, such as hardness, maximum elongation and maximum weight, of rats' *gastrocnemius* after inducing muscle contusion. Secondly, it was of our interest to analyze skeletal muscle mitochondrial energetic of rats' *gastrocnemius* after induced muscle contusion, by determining end points related to oxygen consumption, transmembrane electric potential and permeability transition pore susceptibility in isolated mitochondria. At last, our last experimental work aimed to verify if HBO has the ability to improve neovascularization, through the analysis of vascular endothelial growth factor (VEGF) as well the proliferation and protein production, of rabbit ruptured ACL. HBO seems to play an important role in the recovery of muscle injuries, more specifically, muscle contusion in rats, by improving muscle biomechanical properties, such as hardness and maximum weight and in mitochondria energetic, where the time until large scale swelling initiates in mitochondria was lower in HBO and the swelling amplitude was higher, which delayed mitochondria apoptosis. However, concerning to ACL rupture, HBO increased neovascularization by activating VEGF, contributing for the increasing of capsule thickness.

Key-words: Hyperbaric oxygen therapy, *gastrocnemius*, muscle contusion, muscle biomechanical properties, mitochondrial energetic, anterior cruciate ligament, neovascularization, Type I collagen.

RESUMÉ

Les lésions sportives sont un grand problème en ce qui concerne la rapidité de sa réhabilitation. De nombreux études essayent de trouver la plus rapide et moins douloureuse technique capable d'accélérer le processus de récupération. L'oxygénothérapie hyperbare (OHB) consiste à l'inhalation de 100% d'oxygène dans un caisson étanche avec une pression plus élevée que celui de la mer. Il a été démontré que l'inhalation de l'OHB se comporte comme une drogue à multiple facette, qui permet d'agir sur l'ischémie tissulaire qu'elle qu'en soit la cause : vasculaire, traumatique, toxique, ou infectieuse. Pourtant, l'objectif de cette étude a été d'analyser l'influence de l'OHB dans la récupération des lésions sportifs, l'ecchymose du ligament croisé antérieur post rupture. Dans un premier temps, on a vérifié si l'utilisation de l'OHB améliore les propriétés biomécaniques, comme la rigidité, l'étirement maximum et le poids maximum, des Gastrocnémiens chez les rats après induire une ecchymose. Deuxièmement, cela été dans notre intérêts d'analyser, après induire une ecchymose des Gastrocnémiens chez les rats, l'influence du OBH dans la bioénergétique mitochondriale, évalué à travers la consommation d'oxygène, le potentiel transmembranaire et la susceptibilité d'induction du pore de transition de perméabilité mitochondrial, des mitochondries isolés. Pour finir, lors du dernier travail expérimental, nous avons essayé de vérifier si l'application de l'OHB a la capacité d'amélioré la néo vascularisation, en analysant le facteur de croissance de l'endothélium vasculaire (en anglais Vascular endothelial growth factor, VEGF), en analysant aussi la prolifération et la production de protéine, sur des lapins avec rupture du LCA. L'OHB semble avoir un rôle important dans la récupération des lésions musculaires, plus précisément sur l'ecchymose chez les rats, améliorant ainsi les propriétés biomécaniques musculaires, comme la rigidité, le poids maximum et la bioénergétique mitochondriale. Le temps pour que l'œdème dans la mitochondrie arrive à grande échelle a été plus faible dans le groupe soumis à l'OHB, comme l'amplitude de l'œdème fut plus grand, ce qui a retardé

l’apoptose mitochondrial. Toutefois, en ce qui concerne la rupture du LCA, l’OHB a promu la néo vascularisation, activant le VEGF, contribuant à une capsule épaisse.

Mots clés : Oxygénothérapie hyperbare, gastrocnémiens, Ecchymose, propriétés biomécaniques, bioénergétique mitochondriale, ligament croisé antérieur, néo vascularisation, collagène du type I.

DEDICATION

"Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
mas não esqueço de que minha vida é a maior empresa do mundo.
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver
apesar de todos os desafios, incompreensões e períodos de crise...
Ser feliz é deixar de ser vítima dos problemas e
se tornar um autor da própria história.
É atravessar desertos fora de si, mas ser capaz de encontrar
um oásis no recôndito da sua alma ..
É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um 'não'.
É ter segurança para receber uma crítica, mesmo que injusta.
Pedras no caminho?
Guardo todas, um dia vou construir um castelo..."

Fernando Pessoa

To my father, Digner, mothers, Sãozinha and Malai, brothers, Pedro and Nuno, husband, Bruno and forever babies Beatriz and Afonso, thank you for helping me to build this castle.

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INDEX

FIGURES

Theoretical Background

Experimental work
Paper I

Paper II

Paper III

TABLES

ABREVIATIONS AND SYMBOLS

ACL Anterior cruciate ligament

ATA Atmospheric absolute

C Mass of a gas

Ca^{2+} Calcium

CAT Catalyze

CCCP Carbonyl cyanide m-chlorophenylhydrazone

CNS Central Nervous System

CO_2 Carbon dioxide

CPK Creatine Phosphokinase

DCS Decompression sickness

DOMS Delayed onset muscle soreness

EBM Evidence-Based Medicine

ECHM European Committee for Hyperbaric Medicine

ETC Electron transport chain

FELASA Federation for Laboratory Animal Science Associations

FiO_2 Inspired oxygen fraction

H Hardness

Hb Hemoglobin

HB Hyperbaric

HBO Hyperbaric Oxygen Therapy

HIF Hypoxia inducible factor

LDH Lactate dehydrogenase

ME Maximum Elongation

MPTP Mitochondrial permeability transition pores

MW Maximum Weight

N_2 Nitrogen

NBO Normobaric oxygen

NO Nitric oxide

NOS Nitrous oxide systems

O_2 Oxygen

P Pressure

$PaCO_2$ Arterial pressure of carbon dioxide

paO_2 Alveolar oxygen tension

PaO_2 Arterial oxygen partial pressure

PMNs Polymorphonuclear neutrophils

pO_2 Oxygen partial pressure

pO_2 Partial pressure of oxygen

PtO_2 Tissue oxygen tension

RCR Respiratory control ratio

ROS Reactive oxygen species

RQ Respiratory quocient

SaO_2 Arterial oxygen saturation

SOD Superoxide dismutase

SPCs Stem Progenitor Cells

SPSS Statistical Package for the Social Sciences

T Temperature

TPP^+ Tetraphenylphosphonium

UHMS Undersea and Hyperbaric Medical Society

V Volume

VEGF Vascular endothelial growth factor

$\Delta\psi$ Transmembrane potential

CHAPTER I

GENERAL INTRODUCTION

I. General Introduction

Hyperbaric Oxygen Therapy (HBO) is the therapeutic administration of 100% oxygen at pressures higher than one absolute atmosphere (ATA). It is administered by placing the patient in a multiplace or in a monoplace (one man) chamber and typically the vessels are pressurized to 1.5 to 3.0 ATA for periods between 60 and 120 minutes once or twice daily (Bennett et al., 2005a). In the monoplace chamber the patient breathes the oxygen directly from the chamber but in the multiplace chamber this is done through a mask.

Oxygen is a colorless, odorless and tasteless gaseous chemical element that is essential for body function to sustain life (Singh et al., 2001).

In the body, oxygen moves towards a partial pressure gradient of inspired air to body cells and their mitochondria. Atmospheric air usually contains 20.9% of oxygen (at normal barometric pressure), which is equivalent to a partial pressure of 159 mmHg (Mathieu, 2006).

The ultimate consumption of the oxygen (90%) is realized within the mitochondrion which is the intracellular organelle responsible for oxidation of end-products of energy substrates. Thus, it is necessary to have correct supplemental oxygen for energy metabolism (Mathieu, 2006).

Oxygen is a universal electron that enables the body to use the energy stored in carbohydrates, lipids and proteins (Ji, 1999). This catabolic process can generate oxygen free radicals and other reactive oxygen species (ROS). At normal conditions, the majority of ROS is produced in the mitochondrial electron transport chain (ETC), since 90% of the oxygen consumption by the body is reduced to water in the mitochondria (Ames et al., 1995).

Oxygen therapy, at normal pressure (normobaric), must be given continuously and should not be abruptly interrupted until full recovery of the patient, because it may

cause a decrease in the alveolar oxygen tension. The partial pressure of oxygen (pO_2) can be measured in arterial blood and this pressure of 60 mmHg corresponds to about 90% saturation of arterial blood, but if acidosis is present, it takes more than 80 mmHg. A small increase in arterial oxygen tension results in a significant increase in hemoglobin saturation. In normal situations, no additional benefit is guaranteed to raise the level of pO_2 over 60-80 mmHg. Higher concentrations of inspired oxygen fraction (FiO_2) increases oxygen dissolution in plasma, resulting in small increase in transport capacity and oxygen flow to tissue (Mathieu, 2006). An increase in oxygen concentration of 1% raises oxygen tension over 7 mmHg (Singh et al., 2001). By breathing 100% oxygen in a hyperbaric environment at 2.0 ATA, blood oxygen content is increased 2.5% and sufficient oxygen becomes dissolved in plasma to meet tissue needs in the absence of hemoglobin-bound oxygen, increasing 10-fold (1000%) oxygen tissue tensions (Staples and Clement, 1996).

The administration of pure oxygen at atmospheric pressures higher than sea level originated from the treatment of decompression illness over a hundred years ago. During the last fifty years it has been proposed to address a wide variety of medical and surgical problems for many decades (Grim et al., 1990, Tibbles and Edelsberg, 1996, De Laet et al., 2008). However, a sense of controversy continues to pervade the field of hyperbaric medicine that is slowly fading way. Only a restricted number of indications have been accepted by the two main scientific hyperbaric societies, the Undersea and Hyperbaric Medical Society and the European Committee for Hyperbaric Medicine, because there is a lack of evidence demonstrated in several clinical trials. However, there are ongoing international studies, aiming to clarify the efficacy of HBO for other specific indications, a trend that is clearly increasing every year.

Hyperbaric therapy units are managed by different medical specialties, depending on its indication, accepted by the two societies mentioned above. Many of the units are military, mostly in the navy, for historical reasons, due to the need for a treatment facility for diving incidents. Apart from this, most units are linked to departments of anesthesiology and intensive care. HBO therapy requires high acknowledgement in physics, gas laws, pharmacology and physiology effects (Mortensen, 2008).

HBO has a number of physiological and pharmacological pathways of action. These therapeutic mechanisms of action are based on the increase of both the partial pressure of oxygen and hydrostatic pressure (Thom, 2009). These properties constitute the basic rationale for treatment of a number of different conditions.

However, hyperbaric oxygen is remarkably free of side effects. Complications such as oxygen toxicity, middle ear barotrauma and confinement anxiety are well controlled with appropriate pre-exposure orientations (Mekjavic et al., 2000).

In the last decade, competitive sports have taken on a whole new meaning, where its intensity increased as well as the incidence of injuries in the athletes. These sports injuries, ranging from broken bones to disrupted muscles, tendons, and ligaments, may be a result of acute impact forces in contact sports or the everyday rigors of training and conditioning (Babul et al., 2003).

Skeletal muscle injuries are a major problem that today's athletes face, both recreational and professional. Their incidence varying from 10 to 55% of all injuries sustained in sport events. The majority of muscle injuries (more than 90%) are caused either by contusion or by excessive strain of the muscle (Järvinen et al., 2000). Among the causal factors of muscle injuries several metabolic changes occur: glucose is decreased, the muscle, which is usually alkaline, accumulated acidic products, the bloodstream is reduced and oxygen is not sufficient to neutralize toxic substances that were accumulated (Woods et al., 2007).

One of the most common skeletal muscle disorders can occur as a result of a direct blow or force to the muscle and is known as a muscle contusion (Barata et al., 2011). The spectrum of severity of this muscle injury ranges from focal intracellular damage, such as plasma membrane rupture or myofibril disruption, to segmental damage or necrosis involving the whole length of individual muscle fibers, and finally to injuries affecting the whole muscle bundles. This type usually presents as an acute painful injury experienced during physical activity (Garrett, 1990). However, it depends on the severity of the impact.

Concerning to ligaments, one of the most frequently injured structures during high impact or sporting activities is rupture of the anterior cruciate ligament (ACL) (Ryder et al., 1997). The ACL is a key structure in the knee joint, responsible to resist anterior tibial translation and rotational loads (Beynnon et al., 1997). The ACL does not heal when torn and surgical reconstruction is the standard treatment in the field of sports medicine (Bach et al., 1998). Such reconstruction not only aims to restore the kinematics and stability of the injured knee but also to prevent future degenerative changes (Jacobson, 1977). From its femoral attachment, the ACL runs anteriorly, medially, and distally towards the tibia. The cross-sectional shape of the ACL is irregular and this shape changes with the angle of the knee flexion, but is generally larger in the anterior–posterior direction (Duthon et al., 2006).

ACL differs from others ligaments and tendons due to its complex ultra-structural organization, varied orientation of the bundles, and the abundant elastic system. The ACL is a unique and complex structure able to withstand multiaxial stresses and varying tensile strains (Strocchi et al., 1992). This specificity and complexity may explain the difficulty in reproducing the original ACL following surgical reconstruction.

The importance of the ACL to the normal function of the knee has been emphasized by numerous investigators. Disruption of the ACL often leads to significant disability and its recovery is longstanding. Although both operative and non-operative treatments have been proposed, the optimal management of this injury remains controversial (Scavenius et al., 1999).

However, a critical review of the literature reveals that the success rates reported for ACL surgical reconstruction are between 69% and 95% and over time some patients develop pain, unstable knees due to recurrent laxity or even require revision ACL reconstruction (Fleming et al., 2013, Ristanis et al., 2006).

Therefore, the necessity emerges to discover the best and faster treatment to allow the injured athlete to return to competition faster than the normal course of rehabilitation, fully recovered and with a low risk of re-injury.

In order to understand why these pathologies may benefit from HBO, some explanations are suggested. Breathing pure oxygen under hyperbaric (pressurized) conditions effectively "drives" more O_2 into the tissues to promote faster repair. Delaney and Montgomery (2001) state that this is achieved through a rise in O_2 plasma content (from 0.3 to 6.6 mL/100mL of blood) without a greater saturation of hemoglobin. Gill and Bell (2004) add that this oxygen can reach obstructed areas where blood cells are unable to pass.

It is understandable that this altered physiology may have a positive effect on the previously mentioned pathologies that produce tissue ischemia. However, in recent times it appears that assumptions have been made that this physiology will directly transfer to not only healing muscle and ligament injuries, but also improving endurance performance. However, these assumptions do not appear to have any significant support in the literature at present.

Therefore, the general objective of the present thesis was to analyse the effect of hyperbaric oxygen therapy in sports medicine.

To achieve this general objective, specific purposes were design which correspond to the chapters of the experimental work of this thesis:

i. Evaluate the effect of HBO in the recovery of muscle contusion inflicted to rats by measuring its biomechanical properties and haematological markers of muscle injury.

ii. Analyze the influence of hyperbaric environment with oxygen and with air on skeletal muscle mitochondrial energetic of rats after induced muscle contusion, by determining end points related to oxygen consumption, transmembrane electric potential and permeability transition pore susceptibility in isolated mitochondria.

iii. To study the application of HBO on the recovery of Anterior Cruciate Ligament (ACL) injured, namely by improving neovascularization and changing the pattern of protein production.

CHAPTER II

THEORETICAL BACKGROUND

II. Theoretical Background

2.1. Hyperbaric Oxygen Therapy

i. Definition

Hyperbaric oxygen therapy (HBO) is a clinical intervention, in which patients breathe pure oxygen - 100% - (or exceptionally it can be other gas mixtures) intermittently while inside a dedicated chamber at a pressure higher than sea level pressure, i.e. more than 1 atmosphere absolute (ATA) (Albuquerque and Sousa, 2007).

HBO mode of action is complex, since it is the result of a number of physiological and pharmacological mechanisms: these therapeutics mechanisms of action are based on the elevation of both partial pressure of oxygen and hydrostatic pressure (Thom, 2009). The properties constitute the basic rationale for the treatment of a number of different conditions. HBO has to be seen as part of a therapeutic continuum, without any interruption in the chain of treatment. It should not be considered as an isolated treatment modality. The Undersea and Hyperbaric Medical Society (UHMS), established in 1967 in United States of America, was the first society that selected a Hyperbaric Oxygen Therapy Committee, to identify and classify the various clinical indications for HBO based on scientific evidence. The first Committee Report was published in 1977 and many have followed in later years. European countries at that time followed more or less the indications from the UHMS Committee Report but the contacts between the different hyperbaric centers were rare and restricted to the occasional symposium or congress (Mathieu, 2006). Therefore, the need for a committee that raised the quality and profile of Hyperbaric Medicine emerged in 1989 and the European Committee for Hyperbaric Medicine (ECHM) was created.

The treatment of decompression illness was the original and only use of HBO over an hundred years ago. During the last fifty years, however, several other indications for hyperbaric therapy have been proposed (De Laet et al., 2008), including a wide variety of medical and surgical problems (Grim et al., 1990, Tibbles and Edelsberg, 1996).

Despite the use in various situations for many decades, a sense of controversy continues to prevail in the field of hyperbaric medicine, just a restricted number of indications have been accepted by the two main scientific hyperbaric societies, because a high level of evidence is lacking for the other uses. At this moment international studies are ongoing in order to clarify the efficacy of HBO for some other specific indications.

Hyperbaric therapy units are operated by different medical specialties, depending on the indication for which they were originally established. For historical reasons, the need of treatment facilities for diving accidents, many of these units are military. Apart from this, most units are in the departments of Anesthesiology, since the knowledge in specific fields of interest, like physics, gas laws, and pharmacology and physiology are common and also because these patients often require intensive care therapy (Mortensen, 2008).

ii. History of Hyperbaric Medicine

Hyperbaric therapy was first documented in 1662, when Henshaw built the first hyperbaric chamber. Since this time, reports of beneficial effects from increased pressure have increased, and by 1877, chambers were used widely for many conditions, though there was little scientific rationale or evidence (Gill and Bell, 2004). Serious hyperbaric therapy, however, only began as a treatment of caisson disease, a disease occurring in engineering workers who had to work in caissons under conditions of compressed air, mainly during the construction of tunnels and bridges in the late 19th century (De Laet et al., 2008). The first reports of decompression sickness described this condition as “the bends”, since caisson workers assumed a bent posture to help relieve the pain caused by the nitrogen accrual in their joints (De Laet et al., 2008). Although the physiology of the disease was only understood later, recompression therapy at first with normal air, was proposed in 1854, and for a long time caisson disease, or decompression sickness (DCS), as it was later called, remained the main therapeutic indication for hyperbaric therapy (De Laet et al., 2008).

Paul Bert had an important influence on the history of hyperbaric medicine. His work “La Pression Barométrique” (1878) is universally known and is one of the pillar stones

of Hyperbaric Medicine. He studied its effects on living organisms and insisted on the risk of convulsions when patients were submitted to pressures higher than 3 ATA. Shortly afterwards, Lorrain Smith in Edinburgh found that oxygen, when inhaled during long periods at high pressures, was toxic for the lungs (Mathieu, 2006). Haldane, in 1895, was carrying out an experiment on the effects of carbon monoxide on oxygen tension, recommending as a result the use of HBO for the treatment of carbon monoxide poisoning (Mathieu, 2006).

In 1927, Cunningham reported an improvement in circulatory disorders at sea level and deterioration at altitude, and a patient who was grateful to Cunningham for his recovery after HBO, built a huge "steel ball hospital" chamber, but this was closed when Cunningham failed to produce evidence for its use (Gill and Bell, 2004).

According to Albuquerque and Sousa (2007), Drager, in 1917, was the first to explore the use of pure pressurized oxygen in decompression sickness, and his protocols were put into practice by Behnke and Shaw in 1937.

During World War II interest in hyperbaric physiology and medicine re-emerged due to increased demands not only on divers but also increasingly on aviators and later also astronauts who had to work in both hyperbaric and hypobaric conditions. By then also, the use of normal air in hyperbaric chambers had been replaced by that of 100% oxygen or by different mixtures of oxygen, air or helium (De Laet et al., 2008).

The first real scientifically based therapeutic approaches were made by Boerema in Amsterdam (1959) in the field of cardiac surgery with the asystolic heart, and Brummelkamp (1961) for the increasingly frequent treatment of gas gangrene. Since then the contribution of the Dutch school has been central in research on infections causing soft tissue necrosis, and their treatment by HBO. Moreover, Ledingham in Britain, and Jacobson in the USA, were also among the pioneers of HBO (Mathieu, 2006).

The 1950's and the next 20 years were a period of confusion in hyperbaric oxygen therapy ranging from clinical trials based on sound physiological principles (when 60

indications were listed) to patently overzealous claims. Between 1980 and 1994, the ethical medical community response was to generally discount hyperbaric oxygen as too dangerous or unproved. The years after 1994, have been a phase of rigorous scientific approach (Albuquerque and Sousa, 2007).
In an effort to respond to those shortcomings, medical societies such as the UHMS and the ECHM, that are going to be explored below, were established with the explicit aim to examine the indications for HBO.

iii. State of the art in Hyperbaric oxygen administration

To perform HBO, special facilities are required, like the hyperbaric chambers, with the capacity of withstanding pressures higher than the atmospheric where patients breathe 100% oxygen (Fernandes, 2009).

In monoplace chambers (capacity for only one person) the oxygen is inhaled directly from the camera's environment (Fernandes, 2009). Although being less expensive it has the major disadvantage of reduced access of the health professional to the patient during treatment, however is still possible to monitor a cuff blood pressure, arterial waveform, electrocardiogram, and to provide intravenous medications and fluids. Mechanical ventilation can be possible if chambers are appropriately equipped, although it is not possible to suction patients during treatment. Mechanical ventilation in the monoplace chamber is provided by a modified pressure-cycled ventilator outside of the chamber (Sheridan and Shank, 1999).

In multiplace chambers, the internal atmosphere is room air compressed up to 6 atmospheres. Health professionals in this environment breathe compressed air with a pressure of up to 4.560 mm Hg, acquiring a nitrogen load in their soft tissues, just as a scuba diver breathing compressed air. Health professionals need to decompress by using Navy diving tables after treatment in order to avoid decompression illness. Patients, on the other hand, are breathing oxygen while at pressure. This oxygen can be administered via face mask, a hood or endotracheal tube. The main advantage of these chambers is the ability to attend the patient during treatment. However, the installation and support

costs are very high. These high costs limit the widespread use of multiplace chambers (Sheridan and Shank, 1999).

Typically, it takes approximately 30 minutes to compress a patient to 3 atmospheres, which corresponds in pressure to 20 meters deep, and it takes approximately 30 minutes to decompress. In an emergent circumstance, patients can be decompressed in 3 minutes at 3 atmospheres of pressure. Length of treatment varies, but is typically 90 minutes at pressure; treatment duration and pressure used have been largely empirically derived (Sheridan and Shank, 1999).

Indications recognized by hyperbaric medical societies

There are a number of indications supported by clinical evidence at varying level, and substantial regional differences in what is considered to be well indicated (Mathieu, 2006).

The UHMS published a report (Table 1) identifying the indications with scientifically evidence through experimental and clinical studies carried out with strict methodology and producing significant positive results (Mathieu, 2006).

Table 1: Indications accepted by UHMS (2003)

1	**Air or Gas Embolism**
2	Carbon Monoxide Poisoning
	Carbon Monoxide Poisoning Complicated by Cyanide Poisoning
3	Clostridal Myositis and Myonecrosis (Gas Gangrene)
4	Crush Injury, Compartment Syndrome, and other Acute Traumatic Ischemias
5	Decompression Sickness
6	Enhancement of Healing in Selected Problem Wounds
7	Exceptional Blood Loss (Anemia)
8	Intracranial Abscess
9	Necrotizing Soft Tissue Infections
10	Osteomyelitis (Refractory)
11	Delayed Radiation Injury (Soft Tissue and Bony Necrosis)
12	Skin Grafts & Flaps (Compromised)
13	Thermal Burns

In 2004, the ECHM held its 7th European Consensus Conference on Hyperbaric Medicine, where they also agreed on a list of indications (Table 2) (ECHM).
One of the ways of assessing the efficiency of HBO is by referring to the best data available from basic research, animal studies with control groups and human studies following EBM (Evidence-Based Medicine) procedures. This approach involves: prospective, controlled, randomized clinical studies; quantified results; collection of results through the Cochrane collaboration and meta-analysis from various clinical studies (Mathieu, 2006).

The Jury issued its recommendations using a three-grade scale according to the strength with which each recommendation has been evaluated (ECHM):

Type 1: *Strongly Recommended*. The Jury considers the implementation of the recommendation of critical importance for final outcome of the patient/quality of practice/future specific knowledge.

Type 2: *Recommended*. The Jury considers the implementation of the recommendation as positively affecting final outcome of the patient/quality of practice/future specific knowledge.

Type 3: *Optional*. The Jury considers the implementation of the recommendation as an option.

The Jury also reported the level of evidence supporting the recommendations (ECHM):

Level A: Recommendation supported by level 1 evidence (at least 2 concordant, large, double-blind, controlled randomized studies with no or little methodological bias).

Level B: Recommendation supported by level 2 evidence (double-blind controlled, randomized studies but with methodological flaws; studies with only small samples, or only a single study).

Level C: Recommendation supported by level 3 evidence (consensus opinion of experts).

In order to make the jury discussion and decision more transparent, conditions which were not considered as accepted indications for HBO were also reported with the Jury's evaluation of the existing evidence (Mathieu, 2006).

The scale used in the next table is an extension of that used for accepted indications (ECHM):

Level D: Only uncontrolled studies with no consensus opinion of expert.

Level E: No evidence of beneficial action, or methodological or interpretation bias preclude any conclusion.

Level F: Existing evidence favors not to use HBO.

In many aspects this list is similar to the one from UHMS, although it should be understood that definitions used by different societies do not always completely overlap (De Laet et al., 2008).

In order to understand the mechanism of HBO it is necessary to understand the biochemistry of oxygen, the physical and the physiological basis inherent in its application.

Table 2: List of potential indications for Hyperbaric Oxygen Therapy accepted by ECHM (2004).

CONDITION	Accepted			Non accepted		
	Level of evidence					
	A	B	C	D	E	F
Type I = Strongly recommended						
CO poisoning		X				
Crush syndrome		X				
Prevention of osteoradionecrosis after dental extraction		X				
Osteoradionecrosis (mandible)		X				
Soft tissue radionecrosis (cystitis)		X				
Decompression accident			X			
Gas embolism			X			
Anaerobic or mixed bacterial anaerobic infections			X			
Type II = Recommended						
Diabetic foot lesion		X				
Compromised skin graft and musculocutaneous flap			X			
Osteoradionecrosis (other bones)			X			
Radio-induced proctitis / enteritis			X			
Radio-induced lesions of soft tissues			X			
Surgery and implant in irradiated tissue (preventive action)			X			
Sudden deafness			X			
Ischemic ulcer			X			
Refractory chronic osteomyelitis			X			
Neuroblastoma Stage IV			X			
Type III = Optional						
Post anoxic encephalopathy			X			
Larynx radionecrosis			X			
Radio-induced CNS lesion			X			
Post-vascular procedure reperfusion syndrome			X			
Limb replantation			X			
Burns >20 % of surface area and 2nd degree			X			
Acute ischemic ophthalmological disorders			X			
Selected non healing wounds secondary to inflammatory processes			X			
Pneumatosis cystoides intestinalis			X			
Others indications						
Post sternotomy mediastinitis				X		
Stroke				X		
Sickle cell disease				X		
Malignant otitis externa				X		
Acute myocardial infarction				X		
Femoral head necrosis				X		
Retinitis pigmentosa					X	
Tinnitius					X	
Interstitial cystitis					X	
Facial (Bell's) palsy					X	
Cerebral palsy						X
Multiple sclerosis						X
Fetoplacental insufficiency						X

iv. Biochemistry of Oxygen

Oxygen was discovered in 1774 by Joseph Priestley although it was Antoine Laurent de Lavoisier (1743-1794) who identified its properties (Mathieu, 2006).

Oxygen is a colorless, odorless and tasteless gaseous chemical element that is essential for body function and survival. The air we breathe contains 21% oxygen (O_2), 78% nitrogen (N_2) and 1% of other gases (Powers and Howley, 2000).

Most of the biochemical reactions of aerobic organisms require oxygen. Under normal conditions, 98% of the oxygen is transported in the blood, bound to hemoglobin (Hb), whereas only 2% is dissolved in the plasma. Tissue oxygenation (96-97%) occurs due to the oxygen carried by hemoglobin (Powers and Howley, 2000). Supply of oxygen to tissues depends on many factors like ventilation, diffusion across alveolar-capillary membrane, hemoglobin, cardiac output, and tissue perfusion (Shankar, 1980). When a flaw in this oxygen supply occurs, a therapeutic application may be necessary.

Oxygen is a fundamental drug, extensively used as a therapeutic agent, in the management of patients with respiratory diseases or traumatic injuries and in other conditions associated with tissue hypoxia (Yam, 1993).

Therefore, oxygen therapy is a medical treatment in which supplemental oxygen is provided to the patient at concentrations greater than the surrounding air (Bouak, 2004). However, tissue oxygen delivery depends upon an adequate function of cardiovascular (cardiac output and flow), hematological (Hb and its affinity for oxygen) and the respiratory (arterial oxygen pressure) systems (Singh et al., 2001).

The purpose of using normobaric oxygen (NBO) is to relieve hypoxemia by increasing alveolar tension, to reduce the work of breathing, and to decrease the work of myocardium. However, there are prime considerations that have to be taken into account before administering oxygen in a hypoxemic patient like: the amount required, the exact inspired oxygen concentration and the need for humidification. Arterial blood

gases should be measured repeatedly in patients with acute respiratory failure on oxygen therapy.

The minimal mitochondrial oxygen tension required for aerobic metabolism (0.5 to 1 mmHg) corresponds to an arterial oxygen partial pressure (PaO_2) of 25 mmHg, or arterial oxygen saturation (SaO_2) of 40%. However, even mild degrees of hypoxemia (low arterial oxygen tension below the normal expected value 85-100 mmHg) have subtle but important adverse effects on cellular metabolism, which are enhanced by impaired circulation or increased metabolic demand. Thus, correction of hypoxemia is frequently required before PaO_2 falls below 60 mmHg (SaO_2 90%) (Yam, 1993). Therefore, oxygen should be given continuously, in low dose till a small increase in FiO_2 (fraction of inspired oxygen) causes an increase in PaO_2 (Singh et al., 2001).

