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Effect of intermittent hypobaric hypoxia on induced muscle injury repair in laboratory rats

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Cover illustration: The Vitruvian Rat (greenlabrat.deviantart.com)

Thank you to Dr. Henrique Girão and Dr. Paulo Pereira, together they gave me the motivation I needed by changing my world with a piece of their knowledge.

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Abbreviations

A	Analytical phase
ANOVA	Analysis of variance
BM	Bone Marrow
CD133	Cluster of Differentiation 133
CD14	Cluster of Differentiation 14
CD34	Cluster of Differentiation 34
CD45	Cluster of Differentiation 45
CK	Creatine Kinase
CK	Creatine Kinase
CLPs	Common Lymphoid Progenitors
CMPs	Common Myeloid Progenitors
CNS	Central Nervous System
COOH-	Carboxil-
Ctrl	Control group
Ctrl-	Negative Control Group
Ctrl+	Positive Control Group
D	Muscle Damage
Dist.	Distance ran
EHIP	Hypoxia plus Exercise group
EPC	Endothelial Progenitor Cells
EPO	Erythropoietin
ErP	Erythrocyte precursor
FACS	Fluorescence Activating Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein

FSC	Forward Scatter
GMPs	Granulocyte Macrophage Precursors
Hb	Hemoglobin
HCT	Hematocrit
HIF-1	Hypoxia-inducible factor 1
HIP	Hypoxia Group
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
i.e.	*id est* (that is)
IHHE	Intermittent Hypobaric Hypoxia Exposure
I_i	Initial intensity
I_{max}	Maximal intensity
I_{min}	Minimal intensity
$I_{u, prev}$	Intensity from the previous session
MEP	Megakaryocyte Erythrocyte precursor
MNCs	Mononucleated Cells
MSCs	Mesenchymal Stem Cells
n.s	No significant difference
NK	Natural Killer
Number-S	Number of shocks
Pb	Barometric Pressure
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PE	Phycoetrythrin
PKC	Protein Kinase C
Po2	Partial Pressure of Oxygen
PTK	Protein-Tyrosine Kinase
PTPase	Protein-Tyrosine Phosphatase

R1	Reference phase
ROS	Reactive Oxygen Species
SDF-1	Stromal-Derived Factor 1
Src	Sarcoma
SSC	Side Scatter
T	Time after induced damage
T0	Day before induced damage
T1	One day after induced damage
T14	Fourteen days after induced damage
T3	Three days after induced damage
T7	Seven days after induced damage
Time-S	Total time of shock
VEGF	Vascular Endotelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor 2
WBC	White Blood Cell

Abstract

Our work hypothesis is that hypobaric hypoxia exposure can be a stimulus for the mobilization and increase of hematopoietic stem cells (HSC) in peripheral circulation. Once stem cells has the capacity to self-renew and differentiate it is indisputable its role in muscle regeneration process. Therefore the aim of this study is to establish the possible efficiency of intermittent hypobaric hypoxia (IHHE) in the recovery of induced muscle damage in rats.

Muscle damage was induced in rat posterior limbs by means of one session of downhill forced running according to well-known and accepted protocols. Control (Ctrl) animals followed a passive recovery by means of sedentary behavior. Both experimental groups were submitted to hypobaric hypoxia exposure. The difference between the two experimental groups was that the first group (HIP) followed only the IHHE protocol, whilst the second experimental group (EHIP) was submitted to IHHE and, in addition, an exercise protocol. In order to have a clear observation of the evolution of recuperation period the samplings were made one day before damage (T0), one day after damage (T1), three days after damage (T3), seven days after damage (T7) and fourteen days after damage (T14).

Stem cells were assessed by flow cytometry with CD34-PE and CD45-FITC antibodies, with a protocol that was optimized with the time.

Despite of the few samples number for group there are evidences of the effect of hypoxia in the increase of circulating stem cells and in the inflammatory response. These results open a new therapeutic strategy not only for the reparation of tissue lesions in athletes but also in other fields of medicine.

Key-words: Hypobaric hypoxia exposure, stem cell, muscle damage, recuperation, flow cytometry.

1. Introduction

1.1. Intermittent Exposure to Hypobaric Hypoxia

Oxygen is the substrate of multicellular life. Reduced oxygen activity (hypoxia) is sensed and triggers homeostatic responses, which impact on virtually all areas of biology and medicine.

Hypoxia is an important environmental condition. The transcription factor hypoxia-inducible factor 1 (HIF-1) is a heterodimer composed of one stable β subunit and one α subunit, and is one of the central mediators of the cellular response to hypoxia. Under normoxic conditions, α subunit is rapidly hydroxylated by prolyl hydroxylases on proline residues, leading to polyubiquitination and rapid degradation by proteasome. Under hypoxic conditions, the HIF-1α subunits are not hydroxylated and are consequently stable, persisting as active heterodimeric HIF-1 in the nucleus (Figure 1). The functional status of HIF-1 is determined by the expression and activity of its α subunit (Liu *et al*., 2011). It regulates the transcription of a number of genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which play important roles in cellular and systemic oxygen homeostasis (Semenza, 2000).

Since the purification and molecular cloning of HIF-1 in 1995, the scientific community has witnessed the explosive expansion of this field. This last years it was extended the understanding of the molecular mechanisms underlying responses to hypoxia; the role of hypoxia as a stimulus for normal developmental and physiological processes, including hypoxia-induced changes in vascularization; the role of hypoxia in the pathogenesis of common human diseases including cardiovascular disease, cancer, chronic lung disease, and diabetes, which account for the majority of deaths on developed countries; and the development of novel therapeutic strategies that target hypoxia response pathways (Zhang *et al*., 2008). In particular, this study will be directed to the role of hypoxia in sports biomedicine, more specially, its effect in muscle damage repair.

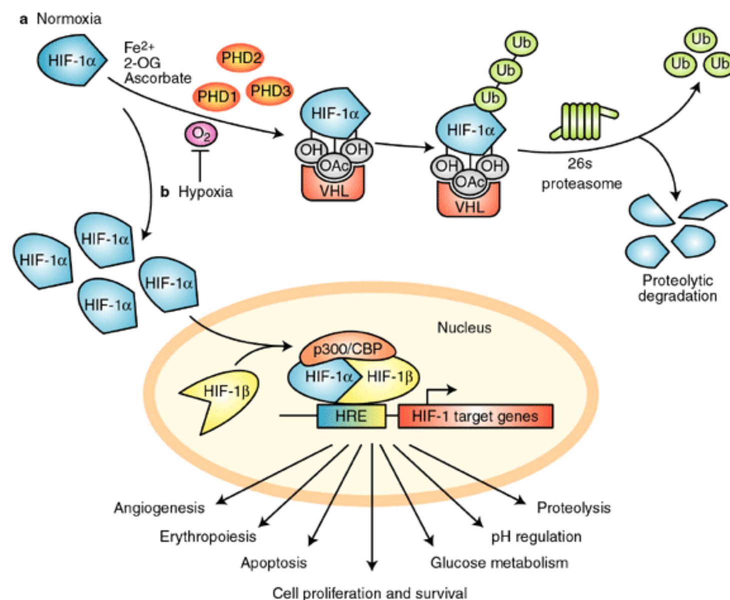


Figure 1. HIF-1α regulation by proline hydroxylation. In normoxia, the α subunit is hydroxylated by prolyl hydroxylases on proline residues, leading to polyubiquitination and degradation by proteasome. In hypoxia, the HIF-1α subunits are not hydroxylated, persisting as active heterodimeric HIF-1 in the nucleus (Adapted from Carroll *et al*., 2005).

Historically, intermittent hypoxia exposure sessions have been used to improve the physical condition and to treat several illnesses, mostly in the countries of the former Soviet Union, although this has been done without a clear understanding of their holistic effects (Serebrovskaya, 2002). At all events, this practice has now become widespread in the sport world. Intermittent hypoxic training refers to the discontinuous use of normobaric or hypobaric hypoxia, in an attempt to reproduce some of the key features of altitude acclimatization, with the ultimate goal to improve sea-level athletic performance (Levine, 2002). Acclimatization to altitude is a relatively slow process, usually attained by staying several days or weeks at progressively higher altitudes. Athletes training at altitude work less intensely which reduces the benefits obtained from altitude acclimatization (Rodriguez *et al*., 1999) that is why the most widely-adopted strategy at present is the living-high training-low model (Levine *et al*., 1997). This combines moderate altitude acclimatization (2500 m) with low altitude training to get the optimal effect. Summarizing, a small specific

altitude effect on performance seems to exist, which may be counteracted by negative influences like reduced stimulation of muscular metabolism. A series of single physiological changes at altitude might have positive or negative implications on training success: training of respiratory muscles, increase of hypoxic ventilator stimulation, reduced heart training by vegetative “braking”, increase of red cell and plasma volume, right shift of the oxygen dissociation curve, increase of oxidative muscle enzymes (only after hypoxia training), shift from fat and muscle glycogen to blood glucose combustion, reduced lactic acid and ammonia production, increase in buffer capacity (Boning, 1997). However, not all athletes or teams have the resources to travel to high altitude environments on a regular basis. Moreover, issues such as availability of adequate training facilities have limited the use of mountain-based altitude training. In the last few years, there has been a remarkable increase in the number of techniques designed to “bring the mountain to the athlete”. Nitrogen houses, hypoxia tents, and special breathing apparatuses to provide inspired hypoxia at rest and during exercise, all have been developed and promoted to simulate what are perceived as the critical elements of altitude training (Levine, 2002). Like those, exposure to hypoxia in hypobaric chambers has been used as an alternative procedure to induce acclimation to altitude (Richalet *et al*., 1992), and also to improve performance at sea level by training at different simulated altitudes (Terrados, 1992). The importance of oxygen transport and consumption in the body for endurance performance is the reason why altitude training as a preparation for competitions at sea level has become popular (Boning, 1997).

The group I had work with, they have studied the effect of intermittent simulated altitude on some functional parameters commonly used to quantify physical fitness in humans (H. Casas *et al*., 2000; M. Casas *et al*., 2000; Rodriguez *et al*., 1999, 2000). However, the efficiency of the different hypoxic exposure methods on well-trained humans remains controversial (Truijens *et al*., 2008). According to the symmorphosis principle, the formation of structural elements in animals’ organisms is oriented to fit but not to exceed their functional requirements (Taylor & Weibel, 1981). Although this principle was first proposed in a study of the relationship between structure and function in the mammalian

respiratory system, it has since been established as a general hypothesis of economic design (Weibel, 2000). Thus, it can be hypothesized that the adaptative responsed elicited by hypoxia in humans at the hematological and central levels (respiratory and cardiac control) must be accompanied by corresponding adjustments at the peripheral level. However, non-erythropoietic adaptative responses to intermittent hypoxia in cardiac and muscular parameters cannot be evaluated in humans for ethical and technical reasons. Therefore, it was developed by the group an intermittent hypobaric hypoxia exposure protocol for laboratory rats to study morphofunctional and metabolic parameters in myocardial and skeletal muscle (Esteva *et al*., 2009). And now, with the same protocol we want to see if, after induced muscle damage in rats, hypoxia helps in the repair of the injury by evaluating specific stem cells.

1.2. Effects of Altitude on Human Physiology

During exercise, the oxygen requirements of the muscle increase dramatically. In hypoxic conditions the oxygen availability decreases, which is the main cause for lower physical performance capacity (Cymerman *et al*., 1989).The most serious effects of high altitude on human physiology are due to the low oxygen partial pressure of the inspired air; consequently, several adjustments are needed to improve the tissue oxygen availability (Leon-Velarde *et al*., 2000). Hypobaric hypoxia increases ventilation, arterionevous O_2 difference, hemoglobin concentration, and hematocrit (Rodriguez *et al*., 1999).It also has profound effects on the structure and function of skeletal muscle tissue (Panisello *et al*., 2008), it induces acid-base alterations and affects the affinity of hemoglobin for oxygen (Cerretelli *et al*., 2003), and it raises EPO levels (Eckardt *et al*., 1989). Moreover, prolonged exposure to hypobaric hypoxia also induces physical deterioration denoted by weight loss (reduction in muscle mass), fatigue, slowing of mental processes, and impaired cognitive function, which increases with altitude (Kayser, 1994; Milledge, 2003; Terrados, 1992). Also, due to the augment in red cell mass, the viscosity of blood (apparent viscosity)

can be increased with the possibility of a subsequent reduction in oxygen transport capacity.