Thus, an understanding of the mechanism of uptake and delivery of oxygen to the body is required like the factors that alter its delivery to intracellular systems in vital organs, the dose dependent toxic effects of oxygen at partial pressures greater than that in room air, and at last the performance characteristics of oxygen therapy devices (Davis, 1998).

Oxygen therapy is dependent on the type of delivery or delivery system. Currently, two types are used as a source of O_2, pressurized tanks or chemical generators. While the first type may be restricted or not allowed in certain places, due to storage problems or weight or modes of transportation (e.g., danger of fire on planes), the second is very limited in terms of gas consumption (Bouak, 2004).

Oxygen therapy equipment needs to control the inspired oxygen concentration, to prevent carbon dioxide accumulation, to have minimal resistance to breathing, to be efficient and economic, to have adaptability to different gas mixtures or different modes of respiration. Understanding the performance characteristics of oxygen therapy devices enables better selection of equipment for several applications (Davis, 1998).

NBO has several advantages: it is simple to administer, noninvasive, inexpensive, widely available, and can be started promptly after the beginning of a stroke, for example (Singhal et al., 2005).

For acute conditions, NBO is recommend for simple alveolar hypoventilation, e.g., narcotics overdose, paralytic states, postoperative respiratory depression, where $PaCO_2$ (arterial pressure of carbon dioxide) is high; for ventilation-perfusion mismatch, intrapulmonary shunting, normal ventilatory drive, e.g., acute severe asthma, pulmonary edema, extensive pneumonia, adult respiratory distress syndrome, where $PaCO_2$ is low initially from hyperventilation, becoming normal or high later due to exhaustion; for ventilation-perfusion mismatch, reduced ventilatory drive, e.g., chronic obstructive airways disease with acute exacerbation where $PaCO_2$ is high and, for normal PaO_2 and SaO_2 (saturation of oxygen), abnormal circulatory states, or impaired tissue oxygen delivery, e.g., shock, severe anaemia, carbon monoxide poisoning, where $PaCO_2$ is normal (Yam, 1993).

Concerning to chronic conditions, NBO is recommended for hypercapnic due to chronic respiratory failure; interstitial lung disease and nocturnal desaturation, when normal sleep is characterized by depressed ventilation with mildly elevated $PaCO_2$ and decreased PaO_2.

v. Physical basis of Hyperbaric Pressure

Hyperbaric is governed by physic rules that are presented in the following lines (adapted from Mathieu, 2006).

Boyle's law

First described independently by Sir Robert Boyle (1627-1691) and Edme Mariotte (1620–1684), it is also called the 'Boyle-Mariotte Law': *'The product of pressure (p) and volume (V) in a confined amount of gas at equal temperature (T) remains constant.'*

$$p \times V = constant \qquad \text{(Eq 1)}$$

Inside hyperbaric chambers any confined gas volume in the human body and in (medical) equipment is subject to this law. In gas filled spaces with rigid walls, this effect has to be accommodated during compression to and decompression from higher

pressures. This is most important between 1bar and 1.5bar (100kPa - 150kPa) where changes of pressure cause the biggest relative changes of volume (Mathieu, 2006). The pressure variations which can be within a hyperbaric chamber make the volumes of all the air cavities, that are or may be closed (digestive tract, ear, sinuses), vary inversely. All hollow objects will suffer the same volume changes (Fernandes, 2009).

Amontons' law

It was discovered by Guillaume Amontons (1663-1705) and published in detail by Thomas Graham (1805–1869), it is also called 'Graham's Law'. It states: *'The quotient of pressure (p) and temperature (T) in a confined amount of gas at equal volume (V) remains constant.'*

$$\frac{p}{T} = const. \quad \text{(Eq 2)}$$

During (rapid) compression the compressed gas inside a hyperbaric chamber warms up. When the target pressure is reached and all valves have been closed, the compressed gas is slowly cooled down to ambient temperature by temperature exchange through the chamber wall. According to Amontons' Law, this is accompanied by a drop in pressure, for which a correction must be made in order to keep the pressure at therapeutic levels (Mathieu, 2006).

Dalton's Law

First described by John Dalton (1766–1844) in 1801, this gas law is also called '*Dalton's law of partial pressure'.* It states that: *'The total pressure exerted by a gaseous mixture is equal to the sum of the pressures that would be exerted by the gases if they alone were present and occupied the total volume.'*

$$P_{tot} = p_1 + p_2 + \ldots + p_n \quad \text{(Eq 3)}$$

Gases which are non toxic when inhaled at ambient pressure in a certain percentage of a gaseous mixture (Vol. %) may become toxic when inhaled at elevated total pressure because the partial pressure, and not the percentage in a gaseous mixture, causes toxicity (Mathieu, 2006).

Henry's Law

First formulated by William Henry (1775-1836) in 1803 this law states:
'The mass of a gas (C) that dissolves in a defined volume of liquid is directly proportional to the pressure of the gas (P) (provided the gas does not react with the solvent)'.

$$\alpha \times \frac{p}{C} = const. \quad \text{(Eq 4)}$$

The pressure dependent solubility of inert gases in body liquids and tissues is crucial for the development of decompression sickness (DCS) due to supersaturation of tissues in relation to reduced ambient pressure after exposure (Mathieu, 2006). While breathing pure oxygen in a hyperbaric environment at 3 ATA, there is an increased arterial oxygen pressure that can overcome 2000mmHg and, therefore, the volume of dissolved oxygen and transported by plasma, which is minimal at atmospheric pressure, increases over 22 times (Fernandes, 2009).

Gas diffusion

Fick's Laws of Diffusion were derived by Adolf Fick in 1858. Fick's First Law is used in steady state diffusion. This law states the rate of diffusion of a gas across a membrane (Mathieu, 2006).

At various places in the human body partial pressures (or concentrations) of dissolved gases, such as oxygen or nitrogen, depend on diffusion. According to Fick's First Law of Diffusion it can identify the variables for diffusion of gases as size of diffusion area, thickness of diffusion barrier (or distance), and differential gas partial pressure.

According to Fick's Second Law of Diffusion, the time needed for diffusion is dependent on size of molecules, allowing smaller gas molecules like helium to diffuse faster than larger ones (Mathieu, 2006).

Adiabatic processes

Adiabatic processes happen without external heating or cooling. In Hyperbaric Medicine, the Joule-Thomson effect and adiabatic compression are of interest (Mathieu, 2006):

Joule-Thomson effect

'When letting a gas expand adiabatically (i.e. without external heating), the gas will cool down.'

The Joule-Thomson effect was first described by James Prescott Joule (1818-1889) and Sir William Thomson (1824–1907). During adiabatic decompression, most gases at atmospheric pressure behave like this, the only gas which warms upon expansion under standard conditions being hydrogen (Mathieu, 2006).

Adiabatic compression

'When compressing a gas adiabatically (i.e. without external cooling), the gas will warm up.'

Adiabatic compression describes the opposite effect. During compression, the gas inside a hyperbaric chamber warms up. The faster the compression, the more the compressed gas will warm up. Compression of a hyperbaric chamber for treatment of DCS to 280kPa "as fast as possible" may lead to a temperature of 40°C or more. Rapid decompression has the opposite effect (Mathieu, 2006).

vi. Biochemical and cellular effects

The O_2 tension in a given tissue depends on the tissue's level of O_2 consumption, local blood stream, and relative distance from the nearest arteriole and capillary. In fact, O_2 consumption causes oxygen partial pressure (pO_2) to fall rapidly between arterioles and venules. This emphasizes the fact that in tissues there is a distribution of oxygen tensions according to a gradient. Such a gradient also exists at the level of the cell such as in the mitochondrion, the terminal place of oxygen consumption where, O_2 concentrations range from 1.5 to 3 μM (Mathieu, 2006).

Oxygen moves down a pressure gradient from inspired to alveolar gas, arterial blood, the capillary bed, across the interstitial and intercellular fluid to the sites of utilization within the cell (perioxome, mitochondria, endoplasmic reticulum). Under normobaric conditions, the gradient of pO_2 known as the "oxygen cascade" starts at 21.2kPa (159 mmHg) and ends up at 0.5-3kPa (3.8-22.5 mmHg) depending on the target tissue (Mathieu, 2006). The arterial oxygen tension (PaO_2) is approximately 90 mm Hg and the tissue oxygen tension (PtO_2) is approximately 55 mmHg (Sheridan and Shank, 1999). These values are markedly increased by breathing pure oxygen at greater than atmospheric pressure.

Hyperbaric oxygen therapy is limited by toxic oxygen effects to a maximum pressure of 300kPa (3 ATA). $PaCO_2$, water vapour pressure and respiratory quotient (RQ) do not vary significantly between 100kPa and 300kPa (1 - 3 ATA). Thus, for example, the inhalation of 100% oxygen at 202.6kPa (2 ATA) provides an alveolar PO_2 of 1423 mmHg and, consequently, the alveolar oxygen passes the alveolar-capillary space and diffuses into the venous pulmonary capillary bed according to Fick's Laws of Diffusion (Mathieu, 2006).

Hyperoxigenation

Oxygen is transported by blood in two ways: chemically, bound to the hemoglobin and physically dissolved in plasma. During normal breathing, or the environment we live in, hemoglobin has an oxygen saturation of 97%, representing a total oxygen content of

about 19.5 O_2/100mL of blood (or 19.5vol %), because 1g of 100% saturated hemoglobin carries 1.34mL oxygen. In these conditions the amount of oxygen dissolved in plasma is 0.32vol%, giving a total of 19.82vol% oxygen (Table 3). When we offer 100% oxygen through a mask, or endotracheal intubation for a patient to breathe, the oxygen content can reach values up to 22 to 22.2vol% (Jain, 2004).

The principle effect of HBO is the achievement of hyperoxia. During this therapy, oxygen is dissolved physically in blood plasma. At an ambient pressure of 2.8 ATA and breathing 100% oxygen, the alveolar oxygen tension (paO_2) is approximately 2.180 mmHg, the PaO_2 is at least 1.800 mmHg, and the tissue concentration (PtO_2) is at least 500 mmHg. The oxygen content of blood is approximately ([1.34 x Hb x SaO_2] + [0.0031 x PaO_2]), where Hb is serum hemoglobin concentration and SaO_2 is arterial oxygen saturation (Sheridan and Shank, 1999). At a PaO_2 of 1.800 mmHg, the dissolved fraction of oxygen in plasma (0.0031 x PaO_2) is approximately 6vol%, which means that 6ml of oxygen will be physically solved in 100ml of plasma, reaching a total volume of oxygen in the circulating blood volume equal to 26.9vol%, equivalent to basic oxygen metabolic needs, and the PaO_2 in the arteries can reach 2.000 mmHg. With a normal lung function and tissue perfusion, a pO_2 > 1,000 mmHg could be reached (Mayer et al., 2005). Breathing pure oxygen environment at 2 ATA, the oxygen content in plasma is 10 times higher that breathing air at sea level. Under normal conditions the pO_2 is 95 mmHg, however, under conditions of a hyperbaric chamber, the pO_2 can reach values greater than 2000 mmHg (Jain, 2004). Consequently, during HBO, Hb is also fully saturated on the venous side, and the result is an increased oxygen tension throughout the vascular bed. Since diffusion is driven by a difference in tension, oxygen will be forced further out into tissues from the vascular bed (Mortensen, 2008) and diffuses to areas inaccessible to molecules of this gas when transported by hemoglobin erythrocyte (Albuquerque and Sousa, 2007).

Table 3. Dissolution of oxygen in plasma at normal air, normobaric and hyperbaric environment

Pressure ATA	**1**	**1**	**3**
FiO_2	**0,21**	**1**	**1**
PaO_2 (mmHg)	**104**	**673**	**2193**
O_2 – OxiHg (mL/100mL)	**19,7**	**20,1**	**20,1**
O_2 – Plasma (mL/100mL)	**0,3**	**1,88**	**6**

After removal from the hyperbaric oxygen environment, the PaO_2 normalizes in minutes, but the PtO_2 may remain elevated for a variable period. The rate of normalization of PtO_2 has not been clearly described, but is likely measured in minutes to a few hours, depending on tissue perfusion (Sheridan and Shank, 1999).

The physiological effects of HBO include short-term effects like vasoconstriction and enhanced oxygen delivery, reduction of edema, and phagocytosis activation, and it has an anti-inflammatory effect (enhanced leukocyte function). Long-term effects are neovascularization (angiogenesis in hypoxic soft tissues), osteoneogenesis as well as stimulation of collagen production by fibroblasts. The clinical results are, therefore, wound healing and recovery of injured tissue (Mayer et al., 2005, Sheridan and Shank, 1999).

Vasoconstriction

In normal tissues, the primary action of oxygen is to cause general vasoconstriction (with special impact on kidneys, skeletal muscle, brain and skin), which elicits a "Robin Hood effect" through a reduction of blood flow to well oxygenated tissue (Mortensen, 2008).

This selective vasoconstriction occurs at healthy tissue level to redistribute blood and O_2 to hypoxic tissues, which, in turn contributes to a better O_2 supply (Albuquerque and Sousa, 2007). HBO reduces edema, partly because of vasoconstriction, partly due to improved mechanisms of homeostasis.

A high gradient of oxygen is a potent stimulator of angioneogenesis, an effect considered to be of significant importance to stimulation of reparative and regenerative processes in some conditions (Mortensen, 2008).

Leukocyte oxidative killing

Many cell and tissue functions depend on oxygen. Of special interest are leukocyte ability to kill bacteria, cell replication, collagen formation, and mechanisms of homeostasis, such as active membrane transport, e.g. the sodium–potassium pump.
HBO inhibits leukocyte adhesion to endothelium, decreasing tissue damage, and enhances leukocyte motility, resulting in improved microcirculation (Mortensen, 2008). This occurs when the presence of gaseous bubbles in the venous vessels blocks the flow and induces hypoxia which causes endothelial stress followed by the release of nitric oxide (NO) that reacts with superoxide anion to form peroxynitrite. This, in turn, provokes oxidative perivascular stress and leads to the activation of leukocytes and their adhesion to the endothelium (Antonelli et al., 2009).

Antimicrobial effect

HBO, by reversing tissue hypoxia and cellular dysfunction, restores the cells defenses and increases the ability to phagocyte some bacteria by working synergistically with antibiotics. It also inhibits the growth of a number of anaerobic and aerobic organisms at wound site. There is evidence that hyperbaric oxygen is bactericidal for *Clostridium perfringens*, besides promoting a definitive inhibitory effect on the growth of toxins in most aerobic and microaerophilic microorganisms. The action of HBO on the anaerobes is based on the generation of free radicals like superoxide, dismutase, catalase and peroxidase (Mader et al., 1980).

There have been identified over 20 different clostridial exotoxins, and the most prevalent is the alfatoxine (fosfolipase C), which is hemolytic, tissue necrotizing, and lethal. Other toxins, acting in synergy, promote anemia, jaundice, renal failure, cardiotoxicity and brain dysfunction. The thetatoxine is responsible for vascular injury and consequent acceleration of tissue necrosis. HBO blocks the production of fosfolipase C and thetatoxine and inhibits bacterial growth (Jain, 2004).

Neovascularization/Angiogenesis

Hypoxia is the major factor stimulating angiogenesis. However, deposition of collagen is increased by hyperoxygenation, and is the collagen matrix that provides the support base for the growth of new capillary bed. Two hour daily treatments with HBO are apparently responsible for stimulating the oxygen in the synthesis of collagen, the remaining 22h of hypoxia real or relative, in which the patient is not submitted to HBO, provide the stimulation of angiogenesis. Thus, the alternation of states of hypoxia and hyperoxia, observed in patients during treatment with intermittent HBO is responsible for maximum stimulation of fibroblast activity in ischemic tissues, producing the development of the matrix of collagen, essential for neovascularization (Jain, 2004).

In addition to promoting an environment less hospitable to anaerobes, the presence of oxygen is known to speed the process of wound healing, whether from being required for the production of collagen matrix and subsequent angiogenesis, from the presence and beneficial effects of ROS, or from yet undetermined means (Kunnavatana et al., 2005).

Dimitrijevich et al. (1999) studied the effect of HBO on human skin cells in culture and in human dermal and skin equivalents. In that study, normal human dermal fibroblasts, keratinocytes, melanocytes, dermal equivalents, and skin equivalents were exposed to HBO at pressures up to 3 ATA for up to 10 consecutive daily treatments lasting 90 minutes each. An increase in fibroblast proliferation, collagen production, and keratinocyte differentiation was observed at 1 and 2.5 ATA of HBO, but no benefit at 3 ATA. Kang et al. (2004), reported that HBO treatment up to 2.0 ATA enhances proliferation and autocrine growth factor production of normal human fibroblasts grown in a serum-free culture environment, but showed no benefit beyond or below 2 ATA of HBO. This indicates that there is a delicate balance between having enough and too much oxygen and/or atmospheric pressure when promoting fibroblast growth (Kunnavatana et al., 2005).

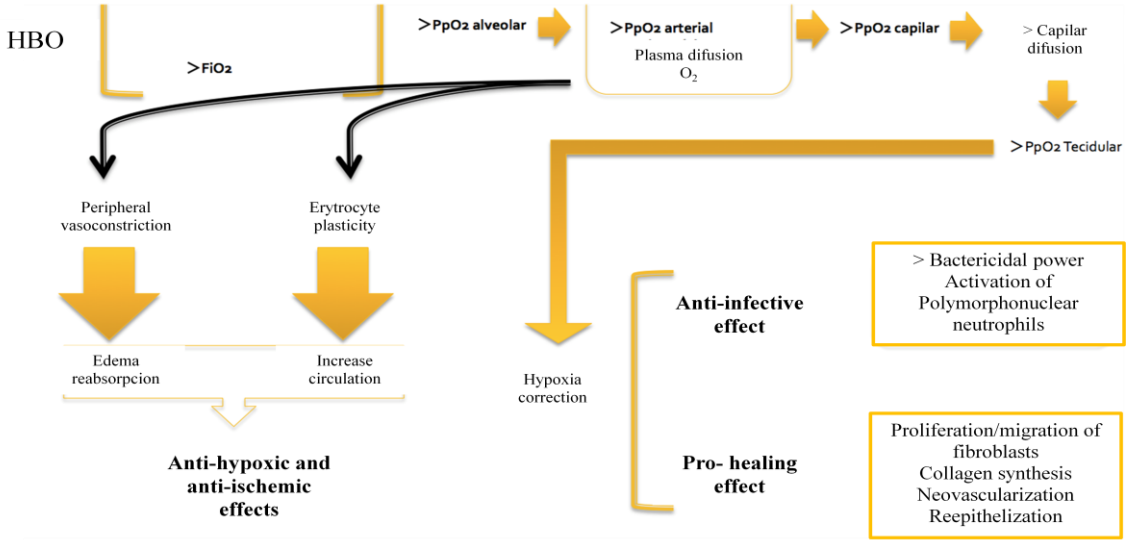

Figure 1. Representative effects of HBO (adapted from Camacho, 2013)

vii. Potential harms, safety and precautions

Oxygen, like any other drug, has its own therapeutic range and ratio, and therefore an understanding of which is essential to its proper administration is needed. At pressures ≤ 1 bar within the first 12-24 hours of administration of 100% oxygen toxicity problems are almost irrelevant to the practice of emergency medicine (Davis, 1998). On the other hand, oxygen becomes toxic to the body when breathed at increased partial pressure (more than atmospheric conditions 0.21). Thus, pulmonary oxygen toxicity can occur when the inspired oxygen partial pressure exceeds 0.6 ATA for prolonged periods. We need to be aware of the common signs and symptoms from this problem like pulmonary symptoms (chest pain, dry coughing, lung irritation, chest tightness and dyspnea), headaches, dizziness, nausea, numbness in fingertips and toes, and a dramatic reduction in aerobic capacity (Bouak, 2004).

Therefore, in normobaric conditions, there are three types of danger by administering oxygen therapy (Singh et al., 2001):

- **Physical risks**

By using oxygen there is always the risk of fire and explosion due to combustion. This can happen when it is used at high concentrations, in pressure chambers, and smokers. Also, catheters and masks can cause injury to the nose and mouth. Dry and non-humidified gas can cause dryness and crusting.

- **Functional risks**

Patients who have lost sensitivity to CO_2 and can easily fall into depression ventilator. Hypoventilation can lead to hypercapnia and CO_2 narcosis, although the risk is small with low flow oxygen therapy. Arterial pH may be a better guide than $PaCO_2$ for monitoring oxygen therapy. As long as arterial pH does not accuse acidosis, long term oxygen therapy can benefit the patients with CO_2 retention.

- **Cytotoxic damage**

Patients with long-term treatment of oxygen after being autopsied showed proliferative and fibrotic changes in the lungs. In acute conditions, most of the structural damage occurs from high FiO_2, and thus, oxygen can cause release of various reactive species.

Concerning to hyperbaric oxygen environment, this is not completely harmless and may have some side effects, complications and others injuries caused by oxidative stress, that are going to be explored:

Barotrauma

Although human tissues may support great pressure, tissue injury can occur, resulting from the failure of a gas-filled space to equalize its internal pressure with ambient pressure changes, according to Boyle's law. This phenomenon is called barotrauma. Pressure-related injury or barotrauma may affect all those who participate in diving activities, compressed-air work, aviation and hyperbaric therapy. Injury is most likely

when the pressure-volume changes are great or the onset is rapid – as occurs during the initial parts of pressurization or descent from atmospheric pressure (Mathieu, 2006).

Two conditions must be present for barotrauma to occur: (1) a change in ambient pressure and (2) transfer of this pressure to a non or partially collapsible airspace. Gas expansion may tear tissue while gas volume reduction may cause vascular engorgement, mucosal swelling and hemorrhage. In particular this may affect the middle ear and sinuses; lung; intestines; teeth; eye, particularly when surrounded by an air space like a face mask; and other physiological or pathological gas spaces (Mathieu, 2006).

Middle ear barotrauma is the most commonly reported acute side effect of HBO, and it was reported to occur in 2% of patients (Feldmeier, 2003). A prospective study reported that almost one-fifth of all patients experienced some ear pain or discomfort related to problems in middle ear pressure equalization, while visual ontological examination confirmed barotraumatic lesions in 3.8% of patients (Plafki et al., 2000).

Pulmonary barotrauma is a potential problem mainly during the decompression phase of HBO since gas volume in the lungs increases due to the reduced pressure and this extra volume needs to be breathed out. However, the occurrence of this complication has only been reported in sporadic cases (Feldmeier, 2003).

Patients who need to be treated with HBO should be carefully clinically evaluated in order to prevent barotrauma. Otolaryngological, pulmonary, cardiovascular, neurological and other systems should be screened properly in order to identify any disease state and minimize the potential risk of treatment (Mathieu, 2006).

A careful clinical history, chest X-ray and otoscopy are mandatory parts of the examination. Tympanometry is a very useful tool and should be regularly performed whenever there is doubt about tympanic membrane elasticity. Myringotomy or ventilation tube insertion should be done when there is an inability to equalize middle ear pressure. All medical and paramedical staff of a hyperbaric facility should be educated and trained to quickly identify signs of developing barotrauma. Compression and decompression rates should be slow and carefully controlled. When a relevant

symptom is discovered during the compression phase, this should be halted followed by slight depressurization to a point of pain relief (Mathieu, 2006).

Equalizing and decongestion may be attempted to alleviate the problem, but in many cases either the hyperbaric treatment or the underwater excursion will need to be aborted in favor of further medical intervention (myringotomy and tympanostomy) or to allow spontaneous recovery of Eustachian tube function (divers) (Mathieu, 2006).

Prevention of middle and inner ear barotrauma should be familiar to every person involved in hyperbaric treatments: When possible, treatments should be avoided in the presence of: significant nasal congestion, nasal discharge or upper respiratory tract infections; if possible have patients sitting rather than lying; nasal or systemic decongestants may be used judiciously; ascent and descent rates should be appropriately slow; and patients should be taught and observed to apply the various equalizing techniques of modified yawning and swallowing; Frenzel and Toynbee techniques; soft palate contraction and gentle Valsalva (Hamilton-Farrell and Bhattacharyya, 2004).

Patients in coma present a special challenge. Some authors propose a routine myringotomy for all intubated and non-cooperative patients, especially neurosurgical ones where the pain may otherwise result in an increase in intracranial pressure (Rockswold et al., 1992). Although 25% of United States Centers perform myringotomy on intubated patients, there is no actual standard of care in the literature regarding middle and inner ear barotrauma prophylaxis prior to HBO therapy (Capes and Tomaszewski, 1996). With a very low incidence of serious barotrauma, many authorities do not recommended a routine myringotomy in this kind of patient (Sloan et al., 1989). In European practice, this is not a routine procedure. Relaxation of the Eustachian tube muscles appears to permit easier middle ear pressure equilibration in comatose patients, with a corresponding low incidence of barotraumatic injuries. Properly controlled, slow descents and careful surveillance by the medical staff in a multiplace hyperbaric chamber contributes to a reduction in barotraumas incidence risk (Mathieu, 2006).

Oxygen toxicity

The awareness to oxygen toxicity has emerged with the widespread use of high oxygen pressure in medicine, the accumulated experience in the field of hyperbaric medicine and in military and professional diving. The most dramatic manifestations of oxygen toxicity are pulmonary oxygen toxicity (Lorraine Smith effect), Central Nervous System (CNS) oxygen toxicity (Paul Bert effect) and ocular oxygen toxicity. Yet the toxic effect of oxygen can be found in almost every organ and tissue, and it is only a matter of time until it affects all body tissues and organs (Mathieu, 2006).

Oxygen has to be considered as a drug and it can give rise to the formation of free radicals during high dose oxygen breathing. These free radicals can lead to the oxidation of chemical tissue components (De Laet et al., 2008).

Most importantly, the pathophysiological sequel of oxidative stress has been notoriously difficult to quantify. Despite these impediments, the medical significance of oxidative stress has become increasingly recognized to the point that it is now considered to be a component of virtually every disease process. The ascendancy of free radical biology is attributable to several major factors. First, new techniques have been invented (and are still being invented) to quantify oxidative stress in vivo, although the existing technology is poorly suited for routine clinical applications. Second, the inseparable relationship of oxidative stress to inflammation has become incontrovertible along with the recognition that certain reactive ROS function as messenger molecules to propagate inflammatory signals. Third, the discovery of nitric oxide (NO) as a vasodilator and immune mediator has stimulated the interest of mainstream biologists and clinicians to an almost unprecedented degree (Hensley et al., 2000).

Narkowicz et al. (1993) analyzed blood from persons submitted to HBO exposure, using low temperature electron spin resonance (ESR) spectroscopy, and found that HBO exposure increases ascorbate radical levels in blood, which is likely to reflect increased ascorbate turnover in human red blood cells, being, probably, the cause of the increased free radical levels also measured in the blood following HBO treatment.

Ocular toxicity

Palmquist et al. (1984) reported forms of ocular toxicity are reversible myopia and cataract development.

Central Nervous System toxicity

Central Nervous System (CNS) oxygen toxicity was first described by Paul Bert (1878). CNS oxygen toxicity is expressed in humans in much higher oxygen pressures than pulmonary toxicity and is the main limitation for the use of HBO in diving and hyperbaric treatments (Mathieu, 2006).

Hyperoxia-induced convulsions are defined and classified as generalized, tonic-clonic seizures although this condition appears to be self-limiting without apparent long-term sequels (Plafki et al., 2000).

Seizures, thought to be secondary to oxygen toxicity, have been reported to occur in as many as 10% of patients undergoing hyperbaric oxygen treatment for 90 minutes at 3 atmospheres, especially in monoplace chambers. However, the incidence of seizures has been dramatically reduced with the routine use of “air breaks”. This is a maneuver in which the patient breathes compressed room air for 10 to 15 minutes. This technique can be performed in a monoplace chamber by having the alert patient breathe compressed air through a mask that is supplied by a tank of compressed air outside the chamber. The patient who is being mechanically ventilated can also be given 10-minute “air breaks” every 30 minutes with a relatively simple modification of the ventilator. Seizures are reported to occur more frequently in patients who are febrile. If a seizure be noticed in the chamber, it is important to wait until the seizure has ceased before beginning emergent decompression; rapid decompression with a closed glottis may result in bilateral tension pneumothorax, because the pressure in the lungs is not able to equalize with the falling chamber pressure. Complications related to restricted patient access associated with monoplace chambers lead us to the importance of careful selection of the critically ill for hyperbaric treatment (Sheridan and Shank, 1999).

Nitric oxide (NO) is emerging as an additional possible mediator of the toxic effects of oxygen (Clark and Thom, 2003). Furthermore, inhibition of nitric oxide synthase (NOS) was demonstrated to protect against CNS oxygen toxicity (Oury et al., 1992). Thom et al. (2002) tried to determine the impact of elevated partial pressures of O_2 on the steady state concentration of NO in the cerebral cortex of rats after HBO treatment and concluded that hyperoxia causes an increase in NO synthesis, in the cerebral cortex, as part of a response to oxidative stress, without predicting if similar changes occur in deeper brain structures.

The literature contains contradictory reports on the potential of various antioxidants to protect against CNS oxygen toxicity. For example, two of the most studied antioxidant enzymes – superoxide dismutase (SOD) and catalyses (CAT) – were found to be protective by Puglia and Loeb (1984), yet according to Block (1977), these same antioxidants failed to exhibit protective effect. When entrapped in liposomes, SOD and CAT inhibited hyperoxia-induced seizures (Yusa et al., 1984).

Pulmonary toxicity

Initially described in 1899 by Lorrain Smith, it is currently well established that exposure to oxygen at high ambient partial pressures causes alterations throughout the entire respiratory tract including the airway epithelium, microcirculation, alveolar septa and pleural space. A typical pathologic and clinical picture of diffuse alveolar damage and/or necrotizing bronchitis (bronchiolitis) occurs within three days to one week of continuous exposure to 0.08-0.1 MPa (0.8-1.0 ATA) of oxygen or within shorter periods of time at higher partial pressures, as a result of direct pulmonary oxygen toxicity (Mathieu, 2006).

Clark et al. (1999) in a study of human organ O_2 tolerance on pulmonary function after HBO at different pressure found that the rate of pulmonary symptom development and lung volume reduction increased progressively with elevation of O_2 pressure. Average rates of vital capacity reduction over a useful range of O_2 pressures provided a valuable general description of pulmonary O_2 tolerance in humans. However, the existence of multiple pulmonary effects of O_2 toxicity and the complexity of their interactions

require awareness that deviations from the average relationships may occur in different individuals or under varying conditions of O_2 exposure and subsequent recovery. The associated pulmonary function deficits may represent responses to a composite of direct and indirect effects of O_2 poisoning, along with related consequences and subsequent reactions to those effects.

Thorsen et al. (1998) studied the effects of a standard hyperbaric oxygen treatment protocol, on pulmonary function and found a reduction in small airways conductance that is consistent with other studies where total oxygen exposures have been below the toxic pulmonary threshold effects traditionally measured as a reduction in vital capacity. This effect is not considered to be of any clinical relevance for patients treated with hyperbaric oxygen unless repeated treatment series are to be given.

The direct effect of oxygen on the lung predominates in normobaric hyperoxia, whereas CNS effects prevail in hyperbaric conditions. The former develops slowly, and because the entire surface of the lung is directly exposed to the hyperoxic environment for many hours, the inflammatory response is diffuse with destruction of the alveolar-capillary barrier, edema, impaired gas exchange, respiratory failure, and death. However, at hyperbaric exposures, pulmonary damage develops more rapidly and is presaged by events in the brain. This more acute injury is driven by extrapulmonary mechanisms in which NO derived from NOS may link elevated PO_2 in brain to accelerated damage in the lung via central autonomic pathways (Demchenko et al., 2007).

Other complications

Hyperbaric-oxygen-induced alterations of renal function, manifested by reductions of sodium reabsorption and oxygen consumption and diuresis with increased sodium excretion, have suggested a toxic effect of excessive oxygen on renal tubular cells. These changes have occurred under conditions of hyperoxia that significantly reduce renal blood flow and increase renal vascular resistance. However, renal arterial and venous as well as urinary oxygen tensions increases hyperbaric oxygenation, indicating that renal function is probably altered due to excessive oxygenation and not by ischemia (Greene et al., 1979).

Clinical and experimental evidence does not support claims that HBO during pregnancy can cause a range of fetal complications, including spina bifida and limb defects (Van Hoesen et al., 1989).

Decompression illness itself is a risk inherent to HBO for care personnel inside the pressurized chamber even when not breathing oxygen, but can be avoided by careful usage of compression and decompression schemes, already mentioned above (De Laet et al., 2008).

The confinement to a relatively small and closed container can give rise to claustrophobia which in severe cases can make HBO impossible. Distraction schemes or occasionally light sedation can help to overcome these problems. However, in severe cases that can not be possible (De Laet et al., 2008).

The most important serious risk is fire. Oxygen under pressure constitutes a serious fire hazard, and special precautions have to be made to meet this hazard. Therefore, there are very rigorous restrictions of what is allowed inside a monoplace chamber, and the following are prohibited: any kind of electrical equipment, grease (including many forms of make up), newspapers, and some types of clothing (only cotton is allowed) (Kot et al., 2004). In multiplace chambers, expired oxygen is led out of the chamber, maintaining a low oxygen fraction in the chamber atmosphere, reducing the risk and the restrictions. Effective fire extinguishing systems are mandatory in multiplace chambers (Mortensen, 2008).

viii. Contraindications

The only absolute contraindications are untreated pneumothorax and some chemotherapeutics, especially Bleomycin, because of the risk of pulmonary fibrosis. Relative contraindications are poorly controlled epilepsy, overt heart failure, and some airway problems, such as acute upper airway infection, emphysema, and former spontaneous pneumothorax (Mathieu, 2006).

There have been some concerns that HBO could stimulate malignant growth by increasing tumour oxygenation. This was not supported by Feldmeier in his review in 2003, where the author concluded that a history of malignancy should not be a contra-indication for HBO (Feldmeier, 2003). Shi et al. (2005) also found the same results studying the effect of HBO treatment on tumor hypoxia and vasculature, on head and neck squamous cell carcinoma, by immunohistochemical analysis: HBO treatment increased tumor oxygenation during the treatment interval but did not promote the growth of either irradiated or unirradiated tumors. No increase in tumor vascular endothelial growth factor expression or vascularization was detected.

In conclusion, Plafki et al. (2000), in a report, analyzed the medical safety of HBO therapy, reviewed complications and side effects of 782 patients treated for various indications with a total of 11.376 HBO therapy sessions within a multiplace chamber and concluded that patients scheduled for HBO therapy need a careful examination and monitoring. If safety guidelines are strictly followed, HBO therapy is a modality with an acceptable rate of complications. Furthermore, the predominant complication is represented by pressure equalization problems within the middle ear and serious complications rarely occur.

ix. Reactive Oxygen Species

Recent publications suggest that finding the balance requires an understanding that low concentrations of Reactive Oxygen Species (ROS) may play a beneficial role in wound healing, contrasting the common view that all ROS are damaging to cells (Kunnavatana et al., 2005). Oxidizing species such as free radicals and hydrogen peroxide may serve as cellular messengers mediating complex redox-sensitive processes such as extracellular matrix formation, cytokine action, angiogenesis, and cell motility (Hink and Jansen, 2001). However, high levels of ROS and hydrogen peroxide (3% is used as a clinical disinfectant) still prove toxic to cells (Kunnavatana et al., 2005).

Glutathione is a cysteine-containing tripeptide whose redox properties are essential for the viability and function of virtually all cells, ranging from intracellular defense against oxidative stress to regulation of signal transduction and systemic defense against drug

toxicity. In periods of oxidative stress, such as the presence of overwhelming ROS, glutathione levels in the cell decrease. In addition, studies have shown that normal glutathione levels decrease from 60% to 70% after wound insult. *N*-acetylcysteine (NAC) is a prodrug that supplies bioavailable cysteine for glutathione replenishment and prevents oxidative damage (Kunnavatana et al., 2005, Shan et al., 1990).

It is known that ROS are a trophic stimulus to Stem Progenitor Cells (SPCs) (Milovanova et al., 2008). For instance, under normal physiological conditions, increasing the tissue lactate concentration will increase cellular NADH through the action of lactate dehydrogenase (LDH), and this secondarily increases intracellular ROS production by stimulating NAD(P)H oxidase enzymes. Lactate can also enhance free radical formation via Fenton-like reactions (Gupte et al., 1999). Lactate metabolism by SPCs accelerates progressive SPCs recruitment to target sites remote from the bone marrow. This occurs because of a complex set of responses initiated by thioredoxin (Trx)-1 (Trx1) synthesis in response to oxidative stress, which elevates hypoxia inducible factor-1 (HIF-1) and HIF-1-dependent growth factors (Milovanova et al., 2008). Local tissue lactate concentration is elevated in wounds and reaches near 6–15 mM, in contrast to a concentration of 1.8–2 mM under nonwounded conditions (Gladden, 2004). By causing an oxidative stress, HBO activates a physiological redox-active autocrine loop in SPCs that stimulates vasculogenesis. Thioredoxin system activation leads to elevations in HIF-1 and -2, followed by synthesis of HIF-dependent growth factors. HIF-3 has a negative impact on SPCs (Thom, 2009).

Thom's review highlighted some of the beneficial actions of HBO and data suggested that oxidative stress brought by hyperoxia can have therapeutic effects. This author also states that HBO also improves ischemic tolerance when used in a prophylactic manner. The basis for these effects is only partially understood. Augmented synthesis of reactive species temporarily inhibits endothelial sequestration of neutrophils by inhibiting β2-integrin function and in many tissues HBO will induce antioxidant enzymes and anti-inflammatory proteins (Thom, 2009). However, the author states that this issue requires additional investigation.

2.2. Sports injuries

The healing of a sports injury has its natural recovery, and follows a fairly constant pattern irrespective of the underlying cause. Three phases have been identified in this process: the inflammatory phase, the proliferative phase and the remodeling phase. In each one of these phases oxygen has an important role (Ishii et al., 2005):

In the inflammatory phase, the hypoxia-induced factor-1α, which promotes, for example, the glycolytic system, vascularisation and angiogenesis has been shown to be important. However, if the oxygen supply could be controlled without promoting blood flow, the blood vessel permeability could be controlled to reduce swelling and consequently sharp pain.

In the proliferative phase, in musculoskeletal tissues (except cartilage), the oxygen supply to the injured area is gradually raised and is essential for the synthesis of extracellular matrix components such as fibronectin and proteoglycan.

In the remodeling phase, tissue is slowly replaced over many hours using the oxygen supply provided by the blood vessel already built into the organization of the musculoskeletal system, with the exception of the cartilage. If the damage is small, the tissue is recoverable with nearly perfect organization but, if the extent of the damage is large, a scar (consisting mainly of collagen) may replace tissue. Consequently, depending on the injury, this collagen will become deficiently hard or loose in the case of muscle or ligament repair, respectively.

For our interest a more detailed description of muscle contusion injury and anterior cruciate ligament injury is going to be explored.

i. Muscle contusion

Muscle injury presents a challenging problem in traumatology and is frequently encountered in sports medicine. The injury can occur via a variety of mechanisms, ranging from direct mechanical deformation (as in contusions, lacerations, and strain) to

indirect causes (such as ischemia and neurological damage) (Li et al., 2001). Indirect injuries can be either complete or incomplete (Petersen and Hölmich, 2005).

In sport events in the United States the incidence of all injuries ranges from 10 to 55%. The majority of muscle injuries (more than 90%) are caused either by excessive strain or by contusion of the muscle (Järvinen et al., 2000).

Muscle contusion occurs when a muscle is subjected to a sudden, heavy compressive force, such a direct blow (Järvinen et al., 2005, Järvinen et al., 2007). Muscle contusion is produced by objects that collide with the body or sometimes on the opposite and worsens if during the aggression the muscle is in contraction (Lopes et al., 1993). This aggression reaches both the skin and the subcutaneous tissue, without interruption of the skin. The vessels, connective tissues and muscle fibers suffer ruptures in different degrees of intensity. Therefore, the healing process depends on the magnitude of bleeding, which leads to an interstitial hematoma. In muscle contusion, due to the rupture of small blood vessels of the subcutaneous tissue or dermis it gives rise to equimosis. However, concerning to the rupture of the larger vessels, blood can seep into the subcutaneous tissue and muscles, remaining collected between intermuscular septum or between muscle and bone, leading to a larger hematoma (Lopes et al., 1993).

Muscle contusion is manifested by intense localized pain, immediate muscle contracture that gets worse within 24 hours after trauma causing also a decrease in the range of motion. According to Lopes et al. (1993), muscle contusion are classified into mild, moderate and severe, depending on its pathological changes produced by direct trauma:

- in mild contusion, after direct trauma, pain is immediately well localized, but not interfere with its mobilization. Usually, cryotherapy allows immediate analgesia and normally does not interfere in day-to-day life of the individual. However, 12 hours after the accident, pain tends to evolve into more severe and subjects can have intermittent claudication.

- in moderate contusion, pain is intense, localized, tending to widespread, and depending on its location can take antalgic postures, with decreased amplitude. The individual must stop immediately and normally needs crutches.

- in severe contusion, pain is also intense, localized or widespread, functional impotence and antalgic posture, with a significant decrease in amplitude. The individual must stop immediately and avoid walking. The treatment is usually initiated after an observation period of 12-24 hours. In the meantime, the site of the injury swells, making it impossible to do muscle palpation due to its stiffness and often can cause clinical signs of compartment syndrome.

Ultrasonography is the test indicated for the differential diagnosis to define the best treatment to be used: conservative or surgical. The mild and moderate contusions are seen as hypoechoic mass corresponding to lymphoplasmacytic infiltration and edema that had in muscle installed due to injury. Also, myofibriles are thickness and irregular and muscle has a non defined structure. In severe contusions, ultrassonography also shows a hypoechoic mass due to myofibril rupture and due to the blood affluence (Lopes et al., 1993).

Despite the high incidence of muscle injuries by contusion, there are limited studies in this area, concerning to its treatment, due to the high variability of the lesion.

Once established and standardized the methodology of inducing muscle contusion it is possible to compare techniques and procedures to speed muscle recovery.

The techniques are varied, including physical agents, such as the ice but, however, it was shown that only assists in reducing inflammation having no influence in tissue regeneration (Lee et al., 2005). The same effect was found by administering analgesic/anti-inflammatory drugs (Beiner and Jokl, 2002). The application of mechanical agents, such as immobilization has shown that the repair process is delayed by its immobilization (Järvinen and Lehto, 1993). When there is an application of electrotherapy, such as ultrasound at low intensity the authors concluded that it did not influence the acceleration of the repair process (Rantanen et al., 1999). However, when

laser is applied, with only an average of five daily sessions of laser therapy cause similar results to the non injured muscle, depending on the type of laser and the characteristics used (Oliveira et al., 1999, Amaral et al., 2001, Iyomasa et al., 2009).

However, the best technique is not fully understood and there are divergent results in the literature. Thus, the need arises to find a quickest method to prevent one of the consequences of muscle contusion which is myositis ossificans.

ii. Anterior Cruciate Ligament injuries

There are several ligaments in every joint in the human skeleton being responsible for joint restraint. Ligaments are also sensory organs and have significant input to sensation and reflexive/synergistic activation of muscles. Therefore, the muscles also have a significant role as restraints. In some joints, such as the intervertebral joints of the spine, the role of the muscles as restraints is amplified. The role of ligaments as joint restraints is rather complex when considering the multitude of physical activities performed by individuals in routine daily functions, work and sports, the complexity of the anatomy of the different joints and the wide range of magnitude of the external loads. The functional complexity of ligaments is amplified when considering their inherent viscoelastic properties such as creep, tension–relaxation, hysteresis and time or frequency dependent length–tension behavior. As joints go through their range of motion, with or without external load, the ligaments ensure that the bones associated with the joint can make anatomical tracks, keeping full contact in the articular surface by preventing separation of the bones, ensuring stable motion (Solomonow, 2004).

The Anterior Cruciate Ligament (ACL) is a short, strong, intra-articular, extrasynovial structure which controls rotational movements and anterior translation of the femur upon the fixed tibia (Deehan and Cawston, 2005). From the origin at the lateral femoral condyle, fibers of the ACL run obliquely to its insertion at the medial and lateral tibial spine and to the area between the spines. The direction from the origin at the femoral condyle is distal– anterior–medial towards the tibia (Zantop et al., 2006).

The ACL is a dense, highly organized, cable-like tissue composed of different types of collagens. Type I, are oriented parallel to the longitudinal axis of the ligament and is responsible for the tensile strength of the ligament. Type II, is the typical collagen of the cartilage and is normally not found in ligaments but exceptionally can be found in the fibrocartilaginous regions of ACL, in the binding support, being exposed to pressure or shear force elastin. Type III, is located in the loose connective tissue that divides the type I, being important for the elasticity of the ligament proteoglycans. Type IV is found in all vascular basement membranes, mainly in the binding support which is less vascularized. Type VI collagen has an orientation parallel to type III collagen, which is responsible for the gliding component between functional fibrillar units. This ligament also has a high concentration of water (*glycosaminoglicans*), and cells that allow extreme distance changes during motion and function to attract and couple key elements in normal, healing, and growing tissues (Duthon et al., 2006, Solomonow, 2004).

Table 4. Representative types of collagen, according to its constitution, localization and function (adapted from Duthon et al., 2006).

Collagen	Cells	Localization	Function
Type I	Fibroblasts	Skin, tendon, bone, ligaments	Tensile strength
Type II	Condrocytes	Cartilage	Exposure to pressure or shear force
Type III	Quiescent hepatocytes, epithelial; fibroblats	Skin, muscle, ligaments (with type I)	Pliability
Type IV	All epithelial cells, endothelial cells, regenerating hepatocytes	All basal lamina	Binding support
Type VI	Quiescent fibroblasts	Most interstitial tissue (with type I)	Gliding component

The ACL is composed of multiple fascicles, a band of dense collagen tissue, that fascicles range from 250 μm to several millimeters being surrounded by a connective tissue known as the paratenon. Each fascicle is composed of 3–20 subfasciculi which are enclosed by an epitenon. The subfasciculi have an undulating course and consist of groups of subfascicular units (100–250 μm in diameter) surrounded by loose connective tissue, the endotenon, consisting of collagen type II. These subfascicular units are composed with fibers (1–20 μm in diameter) which are made up of collagen fibrils (25–

250 nm in diameter) (Strocchi et al., 1992). Overall, the ACL's microstructure seems to be similar to other soft connective tissues.

However, the parallel, dense, and regular organization of ACL fibrils is unique. It is a combination of helical and planar, parallel or twisted, nonlinear networks. The centrally located fascicles in the ACL are either straight or undulated in a planar wave pattern, whereas those located at the periphery are arranged in a helical wave pattern. The purpose of the wave and nonlinear pattern of the fibrils has been interpreted as "crimp" and "recruitment", respectively (Smith et al., 1993). Crimp enable slight longitudinal elongation may occur without fibrous damage due to its regular sinusoidal pattern in the matrix. It also controls its tension and acts as a shock-absorber along the length of the tissue. Hence, during tensile stretch, fibril crimp is first straightened out by small loads, after which larger loads are needed to elongate these fibrils (Duthon et al., 2006).

Joint stability, therefore, is the general role of ligaments without which the joint may subluxate, cause damage to the capsule, cartilage, tendons, nearby nerves and blood vessels, and to the ligaments themselves. Such injury may debilitate the individual by preventing the use of the joint and loss of function. Unstable joints are also known to drastically modify the intra-articular pressure and the muscular activity about the joint, resulting in early onset of osteoarthritis, pain, disability and eventually the need for joint replacement surgery. Dysfunctional or ruptured ligaments, therefore, result in a complex syndrome, various sensory–motor disorders and other long-term consequences which impact the individual's well-being, employer, skilled work force pool and national medical expenses (Solomonow, 2004).

Disruption of the ACL is more frequently a result of a non-contact injury. The mechanism of the injury may be flexion valgus plus external rotation movement, flexion varus plus internal rotation loading, forced external rotation or hyperextension (Deehan and Cawston, 2005).

Four phases in the response to injury of the ruptured human ACL are observed histologically; these include an inflammatory phase, an epiligamentous repair phase, a proliferative phase, and a remodeling phase (Murray et al., 2000).

ACL does not appear to form a bridging scar after rupture (Hefti et al., 1991). After rupture, the human ACL appears to retract initially, with no evidence of healing (Warren, 1983). In the inflammatory phase, within 72 h of the injury, when there are inflammatory signs as swelling, redness, elevated temperature and pain demonstrate that a healing process is underway. The collagen fibers are undergoing changes in cellular, metabolic and vascular condition in order to improve the mechanical properties of the ligament. The inflammation also manages the breakdown and removal of damaged protein and the importation of new protein to repair and reconstruct the micro-damage and hypertrophy the tissue (Solomonow, 2004). During the epiligamentous repair phase that lasts eight to twelve weeks, synovial tissue was refined and covered the ends of the ruptured ligament. Most of the synovial lining cells were myofibroblast-like cells that contained α-smooth muscle actin, suggesting that the early formation of synovial tissue over the ends of the ligament combined with the formation of myofibroblast-like cells with contractile properties in this synovial tissue might be responsible in part for the retraction of the ligament and its poor healing. Other reported characteristics of the healing of dense connective tissue, such as fibroblast proliferation, expression of α-smooth muscle actin, and angiogenesis, all occur in the human ACL. The proliferative fibroblast response, while similar to that seen in tissues that heal, occurs at a later timepoint after ruptures of this ligament (Murray et al., 2000).

The biology of the response of the human ACL to complete rupture is a complex process. It differs from that of tissues that successfully heal however in that ligament there is an epiligamentous reparative phase and a lack of any bridging scar formation. The ACL is different from other intra-articular tissues that fail to heal. The poor wound-healing response has been noted both histologically in animal models and clinically in humans. Therefore, is important to understand why the ACL has difficulty to heal after rupture (Murray et al., 2000).

2.3. HBO applications in Sports Medicine

Apart from the above mentioned accepted indications by ECHM, UHMS or both, there are numerous other indications, often based on little or no evidence, as sports injuries (De Laet et al., 2008).

The application of HBO for the treatment of sports injuries has recently been suggested in the scientific literature as a therapy modality: a primary or an adjunct treatment (Babul et al., 2003). Although results have proven to be promising in terms of using HBO as a treatment modality in sports-related injuries, these studies have been limited due to small trials, lack of blinding and randomization problems (Babul and Rhodes, 2000).

Even less studies referring the use of HBO in high level athletes can be found in the literature. Ishii et al. (2005) reported the use of HBO as a recovery method for muscular fatigue during the Nagano Winter Olympics. In this experiment seven Olympic athletes received HBO treatment during 30-40 minutes at 1.3 ATA with a maximum of six times per athlete and an average of two. It was found that all players benefited from the HBO treatment presenting faster recovery rates. These results are concordant with those obtained by Fisher et al. (1999) and Haapaniemi et al. (1996) that suggested that lactic acid and ammonia were removed faster with HBO treatment leading to shorter recovery periods.

i. Muscle Injuries

Oriani et al. (1982) first suggested that HBO might accelerate the rate of recovery from injuries suffered in sports. However, the first clinical report appeared only in 1993 where results suggested a 55% reduction in lost days to injury, in professional soccer players in Scotland suffering from a variety of injuries following the application of HBO. These values were based on a physiotherapist's estimation of the time course for the injury versus the actual number of days lost with routine therapy and HBO treatment sessions (James et al., 1993). Although promising, this study needed a control group and required a greater homogeneity of injuries as suggested by Babul et al. (2000).

Delayed Onset Muscle Soreness

Delayed onset muscle soreness (DOMS) describes a phenomenon of muscle pain, muscle soreness or muscle stiffness that is generally felt 12-48 hours after exercise, particularly at the beginning of a new exercise program, after a change in sport

activities, or after a dramatic increase in the duration or intensity of exercise. DOMS is accompanied by a sensation of discomfort within the skeletal muscle experienced by the novice or elite athlete. The intensity of discomfort increases within the first 24 hours following cessation of exercise, peaks between 24 and 72 hours, subsides and eventually disappears by 5–7 days post-exercise (Cervaens and Barata, 2009).

Staples et al. (1995) in an animal study, used a downhill running model to induce damage, and observed significant changes in the myeloperoxidase levels in rats treated with hyperbaric oxygen compared to untreated rats. It was suggested that hyperbaric oxygen could have an inhibitory effect on the inflammatory process or the ability to actually modulate the injury to the tissue.

In 1999, the same author (Staples et al., 1999) conducted a randomized, controlled, double-blind, prospective study to determine whether intermittent exposures to hyperbaric oxygen enhanced recovery from delayed-onset muscle soreness of the quadriceps by using 66 untrained men between the ages of 18 and 35 years. After the induction of muscle soreness, subjects were treated in a hyperbaric chamber over a 5-day period in two phases: the first phase with four groups (control, hyperbaric oxygen treatment, delayed treatment, and sham treatment); and in the second phase three groups (3 days of treatment, 5 days of treatment, and sham treatment). The hyperbaric exposures involved 100% oxygen for 1 hour at 2.0 ATA. The sham treatments involved 21% oxygen for 1 hour at 1.2 ATA. There was noted, in phase 1, a significant difference in recovery of eccentric torque in the treatment group compared with the other groups as well as in phase 2, where there was also a significant recovery of eccentric torque for the 5-day in the treatment group comparing to sham group, immediately after exercise and upper to 96 hours after exercise. However, there was no significant difference in pain in either phase. The results suggested that treatment with hyperbaric oxygen may enhance recovery of eccentric torque of the quadriceps muscle from delayed-onset muscle soreness. This study had a complex protocol and the experimental design wasn’t entirely clear (exclusion of some participants and the allocation of groups wasn’t clarified) which makes interpretation difficult (Bennett et al., 2005a).

In 2000, another clinical trial did not find any recovery from DOMS after HBO (Mekjavic et al., 2000). They studied 24 healthy male subjects who were randomly assigned to a placebo group or a HBO group after being induced DOMS in their right elbow flexors. The HBO group was exposed to 100% oxygen at 2.5 ATA and the sham group to 8% oxygen at 2.5 ATA both for 1 hour per day and during 7 days. Over the period of 10 days there was no difference in the rate of recovery of muscle strength between the two groups as well as the perceived pain. Although being a randomized, double-blind trial, this was a small study (Bennett et al., 2005a).

Harrison et al. (2001) also studied the effect of HBO in 21 healthy male volunteers after inducing DOMS in the elbow flexors. The subjects were assigned inequitable into three groups: control, immediate HBO and delayed HBO. These two last groups were exposed to 2.5 ATA, during 100 min with 3 periods of 30 min at 100% oxygen intercalated with 5 min with 20.93% oxygen between them. The first group began the treatments with HBO after 2h and the second group 24h post-exercise and both were administered daily during 4 days. The delayed HBO group also suffered a sham treatment with HBO at the day 0 during the same time as the following days but with 20.93% oxygen at minimal pressure. The control group had no specific therapy. There were no significant differences between groups in serum Creatine Kinase (CK) levels, isometric strength, swelling and pain, which suggested that HBO was not effective on DOMS. This study also presented limitations like small sample size and just partial blinding (Bennett et al., 2005a).

Webster et al. (2002) wanted to determine if HBO accelerated recovery from exercise induced muscle damage in 12 healthy male volunteers that underwent strenuous eccentric exercise of the gastrocnemius muscle. The subjects were randomly assigned to 2 groups, where the first was the sham group who received HBO with atmospheric air at 1.3 ATA, and the second with 100% oxygen with 2.5 ATA, both for 60 minutes. The first treatment took place 3 to 4 hours after damage followed by treatments after 24h and 48 hours. There was little evidence in the recovery measured data, highlighting a faster recovery in the HBO group in the isometric torque, pain sensation and unpleasantness. However, it was a small study with multiple outcomes and some data were not used due to difficulties in interpretation (Bennett et al., 2005a).

Babul et al. (2003) also conducted a randomized, double-blinded study in order to find out if HBO accelerated the rate of recovery from DOMS in the quadriceps muscle. This exercise-induced injury was produced in 16 sedentary female students that were assigned into 2 groups: control and HBO. The first was submitted to 21% oxygen at 1.2 ATA, and the second to 100% oxygen at 2.0 ATA for 60 minutes at 4, 24, 48 and 72 hours post-injury. There were no significant differences between the groups in the measured outcomes. However, again this was also a small study with multiple outcomes, with a complex experimental design with 2 distinct phases with somewhat different therapy arms (Bennett et al., 2005a).

Germain et al. (2003) had the same objective as the previous study but this time the sample had 10 female and 6 male subjects that were randomly assigned into 2 groups: the control group that did not undergo any treatment and the HBO group that was exposed to 95% oxygen at 2.5 ATA during 100 minutes for 5 sessions. There were no significant differences between both groups which lead to the conclusion that HBO did not accelerate the rate of recovery of DOMS in the quadriceps. Once again, this was a very small and unblinded study that presented multiple outcomes (Bennett et al., 2005a).

Muscle stretch injury

In 1998, a clinical trial wanted to analyze if HBO improved functional and morphologic recovery after a controlled induced muscle stretch in the *tibialis anterior* muscle-tendon unit (Best et al., 1998). They used a rabbit model of injury and the treatment group was submitted to a 5-day treatment with 95% oxygen at 2.5 ATA for 60 minutes. Then, after 7 days, this group was compared to a control group that did not suffer HBO treatment. The results suggested that HBO administration may play a role in accelerating recovery after acute muscle stretch injury.

Ischemia

Another muscle injury that is often a consequence of trauma is ischemia. Normally it is accompanied by anaerobic glycolysis, the formation of lactate and depletion of high-

energy phosphates within the extracellular fluid of the affected skeletal muscle tissue. When ischemia is prolonged it can result in loss of cellular homeostasis, disruption of ion gradients and breakdown of membrane phospholipids. The activation of neutrophils, the production of oxygen radicals and the release of vasoactive factors, during reperfusion, may cause further damage to local and remote tissues. However, the mechanisms of ischemia–reperfusion induced muscle injury are not fully understood (Bosco et al., 2007). These authors aimed to see the effects of HBO in the skeletal muscle of rats after ischemia-induced injury and found that HBO treatment attenuated significantly the increase of lactate and glycerol levels caused by ischemia, without affecting glucose concentration, and modulating anti-oxidant enzyme activity in the post-ischemic skeletal muscle.

A similar study was performed in 1996 in which the authors concluded that HBO had positive aspects for at least 48 hours after severe injury, by raising the levels of high-energy phosphate compounds which indicated a stimulation of aerobic oxidation in the mitochondria (Haapaniemi et al., 1996). This maintains the transport of ions and molecules across the cell membrane and optimizes the possibilities of preserving the muscle cell structure.

Gregorevic et al. (2000), induced muscle degeneration in rats in order to see if HBO hastens the functional recovery and myofiber regeneration of the skeletal muscle. The results of this study demonstrated that the mechanism of the improved functional capacity is not associated with the reestablishment of a previously compromised blood supply or with the repair of associated nerve components, as seen in ischemia, but with the pressure of oxygen inspired with a crucial role in improving the maximum force-producing capacity of the regenerating muscle fibers after this myotoxic injury. In addition, there were better results following 14 days of HBO treatment at 3 ATA than at 2 ATA.

ii. Ligament injuries

In 1995, a study conducted at the Temple University suggested that patients treated with HBO returned approximately 30% faster than the control group after ankle sprain. The

authors, however, stated that there was a large variability in this study design due to the difficulty in quantifying the severity of sprains (Staples and Clement, 1996).

Interestingly, Borromeo et al. (1997), in a randomized, double-blinded study, observed in 32 patients who had acute ankle sprains the effects of HBO in its rehabilitation. The HBO group was submitted to 100% oxygen at 2 ATA for 90 minutes the first session and the other two for 60 minutes. The placebo group was exposed to ambient air, at 1.1 ATA for 90 minutes, both groups for 3 sessions over 7 days. The HBO group had an improvement in joint function. However, there were no significant differences between groups in the subjective pain, edema, passive or active range of motion or time to recovery. This study included an average delay of 34 hours from the time of injury to treatment, and it had short treatment duration (Bennett et al., 2005a).

Medial Collateral Ligament

Horn et al. (1999) in an animal study surgically lacerated medial collateral ligament of 48 rats. Half of them were the control group without intervention, the other half were exposed to HBO at 2.8 ATA, for 1.5 hours a day during 5 days. Six rats from each group were euthanized at 2, 4, 6, and 8 weeks and at 4 weeks a statistically greater force was required to cause failure of the previously divided ligaments for those exposed to HBO than in the control group. After 4 weeks an interesting contribution from HBO could be seen by promoting the return of normal stiffness of the ligament.

Ishii et al. (2002) induced ligament lacerations in the right limb of 44 rats and divided them into 4 groups: the control group, animals that breathed room air at 1 ATA for 60 min; HBO treatment at 1.5 ATA for 30 min once a day; HBO treatment at 2 ATA for 30 min once a day; and 2 ATA for 60 min once a day. After 14 days post-induced injury, of the three exposures the last group was more effective in promoting healing by enhancing extra-cellular matrix deposition as measured by collagen synthesis.