Some data were presented regarding the distinct adaptations of Andean, Tibetan and East African indigenous high-altitude populations. Andean people have higher hemoglobin concentrations than East Africans. Arterial O_2 content is also remarkably different among these groups. Tibetans have higher NO levels, higher pulmonary blood flow, and very high plasma nitride and nitrate concentrations, which are unprecedented for healthy people (100 times higher than lowlanders) (Zhang *et al*., 2008).

According to this later information, it is important to prevent negative effects of chronic hypoxia exposure so it have been proposed several procedures alternating short hypoxia exposure with immediate recovery in normoxia (Brugniaux *et al*., 2006; Robach *et al*., 2006). These intermittent hypoxia-exposure procedures are performed in hypoxic chambers and have led to relevant findings, such as the efficiency of a hypoxic stimulus to elicit an erythropoietic response and also other nonerythropoietic physiological adjustments affecting aerobic capacity. Thus, these exposure protocols have been considered as efficient methods for high altitude acclimatization (Richalet *et al*., 1992).

1.3. Sport and Muscle Injury

A number of studies have shown that exercise improves the function and regeneration of the cardiovascular system and skeletal muscle by activating and mobilizing organ-resident stem cells (Crameri *et al*. 2007) or by recruiting blood-circulating stem or progenitor cells (Laufs *et al*., 2004) (Figure 2).

Exercise provokes a number of stimuli: mechanical, metabolic and hypoxic. It also induces the release of various growth factors, cytokines and hormones. Physical activity results in the induction of molecular adaptations that improve physical performance, fitness and/or health whether under power sport conditions or situations of leisure sport, prevention or rehabilitation. This implies growth processes must occur for both heart and skeletal muscle

cells. This in turn depends on the formation of new blood vessels and the repair or replacement of cells that were physically stressed so much they are damaged or undergo cellular apoptosis (Laufs *et al*., 2004).

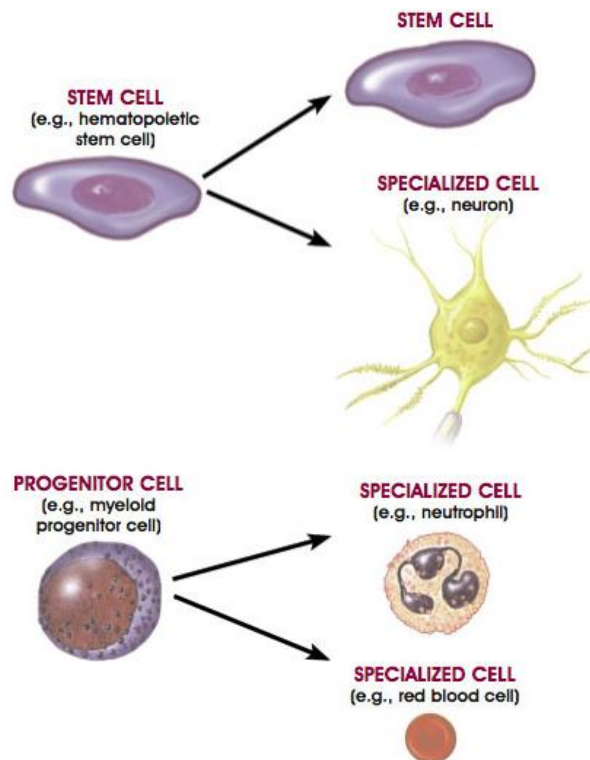


Figure 2. Distinguishing features of progenitor cells and stem cells. A stem cell is an unspecialized cell that is capable of replicating or self-renewing itself and developing into specialized cells that has the same capabilities of the originating cell. Shown here is an example of an hematopoietic stem cell producing a second generation stem cell and neuron. A progenitor cell is an unspecialized or has partial characteristics of a specialized cell that is capable of undergoing cell division and yielding two specialized cells. Shown here is an example of a myeloid progenitor undergoing cell division to yield two specialized cells (a neutrophil and a red blood cell) (© 2001 Terese Winslow, Lydia Kibiuk) (stemcells.nih.gov).

The injuries of the muscle-tendon system have an important effect in our society. Although, from a mediatic point of view, the injuries which receive more attention are those of elite athletes, we can’t forget the incidence of these in the labor market, affecting all the productivity.

The difficulty of studying this subject is that the injuries of this type, although abundant, are hardly reproducible in severity and localization, so that only allow a retrospective study by epidemiologic techniques. On the other hand the injuries are always a result of an accident or an unpredictable event, complicating a systematic and controlled study.

Our proposal is to study experimental animals (laboratory rats), in order to guarantee an experimental design that allows an equilibrated and clear observation of the evolution after an induced injury with homogeneous characteristics in groups of animals submitted to different conditions of recuperation.

The lengthening of a contracting muscle, for example, during downhill running, is well known to be linked to eccentric-induced muscle damage. The terms “eccentric” or “lengthening” actually illustrate the imbalance between the external strength imposed to the muscle and the strength produced by the muscle itself (Faulkner, 2003). Muscles operate eccentrically to either dissipate energy for decelerating the body or to store elastic recoil energy in preparation for shortening (concentric) contraction. The muscle forces produced during this lengthening behavior can be extremely high, despite the requisite low energy cost (LaStayo *et al*., 2003). This situation is quite common during downhill running, activation of an antagonist muscle group, and when muscles are exposed to unaccustomed or high levels of repeated activity. It is invariably linked to structural and functional muscle damage (Friden *et al*. 1992).

Therefore it’s widely accepted that an intense effort with a high charge of eccentric muscle contraction causes muscle damage (Komulainen *et al*. 1994). This is the mechanisms of injury that we propose but the study would be perfectly feasible in animals that had suffered a muscle injury through others different ways, and in general terms the project would also be applicable with other experimental models of induced muscular damage.