Mashitori et al. (2004) removed a 2-mm segment of the medial collateral ligament in 76 rats. Half of these rats were exposed to HBO at 2.5 ATA for 2 hours during 5 days per week and the remaining rats were exposed to room air. The authors observed that HBO

promotes scar tissue formation by increasing Type I procollagen gene expression, at 7 and 14 days after the injury, which contribute for the improvement of their tensile properties.

In a randomized, controlled and double-blinded study the author examined the effect of HBO at the recovery of a grade II medial ligament of the knee presented in patients within 72 hours of injury. After one group being exposed to HBO at 2 ATA for 1 hour and the control group at 1.2 ATA, room air, for 1 hour, both groups during 10 sessions, the data suggested that, at 6 weeks, HBO had positive effects on pain and functional outcomes, like decreased the volume of edema, a better range of motion and maximum flexion improvement, comparing to sham group (Soolsma, 1996).

Anterior Cruciate Ligament

Yeh et al. (2007) used an animal model to investigate the effects of HBO on neovascularization at the tendon-bone junction, collagen fibers of the tendon graft, and the tendon graft-bony interface which is incorporated into the osseous tunnel. The authors used 40 rabbits that were divided into two groups: the control group that was maintained in cages at normal air and the HBO group that was exposed to 100% oxygen at 2.5 ATA for 2 hours, during 5 days. The authors found that HBO group had significantly increased the amount of trabecular bone around the tendon graft, increasing its incorporation to the bone and therefore increasing the tensile loading strength of the tendon graft. They assumed that HBO contributes for the angiogenesis of blood vessel, improving the blood supply which leads to the observed outcomes.

Takeyama et al. (2007) studied the effects of HBO on gene expressions of procollagen and tissue inhibitor of metalloproteinase (TIMPS) in injured anterior cruciate ligaments in rats. After surgical injury animals were divided into a control group and a group that was submitted to HBO, 2.5 ATA for 2 hours, during 5 days. It was found that even though none of the lacerated ACLs united macroscopically, there was an increase of the gene expression of type I procollagen and of TIMPS 1 and 2 for the group treated with HBO. These results indicate that HBO enhances structural protein synthesis and inhibits degradative processes. Consequently using HBO as an adjunctive therapy after primary

repair of the injured ACL is likely to increase success, situation that is confirmed by the British Medical Journal Evidence Center (Minhas, 2010).

iii. Fractures

Classical treatment with osteosynthesis and bone grafting is not always successful and the attempt to heal nonunion and complicated fractures where the chance of infection is increased is a challenge.

A Cochrane review (Bennett et al., 2005b) stated that there is not sufficient evidence to support hyperbaric oxygenation for the treatment of promoting fracture healing or nonunion fracture as no randomized evidence was located. During the last ten years this issue has not been the subject of many studies.

Okubo et al. (2001) studied in a rat model, where recombinant human bone morphogenetic protein-2 was implanted in the form of lyophilised discs, the influence of HBO. The group treated with HBO, exposed to 2 ATA for 60 min daily, had significantly increased new bone formation, the cartilage was present at the outer edge of the implanted material after 7 days, comparing to group control.

Komurcu et al. (2002) reviewed retrospectively 14 cases of infected tibial nonunion that were treated successfully. Management included aggressive debridement and correction of defects by corticotomy and internal bone transport. The infection occurred in two patients after the operation which was successfully resolved after 20 - 30 sessions of HBO.

Muhonen et al. (2004) aimed to study, in a rabbit mandibular distraction osteogenesis model, the osteogenic and angiogenic response to irradiation and HBO. One group was exposed to 18 sessions of HBO till the operation that was performed 1 month after irradiation. The second group did not receive HBO and the controls underwent surgery receiving neither irradiation nor HBO. The authors concluded that previous irradiation suppresses osteoblastic activity and HBO changes the pattern of bone forming activity towards that of non-irradiated bone.

Wang et al. (2005), in a rabbit model, were able to demonstrate that distraction segments of animals treated with HBO had increased bone mineral density and superior mechanical properties comparing to the controls and yields better results when applied during the early stage of tibial healing process.

In summary, oxygen can be considered as a drug and it can be administered easily under normobaric conditions, but administering oxygen at pressures higher than 1 ATA requires compression. Due its physiological benefits it was tempting to analyze its effects on the recovery of different sports injuries. Each chapter that follows corresponds to independent trial studies with specific objectives.

CHAPTER III

EXPERIMENTAL WORK: PAPER I

Hyperbaric oxygen therapy treatment for the recovery of muscle injury induced in rats

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Abstract

Objective: The purpose of this work was to evaluate the effect of hyperbaric oxygen therapy (HBO) in the recovery of muscle injury in Wistar rats.

Methods: Twelve female Wistar rats, weighting 200-250 g were submitted to contusion of the right gastrocnemius. Animals were then randomly assigned to two groups, an untreated control group and an HBO-treated group. The HBO group was given three 60-minute session of HBO at 253 kPa 24, 48 and 72 hours after the injury. After the last session the animals were sacrificed and the right gastrocnemius removed and blood samples taken for creatine phosphokinase (CPK). The left hamstring muscle was used as an internal control. The muscles were analysed for their biomechanical properties, hardness, maximum elongation and maximum weight using a standard technique on a traction machine.

Results: Significant differences were found between uninjured and injured muscles and between untreated and HBO groups in maximum weight and hardness: maximum weight in the non-treated group 18.27± 2.99 N versus 26.18 ± 2.84 N in the HBO group (P = 0,007) . For hardness 2.24 ± 0.38 10^3 N/m for the non-treated group versus 3.19 ± 0.32 10^3 N/m in the HBO group (P = 0,001). CPK was significantly different between the two groups (non-treated 6445.0 ± 387.3 UI/l; HBO group 4550.7± 79.5 UI/l; P = 0,009).

Conclusions: HBO seem to play a positive role in the recovery of induced muscle injury in rats. However relevant, these results cannot be extrapolated to humans, for whom further clinical studies are warranted.

Key-words: Hyperbaric oxygen, hyperbaric oxygen therapy, muscle-skeletal, injuries, research

1. Introduction

Hyperbaric oxygen therapy (HBO) is a clinical intervention, in which patients breathe pure oxygen (100%) intermittently while inside a dedicated chamber at a pressure higher than sea level pressure, i.e., more than 101.3 kPa. The mode of action of HBO is complex, the result of a number of physiological and pharmacological mechanisms based on elevation of both the partial pressure of oxygen and of the hydrostatic pressure (Thom, 2009).

Muscle injury presents a challenging problem in traumatology and is very common in sports medicine (Järvinen et al., 2000). This injury may be a consequence of direct mechanical deformation (e.g., contusions, lacerations, and strain) or of indirect causes (e.g., ischaemia and neurological damage) (Li et al., 2001). According to Järvinen et al. (2000), more than 90% of muscle injuries are caused either by excessive strain or by contusion of the muscle. A muscle suffers a contusion when it is submitted to a sudden, heavy compressive force, such as a direct blow (Järvinen et al., 2007), that is classified as mild, moderate or severe. The first represents a tear in a few muscle fibers with few symptoms that do not interfere with mobility. The second involves greater damage to the muscle affecting its function. In severe injuries, a tear extends across the entire cross-section of the muscle, preventing normal function (Järvinen et al., 2007).

There is an emerging need for improved therapies that allow the injured athlete to return to competition faster and with a low risk of re-injury. The role of HBO in the recovery of muscle injuries has been debated for several years, but remains poorly understood (Crisco et al., 1994, Barata et al., 2011). Therefore, considering this lack of consistent research on HBO we aimed to evaluate the effect of HBO in the recovery of muscle contusion inflicted to rats by measuring its biomechanical properties and haematological markers of muscle injury.

2. Materials and methods

2.1. Animals

Twelve female rats, *Rattus norvegicus albinos*, Wistar type, weighting 200−250 g, were studied. The animals were kept in the facilities of the Laboratory of the Faculty of Pharmacy, University of Porto, in collective cages with two animals per cage, at room temperature, receiving water and standard food *ad libitum*. All procedures were performed according to the FELASA recommendations for animal welfare and according to Portuguese legislation.

2.2. Procedure for induction of the injury

Prior to the induction of the injury, the animals were anesthetized using 60 mg/kg Ketamine (IP) and 8 mg/kg Xylazine (IP). They were then positioned at the base of the lesion production equipment, in the ventral *decubitus* position, with their knee at maximal extension and ankle in neutral position (90º). In order to cause the lesion, a 171 g weight was released from a 102 cm height onto the belly of the right gastrocnemius muscle according to other studies (Crisco et al., 1994) (Figure 1).

Figure 1. Model of muscle contusion in rats

Animals were then randomly assigned in two groups, the control group received no treatment and the treatment group received three 60-minute sessions of HBO at 253 kPa at 24, 48 and 72 hours after the injury (Figure 2). After the third session, the animals were sacrificed and the right gastrocnemius was surgically removed and blood samples withdrawn. The left gastrocnemius was also removed and used as an uninjured, untreated internal control.

Figure 2. Rats in Hyperbaric chamber

2.3. Traction mechanical essay

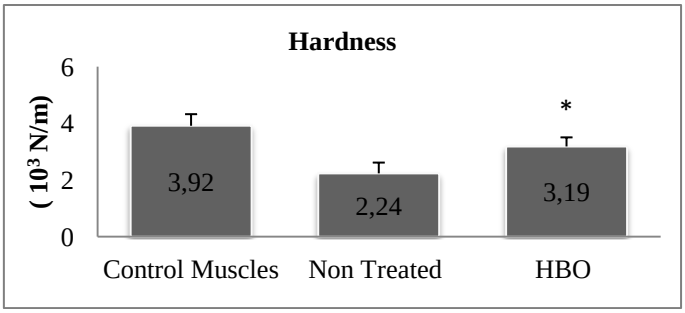
The removed muscle was biomechanically analysed using a traction machine (EMIC, DL 10000) parameters as maximum elongation (ME), maximum weight (MW) and hardness (H). Blood samples were analysed for creatine phosphokinase (CPK). For the traction experiment, the machine was loaded with a charge of 50 kgf, and pre-charge of 200 g was applied during 30 seconds for system accommodation. Afterwards, the assays were performed at a speed of 10 mm min^{-1}. ME corresponds to the maximum length of the muscle string before rupture; MW represents the maximum of imposed load before muscle rupture and H is a property obtained by the software (Tesc) that is determined by the slope of the line obtained in the elastic phase of the process.

2.4. Statistical analysis

The statistical analysis was performed through the BioEstat® program v. 2.0. The Kolmogorov Smirnov normality test was performed. An Anova analysis combined with a Bonferroni post hoc test were used to evaluate differences between the groups. A pre-established significance level of 0.05 was used.

3. Results

For hardness, the internal control muscles had $3.92 \pm 0.41\ 10^3$ N m^{-1}, the non-treated group $2.24 \pm 0.38\ 10^3$ N m^{-1} and the HBO group $3.19 \pm 0.32\ 10^3$ N m^{-1} (Figure 3).



* p=0,001 HBO vs. Non Treated

Figure 3. Mechanical properties for hardness (10^3 N m^{-1}) in a non-injured control muscle and injured non-treated and HBO-treated groups

For maximum elongation, the internal control muscles had 13.40 ± 1.61 10^{-3} m, the non-treated group 10.91 ± 2.20 10^{-3} m and the HBO group 11.70 ± 2.32 10^{-3} m (Figure 4).

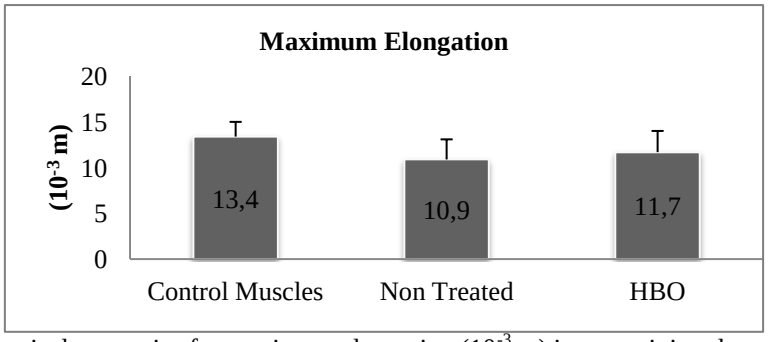
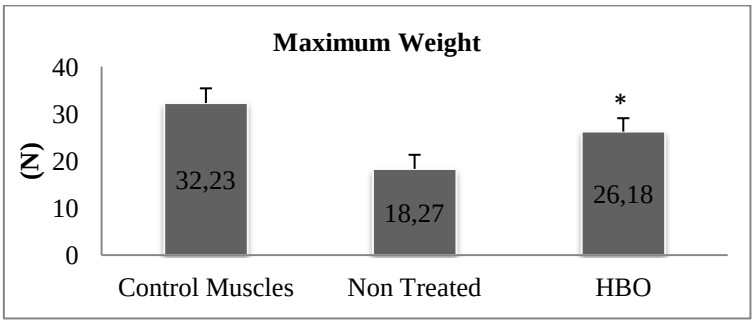


Figure 4. Mechanical properties for maximum elongation (10^{-3} m) in a non-injured control muscle and injured non-treated and HBO-treated groups

For maximum weight, the results were 32.23 ± 3.12 N for the internal control muscles, 18.27 ± 2.99 N for the non-treated group and 26.18 ± 2.84 N for the HBO group (Figure 5).



* p=0,007 HBO vs. Non Treated

Figure 5. Mechanical properties for maximum weight (N) in in a non-injured control muscle and injured non-treated and HBO-treated groups

For CPK, significant differences were found between the non-treated (6445.0 ± 387.3 IU L^{-1}) and the HBO group (4550.7 ± 79.5 IU L^{-1}).

4. Discussion

Not surprisingly, the non-injured muscle always had better results than the injured muscle independently of whether the animal received HBO. This indicates that the injury protocol used was effective.

There were significant differences in hardness (p=0,001) and maximum weight (p=0,007) between the non-treated with HBO groups. Furthermore, the non-treated group demonstrated a significant elevation of CPK (p=0,009), a marker of muscle injury (Cheung et al., 2003) compared to the HBO group but not the non-treated group, indicating that HBO had a positive effect on muscle injury recovery.

There is a lack of studies about the effect of HBO on biomechanical properties of muscle injuries, particularly regarding to contusion. Therefore, it is necessary to discuss other types of muscles injury as well as other measured parameters in other studies. Best et al. (1998) analyzed the use of HBO after an acute muscle stretch injury in rabbits. The 18 animals of the study were submitted to a partial stretch injury to the left *tibialis anterior* muscle-tendon unit. After being randomly assigned into two groups, after 24 hours, the first group received HBO at 253 kPa for 60 minutes daily for five days while the second group had no treatment. Seven days after the injury, having the right leg as a sham-operated control, the HBO group had a minor deficit of ankle isometric torque comparing to the non-treated group and more complete recovery, suggesting that HBO may play a role in accelerating recovery after acute muscle stretch injury. Similarly, Haapaniemi et al. (1996) examined the effect of HBO on muscle ischaemia in Sprague-Dawley rats. After 4 hours of ischaemia, the changes in levels of intracellular muscle compounds adenosine triphosphate, phosphocreatine, and lactate were less in the HBO-treated rats than in the untreated animals.

In humans, Borromeo et al. (1997) performed a randomized double-blind trial in 32 subjects with acute ankle sprains. Treatment consisted of three HBO sessions at 203 kPa. With this regimen, HBO did not influence ankle oedema, subjective pain indices, passive motion indices or time to recovery. Staples et al. (1999) ran a randomized placebo-controlled trial in 66 patients with muscle soreness of the quadriceps. They compared a control group, sham, immediate and delayed HBO groups. Delayed treatment and delayed sham were done at three or five days following injury using 203 kPa. Immediate HBO patients had a better recovery than those treated at three days, while a delay of five days to HBO provided the best result. In all groups, pain was similar.

Staples and Clement (1996) reported that if HBO is applied within eight hours after tissue injury, accelerated recovery from soft tissue injury resulted. This was attributed to known actions of HBO such as reduction of local hypoxia and inflammation, promotion of vasoconstriction, reduction of the adhesion of neutrophils, extinction of free radicals, control of oedema, increased leukocyte activity and promotion of procedures for the synthesis of collagen and blood vessel growth. In a Cochrane review examining the effect of HBO on delayed onset muscle soreness in untrained individuals no conclusive evidence of benefit was found on the speed of recovery in muscular pain (Crisco et al., 1994, Babul et al., 2003, Germain et al., 2003, Mekjavic et al., 2000, Webster et al., 2002). This review stated that more and larger randomized trials were needed to determine the role of HBO in the treatment of sport injuries.

Balestra et al. (2004), demonstrated that oxygen is involved in positive effects on lymphatic vessel metabolism and edema reduction, with interesting clinical applications namely due to the enhanced protein removal by the lymphatic system.

However, the lack of current scientific literature concerning biomechanical properties of skeletal muscle following injury, and the use of HBO as a therapeutic tool complicate the interpretation of results. In the present study, we used rats not only as an established animal injury model but also because, according to some authors they exhibit musculoskeletal structure similar to humans (Järvinen et al., 1992). However, the results

of this study cannot be transferred to humans, but should give guidance to further research.

5. Conclusions

Analysing three biomechanical properties of injured muscle and CPK as a systemic marker of muscle injury, HBO seems to play a positive role in the recovery of induced muscle injury in rats. However, there is still much we need to understand and optimize concerning the use of HBO in sports injuries treatments and therefore more studies are warranted.

6. Acknowledgments

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7. References

BABUL, S., RHODES, E., TAUNTON, J. & LEPAWSKY, M. (2003) Effects of Intermittent Exposure to Hyperbaric Oxygen for the Treatment of an Acute Soft Tissue Injury. *Clinical Journal of Sports Medicine,* 13**,** 138-147.

BALESTRA, C., GERMONPRÉ, P., SNOECK, T., EZQUER, M., LEDUC, O., LEDUC, A., WILLEPUT, F., MARRONI, A., CALI CORLEO, R. & VANN, R. (2004) Normobaric oxygen can enhance protein captation by the lymphatic system in healthy humans. *Undersea and Hyperbaric Medicine,* 31**,** 59-62.

BARATA, P., CERVAENS, M., RESENDE, R., CAMACHO, O. & MARQUES, F. (2011) Hyperbaric oxygen on sports injuries. *Therapeutic Advances in Musculoskeletal Disease,* 3**,** 111-121.

BEST, T., LOITZ-RAMAGE, B., CORR, D. & VANDERBY, R. J. (1998) Hyperbaric oxygen in the treatment of acute muscle stretch injuries: results in an animal model. *American Journal of Sports Medicine,* 26**,** 367-372.

BORROMEO, C. N., RYAN, J. L., MARCHETTO, P. A., PETERSON, R. & BOVE, A. A. (1997) Hyperbaric oxygen therapy for acute ankle sprains. *American Journal of Sports Medicine,* 25**,** 619-625.

CHEUNG, K., HUME, P. A. & MAXWELL, L. (2003) Delayed onset muscle soreness: treatment strategies and performance factors. / La douleur musculaire differee: strategies de therapie et facteurs de performance. *Sports Medicine,* 33**,** 145-164.

CRISCO, J., JOKL, P., HEINEN, G., CONNELL, M. & PANJABI, M. (1994) A muscle contusion injury model. Biomechanics, physiology, and histology. *American Journal of Sports Medicine,* 22**,** 702-10.

GERMAIN, G., DELANEY, J., MOORE, G., LEE, P., LACROIX, V. & MONTGOMERY, D. (2003) Effect of hyperbaric oxygen therapy on exercise-induced muscle soreness. *Undersea and Hyperbaric Medicine,* 30**,** 135-145.

HAAPANIEMI, T., NYLANDER, G., SIRSJÖ, A. & LARSSON, J. (1996) Hyperbaric Oxygen Reduces Ischemia-Induced Skeletal Muscle Injury. *American Society of Plastic Surgeons,* 97**,** 602-607.

JÄRVINEN MJ, EINOLA SA & EO, V. (1992) Effect of the position of immobilization upon the tensile properties of the rat gastrocnemius muscle. *Archives of Physical Medicine and Rehabilitation,* 73**,** 253-7.

JÄRVINEN, T., JÄRVINEN, T., KÄÄRIÄINEN, M., ÄÄRIMAA, V., VAITTINEN, S., KALIMO, H. & JÄRVINEN, M. (2007) Muscle injuries: optimising recovery. *Best Practice & Research Clinical Rheumatology,* 21**,** 317-331.

JÄRVINEN, T., KÄÄRIÄINEN, M., JÄRVINEN, M. & KALIMO, H. (2000) Muscle strain injuries. *Current Opinion in Rheumatology,* 12**,** 155-161.

LI, Y., CUMMINS, J. & HUARD, J. (2001) Muscle Injury and Repair. *Current Opinion in Orthopaedics,* 12**,** 409-415.

MEKJAVIC, I. B., EXNER, J. A., TESCH, P. A. & EIKEN, O. (2000) Hyperbaric oxygen therapy does not affect recovery from delayed onset muscle soreness. *Medicine & Science in Sports & Exercise,* 32**,** 558-563.

STAPLES, J. & CLEMENT, D. (1996) Hyperbaric oxygen chambers and the treatment of sports injuries. *Sports Medicine,* 22**,** 219-227.

STAPLES, J., CLEMENT, D., TAUNTON, J. & MCKENZIE, D. (1999) Effects of Hyperbaric Oxygen on a Human Model of Injury. *The American Journal of Sports Medicine,* 27**,** 600-605.

THOM, S. R. (2009) Oxidative stress is fundamental to hyperbaric oxygen therapy. *Journal of Applied Physiology (Bethesda, Md.: 1985),* 106**,** 988-995.

WEBSTER, A., SYROTUIK, D., BELL, G., JONES, R. & HANSTOCK, C. (2002) Effects of Hyperbaric Oxygen on Recovery From Exercise-Induced Muscle Damage in Humans. *Clinical Journal of Sports Medicine,* 12**,** 139-150.

CHAPTER III

EXPERIMENTAL WORK: PAPER II

The influence of hyperbaric environment skeletal muscle mitochondrial energetic of rats after induced muscle contusion

Abstract

Purpose: Analyze the influence of hyperbaric environment with oxygen and with normal air on skeletal muscle mitochondrial energetic of rats after induced muscle contusion, by determining end points related to oxygen consumption, transmembrane electric potential and permeability transition pore susceptibility in isolated mitochondria.

Methods: Twelve female Wistar rats were randomly assigned into three groups. All rats were submitted to muscle contusion in the right *gastrocnemius*. The control group (C) had no treatment, the hyperbaric group (HB) and the hyperbaric oxygen (HBO) group had four 60 minutes session of hyperbaric therapy at 253.25 kPa (2.5 ATA), receiving normal air or 100% oxygen, respectively. The animals were sacrificed 48h after muscle injury and both muscles (injured and non injured) were analyzed. Muscle mitochondrial respiratory activity, membrane potential and mitochondrial swelling during mitochondrial permeability transition pores (MPTP) induction were evaluated.

Results: In all parameters there were significant differences between the injured *gastrocnemius* (right) and the non injured *gastrocnemius* (left) in C group. HB group have significantly achieved better results in terms of respiratory chain in complex I and II on the right *gastrocnemius* but in the HBO group, the time to V_{max} was significantly higher than other groups and, the swelling amplitude was significantly smaller than the other groups which can suggest a lower susceptibility to MPTP opening.

Conclusion: This study concluded that HBO had an important role in mainting the integrity of mitochondria by delaying the time of sweeling.

Keywords: muscle contusion, hyperbaric oxygen, mitochondrial energetic, permeability transition pore

1. Introduction

Muscle injuries and damage are some of the main concerns of both sports medicine and sports performance. Contusion injuries have been reported to be common in contact sports (Smith et al., 2008) and its severity can range from focal intracellular damage to segmental damage or necrosis affecting the whole muscle bundles (Järvinen et al., 2007). In experimental animals, skeletal muscle contusion injury models were firstly suggested in 1974 (Kvist et al., 1974). Regardless some minor variations depending on the purposes and labs, the most commonly described modeuses of a single impact trauma to the skeletal muscle (Stratton et al., 1984, Crisco et al., 1994). At the ultrastructural level, these injuries can cause capillary rupture and infiltrative bleeding, edema, and inflammation leading to hematoma formation (Beiner et al., 1999), similar to what is seen in the human muscle (Jackson and Feagin, 1973). Furthermore, disruption of the basal lamina and sarcolemma, loss of the membrane potential and segmental degeneration of sarcomeres may occur in myofibrils, indicating that several forms of cellular death and consequent tissue turnover initiates (Carlson and Faulkner, 1983).

Among the several mechanisms implicated in these processes, mitochondrial-associated alterations have been suggested to play a pivotal role. In fact, isolated mitochondria have been proved to be useful experimental model to assess muscle tissue damage under a variety of pathological and stressful conditions (Magalhães et al., 2013a, Magalhães et al., 2013b).

Mitochondria are major sources of cellular ATP, being Ca^{2+} the key regulator of mitochondrial function and therefore contribute to ATP synthesis. However, under conditions of Ca^{2+} overload in the mitochondrial matrix and overproduction of reactive oxygen species (ROS), some mitochondrial-based events are activated that result in cell death or remodeling. These include the triggering of apoptosis due to the release of mitochondrial proteins such as cytochrome-*c* from the intermembrane space into the cytosol after permeabilization of mitochondrial membranes (Brookes et al., 2004). As a highly energy demanding tissue, dependent on oxidative metabolism to cope with the

ATP requirements, it is tempting to speculate that skeletal muscle mitochondrial structural and functional integrity assume a pivotal role in the recovery from muscle injury.

Therefore, any therapeutic modalities that possibility decrease the recovery time and improve muscle functional parameters from these injuries have raised interest.

The application of Hyberbaric Oxygen Therapy (HBO) for the treatment of soft tissue injuries has recently been suggested in the scientific literature as an adjunct therapy (Barata et al., 2011).

HBO is a clinical intervention, in which patients breathe pure oxygen (100%) or sometimes normal air intermittently while inside a dedicated chamber at a pressure higher than 1 atmosphere absolute (Albuquerque and Sousa, 2007, De Laet et al., 2008).

During the last fifty years, several indications for hyperbaric therapy have been proposed including a wide variety of medical and surgical problems (Tibbles and Edelsberg, 1996). HBO mode of action is complex, since it is a result of a number of physiological and pharmacological mechanisms: these therapeutics mechanisms of action are based on elevation of both the partial pressure of oxygen and hydrostatic pressure (Thom, 2009). Clinical trials detected that oxygen, when inhaled in its pure state at hyperbaric environment, behaves like a multifaceted drug endowed with anti-ischemic, anti-hypoxic, anti-edematous, pro-healing and anti-infective effects (Staples and Clement, 1996). Although some reports suggesting that the application of 100% of oxygen in a hyperbaric environment may cause an increase in oxidative stress and therefore can retard skeletal muscle regeneration (Babul et al., 2003), in our recent experimental study (Cervaens et al., 2011), it was observed that 3 sessions of HBO at 2.5 ATA had positive effect on the biomechanical parameters of the skeletal muscle, like hardness and maximum weight, after inducing contusion injury in the *gastrocnemius* of rats. However, it is still unclear whether hyperbaric environment exposure modulates skeletal muscle mitochondrial bioenergetics after muscle contusion, knowing that this muscle trauma in rats' *gastrocnemius* induces a pronounced apoptotic

response in the beginning and at the end of the regenerative process (Hurme and Kalimo, 1992, Schaser et al., 2007).

Therefore, the purpose of the present study was to analyze the influence of hyperbaric environment with 100% oxygen and with normal air on skeletal muscle mitochondrial energetic of rats after induced muscle contusion, by determining end points related to oxygen consumption, transmembrane electric potential and permeability transition pore susceptibility in isolated mitochondria. The results of this study will contribute to a better understanding of how skeletal muscle bioenergetics and mitochondrial-mediated mechanisms are involved in the possible skeletal muscle regeneration through hyperbaric environment exposure after mechanical trauma.

2. Methods

2.1. Animals

Twelve female rats, *Rattus norvegicus albinos*, Wistar type rats, were used in this study. The animals were kept during the experimental protocols in collective cages with two animals per cage, at room temperature, receiving water and standard food *ad libitum*. All procedures were performed at the laboratory of the Faculty of Sport, University of Porto, Portugal and followed the recommendations for animal welfare according to the Federation of European Laboratory Animal Science Associations and the Portuguese legislation.

2.2. Muscle injury induction

Prior to the induction of the injury, the animals were anesthetized using 60 mg/kg Ketamine (IP) and 8 mg/kg Xylazine (IP). They were then positioned at the base of the lesion production equipment, in ventral *decubitus*. In order to cause the lesion, a 171*g* weight was released from a 102 cm height onto the right *gastrocnemius* muscle according to Crisco and colleagues (Crisco et al., 1994). The left *gastrocnemius* had no injury and was used as internal control.

2.3. Hyperbaric protocol

Animals were randomly assigned, using a computer program, in three groups, the control group that had no treatment, the hyperbaric (HB) group and the HBO group. These last two groups were submitted to four 60 minutes session of hyperbaric therapy at 253.25 kPa (2.5 ATA), receiving normal air or 100% oxygen, respectively. The sessions occurred 6, 12, 24 and 48 hours after the injury.

2.4. Preparation of Skeletal Muscle Mitochondria

After the fourth session, the animals were sacrificed by cervical dislocation while still anaesthetized and both *gastrocnemius* were surgically removed, using a scalp and appropriate techniques, for the preparation of isolated mitochondria. Skeletal muscle mitochondria were prepared by conventional methods of differential centrifugation, as previously described by Tonkonogi and Sahlin (1997).

After being immediately excised, for the preparation of isolated mitochondria, the muscles were minced in ice-cold isolation medium containing (in mM) 100 sucrose, 0.1 EGTA, 50 Tris-HCl, 100 KCl, 1 KH_2PO_4, and 0.2% BSA (bovine serum albumin), pH 7.4. Minced blood-free tissue was subsequently rinsed and suspended in 10 mL of fresh medium containing 0.2 mg/mL bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma) and stirred for 2 min. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjem homogenizer and a Teflon pestle. After homogenization, three volumes of Nagarse-free isolation medium were added to the homogenate, which was then fractionated by centrifugation at 700*g* for 10 min. The resulting pellet was removed, and the supernatant suspension was centrifuged at 10 000*g* for 10 min. The supernatant was decanted, and the pellet was gently resuspended in isolation medium (1.3 mL per 100 mg initial tissue) and centrifuged at 7 000*g* for 3 min. The supernatant was discarded, and the final pellet, containing the mitochondrial fraction, was gently resuspended (0.4 mL/mg initial tissue) in a medium containing (in mM) 225 mannitol, 75 sucrose, 10 Tris, and 0.1 EDTA (pH 7.4). All mitochondrial isolation procedures were performed at 0–4°C (Magalhães et al., 2005b).