This project complements previous work in humans developed by the group I integrated this last year. It was possible with a big sized hypobaric chamber taking place in the Centre de Barcelona del Instituto Nacional de Educación Física (Unidad de Hipobaria INEFC-UB), until 2007. It was demonstrated that the intermittent hypobaric hypoxia exposition is

an efficient way of stimulating erythropoiesis, which means it improves the aerobic capacity in humans and animals (Rodriguez *et al*. 1999; Rodriguez *et al*. 2000, Marin *et al*.2008). More recently, a new hypobaric chamber has been sited at Health Campus of Bellvitge and research work and service have been continued at this new location with a second generation chamber. These renewed facilities are mainly oriented to biomedical applications.

1.3.1. Markers of Muscle Damage

Exercise-induced muscle injury in humans frequently occurs after unaccustomed exercise, particularly if the exercise involves a large amount of eccentric (muscle lengthening) contractions. Several indirectly assessed markers of muscle damage after exercise include prolonged decreases in force production measured during both voluntary and electrically stimulated contractions (particularly at low stimulation frequencies), increases in inflammatory markers both within the injured muscle, in the blood, and muscular soreness. Although the exact mechanisms to explain these changes have not been delineated, the initial injury is ascribed to mechanical disruption of the fiber, and subsequent damage is linked to inflammatory processes and to changes in excitation-contraction coupling within the muscle. From a study in humans, it seems that the initial insult of the exercise creates an insult to fibers, which results in damage to the ultrastructure, extracellular matrix, and possibly to capillaries (Stauber WT *et al*., 1990). Many studies have assessed the appearance of muscle proteins in the blood after eccentric exercise to provide indirect evidence of muscle damage. The muscle enzymes lactate dehydrogenase, aspartate aminotransferase, carbonic anhydrase isoenzyme II, and creatine kinase (CK) have been evaluated (Sorichter *et al*., 1999). Other muscle proteins have also been used as indicators of damage, including myoglobin, heart fatty acid binding protein, troponin, and myosin heavy chain (Sorichter *et al*., 1999). Although all of these have been shown to increase after damage-inducing exercise, CK has received the most attention, perhaps because the magnitude of increase is so great relative to other proteins. There has been no systematic

study of all of these markers together, so we know little about how they all relate to one another in time course or extent of appearance in the blood. The use of any muscle protein in the blood as a marker of muscle damage, however, is problematic, because blood concentration is a function of what is being produced in the muscle and what is being cleared from the blood. Analysis of muscle proteins in the blood provides only a qualitative indicator of damage (Clarkson *et al*., 2002). In this study it was used as markers of damage levels of myoglobin and CK activity.

1.4. Muscle Damage Repair

Upon muscle injury a finely orchestrated set of cellular responses is activated, resulting in the regeneration of a well-innervated, fully vascularized, and contractile muscle apparatus. The advances of molecular biology techniques combined with the identification and development of rodent models for muscular dystrophy have contributed to the identification of molecular pathways involved in muscle regeneration. In particular, the identification of muscle satellite cells has led to major advances in our understanding of muscle regeneration. More recently, the identification of multipotent stem cells capable of myogenic differentiation in the course of muscle regeneration has extended the view on the muscle regenerative process and opened new perspectives for the development of novel therapies (Chargé *et al*., 2004).

During embryonic development, specification of mesodermal precursor cells to the myogenic lineage is regulated by positive and negative signals from surrounding tissues. Mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage. Whether the muscle injury is inflicted by a direct trauma (i. e. extensive physical activity and specially resistance training) or an innate genetic defect, muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase. The initial event of muscle degeneration is necrosis of the muscle fibers. The early phase of muscle injury is usually accompanied by the activation of mononucleated cells (MNCs), principally inflammatory cells (Rapolee *et al*., 1992; Tidball,

1995). Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as early as 1-6h after exercise-induced muscle damage therapies. After neutrophil infiltration and ~48h postinjury, macrophages become the predominant inflammatory cell type within the site of injury. Macrophages infiltrate the injured site to phagocytose cellular debris and may affect other aspects of muscle regeneration (Chargé *et al*., 2004).

1.4.1. Inflammation

Inflammation after muscle injury occurs to clear debris from the injured area in preparation for regeneration. This inflammation response is thought to be active by the initial mechanical trauma and is characterized by infiltration of fluid and plasma proteins into the injured tissue and increases in inflammatory cell populations (Pedersen *et al*., 2000). The proliferation of inflammatory cells is thought to amplify the initial muscle injury through increased release of reactive oxygen species (ROS) and activation of phospholipases and proteases at the injury site (MacIntyre *et al*., 1995). The actual time-course of the inflammation response after exercise is variable, dependent on several factors such as exercise mode, intensity or duration, and the muscle groups utilized (Clarkson *et al*., 2002).

Modified muscle use or injury typically initiates a rapid and sequential invasion of muscle by inflammatory cell populations (neutrophils rapidly invade, followed by macrophages) that can persist for days or weeks, while muscle repair, regeneration, and growth occur, involving activation and proliferation of satellite cells, followed by their terminal differentiation. This relationship between inflammation and muscle repair or regeneration has suggested that they may be mechanistically related and provides the basis for teleological arguments that muscle inflammation after modified muscle use is a functionally beneficial response. However, experimental observations have only recently begun to test that expectation and to distinguish between features of muscle inflammation that promote injury and those that promote growth and repair (Tidball, 2005). A local production of inflammatory mediators can be detected 3 h after the end of exercise,

whereas muscle edema and significant increase in blood enzyme activities have been described 2-5 days after exercise as a sign of systemic inflammatory response. Muscle regeneration then starts 4-6 days after the eccentric session and can last several weeks (Warhol *et al*., 1998).

It was described the involvement of mast cells in hypoxia-induced microvascular inflammation in the mesenteric microcirculation. Hypoxia resulted in rapid mast cell degranulation and blockade of mast cell degranulation with cromolyn prevented or attenuated hypoxia-induced increases in ROS, leukocyte adherence, and vascular permeability (Zhang *et al*., 2008).