Mitochondrial protein content was determined by the Biuret method calibrated with BSA. The remaining fresh mitochondrial suspensions were used within 4 h (maintained on ice at 0–4 °C throughout this period) for in vitro assays of mitochondrial oxygen consumption, transmembrane potential and spectrophotometric osmoting swelling (Lumini-Oliveira et al., 2011).

2.5. Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was polarographically measured, using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clark-type oxygen electrode (HansatechDW1, Norfolk,UK). Reactions were conducted in 0.75 mL closed thermostated (at 25ºC) and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in a respiration buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 μM EGTA, 2.5mM KH_2PO_4, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding malate plus pyruvate to a final concentration of 2 and 5 mM each, respectively, or succinate (10 mM) plus rotenone (4 μM). State 3 respiration was determined after adding ADP (400 μM) and state 4 respiration was measured as the rate of oxygen consumption after ADP phosphorylation. The respiratory control ratio (RCR) between state 3 and state 4, were calculated according to Estabrook (Estabrook, 1967), using 235 nmol O_2 per mL as the value for oxygen solubility at 25°C in doubly distilled water. Higher RCR is expected with malate/pyruvate as substrate for complex I in comparison with succinate as a substrate for complex II (Lumini-Oliveira et al., 2011).

In an independent assay, basal respiration was also measured in the presence of succinate, oligomycin (1.5 μg) and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) (2 μM) were subsequently added to inhibit ADP-induced state 3 and to induce uncoupled respiration, respectively (Lumini-Oliveira et al., 2011).

2.6. Mitochondrial transmembrane potential

Mitochondrial transmembrane potential ($\Delta\psi$) was indirectly monitored based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+), using a TPP^+ selective electrode prepared in our laboratory as described by Kamo et al. (1979) and with an AgCL reference electrode (Tacussel, Model MI 402). Both the TPP^+ electrode and the reference electrode were inserted into an open vessel with magnetic stirring and connected to a pH meter (Jenway, Model 3305). The signals were fed to a potentiometric recorder (Kipp & Zonen, Model BD121). Reactions were carried out in 1 mL of reaction buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 μM EGTA, 2.5 mM KH_2PO_4, pH 7.4, supplemented with 3 μM TPP^+ and 0.5 mg/mL of protein at 25 °C. For measurements of $\Delta\psi$ with complex I-linked substrates, energization was carried out with 5 mM of malate and 2 mM of pyruvate and ADP-induced phosphorylation was achieved by adding 400 nmol ADP. For measurements of $\Delta\psi$ with complex II-linked substrates, 10 mM succinate supplemented with 4 μM rotenone was added to the medium containing 3 μM TPP^+ and mitochondria. The lag phase, which reflects the time (in seconds) the mitochondria suspension needed to phosphorylate the added ADP, was also measured for both substrates (Lumini-Oliveira et al., 2011).

2.7. Determination of mitochondrial swelling during mitochondrial permeability transition pores (MPTP) induction

We performed all studies involving MPTP induction with succinate as the substrate. Mitochondrial osmotic volume changes were followed by monitoring the classic decrease of absorbance at 540 nm with a Jasco V-560 spectrophotometer. Swelling amplitude and time until maximal absorbance decrease upon Ca^{2+} addition were considered as MPTP susceptibility indexes. The reaction was continuously stirred and the temperature was maintained at 25 °C. The assays were performed in 2 mL of reaction medium containing 200 mM sucrose, 10 mM Tris, 10 μM EGTA, 5 mM KH_2PO_4 (pH 7.4) and supplemented with 4 μM rotenone, 10 mM succinate and a single pulse of 400 nmol of Ca^{2+} with 0.5 mg/mL mitochondrial protein (Broekemeier et al., 1989).

2.8. Statistical Analysis

Mean and standard error of mean were calculated for all variables in each group. After confirming that the sample comes from a normal distribution, through an analysis of variance test for normality, Shapiro-Wilk, one-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. In order to analyze the internal control, between the right and the left *gastrocnemius*, Student's test for paired samples was used.

Statistical Package for the Social Sciences (SPSS Inc, version 20.0) was used for all analysis. The significance level was set at 5%.

3. Results

Rats' body weight and *gastrocnemius* weights as well as *gastrocnemius* total protein content are presented in Table 1. No significant changes were observed between the different groups.

Table 1. Rats' body weight, *gastrocnemius* weights and muscle/body ratio in control, hyperbaric (HB) and hyperbaric oxygen therapy (HBO) groups

	Body weight (g)	**Right muscle weight (g)**	**Left muscle weight (g)**	**Right muscle/body ratio (mg/g)**	**Left muscle/body ratio (mg/g)**
Control	310,00 ± 41,14	1,73 ± 0,35	1,73 ± 0,48	25,25 ± 6,04	26,75 ± 3,15
HB	305,67 ± 60,18	1,89 ± 0,61	1,86 ± 0,34	20,95 ± 5,78	19,53 ± 8,30
HBO	312,85 ± 17,13	1,97 ± 0,28	1,83 ± 0,09	27,85 ± 7,36	28,75 ± 7,15

Note: Data are means ± SE

The oxygen consumption, after skeletal muscle contusion was evaluated by using complex I-linked substrates with 2 mM malate and 5 mM pyruvate (Table 2). As shown there were significant differences between the injured *gastrocnemius* (right) and the non injured *gastrocnemius* (left) in control group. HB had better results on the right *gastrocnemius*, comparing to other groups, in all parameters with the exception of oligomycin state. We can observe that HBO had the worse results, even in the non injured side.

Table 2. Effect of a contusion injury in the right *gastrocnemius* on the respiratory parameters evaluated in complex I of rat's injured and non injured mitochondria.

	Malate plus pyruvate					
	Control		HB		HBO	
Parameter	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
State 3	179,70±28,56	309,13±19,20[*]	281,03±7,05[#]	327,31±15,06[*]	125,65±39,72	166,28±36,21[*†]
State 4	45,10±6,11	28,70±1,47[*]	25,40±1,99[#]	23,91±7,06	33,00±10,03	31,33±9,45
RCR	4,39±0,74	10,75±0,82[*]	11,26±1,96[#]	14,51±4,05	4,10±1,40	6,18±2,38[†]
Oligomycin	46,40±6,47	23,48±1,98[*]	35,24±1,98	34,56±0,50	32,88±16,2	36,20±17,63
CCCP	152,43±14,01	357,15±30,69[*]	359,56±0,76[#]	427,80±5,82[*]	119,40±31,14	160,15±25,34[†]

Note: Data are means ± SE. States 3, 4, Oligomycin and CCCP, carbonyl cyanide m-chlorophenyl-hydrazone, are stated as natomO/min/mg prot. RCR, respiratory control ratio, (state 3/state 4).
[*]Right vs. Left ($p \leq 0,05$)
[#]HB vs. Control and HBO ($p \leq 0,05$)
[†]HBO vs. Control and HB ($p \leq 0,05$)

In Table 3 we can observe the oxygen consumption data using complex II-linked substrates, 10 mM succinate plus 4 μM rotenone. As expected, lower values were found in RCR, comparing to complex I, with the exception of HBO group. There were also significant differences between the injured and the non injured *gastrocnemius* in control group. No differences were found in the right state 4, however, HBO group had the lowest value on the right and also on the left *gastrocnemius*.

Table 3. Effect of a contusion injury in the right *gastrocnemius* on the respiratory parameters evaluated in complex II of rat's injured and non injured mitochondria.

	Succinate plus rotenone					
	Control		HB		HBO	
Parameter	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
State 3	263,40±57,20	386,23±82,69[*§]	319,53±31,90	322,51±27,28	198,85±66,79	205,56±55,52
State 4	85,68±7,77	55,20±9,21[*]	72,22±6,06	96,17±19,28	57,83±7,25	34,23±12,94[¥]
RCR	3,07±0,87	6,45±1,31[*]	4,33±0,15	3,35±0,33[#]	5,59±3,93	6,62±2,15

Note: Data are means ± SE. States 3 and 4 are stated as natomO/min/mg prot. RCR, respiratory control ratio, (state 3/state 4).
[*]Right vs. Left ($p \leq 0,05$)
[§]Control vs. HBO ($p \leq 0,05$)
[#]HB vs. Control and HBO ($p \leq 0,05$)
[¥]HB vs. HBO ($p \leq 0,05$)

Data presented in Table 4 demonstrate the effect of a contusion injury on the injured (right) and on the non injured (left) side on the mitochondrial $\Delta\psi$ fluctuations energized by malate (2 mM) plus pyruvate (5 mM). There were significant differences between the right and the left *gastrocnemius* in control group. Concerning to HB group it had always the best result in both sides comparing to other groups, highlighting the maximal energization and the lag phase.

Table 4. Effect of a contusion injury in the right *gastrocnemius* on the parameters evaluated on transmembrane electric potential (Δψ) fluctuations of rat's injured and non injured mitochondria energized with complex I.

	Malate plus pyruvate					
	Control		HB		HBO	
Parameter	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
Maximal energization, Δψ (-mV)	176,03 ± 2,76	200,25 ± 3,85*	202,90 ± 3,69#	215,35 ± 4,26	170,60 ± 8,66	168,40 ± 7,43†
ADP depolarization, Δψ (-mV)	160,43 ± 5,54	185,00 ± 2,89*	183,43 ± 2,10	198,30 ± 1,02*	134,78 ± 16,55¥	141,68 ± 13,90†
Repolarization, Δψ (-mV)	188,25 ± 6,79	208,60 ± 3,05*	212,13 ± 0,24	219,05 ± 4,46	171,43 ± 13,17¥	171,83 ± 7,91†
Lag phase, s	118,5 ± 28,08	37,50 ± 2,87*	29,00 ± 0,71#	30,38 ± 5,06	144 ± 13,80	89,25 ± 4,31*†

Note: Data are means ± SE.
*Right vs. Left ($p \leq 0,05$)
#HB vs. Control and HBO ($p \leq 0,05$)
†HBO vs. Control and HB ($p \leq 0,05$)
¥HB vs. HBO ($p \leq 0,05$)

Data on Table 5 describes the effect of a contusion injury on the injured (right) and on the non injured (left) side on the mitochondrial Δψ fluctuations energized by succinate (10 mM) plus rotanone (4 μM). Again, there were significant differences between the right and the left *gastrocnemius* in control group. However, HB group had better results only on the right side, comparing to other groups, with the exception of the lag phase. We can observe that the parameters of the left side of HBO group were also influenced by the hyperbaric oxygen, which is consistent with its systemic effect.

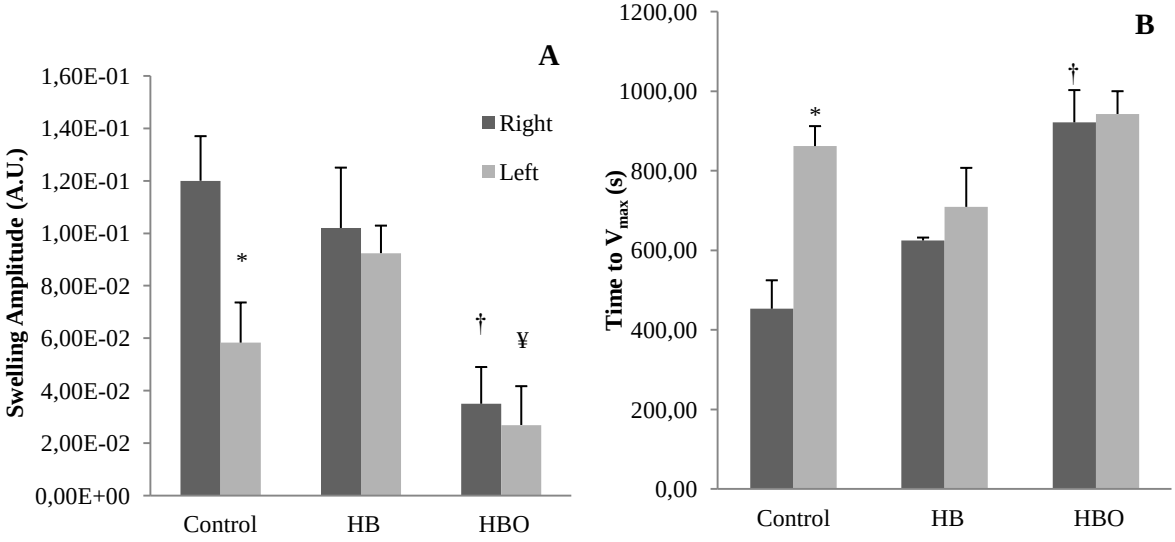
Table 5. Effect of a contusion injury in the right *gastrocnemius* on the parameters evaluated on transmembrane electric potential (Δψ) fluctuations of rat's injured and non injured mitochondria energized with complex II.

	Succinate plus rotenone					
	Control		HB		HBO	
Parameter	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
Maximal energization, Δψ (-mV)	193,80 ± 3,71	235,40 ± 8,66*	217,17 ± 2,86#	227,97 ± 2,18	181,30 ± 4,53	188,65 ± 5,29†
ADP depolarization, Δψ (-mV)	145,10 ± 4,55	177,80 ± 3,95*	184,57 ± 2,60#	186,33 ± 4,20	155,10 ± 5,43	155,85 ± 6,94†
Repolarization, Δψ (-mV)	191,33 ± 6,20	255,80 ± 2,64*§	219,80 ± 2,26#	228,93 ± 2,44	157,28 ± 32,53	193,25 ± 5,01¥
Lag phase, s	108,00 ± 14,39	46,50 ± 4,50*	101,50 ± 37,17	47,50 ± 4,08*	187,50 ± 43,36†	129,00 ± 20,27†

Note: Data are means ± SE.
*Right vs. Left ($p \leq 0,05$)
#HB vs. Control and HBO ($p \leq 0,05$)
§Control vs. HB and HBO ($p \leq 0,05$)
¥HB vs. HBO ($p \leq 0,05$)
†HBO vs. Control and HB ($p \leq 0,05$)

Figure 1 presents the impact of the contusion injury on the right (injured) and on the left (non injured) *gastrocnemius* on in vitro Ca^{2+}-induced MPTP opening. The swelling amplitude (A) was calculated as the difference between the suspension absorbance before Ca^{2+} and the absorbance at the end of the experimental measurement, and the time to V_{max} (B), was the time (in seconds) that elapsed until the faster swelling kinetics starts. The swelling experiments demonstrate that the injured *gastrocnemius* of HBO group developed significantly smaller swelling amplitude comparing to other groups (A) and also exhibited the biggest time to V_{max} (B).

Figure. 1. Effect of a contusion injury in the right *gastrocnemius* on mitochondrial swelling caused by mitochondrial permeability transition pore (MPTP) induction in succinate-energized skeletal muscle mitochondria (A) Swelling amplitude; (B) Time to V_{max}.



*Right vs. Left ($p \leq 0,05$)
†HBO vs. Control and HB ($p \leq 0,05$)
¥HB vs. HBO ($p \leq 0,05$)

4. Discussion

This present study provides new insights into the effect of hyperbaric environment on the recovery of skeletal muscle mitochondria after inducing muscle contusion in rats. Data suggests that hyperbaric oxygen therapy (HBO) contributes significantly to a more resistant apoptotic phenotype.

The muscle selected for the present study was the *gastrocnemius*, due to its location and function because the *gastrocnemius* muscle works under extreme physical activity

conditions and is susceptible to an increased risk of lesions and ruptures (Järvinen et al., 1992).

Skeletal muscle healing due to a direct trauma as contusion injury has three phases, the inflammatory response, then cells proliferate, and at last there is a remodeling of the tissue. The injury model contusion used in this study was firstly suggested by Crisco and colleagues where they showed a good inflammatory response due to the injury (Crisco et al., 1994). This model has been equally used in other studies and its invasiveness is well documented in a recent review (Smith et al., 2008).

It has been proposed by Gregorevic et al. (2000) that HBO can enhance myofiber growth and maximum force-producing capacity, after inducing myotoxic injury in rats. Moreover, in another type of muscle injury, HBO also had positive effect for at least 48 hours after a severe ischemic injury on skeletal muscle of rats, by raising the levels of high-energy phosphate compounds. Apparently, submitting the rats to HBO at 2.2 ATA during 45 minutes, contributed to the stimulation of aerobic oxidation in the mitochondria (Haapaniemi et al., 1996). This was though not evident in the functional parameters analyzed in the present study as both oxygen consumption data and membrane potential measurements were not increased. However, secondary dynamic Ca^{2+} buffering system involving phosphate has been suggested by other authors who could modulate oxidative phosphorylation in response to intracellular calcium signaling and decreased mitochondria susceptibility to apoptosis as seen by the decrease in time to V_{max} and swelling amplitude (Wei et al., 2012).

Respiratory control ratio (RCR) has been considered a good measure for the evaluation of the mitochondrial dysfunction in isolated mitochondria (Brandão et al., 2003), by showing the dependence of the respiratory rate on ADP phosphorylation (Brand and Nicholls, 2011) as the use of ATP levels to analyze mitochondrial integrity can be misleading because an immediate interruption of mitochondrial ATP synthesis as a consequence of reduced oxygen supply that may occur without the occurrence of mitochondrial damage (Brandão et al., 2003). In this study, the RCR increased in the HB group in complex I and II even though only on complex I it had significant differences from the control group. In this complex, the significant increase observed in

state 3 in HB group comparing to control and HBO group was particularly interesting and unexpected and can be attributed to an increase availability of substrates for the electron transport chain (Nulton-Persson and Szweda, 2001) or specific improvements of mitochondrial phosphorylation system (Lumini-Oliveira et al., 2009), however, specific mechanisms are unknown and deserve further investigation. These significant differences in state 3 and RCR were not observed in complex II, although the HB group maintained the highest value comparing to other groups. Concerning to the RCR of the non injured muscle in the control group, it shown higher values similar to a trained rat (Lumini-Oliveira et al., 2009), which can be due to a possible hypertrophy, as observed in the study of Minamoto et al. (2001) which suggested that, after the contusion, there was an overload of the contralateral limb, probably to compensate the hypoactivity of the injured limb in association with an increase in protein synthesis.

The significant increase in CCCP also observed in the present study in the HB group can indicate that the maximal rate of electron transfer improved. Data obtained in HB group in the presence of oligomycin, although not significant, showed a trend and may suggest a good integrity of the mitochondrial inner membrane (Magalhães et al., 2005b), leading into a significant increase in $\Delta\psi$ and in lower state 4 respiration in complex I. The decrease observed in state 4 of HB group would suggest an increase in inner membrane permeability, avoiding proton influx. This suggests an additional stimulus promoted by an increased hyperbaric stimulus but without additional oxygen.

The complementary study of $\Delta\psi$ is essential to analyze the energetic relationships in the mitochondria to maintain cellular homeostasis. There are different functions of $\Delta\psi$ on mitochondrial bioenergetics, including the driving force for cation transport into mitochondria, for pH and redox regulation, and for ATP synthesis (Magalhães et al., 2013b). In the present study, we can notice that the maximal $\Delta\psi$ in HBO group was lower on both complexes and the time needed to phosphorylate the added ADP (lag phase) was higher, regarding to control and HB group. One attractive explanation, as previously suggested by Magalhães et al. (2013b), is an adaptive response to increased proton leakage due to ROS production by the respiratory chain that, in the present study, may have been due to hyperbaric oxygen environment.

The MPTP opening can collapse mitochondrial potential and an amount of Ca^{2+} into the extramitochondrial space, probably induced by oxidative stress. This abnormal opening of this organelle causes its swelling, cytochrome-*c* release, caspase activation and apoptosis or the fall of its potential, ATP depletion and energetic collapse followed by necrotic cell death (Lumini-Oliveira et al., 2010). However, in the present study, concerning to the modulation of the permeability transition pore, in HBO group, the lag phase elapsed until large scale swelling initiates (time to V_{max}) was significantly higher than other groups and, therefore, the swelling amplitude was significantly smaller than the other groups which can guide us to other finding that presents a lower susceptibility to MPTP opening.

Under hypobaric hypoxic conditions, the reduction of oxygen can also contribute to oxidative stress for reducing equivalents in the mitochondria and therefore increase the apoptotic susceptibility (Magalhães et al., 2005a). This situation can also occur when there is an excessive inflammatory response due to an injury such as muscle contusion, which normally is accompanied by an uncontrolled ROS that can affect the normal cell functions (Puntel et al., 2011). However, ROS also serve as signaling molecules in transduction cascades, for a variety of factors and so can generate positive or negative effect that depends on their concentration and intracellular localization (Thom, 2009). In the presence of oxygen, in hyperbaric conditions, it is known that increases ascorbate radical levels which contribute to the afflux of ROS (Tibbles and Edelsberg, 1996). However, oxidative stress is an important factor in hyperbaric oxygen therapy. This augmented synthesis of ROS on a HBO environment improves the outcome from a wide variety of postinflammatory insults and, therefore, temporarily inhibits endothelial sequestration of neutrophils, by inhibiting β_2-integrin function (Thom, 2009). One possible explanation for this fact could be that in the skeletal muscle of the present study, HBO application hypothetically could have induced an increase in antioxidant enzymes and/or mechanism as anti-inflammatory and anti-apoptotic proteins there were not identified for not being the objective of this study. Therefore, further work is needed to truly understand this issue.

5. Summary

Although more is still to be learned about the effect of HBO and HB after skeletal muscle contusion on rats, and the role of ROS in response to HBO application, this study provides a new insight into the impact of the hyperbaric environment. Generically, data supports the fact that hyperbaric environment has a positive contribution to accelerate the recovery after the muscle contusion, by delaying the time for cellular apoptosis, increasing its integrity. However, more studies should follow the present one.

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7. References

ALBUQUERQUE & SOUSA, J. G. (2007) Oxigenoterapia hiperbárica (OTHB). Perspectiva histórica, efeitos fisiológicos e aplicações clínicas. *Revista da Sociedade Poruguesa de Medicina Interna,* 14**,** 219-227.

BABUL, S., RHODES, E., TAUNTON, J. & LEPAWSKY, M. (2003) Effects of Intermittent Exposure to Hyperbaric Oxygen for the Treatment of an Acute Soft Tissue Injury. *Clinical Journal of Sports Medicine,* 13**,** 138-147.

BARATA, P., CERVAENS, M., RESENDE, R., CAMACHO, O. & MARQUES, F. (2011) Hyperbaric oxygen on sports injuries. *Therapeutic Advances in Musculoskeletal Disease,* 3**,** 111-121.

BEINER, J. M., JOKL, P., CHOLEWICKI, J. & PANJABI, M. M. (1999) The Effect of Anabolic Steroids and Corticosteroids on Healing of Muscle Contusion Injury. *The American journal of Sports Medicine,* 27**,** 2-9.

BRAND, M. D. & NICHOLLS, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochemical Journal,* 435**,** 297-312.

BRANDÃO, M. L., ROSELINO, J. E. S., PICCINATO, C. E. & CHERRI, J. (2003) Mitochondrial Alterations in Skeletal Muscle Submitted to Total Ischemia. *Journal of Surgical Research,* 110**,** 235-240.

BROEKEMEIER, K. M., DEMPSEY, M. E. & PFEIFFER, D. R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *Journal of Biological Chemistry,* 264**,** 7826-7830.

BROOKES, P. S., YOON, Y., ROBOTHAM, J. L., ANDERS, M. W. & SHEY-SHING, S. (2004) Ca^{2+}, ATP, and ROS: a mitochondrial love-hate triangle. *American Jounal of Physiology - Cell Physiology,* 287**,** C817–C833.

CARLSON, B. M. & FAULKNER, J. A. (1983) The regeneration of skeletal muscle fibres following injury: a review. *Medicine & Science in Sports & Exercise,* 15**,** 187-198.

CERVAENS, M., CAMACHO, O., RESENDE, R., MARQUES, F. & BARATA, P. (2011) Hyperbaric Oxygen Therapy treatment for the recovery of muscle injuries induced in rats. IN SOCIETY, E. U. B. (Ed.) *37th Annual Meeting of the European Underwater & Baromedical Society (EUBS).* Gdänsk.

CRISCO J, JOKL P, HEINEN GT, CONNELL MD & PANJABI, M. (1994) A muscle contusion injury model. Biomechanics, physiology, and histology. *American Journal of Sports Medicine,* 22**,** 702-10.

DE LAET, C., OBYN, C., RAMAEKERS, D., VAN DE SANDE, S. & NEYT, M. (2008) Hyperbaric Oxygen Therapy: a Rapid Assessment. *Health Technology Assessment.* Brussels, Belgian Health Care Knowledge Centre (KCE).

ESTABROOK, R. (1967) Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods in Enzymology,* 10**,** 41-47.

GREGOREVIC, P., LYNCH, G. S. & WILLIAMS, D. A. (2000) Hyperbaric oxygen improves contractile function of regenerating rat skeletal muscle after myotoxic injury. *Journal of Apllied Physiology,* 89**,** 1477-1482.

HAAPANIEMI, T., NYLANDER, G., SIRSJÖ, A. & LARSSON, J. (1996) Hyperbaric Oxygen Reduces Ischemia-Induced Skeletal Muscle Injury. *American Society of Plastic Surgeons,* 97**,** 602-607.

HURME, T. & KALIMO, H. (1992) Activation of myogenic precursor cells after muscle injury. *Medicine & Science in Sports & Exercise,* 24**,** 197-205.

JACKSON, D. & FEAGIN, J. (1973) Quadriceps contusions in young athletes: Relation of severity of injury to treatment and prognosis. *The Journal of Bone and Joint Surgery,* 55A**,** 95-105.

JÄRVINEN MJ, EINOLA SA & EO, V. (1992) Effect of the position of immobilization upon the tensile properties of the rat gastrocnemius muscle. *Archives of Physical Medicine and Rehabilitation,* 73**,** 253-257.

JÄRVINEN, T., JÄRVINEN, T., KÄÄRIÄINEN, M., ÄÄRIMAA, V., VAITTINEN, S., KALIMO, H. & JÄRVINEN, M. (2007) Muscle injuries: optimising recovery. *Best Practice & Research Clinical Rheumatology,* 21**,** 317-331.

KAMO, N., MURATSUGU, M., HONGOH, R. & KOBATAKE, Y. (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *Journal of Membrane Biology,* 49**,** 105-121.

KVIST, H., JARVINEN, M. & SORVARI, T. (1974) Effect of mobilization and immobilization on the healing of contusion injury in muscle: a preliminary report of a histological study in rats. *Scandinavian Journal of Rehabilitation Medicine,* 6**,** 134-140.

LUMINI-OLIVEIRA, J., ASCENSÃO, A., PEREIRA, C. V., MAGALHÃES, S., MARQUES, F., OLIVEIRA, P. J. & MAGALHÃES, J. (2010) Long-term hyperglycaemia decreases gastrocnemius susceptibility to permeability transition. *European Journal of Clinical Investigation,* 40**,** 319-329.

LUMINI-OLIVEIRA, J., MAGALHÃES, J., PEREIRA, C. V., ALEIXO, I., OLIVEIRA, P. J. & ASCENSÃO, A. (2009) Endurance training improves gastrocnemius mitochondrial function despite increased susceptibility to permeability transition. *Mitochondrion,* 9**,** 454-462.

LUMINI-OLIVEIRA, J., MAGALHÃES, J., PEREIRA, C. V., MOREIRA, A. C., OLIVEIRA, P. J. & ASCENSÃO, A. (2011) Endurance training reverts heart mitochondrial dysfunction, permeability transition and apoptotic signaling in long-term severe hyperglycemia. *Mitochondrion,* 11**,** 54-56.

MAGALHÃES, J., ASCENSÃO, A., SOARES, J., FERREIRA, R., NEUPARTH, M. J., OLIVEIRA, J., AMADO, F., MARQUES, F. & DUARTE, J. (2005a) Acute and chronic exposition of mice to severe hypoxia: the role of acclimatization against skeletal muscle oxidative stress. *International Journal of Sports Medicine,* 26**,** 102-109.

MAGALHÃES, J., ASCENSÃO, A., SOARES, J. M. C., FERREIRA, R., NEUPARTH, M. J., MARQUES, F. & DUARTE, J. A. (2005b) Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. *Journal of Applied Physiology* 99**,** 1247-1253.

MAGALHÃES, J., FALCÃO-PIRES, I., GONÇALVES, I., LUMINI-OLIVEIRA, J., MARQUES-ALEIXO, I., DOS PASSOS, E., ROCHA-RODRIGUES, S., MACHADO, N., MOREIRA, A., MIRANDA-SILVA, D., MOURA, C., LEITE-MOREIRA, A., OLIVEIRA, P., TORRELLA, J. & ASCENSÃO, A. (2013a) Synergistic impact of endurance training and intermittent hypobaric hypoxia on cardiac function and mitochondrial energetic and signaling. *International Journal of Cardiology,* pii: S0167-5273(13)01526-X. doi: 10.1016/j.ijcard.2013.08.001. [Epub ahead of print].

MAGALHÃES, J., FRAGA, M., LUMINI-OLIVEIRA, J., GONÇALVES, I., COSTA, M., FERREIRA, R., OLIVEIRA, P. J. & ASCENÇÃO, A. (2013b) Eccentric exercise transiently affects mice skeletal muscle mitochondrial function. *Applied Physiology, Nutrition and Metabolism,* 38**,** 401-409.

MINAMOTO, V. B., BUNHO, S. R. & SALVINI, T. F. (2001) Regenerated rat skeletal muscle after periodic contusions. *Brazilian Journal of Medical and Biological Research* 34**,** 1447-1452.

NULTON-PERSSON, A. & SZWEDA, L. (2001) Modulation of mitochondrial function by hydrogen peroxide. *Journal of Biological Chemistry,* 276**,** 23357-23361.

PUNTEL, G. O., CARVALHO, N. R., AMARAL , G. P., LOBATO, L. D., SILVEIRA, S. O., DAUBERMANN, M. F., BARBOSA , N. V., ROCHA, J. B. T. & SOARES, F. A. A. (2011) Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion. *Free Radical Research,* 45**,** 125-138.

SCHASER, K., AC, D., STOVER, J., LAUFFER, A., BAIL, H. & MITTLMEIER, T. (2007) Prolonged superficial local cryotherapy attenuates microcirculatory impairment, regional inflammation, and muscle necrosis after closed soft tissue injury in rats. *American Journal of Sports Medicine,* 35**,** 93-102.