1.4.2. Stem Cells

Stem cells are primitive cells with the potential to differentiate into mature cells (Asahara *et al*., 1997). The fabulous ability of an embryo to diversify and of certain adult tissues to regenerate through life is a direct result of stem cells nature's gift to multicellular organisms. Stem cells have both the capacity to self-renew, that is, to divide and create additional stem cells, and also to differentiate along a specified molecular pathway. Adult stem cells are often relatively slow-cycling cells able to respond to specific environmental signals either generate new stem cells or select a particular differentiation program (Figure 3) (Fuchs *et al*., 2000).

The decision of a stem cell to either self-renew or differentiate and the selection of a specific differentiation lineage by mulltipotent progenitor during commitment are intrinsic properties of stem cell progenitor cells and are regulated by stochastic mechanisms (Stella *et al*., 1995).

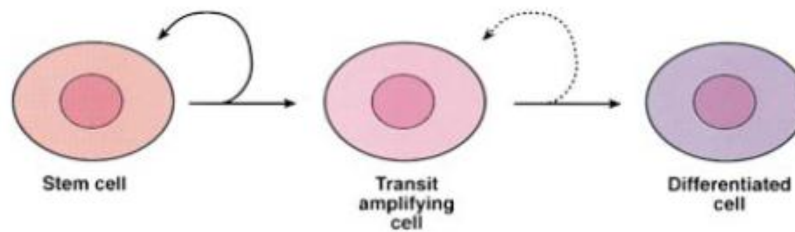


Figure 3. Stem cells self-renew and differentiate to give rise to transit amplifying and fully differentiated cells. Adult stem cells are slow-cycling cells able to respond to specific environmental signals either generate new stem cells or select a particular differentiation program (Fuchs E *et al*., 2000).

An increase in stem cells is observed after various events such as twelve weeks of physical exercise (Hoetzer *et al*., 2007), menstruation (Meng *et al*., 2007), cessation of smoking (Kondo *et al*., 2004) and in animals or human cells subjected to deep hypoxia conditions *in vitro* (Zhu *et al*., 2005; Grayson *et al*., 2007).

Oxygen is a potent signaling molecule which has received increasing recognition for its ability to affect the fundamental characteristics of various types of progenitor cells (Zhu *et al*., 2005).

The human mesenchymal stem cells (MSCs) are obtained primarily from the bone marrow (BM), where they co-exist in a symbiotic relationship with hematopoietic stem cells (HSCs) forming integral parts of each other's microenvironment (Baksh *et al*., 2003). The intimate interactions between these two stem cell types strongly suggest that they reside in similar niches within the BM and are affected by the same environmental cues. HSCs reside in severely hypoxic regions of the BM and maintaining HSCs under hypoxic conditions significantly improves their proliferation and self-renewal capabilities (Ivanovic *et al*., 2000). Not only about HSCs but reduced oxygen levels have been shown to enhance the proliferation of multiple types of stem and progenitor cells (Grayson *et al*., 2007). MSCs have the capacity to differentiate into adipocytes, osteoblasts, chondrocytes, and potentially other cell types including tenocytes, myocytes, and astrocytes (Pittenger *et al*., 1999) (Figure 4).

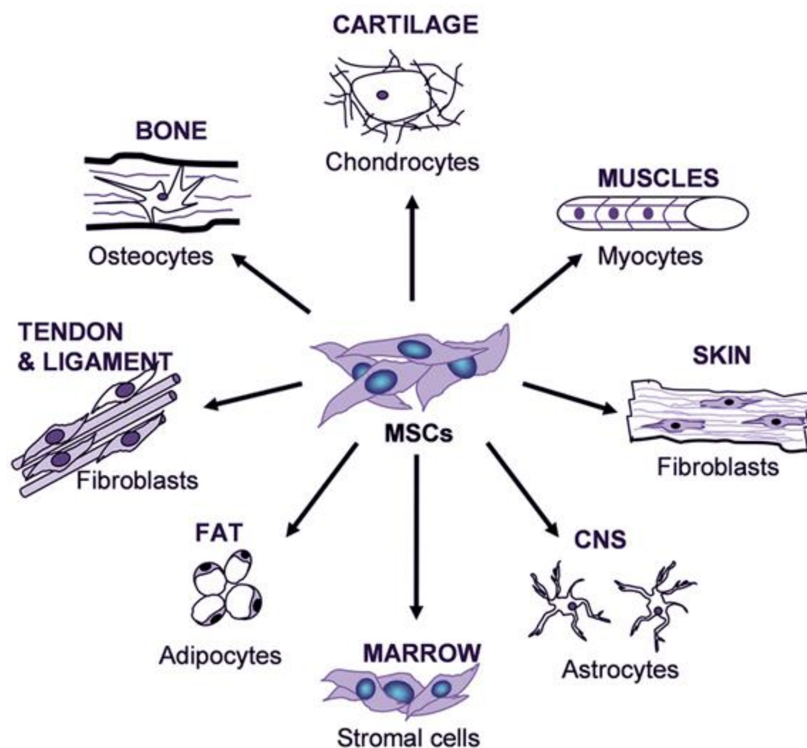


Figure 4. Adult mesenchymal stem cells (MSCs) can differentiate into a variety of cell types. MSCs have the capacity to differentiate into adipocytes, osteoblasts, chondrocytes, cardiomyocytes, and also other types including tenocytes, myocytes and astrocytes from the central nervous system (CNS).