SMITH, C., KRUGER, M. J., SMITH, R. M. & MYBURGH, K. H. (2008) The Inflammatory Response to Skeletal Muscle Injury: Illuminating Complexities. *Sports Medicine,* 38**,** 947-969.

STAPLES, J. & CLEMENT, D. (1996) Hyperbaric oxygen chambers and the treatment of sports injuries. *Sports Medicine,* 22**,** 219-227.

STRATTON, S., HECKMANN, R. & FRANCIS, R. (1984) Therapeutic ultrasound, its effects on the integrity of a nonpenetrating wound. *Journal of Orthopaedics and Sports Physical Therapy,* 5**,** 278-281.

THOM, S. R. (2009) Oxidative stress is fundamental to hyperbaric oxygen therapy. *Journal of Applied Physiology (Bethesda, Md.: 1985),* 106**,** 988-995.

TIBBLES, P. M. & EDELSBERG, J. S. (1996) Hyperbaric-oxygen therapy. *The New England journal of medicine,* 334**,** 1642-1645.

TONKONOGI, M. & SAHLIN, K. (1997) Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. *Acta Physiologica Scandinavica,* 161**,** 345-353.

WEI, A., LIU, T., WINSLOW, R. & O'ROURKE, B. (2012) Dynamics of matrix-free Ca^{2+} in cardiac mitochondria: two components of Ca^{2+} uptake and role of phosphate buffering. *Journal of General Physiology,* 139, 465-78.

CHAPTER III

EXPERIMENTAL WORK: PAPER III

The effects of Hyperbaric Oxygen Therapy on the recovery of Anterior Cruciate Ligament rupture in rabbits

Abstract

Objective: To study the application of HBO on the recovery of Anterior Cruciate Ligament (ACL) injured, namely by improving neovascularization and changing the pattern of protein production in rabbits.

Methods: Ten rabbits, New Zealand White, weighting between 1950 g and 2000 g were induced a transcapsular rupture in ACL. Animals were then randomly assigned in two groups, group A (control group) and B (HBO). The animals in group B were submitted, 24 h after injury, to daily 60 min of HBO, during 21 days, at 2.5 ATA. After 21 days, blood samples were taken from the animals in both groups, and afterwards the animals were sacrificed. Western blots for Vascular Endotelial Growth Factor (VEGF) and type I collagen were obtained and analyzed.

Results: Analyzing the haematological results, there was a decrease in Creatine Phosphokinase and in the lymphocytes in group B. HBO increased VEGF factor. However, the sedimentation velocity was significantly higher in group A and macroscopically, HBO contributed significantly for the increasing of the capsule thickness, increasing its stability and joint mobility.

Conclusions: HBO plays an important role by increasing neovascularization, which contributed for the increase of capsule thickness and knee stability.

Key-words: Anterior Cruciate Ligament, Hyperbaric Oxygen Therapy, neovascularisation, Type I collagen.

1. Introduction

The anterior cruciate ligament (ACL) is the most commonly injured ligament of the knee with over 200,000 patients per year diagnosed with ACL disruptions (Laurencin and Freeman, 2005). The ACL is the major intra-articular ligament of the knee and is critical to normal kinematics and stability. The ACL controls motion by connecting the femur to the tibia and stabilizing the joint, preventing abnormal types of motion. Its main functions are to support and strengthen the knee and prevent excessive anterior translation of the femur as well as an abnormal tibial rotation that could cause a dislocation and fracture of bones in the knee joint (Zantop et al., 2006).

The ACL is a dense, highly organized, cable-like tissue composed of different types of collagen, elastin, proteoglycans, water, and cells (Laurencin and Freeman, 2005). The collagen fibrils are surrounded by connective tissue, which forms multiple fascicles within the ACL. The major collagen of the ACL is type I collagen, responsible for the tensile strength of the ligament while the loose connective tissue consists of type III collagen (Petersen and Tillmann, 2002).

A rupture of the ACL leads to significant knee instability and secondary knee damage including meniscus tears and degeneration of articular cartilage, increasing the stress on other soft tissues (Zantop et al., 2006, Yagi et al., 2002).

After injury, ligament healing can be divided into three phases: inflammation, cellular proliferation and matrix repair, and remodeling. Inflammation occurs mainly within 72 h of the injury. In the cellular proliferation and matrix repair stage, fibroblasts are present and vascular granulation tissue is formed. Collagen is produced an higher ratio of type III to type I collagen, forming the new extracellular matrix. This stage typically lasts for 6 weeks. The remodeling phase, lasts for several months and during this stage, the new extracellular matrix matures into a slightly disorganized hypercellular tissue (Frank et al., 1983).

Healing of the ACL is inhibited by lack of vascularization. Optimal healing is also achieved when the continuity of collagen fibers is maintained. Due to its lack of

vascularization and poor tissue organization, there is a difference in the mechanical properties of the new scar tissue and the original ligament that leads to a reduction in mechanical properties (Laurencin and Freeman, 2005).

Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis that plays an important role in activation, migration, and proliferation of endothelial cells in various pathologic conditions (Ferrara and Davis-Smyth, 1997). VEGF is a growth factor that influences revascularization of healing tissues and has been shown that may contribute to restore the strength of healing ligaments (Kessler et al., 2008).

Hyperbaric therapies are clinical methods used to treat diseases or injuries using pressure higher than local atmospheric pressure inside a hyperbaric chamber. Several clinical trials detected that hyperbaric oxygen therapy (HBO), behaves like a multifaceted drug endowed with anti-ischemic, anti-hypoxic, anti-edematous and pro-healing (Tibbles and Edelsberg, 1996). Strong clinical evidence supports HBO inducement of angiogenesis although the mechanism of its biological action is still not clear. Li et al. (1996) proposed that although lactate and hypoxia stimulate macrophages to produce VEGF, lack of oxygen up-regulates VEGF receptors and the expressing cells may grow faster when exposed to hyperoxia.

The poor wound-healing response of the anterior cruciate ligament has thus been noted both histologically in animal models and clinically in humans. Why the anterior cruciate ligament does not heal after rupture is an important question that remains unanswered (Murray et al., 2000).

ACL reconstruction is not a universally successful procedure, with reported rates of laxity failure at one year of up to 17%. There are traumatic, iatrogenic and atraumatic reasons for recurrent laxity (Deehan and Cawston, 2005), that revealed a high risk of osteoarthritic joint changes (Zantop et al., 2006, Yagi et al., 2002).

Therefore, the objective of the present study was to analyze if the use of HBO on the recovery of Anterior Cruciate Ligament (ACL) injured, namely by improving neovascularization and changing the pattern of protein production, in rabbits.

2. Methods

2.1. Animals

Ten rabbits, New Zealand White, weighting between 1950 g and 2000 g were studied. The animals were kept in the facilities of the Laboratory of the Faculty of Medicine, University of Porto, in collective cages with two animals per cage, at room temperature, receiving water and standard food *ad libitum*. All procedures were ethically approved and were performed according to the FELASA recommendations for animal welfare in the presence of an investigator with category C FELASA. The concept of 3 R (reduce, replace and refinement) was applied in all phases of the study.

2.2. Procedure for inducing injury

Prior to the induction of the injury, the animals were anesthetized using 20 mg/kg Ketamine and 0.5 mg/kg Medetomidine IV.

Afterwards, the intervention area was shaved and then disinfected with povidone-iodine solution. A transcapsular rupture in ACL was then induced by using a 22G needle in the left knee of the back leg. To verify knee instability the Lachman-test was carried out intraoperatively and compared to the contralateral side (Figure 1). The same test was repeated 21 days after the injury.

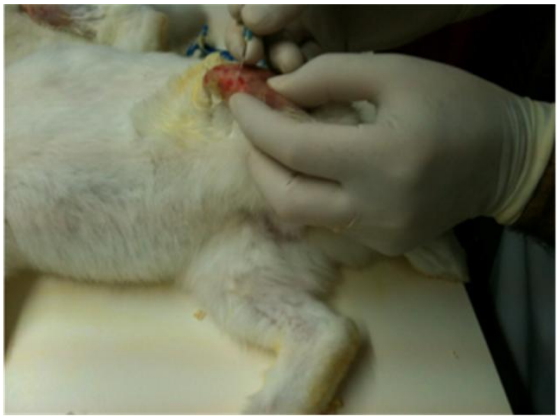


Figure 1. Lachman-test intraoperatively

All animals received a single intramuscular dose of analgesics (0.5 mg/Kg of Butorphanol) and for the reversal of the sedative (0.3 mg/Kg of Atipamezole). Antibiotics were also administered (Enrofloxacin, 1.5 mL/L of H_2O) for a period of 5 days after the procedure as prophylaxis against infection.

Animals were then randomly assigned, using a computer program, in two groups, group A (control group) and B (HBO).

2.3. Protocol of HBO

The animals in group B were submitted 24 h after injury, to 60 min of HBO and the sessions were daily, during 21 days, at 2.5 ATA.

2.4. Preparation of biological samples

After 21 days, blood samples were taken from the animals of both groups, and afterwards the animals were sacrificed.

To re-evaluate knee instability the Lachman-test was performed and compared to the contralateral side. Macroscopic evaluation of the lesion *in situ* was made, by measuring cartilage thickness with a precision caliper and ACL was then harvested and cryopreserved at -80º C.

2.5. Western blots for VEGF and type I collagen

ACL protein was extracted by maceration followed by sonication after adding lysis buffer [NP-40 1%, 500 mM tris HCL, 2.5 M NaCl, 20 mM EDTA (ethylenediamine-tetracetic acid) pH 7.2, protease and fosfatase inhibitors, glicerol 85%].

Proteins were extracted for analysis and electrophoresed on a 4-20% sodym dodecyl sulfate-polyacrymalide gel (SDS–PAGE), followed by blotting on a nitrocellulose membrane, according to the Bradford method (Kruger, 1994). The blots were incubated

with primary antibodies: anti-VEGF, VEGF antibody: sc-507 (1:500; Santa Cruz Biotechnology, Inc.®) and anti-collagen type I (1:1000; abcam®-ab90395) for 2 hours; and then secondary antibodies HRP-conjugated anti-rabbit immunoglobulin G (IgG) and HRP-conjugated with anti- mouse IgG both diluted 1:5000 in a blocking buffer for a period of 1 hour. β-Actin was used as an internal control.

Protein bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine, UK), according to supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software 4.6.5 ® (Bio-Rad Laboratories, Inc).

2.6. Statistical Analysis

The statistical analysis was performed through the IBM® Statistical Package for Social Sciences® (SPSS, Version 20). Firstly, the Kolmogorov Smirnov normality test was performed. All the quantitative data were expressed as mean ± standard deviation. The analysis between group A and B in quantitative and qualitative data was effectuated by using T-student test. The significance level was set at 5%.

3. Results

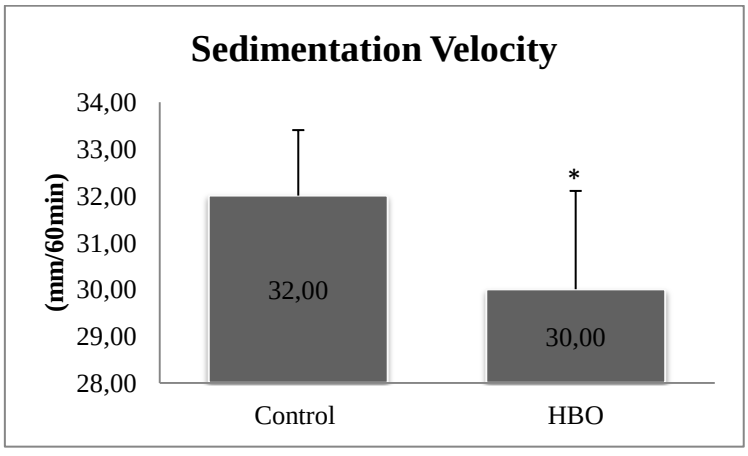
For basic haematological parameters little difference was found between the treated and non-treated groups, but no statistical differences were found between control and HBO group as shown at table 1. However, it can be noticed a decrease of Creatine Phosphokinase (CPK) levels in HBO as well as percentage of lymphocytes and platelets when compared to control group.

Table 1. Observed haematological parameters in HBO (B) and control group (A)

	B (HBO)		A (Control)	
	Average	**SD**	**Average**	**SD**
Eritrocytes (10^12/L)	7.40	0.08	7.34	0.22
Hemoglobin (g/dL)	14.57	0.41	14.43	0.64
Hematocrit (%)	44.50	0.52	44.10	0.68
MCV (fL)	58.37	0.48	59.14	0.57
MCH (pg)	19.87	0.76	19.67	0.32
MCHC (g/dL)	31.23	0.95	31.04	0.83
Leucocytes (10^g/L)	3.40	1.86	5.10	0.73
MPV (μm^3)	6.20	0.45	6.77	0.34
RDW (%)	10.27	0.52	11.50	0.34
GRA (%)	26.50	5.52	24.91	5.41
MON (%)	12.20	2.37	12.11	1.96
LYMP (%)	47.25	5.99	61.03	8.23
Platelets (10^g/L)	534.76	45.21	556.31	37.89
CPK (IU/L)	4200.34	54.12	4875.57	67.12

Note: MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MPV, mean platelet volume; RDW, red cell distribution width; GRA, granulocytes; MON, monocytes; LYMP, Lymphocytes and CPK, Creatine Phosphokinase.

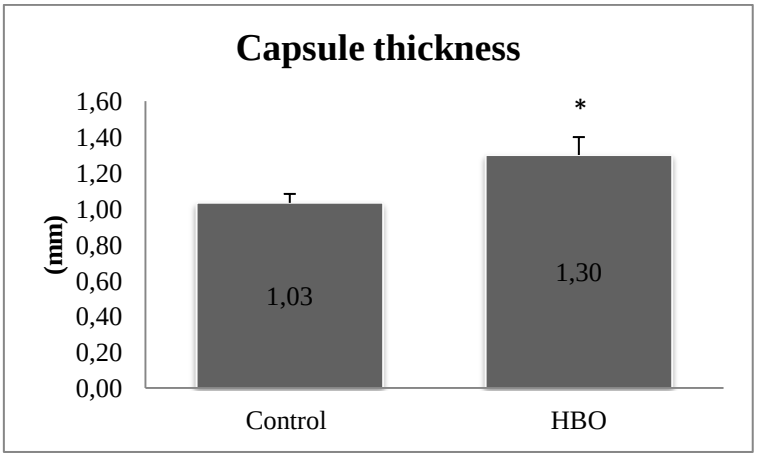
In Figure 2 the sedimentation velocity was significantly higher in control group which can give valuable information about the structure of the macromolecules.



*** p< 0,05**

Figure 2. Sedimentation velocity in Control and HBO group

Capsule thickness was measured *post-mortem* and data are shown in the following bar graph (Figure 3) and seen in Figure 4. The rabbits from both groups still presented instability in Lachman's test, however, the rabbits from group B demonstrated to have more mobility and started to move earlier than the control rabbits.



*** p< 0,05**

Figure 3. Capsule thickness in Control and HBO group

Figure 4. Thickness of the capsule of HBO group

Western blot analysis results on Figure 5 demonstrated the influence of HBO in the production of VEGF.

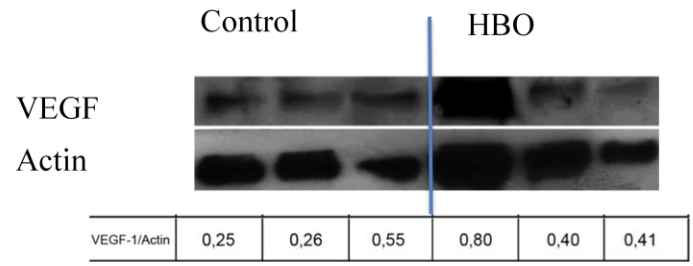


Figure 5. Western blot analysis for VEGF in Control and HBO group

Western blot analysis results on Figure 6 showed that Type I collagen is not present with HBO. The effect of HBO is significantly different between the groups for the production of collagen Type I. However, it can be equally noticed a significant difference in the production of actin.

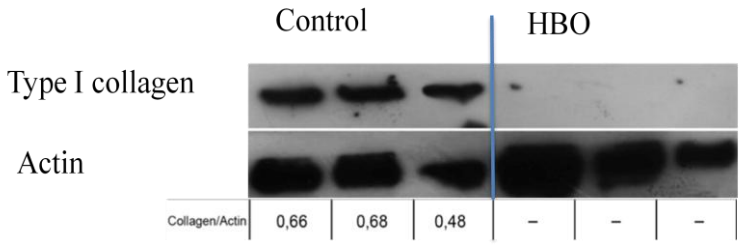


Figure 6. Western blot analysis for Type I collagen in control and HBO group

4. Discussion

This present study provides new insights into the effect of hyperbaric environment on VEGF and the production of type I collagen after ACL rupture in rabbits. Data suggests that the use of hyperbaric oxygen therapy (HBO) contributed for the activation of VEGF, and therefore for the thickness of the capsule and it was noticed that type I collagen is not present.

Angiogenetic response, the sprouting of new blood vessels from preexisting vessels, takes place through migration of the activated endothelial cells, cell division, tube formation, and neo-capillary net development. Strong clinical evidence supports HBO inducement of angiogenesis although the mechanism of its biological action is not clear (Mathieu, 2006). Gibson and Hunt (1998) demonstrated in a Matrigel model

angiogenesis to be stimulated paradoxically by both hypoxia and HBO through released endothelial growth factor (VEGF).

This study shows further beneficial effects of HBO upon angiogenesis as seem to influence the interaction of VEGF and its cellular target, suggesting that had a positive effect on increasing significantly the thickness of the capsule, somehow tempting to affirm that may have contributed to the formation of other type of collagen, present in capsule as type II collagen responsible for support pressure, or other collagen present in the ligament such as type III collagen that divide the type I collagen bundles, more concentrated near the attachments zone and is responsible for the pliability of the ACL or even type IV collagen which is found in all vascular basements membranes, mainly in proximal and distal parts where is less vascularizated (Duthon et al., 2006). Moreover, it was demonstrated that neovascularization contributed for the significant production of actin, which is an important component for joint stability (Straub and Feuer, 1950). Although not significant in HBO group, there was a trend in the inflammatory response group to be lesser than control group, which may have helped to accelerate to the remodeling phase.

Sheikh et al. (2000) used a rat wound model to show that HBO induced 40% of production of VEGF suggesting that this explains in part the angiogenetic action of HBO. Moreover, Lin et al. (2002) presented another study to clarify the angiogenetic HBO effect. They have noted that VEGF in order to promote vessel formation must act synergistically with specific angiopoietins and selectively enhance its action through e-NOS related signaling pathway.

However, as mentioned before, concerning to the production of collagen Type I in ACL healing, HBO seems to have influenced the production of other type of collagen that allowed the HBO rabbits to move earlier than the control group, having a faster recovery. However, due to the lack of collagen Type I, the tissue may be more disorganized and less mechanically resistant. In turn, Mashitori et al. (2004) had the opposite finding in the medial collateral ligament healing where they concluded that the administration of HBO promoted scar tissue formation and increased Type I procollagen gene expression, which improved tensile properties. Another study that

analyzed the effect of HBO in the medial collateral ligament also concluded that the HBO group showed a significantly higher amount of regenerative collagen fibers when comparing to the control group (Ueng et al., 2011). Moreover, when analyzing the recovery from grade II medial collateral ligament injury in humans, the authors concluded that early intervention of HBO had positive effects in the acceleration of its rehabilitation. This study also showed that HBO contributed to decrease edema and increase mobility in the knee.

However, Takayema et al. (2007) analyzed the effect of HBO on the recovery of medial collateral ligament and ACL injury in rats. Although the administration of HBO increased type I procollagen gene expression in both the injured MCL and ACL, the authors have found no scar tissue formation when the ACL was observed macroscopically which indicated that HBO can be insufficient to allow the injured to heal by itself. Therefore, these authors added that if HBO therapy is used as an adjunct with primary repair of the injured ACL, the success rate of the surgery may be increased, hopefully leading to a decreased number of patients requiring ACL reconstruction. In fact, Yeh et al. (2007) analyzed the effects of hyperbaric oxygen (HBO) on neovascularization at the tendon-bone junction in rabbits. It was observed that HBO treatment stimulated the ingrowth of blood vessel formation associated with improvement in blood supply that lead to an increase in trabecular bone around the tendon and improvement in the contacting between tendon and bone at the tendon-bone interface. Furthermore, Yoshikawa et al. (2006) revealed that VEGF promotes angiogenesis in the ACL graft and significantly reduces the stiffness of the ACL graft after ACL reconstruction.

The use of HBO demonstrated that had positive influence on VEGF activation and also for the increasing of thickness of the capsule, both problems that are still remaining after ACL reconstruction. Therefore, we strongly believe that HBO is an important adjuvant therapy for the recovery of reconstructed ACL.

Although the success rate of primary repair of the ACL injury is promising, further work is necessary to analyze its effects with a bigger sample. However, the results of

this study should not be transferred to humans, but should give guidance to further research.

5. Conclusions

HBO can play an important factor to contribute to the increase of neovascularization and therefore for the activation of VEGF which contributed to a thicker capsule in the knee of the rabbits with ACL rupture accelerating its recovery. It would be interesting to analyze the influence of hyperbaric environment in the production of other types of collagen that are also important for ACL healing. Moreover, it seems reasonable to affirm that HBO may have an important role after ACL reconstruction.

6. References

DEEHAN, D. J. & CAWSTON, T. E. (2005) The biology of integration of the anterior cruciate ligament. *The Journal of Bone and Joint Surgery,* 87-B**,** 889-895.

FERRARA, N. & DAVIS-SMYTH, T. (1997) The biology of vascular endothelial growth factor. *Endocrine Reviews,* 18**,** 4-25.

FRANK, C., WOO, S.-Y., AMIEL, D., HARWOOD, F., GOMEZ, M. & AKESON, W. (1983) Medial collateral ligament healing: a multidisciplinary assessment in rabbits. *American Journal of Sports Medicine,* 11**,** 379-89.

GIBSON, J. & HUNT, T. (1998) Hyperbaric oxygen potentiates wound healing. *European Journal of Hyperbaric Medicine* , 5**,** 7-9.

KESSLER, M., BEHREND, H., HENZ, S., STUTZ, G., RUKAVINA, A. & KUSTER, M. (2008) Function, osteoarthritis and activity after ACL-rupture: 11 years follow-up results of conservative versus reconstructive treatment. *Knee Surgery, Sports Traumatology, Arthroscopy,* 16**,** 442-448.

KRUGER, N. J. (1994) The Bradford Method for Protein Quantitation. *Methods in Molecular Biology,* 32**,** 9-15.

LAURENCIN, C. T. & FREEMAN, J. W. (2005) Ligament tissue engineering: An evolutionary materials science approach. *Biomaterials,* 26**,** 7530-7536.

LI, J., BROWN, L., HIBBERD, M., GROSSMAN, J., MORGAN, J. & M., S. (1996) VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *American Journal of Physiology,* 270 1803-11.

LIN, S., SHYU, K., LEE, C., WANG, B., CHANG, C., LIU, Y., HUANG, F. & CHANG, H. (2002) Hyperbaric oxygen selectively induces angiopoietin-2 in human umbilical vein endothelial cells. *Biochemical and Biophysical Research Communications,* 296**,** 710-715.

MASHITORI, H., SAKAI, H., KOIBUCHI, N., OHTAKE, H., TASHIRO, T., TAMAI, K. & SAOTOME, K. M. (2004) Effect of Hyperbaric Oxygen on the Ligament Healing Process in Rats. *Clinical Orthopaedics & Related Research,* 423**,** 268-274.

MATHIEU, D. (2006) *Handbook on hyperbaric medicine,* Kluwer Academic Pub.

MURRAY, M. M., MARTIN, S. D., MARTIN, T. L. & SPECTOR, M. (2000) Histological Changes in the Human Anterior Cruciate Ligament After Rupture. *The Journal of Bone and Joint Surgery,* 82-A**,** 1387-1397.

PETERSEN, W. & TILLMANN, B. (2002) Anatomy and function of the anterior cruciate ligament. *Orthopade,* 31**,** 710-718.

SHEIKH, A., GIBSON, J., ROLLINS, M., HOPF, H., HUSSAIN, Z. & HUNT, T. (2000) Effect of hyperoxia on VEGF levels in a wound model. *Archives of Surgery,* 135**,** 1293-1297.

STRAUB, F. B. & FEUER, G. (1950) Adenosinetriphosphate the functional group of actin. *Biochimica et Biophysica Acta,* 4**,** 455-470.

TAKEYAMA, N., SAKAI, H., OHTAKE, H., MASHITORI, H., TAMAI, K. & SAOTOME, K. (2007) Effects of hyperbaric oxygen on gene expressions of procollagen, matrix metalloproteinase and tissue inhibitor of metalloproteinase in injured medial collateral ligament and anterior cruciate ligament *Knee Surgery, Sports Traumatology, Arthroscopy,* 15**,** 443-452.

TIBBLES, P. M. & EDELSBERG, J. S. (1996) Hyperbaric-oxygen therapy. *The New England journal of medicine,* 334**,** 1642-1645.

UENG, S. W.-N., LEE, M. S.-S., TAI, C.-L., HSU, K.-Y., LIN, S.-S., CHAN, Y.-S. & CHEN, W.-J. (2011) Hyperbaric oxygen therapy improves medial collateral ligament healing in a rabbit model. *Formosan Journal of Musculoskeletal Disorders* 2**,** 7-11.

YAGI, M., WONG, E. K., KANAMORI, A., DEBSKI, R. E., FU, F. H. & WOO, S. L.-Y. (2002) Biomechanical Analysis of an Anatomic Anterior Cruciate Ligament Reconstruction. *The American Journal of Sports Medicine,* 30**,** 660-666.

YEH, W.-L., LIN, S.-S., YUAN, L.-J., LEE, K.-F., LEE, M. Y. & UENG, S. W. N. (2007) Effects of Hyperbaric Oxygen Treatment on Tendon Graft and Tendon-Bone Integration in Bone Tunnel: Biochemical and Histological Analysis in Rabbits. *Journal of Orthopaedic Research*, 25**,** 636-645.

YOSHIKAWA, T., TOHYAMA, H., KATSURA, T., KONDO, E., KOTANI, Y., MATSUMOTO, H., TOYAMA, Y. & YASUDA, K. (2006) Effects of Local Administration of Vascular Endothelial Growth Factor on Mechanical Characteristics of the Semitendinosus Tendon Graft After Anterior Cruciate Ligament Reconstruction in Sheep. *The American Journal of Sports Medicine,* 34**,** 1918-1925.

ZANTOP, T., PETERSEN, W., SEKIYA, J. K., MUSAHL, V. & FU, F. H. (2006) Anterior cruciate ligament anatomy and function relating to anatomical reconstruction. *Knee Surgery, Sports Traumatololgy, Arthroscopy,* 14**,** 982-992.

CHAPTER IV

GENERAL DISCUSSION

General Discussion

Skeletal muscle represents the largest tissue mass in the body, representing 40% to 45% of total body weight. The basic structural element of skeletal muscle is the muscle fiber also known as myofiber. The cytoplasm of the myofiber, contains a cellular matrix and organelles, including the Golgi complex, mitochondria, sarcoplasmic reticulum, lipid droplets, glycogen, and myoglobin. After mechanical trauma, the integrity of the myofiber plasma membrane and basal lamina are destroyed, leading to the ingress of extracellular Ca^{2+} (Järvinen et al., 2000). In the injured muscle, myofibers undergo necrosis by autodigestion mediated by intrinsic proteases (St Pierre and Tidball, 1994).

ACL plays an important role in supporting and restraining some movements in the knee, by controlling rotational movements and anterior translation of the tibia. However, after rupture, the human anterior cruciate ligament appears to retract initially, with no evidence of healing (Kohn, 1986) and therefore, its recovery normally needs surgical intervention.

Soft tissues that heal, progress through a series of inflammatory, proliferative and remodeling phases, after injury, where oxygen supply plays a crucial role in each of these phases.

Active muscle degeneration and inflammation occur in the first few days post-injury (Huard et al., 2002). In this phase, the hypoxia-induced factor-1α, which promotes, for example, the glycolytic system, vascularisation and angiogenesis has been shown to play an important role. However, if the oxygen supply could be controlled without promoting blood flow, the blood vessel permeability could be controlled to reduce swelling and the associated pain (Ishii et al., 2005). During the inflammatory phase of healing, the role of HBO is to reduce blood flow to the local area by up to 20% (Delaney and Montgomery, 2001) and due to this vasoconstriction reduces the edema and sharp pain (Ishii et al., 2005). This mechanism is also outlined by Gill and Bell (2004) who state that vasoconstriction is effective for reducing post-traumatic tissue edema. In fact, in the experimental papers of this thesis where were analyzed

haematological parameters, it was verified a decrease in inflammatory response. However this restricted blood flow is compensated by increased O_2 delivery through the plasma. A separate anti-inflammatory pathway for hyperbaric oxygen involves impaired proinflammatory cytokine production by monocyte-macrophages (Benson et al., 2003). The effect on monocyte-macrophages may be the basis for reduced levels of circulating proinflammatory cytokines under stress conditions (Fildissis et al., 2004). Hence, an oxidative stress response seems to occur. Oxidative stress responses triggered by hyperbaric oxygen improve outcome from a wide variety of postischemic or inflammatory insults. HBO also improves ischemic tolerance when used in a prophylactic manner. Augmented synthesis of ROS temporarily inhibits adherence or sequestration of neutrophils by inhibiting β_2 integrin function, and in many tissues, this therapy will induce antioxidant enzymes and anti-inflammatory proteins (Thom, 2011). The recognition that ROS may stimulate inflammatory signaling pathways comes with considerable clinical ramifications. Once the sources and targets of second-messenger ROS will be identified, new avenues will be open for the development of novel pharmacophores that function both as antioxidants and nonsteroidal anti-inflammatory agents (Hensley et al., 2000).