The combination of their multipotent differentiation potential and paracrine effects means that MSCs are considered to be an attractive candidate for tissue repair and regenerative medicine (Barry *et al*., 2004). The mechanisms responsible for MSCs mobilization are currently unclear, but appear to differ from those for HSCs and endothelial progenitor cells (EPCs) (Levesque *et al*., 2007). Circulating MSCs have been previously identified only at very low frequencies under physiological conditions, and the feasibility of their isolation from peripheral blood (PB) remains a matter of debate (He *et al*., 2007). A recent study reported that circulating MSC pool was increased almost 15-fold when rats were exposed to chronic hypoxia (Rochefort *et al*., 2006), whereas another study showed that MSCs could be released into circulating blood following recurrent obstructive apnea for just 5 h, representing a process of intermittent hypoxia and inflammation (Carreras *et al*., 2009). Local hypoxia of ischemic tissue also mobilizes progenitor cells and facilitates their

recruitment and retention (Ceradini *et al*., 2004). These results suggest that hypoxia is a sensitive stimulus that induces the release of MSCs into the blood. Increasing evidence suggests that HIF-1α is essencial for progenitor cell mobilization and migration into ischemic or tumor tissues (Cerdanini *et al*., 2005) and implies a role for HIF-1α in hypoxia-induced MSC mobilization (Liu *et al*., 2011; Rocheford *et al*., 2006). Following adequate stimulation, stem and progenitor cells may leave the cell niche and circulate into the PB, which is termed mobilization, before migrating to the target tissues.

Regeneration and growth of skeletal muscle are mainly managed by resident stem cells, the so called “satellite cells”. Satellite cells occupy a sublaminar position between the basal lamina and sarcolemma (Mauro, 1961). In contrast to adult stem cells, with considerable proliferative potential, satellite cells only have a limited capacity for self-renewal. This means that under pathological conditions skeletal muscle degenerates (Whal *et al*., 2008). This exercise-induced activation of satellite cells seems to be specifically attributed to eccentric exercise, i. e to a situation when the muscle is activated while it is stretched. It is interesting to note that the forces generated by activation combined with stretch exceed even those of maximal isometric contraction. When cultured, the satellite cells proliferated, giving rise to satellite cell-derived myoblasts that differentiated to produce multinucleated myotubes. Recently, transplantation of such single myofibers into muscle has provided good evidence that the satellite cell indeed acts as myogenic stem cell *in vivo*, able to give rise to both new myofibers and, importantly, also many new satellite cells (Collins *et al*., 2005). The satellite cell therefore fulfills the basic definition of a stem cell, in that it can give rise to a differentiated cell type and maintain itself by self-reweal (Zammit *et al*., 2006). Nevertheless to accomplish their role in muscle maintenance, hypertrophy, and repair, satellite cells must first be activated from this quiescent state to produce myoblast progeny (Charge *et al*., 2004).

According to some studies CD34 is certainly expressed by adult satellite cells (Beauchamp *et al*., 2000). This raises the possibility of a common origin for endothelial cells and some satellite cells (Kardon *et al*., 2002), or that endothelial cells may be able to give rise to satellite cells (Zammit *et al*., 2006). Until recently, muscle satellite cell was presumed to be

the sole source of myonuclei in muscle repair. Thus there is strong evidence for the presence of progenitor cells with myogenic potential other than satellite cells within skeletal muscles. In fact, adult stem cells isolated from various tissues appear to differentiate *in vitro* and *in vivo* into multiple lineages depending on environmental cues (Chargé *et al*., 2004).

Whereas muscle regeneration and new formation under physiological conditions seem to be mainly dependent on the presence of resident stem cells, they are critically dependent on the presence and the function of BM-derived circulating stem and progenitor cells (Wahl *et al*., 2008).

Hypoxia and ischemia initiate a number of angiogenic and vasculogenic processes including the release of growth factors and the release of progenitor cells. Several studies have reported the contribution of BM-derived EPC to neovascularization in ischemic muscle, the influence of hypoxia on EPC (Asahara *et al*., 2004; Murasawa *et al*., 2005), upregulation of adhesion molecules and chemoattractant molecules and changes in proliferation and differentiation of progenitor cells.

Both vasculogenesis and angiogenesis are defined as processes responsible for new blood vessel formation (Asahara *et al*. 1997). For several years, vascular growth and remodeling in postnatal life were traditionally explained as results of the proliferative and migratory capacity of mature endothelial cells. According to this hypothesis, fully differentiated endothelial cells were proposed to participate by sprouting from existing blood vessels and giving rise to new vasculature (Rumpold *et al*., 2004). The discovery of BM-derived EPCs circulating in the PB of adults extended the view of the angiogenic process in humans (Asahara *et al*., 1997). There is a growing body of data that the cells defined as EPCs play a significant role in the reendothelialization and neovascularization of injured endothelium (Urbich *et al*., 2004).Asahara *et al*. provided the first evidence that the PB is a reservoir of BM-derived EPCs circulating in the blood vessel system and exhibiting reparative properties.

After an ischemic or hypoxic event, growth factors/cytokines, such as VEGF, EPO (all regulated by the O_2-dependent transcription factor HIF-1) are released by tissue and stimulate the mobilization of progenitor cells from BM (Fandrey, 2004). Several studies have shown that EPO significantly increases the number of EPCs in the BM and PB. It enhances EPC differentiation and proliferation, and increases ischemia-induced neovascularization (Bahlmann *et al*., 2003). Similar results were shown for VEGF, which augments the number of circulating EPCs and enhances EPC proliferation, adhesion and incorporation into endothelial monolayers (Iwaguro *et al*., 2002) (Figure 5).

The number of EPCs as well as their proliferative potential may change under pathological conditions (Figure 6).

After adhesion, adherent progenitor cells egress into the tissue where they are themselves exposed to hypoxia. Microenvironment – including contact with surrounding cells, the extracellular matrix, the local milieu as well as growth factors – is likely to play a key role in determining stem cell differentiation (Blau *et al*., 2001). Hypoxia, shear stress and strain may represent first-line mediators of complex pathways in exercise-induced stem cell tissue replacement. In addition, exercise may support stem cell-induced regeneration by preconditioning/ optimizing the microenvironment (e.g. pH alterations or a processation of the extracellular matrix).

A better understanding of these mechanisms may make physical activity a useful tool for regulation of stem cell proliferation and differentiation also in minimally invasive stem cell transplantation therapy (Wahl *et al*., 2008).

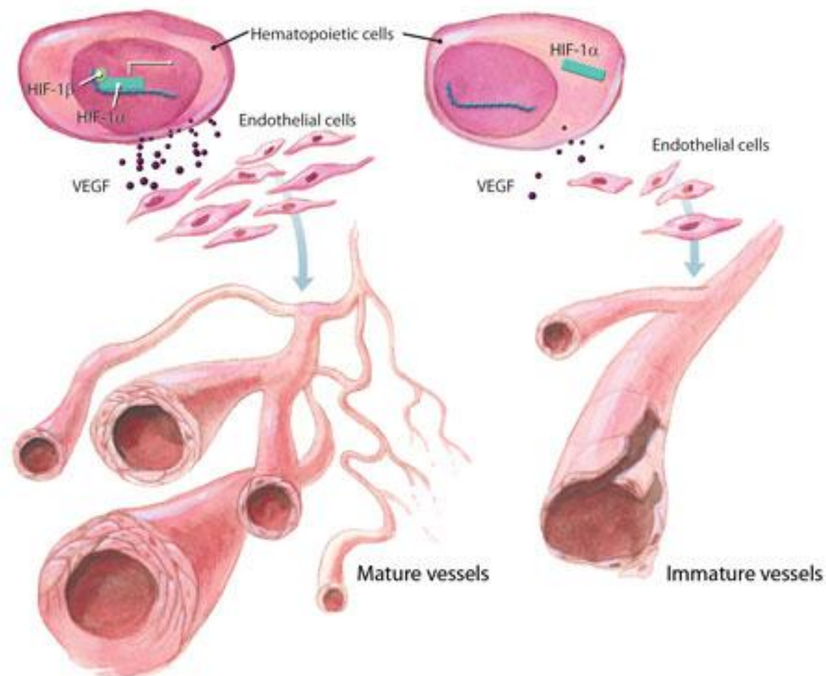


Figure 5. Vascular endothelial growth factor (VEGF) increases the number of circulating endothelial cells and enhances its proliferation. In hypoxic conditions, HIF-1 works as an active heterodimer in the nucleus and provokes the expression of VEGF by hematopoietic cells. This stimulus for vascularization is responsible for the maturation of vessels. (Adapted from Ramirez-Bergeron *et al*., 2006).

There are multiple sources of fibroblast-like cells present in fibrotic lesions and healing wounds (Martin, 1997). In addition to the proliferation of resident fibroblasts, BM-derived hematopoietic precursors present within the blood are attached to sites of injury where they differentiate into spindle-shaped fibroblast-like cells called fibrocytes, and at least in part, mediate tissue repair and fibrosis (Bucala *et al*., 1994). Fibrocytes appear to differentiate from CD14+ PB monocytes (Abe *et al*., 2001). Cell surface analysis suggests that these cells share both leukocytic and connective tissue cell features. In addition to expressing the fibroblast components vimentin, collagen, and fibronectin, fibrocytes also display the leukocyte common antigen CD45 and the hematopoietic stem cell marker CD34. (Bucala *et al*., 1994). At present, there is no single specific marker for fibrocytes. However, several groups have shown that the progressive loss of CD34 and eventually CD45 on fibrocytes

can lead to an underestimation of fibrocyte numbers (Bellini *et al*., 2007; Andersson-Sjoland *et al*. 2008). These data suggest that some combinations of markers to identify fibrocytes do not adequately discriminate different cell populations, and may lead to either over or underestimation of fibrocyte numbers (Andersson-Sjoland *et al*. 2008). Several groups have shown that fibrocytes have a progressive loss of hematopoietic markers as they differentiate from monocytes (Bellini *et al*., 2007; Andersson-Sjoland *et al*., 2008).

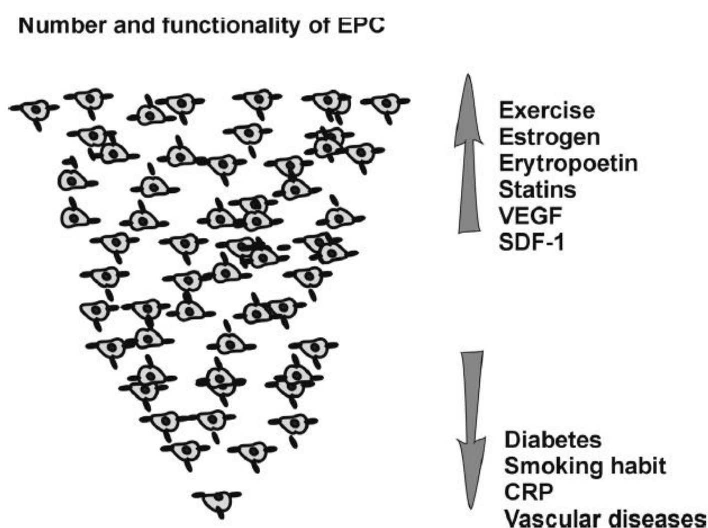


Figure 6. The counts of endothelial progenitor cells (EPCs) in the peripheral blood and their proliferative potential can change under various conditions. Pathological stages, including diabetes, smoking, C-reactive protein (CRP), up-regulation of some factor, and vascular diseases, reduce EPC counts and their reparative ability. However, factors including physical exercise, estrogen, erythropoietin, statins, vascular endothelial growth factor (VEGF), and stromal-derived factor 1 (SDF-1) increase EPC count and function (Miller-Kasprzak *et al*., 2007).

1.4.2.1.CD45

The common leukocyte antigen, CD45 is a transmembrane glycoprotein expressed on most hematopoietic cells including lymphocytes, monocytes, macrophages, and fibrocytes. (Charbonneau *et al*., 1992). A protein-tyrosine phosphatase (PTPase), CD45 is required for the development and activation of T lymphocytes (McFarland *et al*., 1993) and also for the tolerance induction and activation of B lymphocytes via antigen receptors (Justement, 2001). Dephosphorylation of the COOH-terminal tyrosine residue of Src family protein-tyrosine kinases (PTKs) by CD45 PTPase has been shown as a triggering mechanism of the Src family PTK activation (Mustelin *et al*., 1989). The activated Src family PTKs mediate the downstream signals of several extracellular stimuli, such as growth factors, cytokines and antigen stimulation, leading to diversification and amplification of the initial signals (Corey *et al*., 1999).