However, when Staple and Clement (1996) performed HBO treatment on 66 people with muscular pain of the quadriceps femoris, the group who received HBO treatment reported no large difference in recovery, especially regarding subjective sharp pain. Furthermore, when Borromeo et al. (1997) performed the HBO treatment on 32 patients with acute leg joint sprains, they also reported that the mitigation of sharp pain and swelling did not occur appreciably. However, this particular study did use a relatively small sample size and subjects only received 3 treatments after an average delay of 34 hours from the time of injury to treatment. According to Zamboni et al. (1993), when there is an exposition to hyperbaric oxygen environment at 2.5 to 3.0 ATA for at least 45 minutes, the ability of circulating neutrophils to adhere the target tissues is temporarily inhibited. Furthermore, conditions of 2 ATA/60 minutes were compared with 2 ATA/30 minutes and 1.5 ATA/30 minutes on ligament healing. When collagen tissue was compared among the three groups that underwent the HBO treatment, the speed of ligament recovery occurred in the order of 2 ATA/60 minutes first, followed by 2 ATA/30 minutes, and finally 1.5 ATA/30 minutes (Ishii et al., 2002). Moreover, Delaney and Montgomery (2001) state that HBO should be initiated within 24 hours of

injury, in the acute phase. This is also supported by Kanhai and Losito (2003) who concluded that the time between injury and treatment may affect outcomes. On the other hand, no significant results were found after 10 days of treatment in Nelson et al. (1994) trial, where after crushing surgically the *gastrocnemius* of 36 rabbits were submitted 16-18h post-muscle crush and then daily, for 90 min 5 days/wk to either 100% O_2 at 2.5 ATA, 8.5% O_2 and 91.5% N_2 at 2.5 ATA, 100% O_2 at 1 ATA, or 21% O_2 at 1 ATA.

In the proliferative phase, the regeneration process usually occurs seven to ten days after injury and peaks at two weeks and then decreases at three to four weeks post-injury (Huard et al., 2002). In musculoskeletal tissues (with the exception of cartilage), the oxygen supply to the damaged area is gradually raised playing an important role for the synthesis of extracellular matrix components such as fibronectin and proteoglycan. Also, certain processes cannot be performed in the rough endoplasmic reticulum without oxygen, and as a consequence collagen synthesis becomes impossible (Ishii et al., 2005). Fibroblast and osteoclast stimulation is another response to HBO that benefits treatment of fractures and osteomyelitis (Delaney and Montgomery, 2001, Wang et al., 2002). It is tempting to assume that this adaptation would enhance the process of ligamentisation that occurs after a sports injury.

In the remodeling phase, tissue is slowly replaced over many hours using the oxygen supply provided by the blood supply vessel already built into the organization of the musculoskeletal system. If the damage is small, the tissue is recoverable with nearly perfect organization. If the extent of the damage is large, a scar, mainly constituted of collagen, may replace tissue (Ishii et al., 2005). The formation of scar tissue (fibrosis) begins between the second and third weeks post injury, and the scar tissue increases in size over time. The formation of scar appears to be the end product of the muscle repair process (Huard et al., 2002). Consequently, depending on the injury, this collagen will become deficiently hard in the case of muscle repair, or deficiently loose in the case of ligament repair (Ishii et al., 2005). In contrast, in the case of ACL it does not appear to form a bridging scar after rupture even when a primary repair has been performed (O'Donoghue et al., 1971). In this ligament, the resulting chronic inflammation is associated with atrophy and degeneration of the collagen matrix leaving a permanently damaged, weak and non-functional ligament (Solomonow, 2004). As mentioned before,

HBO environment improve oxygen circulation and improve recovery. Therefore, HBO treatment is expected to affect remodeling of damaged tissue, angiogenesis and synthetic production of extracellular matrix components, especially collagen. In fact, in the present thesis, analyzing paper III, which purpose was to analyze the influence of HBO in neovascularization and in the production of protein in injured ACL of rabbits, HBO promoted neovascularization by activating VEGF which probably enhanced the production of collagen process that may had contributed for the thickness of the capsule, probably such as type II, III or IV collagen accelerating rabbits recovery. The rabbits also started to move earlier than the control rabbits, however HBO did not contributed for the presence of collagen Type I in ACL, which can contribute to a more disorganized tissue and mechanically less resistant.

Generally, it is supposed that vascular endothelial growth factors are stimulated, and the low oxygen stimulus in the convalescence of an injury works in the favor of blood vessel regeneration. However, according to the report of Méchine et al. (1999) treatment with HBO resulted in a rise of blood vessel density under granulation, and promoted a blood vessel sprout. Several studies have described angiogenesis as a physiological response to HBO that is beneficial for treating gangrene. The effect of HBO on gas gangrene has been known since the 1960s, as described by Brummelkamp & Boerema (Brummelkamp et al., 1961) and it has been studied in several experimental studies and reviewed afterwards (Hart et al., 1983). This is certainly an adaptation that could be beneficial to the healing of sports injuries, however Gill and Bell (2004) are more detailed stating that this process specifically occurs along the periphery of ischemic wounds. In fact, Kivisaari and Niinikoski (1975) reported that in ischemic wounds intermittent HBO for 2 hours twice daily enhanced the wound closure rate in the final healing stage, however, it had no significant influence on the closure rate of open skin wounds with normal blood supply. Moreover, according to Uhl et al. (1994) it is believed that the effect of the 100% oxygen twice daily HBO treatment (2 ATA/45 minutes) improves re-epithelisation in normal and ischemic skin tissue.

The recovery process of a damaged medial collateral knee ligament has also been investigated. Webster et al. (1996) reported in rats that the elasticity and fracture intensity of a ligament recovered to almost its normal value within 4 weeks with HBO treatment (2.8 ATA/1.5 hours) and Mashitori et al. (2004) also reported that type I

procollagen gene expression at 7 or 14 days after injury was significantly higher in the HBO group and the ultimate load and stiffness was significantly greater at 14 days (2.5 ATA/2 hours). These effects are associated with the improvement of their tensile properties. When Best et al. (1998) examined the influence of the contraction ability of flounder-line damaged reproductive organs on rats, the HBO treatment, at 3 ATA showed that the recovery effect may vary with different types of muscle fibers. Furthermore, while injuries analyzed in this thesis involving muscles and ligaments have shown promising results, HBO treatment also contributed for the increasing of the thickness of the capsule in the knee of rabbits. A previous research from Ishii et al. (1999) in sport injury involved 22 athletes with various injuries including seven leg joint sprains, four knee ligament damages, four partial muscle ruptures, three peripheral nerve injuries, two fractures and two other injuries. Depending on the clinical condition of the patient, atmospheric pressure was set between 1.3–2 ATA, and the exposure time was from 30 to 90 minutes. As a result, more than 70% of the patients experienced improvement. Furthermore, there was a correlation between improvement and time from the injury in which the treatment was administered.

Between muscle injuries, in the present thesis, the recovery from muscle contusion was explored. Although contusion injury is capable of healing, incomplete functional recovery often occurs, depending on the severity of the initial trauma or even on the age (Minamoto et al., 2001). In fact, Neuparth (2005) after inducing muscle contusion in rats observed loss of continuity of the sarcolemma, presence of an infiltrative hemorrhage with erythrocytes in the interstitial space and intracellular edema, contrasting with the normal morphological pattern of the control group, inducing an increase in muscle weight also seen in Minamoto et al. (2001) trial.

A significantly higher number of adherent neutrophils was also observed in Menth-Chiari (1998) study after 300 minutes with muscle contusion. The tissue damage that occurs with contusion consists of an acute component that leads to the stimulation of the endothelial cells and recruitment of rolling (preceding adhesion) by the polymorphonuclear neutrophils (PMNs). Over the midterm to long-term stages of tissue injury associated with contusion, a significant portion of the overall tissue damage is

probably secondary to PMN damage to the microvascular endothelium and movement of the PMNs into the tissue.

Thus, this process can be seen as an expression of deterioration in muscle in response to functional or pathological changes. Therefore, one of the aims of this thesis was to analyze is HBO influence the biomechanics properties of the *gastrocnemius* muscle. There were significant differences in hardness and maximum weight between the non-treated with HBO groups. Furthermore, this type of injury increased significantly the CPK, a marker of muscle injury, in the non-treated group compared to the HBO group, which can suggest that HBO had a positive effect on muscle injury recovery after inducing contusion.

Mitochondria are thought to be an important factor not only in energy transfer but also in the control of apoptosis. There are several pathways involving mitochondria that control apoptosis (Rosse et al., 1998). Cytochrome *c* release and mitochondrial membrane potential change are both considered to be important in the initiation of caspase cascade leading to apoptosis (Yasuhara et al., 2000).

However, although immediately after the injury it can observe zones of intracellular edema, particularly nearby the damaged membrane, the organelles, especially mitochondria remained intact and showed no signs of swelling. At this initial stage there is still myofibrillar disorganization in the impact zone (Neuparth, 2005). However, in the second and fourth day after this injury, myofibrillar disruption and contractile protein denaturation, as well as the intense intracellular edema aspects were evident, inducing mitochondrial swelling and a decrease in ATP synthesis (Neuparth, 2005). In fact, the intrinsic degeneration phenomena observed after induction of injury caused by crushing can be explained, at least in part, to the loss of Ca^{2+} ion homeostasis, since the increase in the intracellular concentration of this ion can initiate the degradation processes to several cell levels (Armstrong et al., 1991). This author adds that with the increasing of the concentrations of Ca^{2+} in the cytoplasm, the buffer function of the mitochondria is activated, in order to maintain skeletal cellular homeostasis.

This general qualitative assessment of morphological changes in damaged muscle fibers was also observed in the study Crisco et al. (1994), which was the model of crushing fiber used in the present study. Moreover, Huard et al. (2002) and MacGregor and Parkhouse (1996) report that this mechanical trauma models of injury, damages the integrity of the plasma membrane and basal lamina, vascular and nervous structures of the affected muscle, allowing the influx of Ca^{2+} into the muscle fiber, with deleterious effects on cell viability.

Therefore, another aim of the present thesis was to analyze the influence of hyperbaric environment on this cellular disintegration, in mitochondria, after muscle contusion. In general, data suggested that HBO contributed to a better integrity in the injured mitochondria by delaying the time for cellular apoptosis. Concerning to the respiratory chain, it was seen that hyperbaric environment influenced positively the results in complex I and II, comparing to control group who had no treatment and HBO group. However, when analyzing the mitochondrial swelling during mitochondrial permeability transition pores (MPTP) induction, in HBO group, the lag phase elapsed until large scale swelling initiates (time to V_{max}) was significantly higher than other groups and, therefore, the swelling amplitude was significantly smaller than the other groups which can guide us to other finding that presents a lower susceptibility to MPTP opening. This was particularly unpredicted as lag phase in $\Delta\psi$ was increased and once more suggests that a phosphate mechanism might be involved (Wei et al., 2012). An interesting possibility arises from p53 target gene phosphate-activated mitochondrial glutaminase (GLS2), a key enzyme in conversion of glutamine to glutamate, and thereby a regulator of glutathione (GSH) synthesis and energy production. The GLS2 expression is induced in response to DNA damage or oxidative stress in a p53-dependent manner, and p53 associates with the GLS2 promoter. Elevated GLS2 facilitates glutamine metabolism and lowers intracellular reactive oxygen species (ROS) levels (Suzuki et al., 2010). On the other hand, another attractive explanation is that HBO application hypothetically could have induced, due to ROS, an increase in antioxidant enzymes and/or mechanism as anti-inflammatory and anti-apoptotic proteins, however there were not identified for not being the aim of this study.

However, these results have shown that HBO environment can increase the integrity of the mitochondria, and therefore delaying cellular apoptosis.

The experimental model of contusion injury used in this study was chosen mainly because of its feasibility, the effect is mostly local and its reproducibility that had already been demonstrated (Crisco et al., 1994). Furthermore, as mentioned before, muscle contusion induced by this method is a high-energy blunt injury that creates a large hematoma and is followed by massive muscle regeneration (Crisco et al., 1994, Kasemkijwattana et al., 1998) healing processes that are very similar to those seen in humans (Diaz et al., 2003). It is also important to reference that these injuries are often encountered in contact sports (Minamoto et al., 2001) and, as such, the study of the regenerative process using this experimental model may have some applicability.

Although these reports suggest that HBO is advantageous for medical treatment, the optimal conditions for treatment (atmospheric pressure, duration of sessions, frequency of session, and duration of treatment) still need to be determined.

Once again, well-designed clinical trials in humans are needed to determine if the placebo effect is significant for soft tissue injury, although at this point it must be recognized that it is difficult to obtain a large sample size due to availability of hyperbaric chambers and obtaining subjects who have similar stage injuries.

However, before deliberating the use of HBO, an appropriate medical screen prior to HBO should enable the practitioner to avoid potentially dangerous situations and ensure the safety of participants.

CHAPTER IV

CONCLUSIONS

Conclusions

Based on the results of the different studies which comprised in this thesis, it seems reasonable to highlight the following conclusions:

a) HBO attenuate the production of CPK, a marker of muscle injury, after muscle contusion, comparing to control group.
b) In the recovery from muscle contusion, HBO had shown significantly better results in the following biomechanical parameters: hardness and maximum weight.
c) HB environment has a positive effect in the respiratory chain of mitochondria, in complex I and II, after muscle contusion.
d) The time until large scale swelling initiates in mitochondria was higher in HBO and the swelling amplitude was lower in HBO group after muscle contusion.
e) HBO contributed to the increase of neovascularization activating VEGF after ACL rupture.
f) HBO enhanced capsule thickness comparing to control group after ACL rupture.

In summary, HBO seems to play an important role in the recovery of muscle injuries, more specifically, muscle contusion in rats, by analyzing muscle biomechanical properties and mitochondria energetic. Concerning to ACL rupture, HBO influenced the neovascularization and changed the collagen production process, which contributed to increase the thickness of the capsule.

Even though further and translations studies are needed to fully understand the mechanisms of HBO, it seems clear that HBO is an important adjuvant therapy for the treatment of sports injuries.

CHAPTER IV

REFERENCES

References

ALBUQUERQUE & SOUSA, J. G. (2007) Oxigenoterapia hiperbárica (OTHB). Perspectiva histórica, efeitos fisiológicos e aplicações clínicas. *Revista da Sociedade Portuguesa de Medicina Interna,* 14**,** 219-227.

AMARAL, A., PARIZOTTO, N. & SALVINI, T. (2001) Dose-dependency of low-energy HeNe laser effect in regeneration of skeletal muscle in mice. *Lasers in Medical Science,* 16**,** 44-51.

AMES, B., SHIGENAGA, M. & HAGEN, T. (1995) Mitochondrial decay in aging. *Biochimica et Biophysica Acta,* 1271**,** 165-170.

ANTONELLI, C., FRANCHI, F., DELLA MARTA, M. E., CARINCI, A., SBRANA, G., TANASI, P., DE FINA, L. & BRAUZZI, M. (2009) Guiding principles in choosing a therapeutic table for DCI hyperbaric therapy. *Minerva anestesiologica,* 75**,** 151-161.

ARMSTRONG, R., WARREN, G. & WARREN, J. (1991) Mechanisms of exercise-induced muscle fibre injury. *Sports Medicine,* 12**,** 184-207.

BABUL, S. & RHODES, E. (2000) The Role of Hyperbaric Oxygen Therapy in Sports Medicine. *Sports Medicine,* 30**,** 395-403.

BABUL, S., RHODES, E., TAUNTON, J. & LEPAWSKY, M. (2003) Effects of Intermittent Exposure to Hyperbaric Oxygen for the Treatment of an Acute Soft Tissue Injury. *Clinical Journal of Sports Medicine,* 13**,** 138-147.

BACH, B. J., LEVY, M., BOJCHUK, J., TRADONSKY, S., BUSH-JOSEPH, C. & KHAN, N. (1998) Single-incision endoscopic anterior cruciate ligament reconstruction using patellar tendon autograft. Minimum two-year follow-up evaluation. *American Journal of Sports Medicine,* 26**,** 30-40.

BALESTRA, C., GERMONPRÉ, P., SNOECK, T., EZQUER, M., LEDUC, O., LEDUC, A., WILLEPUT, F., MARRONI, A., CALI CORLEO, R. & VANN, R. (2004) Normobaric oxygen can enhance protein captation by the lymphatic system in healthy humans. *Undersea and Hyperbaric Medicine,* 31**,** 59-62.

BARATA, P., CERVAENS, M., RESENDE, R., CAMACHO, O. & MARQUES, F. (2011) Hyperbaric oxygen on sports injuries. *Therapeutic Advances in Musculoskeletal Disease,* 3**,** 111-121.

BEINER, J. & JOKL, P. (2002) Muscle contusion injury and myositis ossificans traumatica. *Clinical Orthopaedics and Related Research,* (403 Suppl)**,** S110-9.

BEINER, J. M., JOKL, P., CHOLEWICKI, J. & PANJABI, M. M. (1999) The Effect of Anabolic Steroids and Corticosteroids on Healing of Muscle Contusion Injury. *The American journal of Sports Medicine,* 27**,** 2-9.

BENNETT, M., BEST, T., BABUL-WELLAR, S. & TAUNTON, J. (2005a) Hyperbaric oxygen therapy for delayed onset muscle soreness and closed soft tissue injury. *Cochrane Database of Systematic Reviews,* 19**,** 1-39.

BENNETT, M. H., STANFORD, R. E. & TURNER, R. (2005b) Hyperbaric oxygen therapy for promoting fracture healing and treating fracture non-union. *Cochrane Database of Systematic Reviews,* Issue 1. Art. No.**,** CD004712. DOI: 10.1002/14651858.CD004712.pub2.

BENSON, R., MINTER, L., OSBORNE, B. & GRANOWITZ, E. (2003) Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. *Clinical and Experimental Immunology* , 134**,** 57-62.

BEST, T., LOITZ-RAMAGE, B., CORR, D. & VANDERBY, R. J. (1998) Hyperbaric oxygen in the treatment of acute muscle stretch injuries: results in an animal model. *American Journal of Sports Medicine,* 26**,** 367-372.

BEYNNON, B., JOHNSON, R., FLEMING, B., PEURA, G., RENSTROM, P., NICHOLS, C. & POPE, M. (1997) The effect of functional knee bracing on the anterior cruciate ligament in the weightbearing and nonweightbearing knee. *American Journal of Sports Medicine,* 25**,** 353-359.

BLOCK, E. R. (1977) Effect of superoxide dismutase and succinate on the development of hyperbaric oxygen toxicity. *Aviation, space, and environmental medicine,* 48**,** 645.

BORROMEO, C. N., RYAN, J. L., MARCHETTO, P. A., PETERSON, R. & BOVE, A. A. (1997) Hyperbaric oxygen therapy for acute ankle sprains. *American Journal of Sports Medicine,* 25**,** 619-625.

BOSCO, G., YANG, Z.-J., NANDI, J., WANG, J., CHEN, C. & CAMPORESI, E. M. (2007) Effects of Hyperbaric Oxygen on Glucose, Lactate, Glycerol and Anti-Oxidant Enzimes in the Skeletal Muscle of Rats during Ischaemia and Reperfusion. *Clinical and Experimental Pharmacology and Physiology,* 34**,** 70-76.

BOUAK, F. (2004) Oxygen Therapy - Description and Advances in Oxygen Delivery Systems. Toronto, Defence R&D Canada - Technical Memorandum.

BRAND, M. D. & NICHOLLS, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochemical Journal,* 435**,** 297-312.

BRANDÃO, M. L., ROSELINO, J. E. S., PICCINATO, C. E. & CHERRI, J. (2003) Mitochondrial Alterations in Skeletal Muscle Submitted to Total Ischemia. *Journal of Surgical Research,* 110**,** 235-240.

BROEKEMEIER, K. M., DEMPSEY, M. E. & PFEIFFER, D. R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *The Journal of Biological Chemistry,* 264**,** 7826-7830.

BROOKES, P. S., YOON, Y., ROBOTHAM, J. L., ANDERS, M. W. & SHEY-SHING, S. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *American Journal of Physiology - Cell Physiology,* 287**,** C817–C833.

BRUMMELKAMP, W. H., HOGENDIJK, J. & BOEREMA, I. (1961) Treatment of anaerobic infections (clostridial myositis) by drenching the tissues with oxygen under high atmospheric pressure. *Surgery,* 49**,** 299-302.

CAMACHO, O. (2013) Medicina Hiperbárica e Subaquática. IN ORDEM DOS MÉDICOS *Competência em Medicina Hiperbárica e Subaquática*. Porto.

CAPES, J. P. & TOMASZEWSKI, C. (1996) Prophylaxis against middle ear barotrauma in US hyperbaric oxygen therapy centers. *The American journal of emergency medicine,* 14**,** 645-648.

CARLSON, B. M. & FAULKNER, J. A. (1983) The regeneration of skeletal muscle fibres following injury: a review. *Medicine & Science in Sports & Exercise,* 15**,** 187-198.

CERVAENS, M. & BARATA, P. (2009) Sensação Retardada de Dor Muscular. *Universidade Fernando Pessoa: Revista da Faculdade de Ciências da Saúde,* 6**,** 186-196.

CERVAENS, M., CAMACHO, O., RESENDE, R., MARQUES, F. & BARATA, P. (2011) Hyperbaric Oxygen Therapy treatment for the recovery of muscle injuries induced in rats. IN SOCIETY, E. U. B. (Ed.) *37th Annual Meeting of the European Underwater & Baromedical Society (EUBS).* Gdänsk.

CHEUNG, K., HUME, P. A. & MAXWELL, L. (2003) Delayed onset muscle soreness: treatment strategies and performance factors. / La douleur musculaire differee: strategies de therapie et facteurs de performance. *Sports Medicine,* 33**,** 145-164.

CLARK, J., LAMBERTSEN, C., GELFAND, R., FLORES, N., PISARELLO, J., ROSSMAN, M. & ELIAS, J. (1999) Effects of prolonged oxygen exposure at 1.5, 2.0, or 2.5 ATA on pulmonary function in men (Predictive Studies V). *Journal of Applied Physiology,* 86**,** 243.

CLARK, J. M. & THOM, S. R. (Eds.) (2003) *Oxygen under pressure,* Philadelphia, *Saunders*.

CRISCO, J., JOKL, P., HEINEN, G., CONNELL, M. & PANJABI, M. (1994) A muscle contusion injury model. Biomechanics, physiology, and histology. *American Journal of Sports Medicine,* 22**,** 702-10.

DAVIS, M. (1998) Oxygen therapy equipment - a theoretical review. *South Pacific Medicine Society (SPUMS) Journal,* 28**,** 165-172.

DE LAET, C., OBYN, C., RAMAEKERS, D., VAN DE SANDE, S. & NEYT, M. (2008) Hyperbaric Oxygen Therapy: a Rapid Assessment. *Health Technology Assessment.* Brussels, Belgian Health Care Knowledge Centre (KCE).

DEEHAN, D. J. & CAWSTON, T. E. (2005) The biology of integration of the anterior cruciate ligament. *The Journal of Bone and Joint Surgery,* 87-B**,** 889-895.

DELANEY, J. S. & MONTGOMERY, D. L. (2001) How can hyperbaric oxygen contribute to treatment? *The Physician and Sportsmedicine,* 29**,** 77-84.

DEMCHENKO, I., WELTY-WOLF, K., ALLEN, B. & PIANTADOSI, C. (2007) Similar but not the same: normobaric and hyperbaric pulmonary oxygen toxicity, the role of nitric oxide. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 293**,** L229.

DIAZ, J., FISCHER, D., RETTIG, A., DAVIS, T. & SHELBOURNE, K. (2003) Severe quadriceps muscle contusions in athletes: a report of three cases. *American Journal of Sports Medicine,* 31**,** 289-293.

DIMITRIJEVICH, S. D., PARANJAPE, S., WILSON, J. R., GRACY, R. W. & MILLS, J. G. (1999) Effect of hyperbaric oxygen on human skin cells in culture and in human dermal and skin equivalents. *Wound Repair and Regeneration,* 7**,** 53-64.

DUTHON, V. B., BAREA, C., ABRASSART, S., FASEL, J. H., FRITSCHY, D. & MÉNÉTREY, J. (2006) Anatomy of the anterior cruciate ligament. *Knee Surgery, Sports Traumatology, Arthroscopy,* 14**,** 204-213.

ECHM European Committee for Hyperbaric Medicine. http://www.echm.org/

ESTABROOK, R. (1967) Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods in Enzymology,* 10**,** 41-47.

FELDMEIER, J. J. (2003) Hyperbaric oxygen 2003: indications and results: the hyperbaric oxygen therapy committee report. *Kensington, MD: Undersea and Hyperbaric Medical Society.*

FERNANDES, T. D. (2009) Medicina Hiperbárica. *Acta Medica Portuguesa,* 22**,** 324-334.

FERRARA, N. & DAVIS-SMYTH, T. (1997) The biology of vascular endothelial growth factor. *Endocrine Reviews,* 18**,** 4-25.

FILDISSIS, G., VENETSANOU, K., MYRIANTHEFS, P., KARATZAS, S., ZIDIANAKIS, V. & BALTOPOULOS, G. (2004) Whole blood pro-inflammatory cytokines and adhesion molecules postlipopolysaccharides exposure in hyperbaric conditions. *European Cytokine Network,* 15**,** 217-221.

FISHER, P. (1999) Delayed onset muscle soreness in long-distance runners. *Journal Of Alternative And Complementary Medicine (New York, N.Y.),* 5**,** 119-119.

FLEMING, B. C., FADALE, P. D., HULSTYN, M. J., SHALVOY, R. M., OKSENDAHL, H. L., BADGER, G. J. & TUNG, G. A. (2013) The Effect of Initial Graft Tension After Anterior Cruciate Ligament Reconstruction - A Randomized Clinical Trial With 36-Month Follow-up. *The American Journal of Sports Medicine,* 41**,** 25-34.

FRANK, C., WOO, S.-Y., AMIEL, D., HARWOOD, F., GOMEZ, M. & AKESON, W. (1983) Medial collateral ligament healing: a multidisciplinary assessment in rabbits. *American Journal of Sports Medicine,* 11**,** 379-89.

GARRETT JR, W. E. (1990) Muscle strain injuries: clinical and basic aspects. *Medicine & Science in Sports & Exercise,* 22**,** 436-443.

GERMAIN, G., DELANEY, J., MOORE, G., LEE, P., LACROIX, V. & MONTGOMERY, D. (2003) Effect of hyperbaric oxygen therapy on exercise-induced muscle soreness. *Undersea and Hyperbaric Medicine,* 30**,** 135-145.

GIBSON, J. & HUNT, T. (1998) Hyperbaric oxygen potentiates wound healing. *European Journal of Underwater and Hyperbaric Medicine,* 5**,** 7-9.

GILL, A. L. & BELL, C. N. A. (2004) Hyperbaric oxygen: its uses, mechanisms of action and outcomes. *Quarterly Journal of Medicine,* 97**,** 385-395.

GLADDEN, L. B. (2004) Lactate metabolism: a new paradigm for the third millennium. *The Journal of physiology,* 558**,** 5.

GREENE, W. B., BALENTINE, J. D. & HENNIGAR, G. R. (1979) Selective mitochondrial degeneration in renal tubules following hyperbaric oxygen exposure. *The American Journal of Pathology,* 96**,** 737.

GREGOREVIC, P., LYNCH, G. S. & WILLIAMS, D. A. (2000) Hyperbaric oxygen improves contractile function of regenerating rat skeletal muscle after myotoxic injury. *Journal of Apllied Physiology,* 89**,** 1477-1482.

GRIM, P. S., GOTTLIEB, L., BODDIE, A. & BATSON, E. (1990) Hyperbaric oxygen therapy. *Jama,* 263**,** 2216.

GUPTE, S., RUPAWALLA, T., MOHAZZAB-H, K. & WOLIN, M. (1999) Regulation of NO-elicited pulmonary artery relaxation and guanylate cyclase activation by HADH oxidase and SOD. *American Journal of Physiology, Heart and Circulatory Physiology,* 276**,** H1535-H1542.

HAAPANIEMI, T., NYLANDER, G., SIRSJÖ, A. & LARSSON, J. (1996) Hyperbaric Oxygen Reduces Ischemia-Induced Skeletal Muscle Injury. *American Society of Plastic Surgeons,* 97**,** 602-607.

HAMILTON-FARRELL, M. & BHATTACHARYYA, A. (2004) Barotrauma. *Injury,* 35**,** 359-370.

HARRISON, B., ROBINSON, D., DAVISON, B., FOLEY, B., SEDA, E. & BYRNES, W. (2001) Treatment of exercise-induced muscle injury via hyperbaric oxygen therapy. *Medicine & Science in Sports & Exercise,* 33**,** 36-42.

HART, G. B., LAMB, R. C. & STRAUSS, M. B. (1983) Gas gangrene: I. A collective review. *The Journal of trauma,* 23**,** 991.

HEFTI, F. L., KRESS, A., FASEL, J. & MORSCHER, E. W. (1991) Healing of the transected anterior cruciate ligament in the rabbit. *The Journal of Bone and Joint Surgery,* 73-A**,** 373-383.

HENSLEY, K., ROBINSON, K., GABBITA, S., SALSMAN, S. & FLOYD, R. (2000) Reactive oxygen species, cell signaling, and cell injury. *Free Radical Biology and Medicine,* 28**,** 1456-1462.

HINK, J. & JANSEN, E. (2001) Are superoxide and/or hydrogen peroxide responsible for some of the beneficial effects of hyperbaric oxygen therapy? *Medical hypotheses,* 57**,** 764-769.

HORN, P. C., WEBSTER, D. A., AMIN, H. M., MASCIA, M. F., WERNER, F. W. & FORTINO, M. D. (1999) The Effect of Hyperbaric Oxygen on Medial Collateral Ligament Healing in a Rat Model. *Clinical Orthopaedics & Related Research,* 360**,** 238-242.

HUARD, J., LI, Y. & FU, F. H. (2002) Muscle Injuries and Repair: Current Trends in Research. *The Journal of Bone and Joint Surgery,* 84-A**,** 822-832.

HURME, T. & KALIMO, H. (1992) Activation of myogenic precursor cells after muscle injury. *Medicine & Science in Sports & Exercise,* 24**,** 197-205.

ISHII, Y., DEIE, M., ADACHI, N., YASUNAGA, Y., SHARMAN, P., MIYANAGA, Y. & OCHI, M. (2005) Hyperbaric Oxygen as an Adjuvant for Athletes. *Sports Medicine,* 35**,** 739-746.

ISHII, Y., MIYANAGA, Y. & SHIMOJO, H. (1999) The role of hyperbaric oxygen for the early return to sports activities [in Japanese]. *Journal of Musculoskeletal System* 12**,** 785-789.

ISHII, Y., USHIDA, T., TATEISHI, T., SHIMOJO, H. & MIYANAGA, Y. (2002) Effects of different exposures of hyperbaric oxygen on ligament healing in rats. *Journal of Orthopaedic Research,* 20**,** 353-356.

IYOMASA, D., GARAVELO, I., IYOMASA, M., WATANABE, I. & ISSA, J. (2009) Ultrastructural analysis of the low level laser therapy effects on the lesioned anterior tibial muscle in the gerbil. *Micron,* 40**,** 413-8.

JACKSON, D. & FEAGIN, J. (1973) Quadriceps contusions in young athletes: Relation of severity of injury to treatment and prognosis. *The Journal of Bone and Joint Surgery,* 55A**,** 95-105.

JACOBSON, K. (1977) Osteoarthritis following insufficiency of the cruciate ligament in man: a clinical study. *Acta Orthopaedica Scandinavica,* 48**,** 520-526.

JAIN, K. K. (2004) *Textbook of hyperbaric medicine,* Military Medicine.

JAMES, P. B., SCOTT, B. & ALLEN, M. W. (1993) Hyperbaric oxygen therapy in sports injuries. *Physiotherapy,* 79**,** 571-572.

JÄRVINEN MJ, EINOLA SA & EO, V. (1992) Effect of the position of immobilization upon the tensile properties of the rat gastrocnemius muscle. *Archives of Physical Medicine and Rehabilitation,* 73**,** 253-7.

JÄRVINEN, M. J. & LEHTO, M. U. K. (1993) The Effects of Early Mobilisation and Immobilisation on the Healing Process Following Muscle Injuries. *Sports Medicine,* 15**,** 78-89.

JÄRVINEN, T., JÄRVINEN, T., KÄÄRIÄINEN, M., ÄÄRIMAA, V., VAITTINEN, S., KALIMO, H. & JÄRVINEN, M. (2007) Muscle injuries: optimising recovery. *Best Practice & Research Clinical Rheumatology,* 21**,** 317-331.

JÄRVINEN, T., KÄÄRIÄINEN, M., JÄRVINEN, M. & KALIMO, H. (2000) Muscle strain injuries. *Current Opinion in Rheumatology,* 12**,** 155-161.

JÄRVINEN, T. A. H., JÄRVINEN, T. L. N., KÄÄRIÄINEN, M., KALIMO, H. & JÄRVINEN, M. (2005) Muscle Injuries: Biology and Treatment. *The American Journal of Sports Medicine,* 33**,** 745-764.

JI, L. L. (1999) Antioxidants and oxidative stress in exercise. *Proceedings of the Society for Experimental Biology and Medicine,* 222**,** 283-292.

KAMO, N., MURATSUGU, M., HONGOH, R. & KOBATAKE, Y. (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *The Journal of Membrane Biology,* 49**,** 105-121.

KANG, T. S., GORTI, G. K., QUAN, S. Y., HO, M. & KOCH, R. J. (2004) Effect of hyperbaric oxygen on the growth factor profile of fibroblasts. *Archives of Facial Plastic Surgery,* 6**,** 31-35.

KANHAI, A. & LOSITO, J. M. (2003) Hyperbaric oxygen therapy for lower-extremity soft-tissue sports injuries. *Journal of the American Podiatric Medical Association,* 93**,** 298-306.

KASEMKIJWATTANA, C., MENETREY, J., SOMOGYL, G., MORELAND, M., FU, F., BURANAPANITKIT, B., WATKINS, S. & HUARD, J. (1998) Development of approaches to improve the healing following muscle contusion. *Cell Transplant,* 7**,** 585-598.

KESSLER, M., BEHREND, H., HENZ, S., STUTZ, G., RUKAVINA, A. & KUSTER, M. (2008) Function, osteoarthritis and activity after ACL-rupture: 11 years follow-up results of conservative versus reconstructive treatment. *Knee Surgery, Sports Traumatology, Arthroscopy,* 16**,** 442-448.

KIVISAARI, J. & NIINIKOSKI, J. (1975) Effects of hyperbaric oxygenation and prolonged hypoxia on the healing of open wounds. *Acta Chirurgica Scandinavica* 141**,** 14-19.

KOHN, D. (1986) Arthroscopy in acute injuries of anterior cruciate-deficient knees: fresh and old intraarticular lesions. *Arthroscopy,* 2**,** 98-102.

KOMURCU, M., ATESALP, A. S., BASBOZKURT, M. & KURKLU, M. (2002) The treatment of infected tibial nonunion with aggressive debridement and internal bone transport. *Military Medicine,* 167**,** 978-981.

KOT, J., DESOLA, J., SIMAO, A., GOUGH-ALLEN, R., HOUMAN, R., MELIET, J., GALLAND, F., MORTENSEN, C., MUELLER, P. & SIPPINEN, S. (2004) A European code of good practice for hyperbaric oxygen therapy. *International maritime health,* 55**,** 121.

KRUGER, N. J. (1994) The Bradford Method for Protein Quantitation. *Methods in Molecular Biology,* 32**,** 9-15.

KUNNAVATANA, S. S., QUAN, S. Y. & KOCH, R. J. (2005) Combined Effect of Hyberbaric Oxygen and N-Acetylcysteine on Fibroblast Proliferation. *Archives of Otolaryngology-Head & Neck Surgery,* 131**,** 809-814.

KVIST, H., JARVINEN, M. & SORVARI, T. (1974) Effect of mobilization and immobilization on the healing of contusion injury in muscle: a preliminary report of a histological study in rats. *Scandinavian Journal of Rehabilitation Medicine* 6**,** 134-140.

LAURENCIN, C. T. & FREEMAN, J. W. (2005) Ligament tissue engineering: An evolutionary materials science approach. *Biomaterials,* 26**,** 7530-7536.

LEE, H., NATSUI, H., AKIMOTO, T., YANAGI, K., OHSHIMA, N. & KONO, I. (2005) Effects of cryotherapy after contusion using real-time intravital microscopy. *Medicine & Science in Sports & Exercise,* 37**,** 1093-8.

LI, J., BROWN, L., HIBBERD, M., GROSSMAN, J., MORGAN, J. & M., S. (1996) VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *American Journal of Physiology,* 270 1803-11.

LI, Y., CUMMINS, J. & HUARD, J. (2001) Muscle Injury and Repair. *Current Opinion in Orthopaedics,* 12**,** 409-415.

LIN, S., SHYU, K., LEE, C., WANG, B., CHANG, C., LIU, Y., HUANG, F. & CHANG, H. (2002) Hyperbaric oxygen selectively induces angiopoietin-2 in human umbilical vein endothelial cells. *Biochemical and Biophysical Research Communications,* 296**,** 710-715.

LOPES, A. S., KATTAN, R., COSTA, S. & MOURA, C. E. (1993) Estudo clínico e classificação das lesões musculares. *Revista Brasileira de Ortopedia,* 28**,** 707-717.

LUMINI-OLIVEIRA, J., ASCENSÃO, A., PEREIRA, C. V., MAGALHÃES, S., MARQUES, F., OLIVEIRA, P. J. & MAGALHÃES, J. (2010) Long-term hyperglycaemia decreases gastrocnemius susceptibility to permeability transition. *European Journal of Clinical Investigation,* 40**,** 319-329.

LUMINI-OLIVEIRA, J., MAGALHÃES, J., PEREIRA, C. V., ALEIXO, I., OLIVEIRA, P. J. & ASCENSÃO, A. (2009) Endurance training improves gastrocnemius mitochondrial function despite increased susceptibility to permeability transition. *Mitochondrion,* 9**,** 454-462.

LUMINI-OLIVEIRA, J., MAGALHÃES, J., PEREIRA, C. V., MOREIRA, A. C., OLIVEIRA, P. J. & ASCENSÃO, A. (2011) Endurance training reverts heart mitochondrial dysfunction, permeability transition and apoptotic signaling in long-term severe hyperglycemia. *Mitochondrion,* 11**,** 54-56.

MACGREGOR, J. & PARKHOUSE, W. (1996) The potential role of insulin-like growth factors in skeletal muscle regeneration. *Canadian Journal of Applied Physiology,* 21**,** 236-250.

MADER, J. T., BROWN, G. L., GUCKIAN, J. C., WELLS, C. H. & REINARZ, J. A. (1980) A mechanism for the amelioration by hyperbaric oxygen of experimental staphylococcal osteomyelitis in rabbits. *The Journal of Infectious Diseases,* 142**,** 915-922.

MAGALHÃES, J., ASCENSÃO, A., SOARES, J., FERREIRA, R., NEUPARTH, M. J., OLIVEIRA, J., AMADO, F., MARQUES, F. & DUARTE, J. (2005a) Acute and chronic exposition of mice to severe hypoxia: the role of acclimatization against skeletal muscle oxidative stress. *International Journal of Sports Medicine,* 26**,** 102-109.

MAGALHÃES, J., ASCENSÃO, A., SOARES, J. M. C., FERREIRA, R., NEUPARTH, M. J., MARQUES, F. & DUARTE, J. A. (2005b) Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. *Journal of Applied Physiology,* 99**,** 1247-1253.

MAGALHÃES, J., FALCÃO-PIRES, I., GONÇALVES, I., LUMINI-OLIVEIRA, J., MARQUES-ALEIXO, I., DOS PASSOS, E., ROCHA-RODRIGUES, S., MACHADO, N., MOREIRA, A., MIRANDA-SILVA, D., MOURA, C., LEITE-MOREIRA, A., OLIVEIRA, P., TORRELLA, J. & ASCENSÃO, A. (2013a) Synergistic impact of endurance training and intermittent hypobaric hypoxia on cardiac function and mitochondrial energetic and signaling. *International Journal of Cardiology,* pii: S0167-5273(13)01526-X. doi: 10.1016/j.ijcard.2013.08.001. [Epub ahead of print].

MAGALHÃES, J., FRAGA, M., LUMINI-OLIVEIRA, J., GONÇALVES, I., COSTA, M., FERREIRA, R., OLIVEIRA, P. J. & ASCENÇÃO, A. (2013b) Eccentric exercise transiently affects mice skeletal muscle mitochondrial function. *Applied Physiology, Nutrition and Metabolism,* 38**,** 401-409.

MASHITORI, H., SAKAI, H., KOIBUCHI, N., OHTAKE, H., TASHIRO, T., TAMAI, K. & SAOTOME, K. M. (2004) Effect of Hyperbaric Oxygen on the Ligament Healing Process in Rats. *Clinical Orthopaedics & Related Research,* 423**,** 268-274.

MATHIEU, D. (2006) *Handbook on hyperbaric medicine,* Kluwer Academic Pub.

MAYER, R., HAMILTON-FARRELL, M. R., KLEIJ, A. J. V. D., SCHMUTZ, J., GRANSTRÖM, G., SICKO, Z., MELAMED, Y., CARL, U. M., HARTMANN, K. A., JANSEN, E. C., DITRI, L. & SMINIA, P. (2005) Hyperbaric Oxygen and Radiotherapy. *Strahlentherapie und Onkologie* 181**,** 113-123.

MÉCHINE, A., ROHR, S., TOTI, F., AYSOY, C., SCHNEIDER, F., MEYER, C., TEMPÉ, J. & BELLOCQ, J. (1999) Wound healing and hyperbaric oxygen. Experimental study of the angiogenesis phase in the rat. *Annales de Chirurgie,* 53**,** 307-313.

MEKJAVIC, I. B., EXNER, J. A., TESCH, P. A. & EIKEN, O. (2000) Hyperbaric oxygen therapy does not affect recovery from delayed onset muscle soreness. *Medicine & Science in Sports & Exercise,* 32**,** 558-563.

MENTH-CHIARI, W. A., CURL, W. W., ROSENCRANCE, E. & SMITH, T. L. (1998) Contusion of Skeletal Muscle Increases Leukocyte-Endothelial Cell Interactions: An Intravital-Microscopy Study in Rats. *The Journal of Trauma: Injury, Infection, and Critical Care,* 45**,** 709-714.

MILOVANOVA, T. N., BHOPALE, V., SOROKINA, E., MOORE, J., HUNT, T., HAUER-JENSEN, M., VELAZQUEZ, O. & THOM, S. (2008) Lactate stimulates vasculogenic stem cells via the thioredoxin system and engages an autocrine activation loop involving hypoxia-inducible factor 1. *Molecular and cellular biology,* 28**,** 6248.

MINAMOTO, V. B., BUNHO, S. R. & SALVINI, T. F. (2001) Regenerated rat skeletal muscle after periodic contusions. *Brazilian Journal of Medical and Biological Research* 34**,** 1447-1452.

MINHAS, R. (2010) Best Practice – BMJ Evidence Center. IN MINHAS, R. (Ed.) *Best Practice - BMJ.* London, http://group.bmj.com/products/evidence-centre/best-practice.

MORTENSEN, C. (2008) Hyperbaric oxygen therapy. *Current Anaesthesia & Critical Care,* 19**,** 333-337.

MUHONEN, A., HAAPARANTA, M., GRÖNROOS, T., BERGMAN, J., KNUUTI, J., HINKKA, S. & HAPPONEN, R.-P. (2004) Osteoblastic activity and neoangiogenesis in distracted bone of irradiated rabbit mandible with or without hyperbaric oxygen treatment. *International journal of oral and maxillofacial surgery,* 33**,** 173-178.

MURRAY, M. M., MARTIN, S. D., MARTIN, T. L. & SPECTOR, M. (2000) Histological Changes in the Human Anterior Cruciate Ligament After Rupture. *The Journal of Bone and Joint Surgery,* 82-A**,** 1387-1397.

NARKOWICZ, C. K., VIAL, J. H. & MCCARTNEY, P. W. (1993) Hyperbaric oxygen therapy increases free radical levels in the blood of humans. *Free Radical Research,* 19**,** 71-80.

NELSON, A., WOLF, E. J. & LI, B. (1994) Influence of delayed hyperbaric oxygenation on recovery from mechanically induced damage. *Undersea and Hyperbaric Medicine,* 21**,** 185-191.

NEUPARTH, M. J. G. S. S. (2005) Contribuição das células percursoras miogénicas para a plasticidade muscular esquelética no modelo animal. *Department of Chemistry.* Aveiro, University of Aveiro.

NULTON-PERSSON, A. & SZWEDA, L. (2001) Modulation of mitochondrial function by hydrogen peroxide. *The Journal of Biological Chemistry* 276**,** 23357-23361.

O'DONOGHUE, D. H., FRANK, G. R., JETER, G. L., JOHNSON, W., ZEIDERS, J. W. & KENYON, R. (1971) Repair and reconstruction of the anterior cruciate ligament in dogs. Factors influencing long-term results. *The Journal of Bone and Joint Surgery,* 53-A**,** 710-718.

OKUBO, Y., BESSHO, K., FUJIMURA, K., KUSUMOTO, K., OGAWA, Y. & IIZUKA, T. (2001) Effect of hyperbaric oxygenation on bone induced by recombinant human bone morphogenetic protein-2. *British Journal of Oral and Maxillofacial Surgery,* 39**,** 91-95.

OLIVEIRA, N., PARIZZOTTO, N. & SALVINI, T. (1999) GaAs (904-nm) laser radiation does not affect muscle regeneration in mouse skeletal muscle. *Lasers in Surgery and Medicine,* 25**,** 13-21.

ORIANI, G., BARNINI, C. & MARRONI, G. (1982) Hyperbaric oxygen therapy in the treatment of various orthopedic disorders *Minerva Medica,* 73**,** 2983-2988.

OURY, T. D., HO, Y., PIANTADOSI, C. & CRAPO, J. (1992) Extracellular superoxide dismutase, nitric oxide, and central nervous system O_2 toxicity. *Proceedings of the National Academy of Sciences,* 89**,** 971.

PALMQUIST, B. M., PHILIPSON, B. & BARR, P. O. (1984) Nuclear cataract and myopia during hyperbaric oxygen therapy. *British Journal of Ophthalmology,* 68**,** 113.

PETERSEN, J. & HÖLMICH, P. (2005) Evidence based prevention of hamstring injuries in sports. *British Journal of Sports Medicine,* 39**,** 319-323.

PETERSEN, W. & TILLMANN, B. (2002) Anatomy and function of the anterior cruciate ligament. *Orthopade,* 31**,** 710-718.

PLAFKI, C., PETERS, P., ALMELING, M., WELSLAU, W. & BUSCH, R. (2000) Complications and Side Effects of Hyperbaric Oxygen Therapy. *Journal of Environmental Medicine,* 71**,** 119-124.

POWERS, S. K. & HOWLEY, E. T. (2000) *Fisiologia do Exercício: Teoria e Aplicação ao Condicionamento e ao Desempenho,* São Paulo, Editora Manole, Ltda.

PUGLIA, C. D. & LOEB, G. A. (1984) Influence of rat brain superoxide dismutase inhibition by diethyldithiocarbamate upon the rate of development of central nervous system oxygen toxicity. *Toxicology and applied pharmacology,* 75**,** 258-264.

PUNTEL, G. O., CARVALHO, N. R., AMARAL , G. P., LOBATO, L. D., SILVEIRA, S. O., DAUBERMANN, M. F., BARBOSA , N. V., ROCHA, J. B. T. & SOARES, F. A. A. (2011) Therapeutic cold: An effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion. *Free Radical Research,* 45**,** 125-138.

RANTANEN, J., THORSSON, O., WOLLMER, P., HURME, T. & KALIMO, H. (1999) Effects of therapeutic ultrasound on the regeneration of skeletal myofibers after experimental muscle injury. *American Journal of Sports Medicine,* 27**,** 54-9.

RISTANIS, S., STERGIOU, N., PATRAS, K., TSEPIS, E., MORAITI, C. & GEORGOULIS, A. D. (2006) Follow-up Evaluation 2 Years After ACL Reconstruction With Bone–Patellar Tendon–Bone Graft Shows That Excessive Tibial Rotation Persists. *Clinical Journal of Sport Medicine,* 16**,** 111-116.

ROCKSWOLD, G., FORD, S., ANDERSON, D., BERGMAN, T. & SHERMAN, R. (1992) Results of a prospective randomized trial for treatment of severely brain-injured patients with hyperbaric oxygen. *Journal of Neurosurgery: Pediatrics,* 76**,** 929-934.

ROSSE, T., OLIVIER, R., MONNEY, L., RAGER, M., CONUS, S., FELLAY, I., JANSEN, B. & BORNER, C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature,* 391**,** 496-499.

RYDER, S., JOHNSON, R., BEYNNON, B. & ETTLINGER, C. (1997) Prevention of ACL injuries. *Journal of Sports Rehabilitation,* 6**,** 80-96.

SCAVENIUS, M., BAK, K., HANSEN, S., NORRING, K., JENSEN, K. H. & JORGENSEN, U. (1999) Isolated total ruptures of the anterior cruciate ligament - a clinical study with long-term follow-up of 7 years. *Scandinavian Journal of Medicine & Science in Sports,* 9**,** 114-119.

SCHASER, K., AC, D., STOVER, J., LAUFFER, A., BAIL, H. & MITTLMEIER, T. (2007) Prolonged superficial local cryotherapy attenuates microcirculatory impairment, regional inflammation, and muscle necrosis after closed soft tissue injury in rats. *American Journal of Sports Medicine,* 35**,** 93-102.

SHAN, X., AW, T. Y. & JONES, D. P. (1990) Glutathione-dependent projection against oxidative injury. *Pharmacology & therapeutics,* 47**,** 61-71.

SHANKAR, P. (1980) Oxygen therapy. *Quarterly Medical Review,* 30**,** 1-20.

SHEIKH, A., GIBSON, J., ROLLINS, M., HOPF, H., HUSSAIN, Z. & HUNT, T. (2000) Effect of hyperoxia on VEGF levels in a wound model. *Archives of Surgery,* 135**,** 1293-1297.

SHERIDAN, R. L. & SHANK, E. S. (1999) Hyperbaric oxygen treatment: a brief overview of a controversial topic. *The journal of trauma,* 47**,** 426-435.

SHI, Y., LEE, C., WU, J., KOCH, C., THOM, S., MAITY, A. & BERNHARD, E. (2005) Effects of hyperbaric oxygen exposure on experimental head and neck tumor growth, oxygenation, and vasculature. *Head & neck,* 27**,** 362-369.

SINGH, C., SINGH, N., SINGH, J., BRAR, G. K. & SINGH, G. (2001) Oxygen Therapy. *Journal Indian Academy of Clinical Medicine,* 2**,** 178-184.

SINGHAL, A. B., BENNER, T., ROCCATAGLIATA, L., KOROSHETZ, W. J., SCHAEFER, P. W., LO, E. H., BUONANNO, F. S., GONZALEZ, R. G. & SORENSEN, A. G. (2005) A Pilot Study of Normobaric Oxygen Therapy in Acute Ischemic Stroke. *Stroke - Journal of the American Heart Association,* 36**,** 797-802.

SLOAN, E., MURPHY, D., HART, R., COOPER, M., TURNBULL, T., BARRECA, R. & ELLERSON, B. (1989) Complications and protocol considerations in carbon monoxide-poisoned patients who require hyperbaric oxygen therapy: Report from a ten-year experience. *Annals of emergency medicine,* 18**,** 629-634.

SMITH, B., LIVESAY, G. & WOO, S.-Y. (1993) Biology and biomechanics of the anterior cruciate ligament. *Clinical Journal of Sports Medicine,* 12**,** 637-670.

SMITH, C., KRUGER, M. J., SMITH, R. M. & MYBURGH, K. H. (2008) The Inflammatory Response to Skeletal Muscle Injury: Illuminating Complexities. *Sports Medicine,* 38**,** 947-969.

SOLOMONOW, M. (2004) Ligaments: a source of work-related musculoskeletal disorders. *Journal of Electromyography and Kinesiology,* 14**,** 49-60.

SOOLSMA, S. J. (1996) The effect of intermittent hyperbaric oxygen on short term recovery from grade I I medial collateral ligament injuries (thesis). Vancouver (CA), University of British Columbia.

ST PIERRE, B. & TIDBALL, J. (1994) Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *Journal of Applied Physiology,* 77**,** 290-297.

STAPLES, J. & CLEMENT, D. (1996) Hyperbaric oxygen chambers and the treatment of sports injuries. *Sports Medicine,* 22**,** 219-227.

STAPLES, J., CLEMENT, D., MCKENZIE, D., BOOKER, T., SBEEL, A. & BELCASTRO, A. (1995) The effects of intermittent hyperbaric oxygen on biochemical muscle metabolites of eccentrically-exercised rats. (abstract). *Canadian Journal of Applied Physiology,* 20 Suppl**,** 49.

STAPLES, J., CLEMENT, D., TAUNTON, J. & MCKENZIE, D. (1999) Effects of Hyperbaric Oxygen on a Human Model of Injury. *The American Journal of Sports Medicine,* 27**,** 600-605.

STRATTON, S., HECKMANN, R. & FRANCIS, R. (1984) Therapeutic ultrasound, its effects on the integrity of a nonpenetrating wound. *Journal of Orthopaedic and Sports Physical Therapy,* 5**,** 278-281.

STRAUB, F. B. & FEUER, G. (1950) Adenosinetriphosphate the functional group of actin. *Biochimica et Biophysica Acta,* 4**,** 455-470.

STROCCHI, R., PASQUALE, V. D., GUBELLINI, P., FACCHINI, A., MARCACCI, M., BUDA, R., ZAFFAGNINI, S. & RUGGERI, A. (1992) The human anterior cruciate ligament: histological and ultrastructural observations. *Journal of Anatomy,* 180**,** 515-519.

SUZUKI, S., TANAKA, T., POYUROVSKY, M., NAGANO, H., MAYAMA, T., OHKUBO, S., LOKSHIN, M., HOSOKAWA, H., NAKAYAMA, T., SUZUKI, Y., SUGANO, S., SATO, E., NAGAO, T., YOKOTE, K., TATSUNO, I. & PRIVES, C. (2010) Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proceedings of the National Academy of Sciences U S A.,* 107**,** 7461-6.

TAKEYAMA N, SAKAI H, OHTAKE H, MASHITORI H, TAMAI K & SAOTOME K (2007) Effects of hyperbaric oxygen on gene expressions of procollagen, matrix metalloproteinase and tissue inhibitor of metalloproteinase in injured medial collateral ligament and anterior cruciate ligament. *Knee Surgery, Sports Traumatology, Arthroscopy,* 15**,** 443-452.

TAKEYAMA, N., SAKAI, H., OHTAKE, H., MASHITORI, H., TAMAI, K. & SAOTOME, K. (2007) Effects of hyperbaric oxygen on gene expressions of procollagen, matrix metalloproteinase and tissue inhibitor of metalloproteinase in injured medial collateral ligament and anterior cruciate ligament *Knee Surgery, Sports Traumatology, Arthroscopy,* 15**,** 443-452.

THOM, S. (2011) Hyperbaric Oxygen: Its Mechanisms and Efficacy. *Plastic and Reconstructive Surgery,* 127 (Suppl 1)**,** 131S-141S.

THOM, S., BHOPALE, V., FISHER, D., MANEVICH, Y., HUANG, P. & BUERK, D. (2002) Stimulation of nitric oxide synthase in cerebral cortex due to elevated partial pressures of oxygen: an oxidative stress response. *Journal of neurobiology,* 51**,** 85-100.

THOM, S. R. (2009) Oxidative stress is fundamental to hyperbaric oxygen therapy. *Journal of Applied Physiology (Bethesda, Md.: 1985),* 106**,** 988-995.

THORSEN, E., AANDERUD, L. & AASEN, T. B. (1998) Effects of a standard hyperbaric oxygen treatment protocol on pulmonary function. *European Respiratory Journal,* 12**,** 1442.

TIBBLES, P. M. & EDELSBERG, J. S. (1996) Hyperbaric-oxygen therapy. *The New England journal of medicine,* 334**,** 1642-1645.

TONKONOGI, M. & SAHLIN, K. (1997) Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. *Acta Physiologica Scandinavica,* 161**,** 345-353.

UENG, S. W.-N., LEE, M. S.-S., TAI, C.-L., HSU, K.-Y., LIN, S.-S., CHAN, Y.-S. & CHEN, W.-J. (2011) Hyperbaric oxygen therapy improves medial collateral ligament healing in a rabbit model. *Formosan Journal of Musculoskeletal Disorders* 2**,** 7-11.

UHL, E., SIRSJÖ, A., HAAPANIEMI, T., NILSSON, G. & NYLANDER, G. (1994) Hyperbaric oxygen improves wound healing in normal and ischemic skin tissue. *Plastic and Reconstructive Surgery,* 93**,** 835-841.

VAN HOESEN, K., CAMPORESI, E., MOON, R., HAGE, M. & PIANTADOSI, C. (1989) Should hyperbaric oxygen be used to treat the pregnant patient for acute carbon monoxide poisoning? A case report and literature review. *Jama,* 261**,** 1039.

WANG, I.-C., UENG, S. W.-N., YUAN, L.-J., TU, Y.-K., LIN, S.-S., WANG, C.-R., TAI, C.-L. & WANG, K.-C. (2005) Early Administration of Hyperbaric Oxygen Therapy in Distraction Osteogenesis: A Quantitative Study in New Zealand Rabbits. *Journal of Trauma-Injury Infection & Critical Care,* 58**,** 1230-1235.

WANG, J., LI, F., CALHOUN, J. H. & MADER, J. T. (2002) The role and effectiveness of adjunctive hyperbaric oxygen therapy in the management of musculoskeletal disorders. *Journal of Postgraduate Medicine,* 48**,** 226-231.

WARREN, R. F. (1983) Primary repair of the anterior cruciate ligament. *Clinical Orthopaedics,* 172**,** 65-70.

WEBSTER, A., SYROTUIK, D., BELL, G., JONES, R. & HANSTOCK, C. (2002) Effects of Hyperbaric Oxygen on Recovery From Exercise-Induced Muscle Damage in Humans. *Clinical Journal of Sports Medicine,* 12**,** 139-150.

WEBSTER, D., HORN, P., AMIN, H., WEISSBRICH, O., MASCIA, M. & WERNER, F. (1996) Effect of hyperbaric oxygen on ligament healing in a rat model. *Undersea and Hyperbaric Medicine,* 23**,** Suppl.13.

WEI, A., LIU, T., WINSLOW, R. & O'ROURKE, B. (2012) Dynamics of matrix-free Ca^{2+} in cardiac mitochondria: two components of Ca^{2+} uptake and role of phosphate buffering. *The Journal of General Physiology,* 139**,** 465-78.

WOODS, K., BISHOP, P. & JONES, E. (2007) Warm-Up and Stretching in the Prevention of Muscular Injury. *Sports Medicine,* 37**,** 1089-1099.

YAGI, M., WONG, E. K., KANAMORI, A., DEBSKI, R. E., FU, F. H. & WOO, S. L.-Y. (2002) Biomechanical Analysis of an Anatomic Anterior Cruciate Ligament Reconstruction. *The American Journal of Sports Medicine,* 30**,** 660-666.

YAM, L. Y. C. (1993) Clinical applications of oxygen therapy in hospitals and techniques of oxygen administration — a review. *Journal of the Hong Kong Medical Association,* 45**,** 318-325.

YASUHARA, S., PEREZ, M.-E., KANAKUBO, E., YASUHARA, Y., SHIN, Y.-S., KANEKI, M., FUJITA, T. & MARTYN, J. A. J. (2000) Skeletal muscle apoptosis after burns is associated with activation of proapoptotic signals. *American Journal of Physiology – Endocrinology and Metabolism,* 279**,** E1114-E1121.

YEH, W.-L., LIN, S.-S., YUAN, L.-J., LEE, K.-F., LEE, M. Y. & UENG, S. W. N. (2007) Effects of Hyperbaric Oxygen Treatment on Tendon Graft and Tendon-Bone Integration in Bone Tunnel: Biochemical and Histological Analysis in Rabbits. *Journal of Orthopaedic Research,* 25**,** 636-645.

YOSHIKAWA, T., TOHYAMA, H., KATSURA, T., KONDO, E., KOTANI, Y., MATSUMOTO, H., TOYAMA, Y. & YASUDA, K. (2006) Effects of Local Administration of Vascular Endothelial Growth Factor on Mechanical Characteristics of the Semitendinosus Tendon Graft After Anterior Cruciate Ligament Reconstruction in Sheep. *The American Journal of Sports Medicine,* 34**,** 1918-1925.

YUSA, T., CRAPO, J. D. & FREEMAN, B. A. (1984) Liposome-mediated augmentation of brain SOD and catalase inhibits CNS O2 toxicity. *Journal of Applied Physiology,* 57**,** 1674.

ZAMBONI, W., ROTH, A., RUSSELL, R., GRAHAM, B., SUCHY, H. & KUCAN, J. (1993) Morphologic analysis of the microcirculation during reperfusion of ischemic skeletal muscle and the effect of hyperbaric oxygen. *Plastic and Reconstructive Surgery,* 91**,** 1110-1123.

ZANTOP, T., PETERSEN, W., SEKIYA, J. K., MUSAHL, V. & FU, F. H. (2006) Anterior cruciate ligament anatomy and function relating to anatomical reconstruction. *Knee Surgery, Sports Traumatology, Arthroscopy,* 14**,** 982-992.