Eight isoforms of CD45 are distributed throughout the immune system according to cell type. The variation in these isoforms is localized to the extracellular domain of CD45, while the intracellular domain is conserved. The tyrosine phosphatase activity of CD45 is contained within the conserved intracellular domain (Thomas, 1989).

1.4.2.2.CD34

CD34 is a heavily glycosylated, transmembrane glycoprotein that is expressed on the surface of lymphohematopoietic cells (responsible for the generation and regeneration of blood-forming and immune stem and progenitor systems) (Figure 7), small-vessel endothelial cells, fibrocytes and some cells in fetal and adult nervous tissue. CD34 antigen expression is highest in the most primitive stem cells and is gradually lost as lineage committed progenitors differentiate. The CD34 antigen is also present on capillary endothelial cells and on BM stromal cells. The CD34 cytoplasmic domain has an intracellular domain that contains consensus sites for activated protein kinase C (PKC) phosphorylation as well as serine, threonine and tyrosine phosphorylation consensus sites

(Liu *et al*., 2005). CD34 cells contain receptors for a number of growth factors from two different related families: tyrosine kinase receptors and hematopoietic receptors not containing a tyrosine kinase domain (Stella *et al*.,1995).

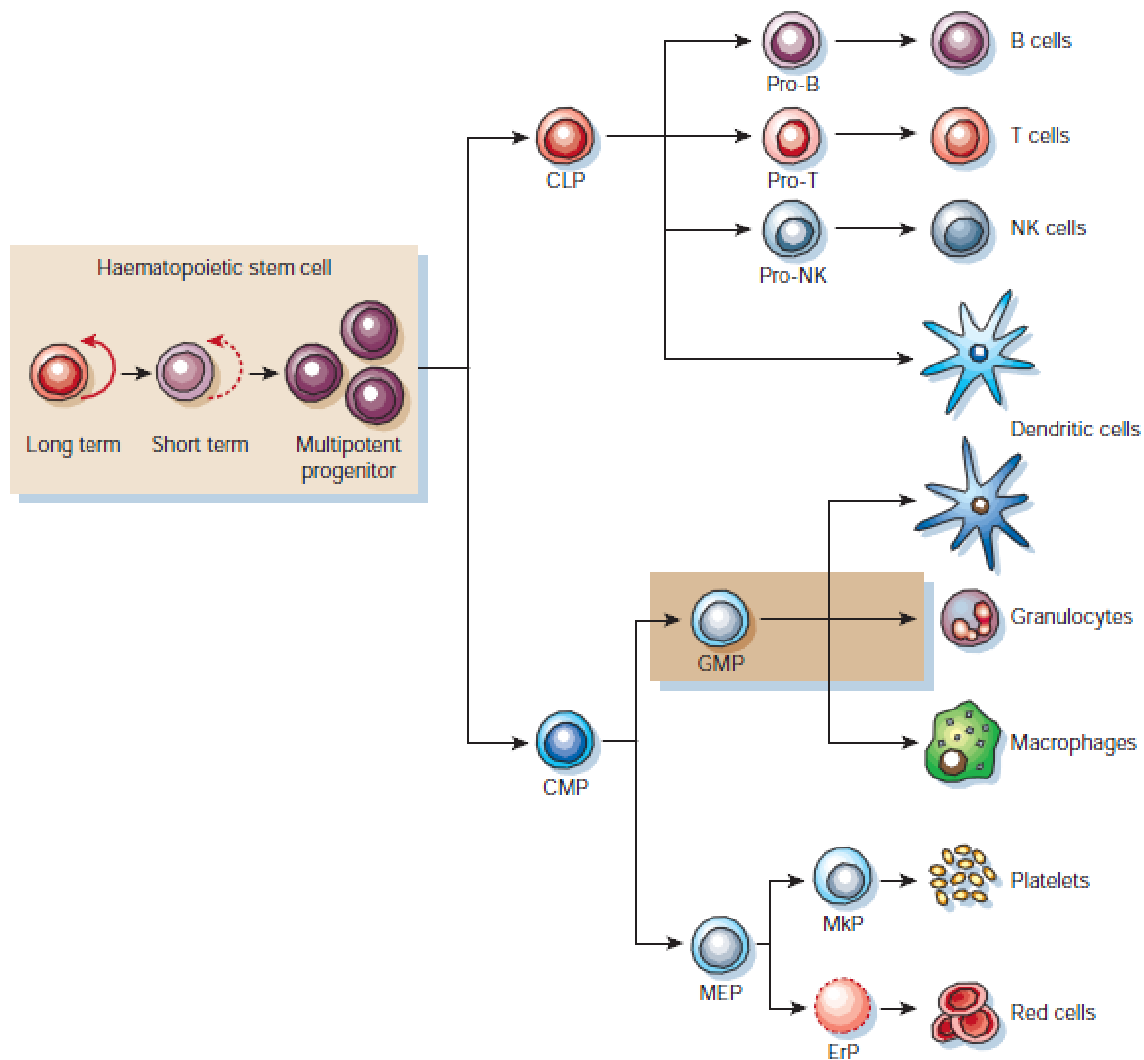


Figure 7. Development of hematopoietic stem cells. HSCs can be subdivided into long-term self-renewing HSCs, short-term self-renewing HSCs and multipotent progenitors (red arrows indicate self-renewal). They give rise to common lymphoid progenitors (CLPs; the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells). Both CMPs/GMPs (granulocyte macrophage precursors) and CLPs can give rise to all known mouse dendritic cells. ErP, erythrocyte precursor; MEP, megakaryocyte erythrocyte precursor; NK, natural killer (Reya *et al*., 2001).

Hematopoietic stem and progenitor cells (HSPCs) represent a small but important population of cells found in the blood circulation. These multipotent adult stem cells differentiate into every major blood cell type of the more than twenty trillion cells in the bloodstream (Gunsilius *et al*., 2001).There is a complex interplay between the positive and the negative regulatory proteins which determines the proliferation or inhibition of early hematopoietic progenitor cells. In general, the activity of inhibitors of hematopoiesis appears to be reversible, lineage-nonspeficic and directed at the early stages of differentiation (Stella *et al*., 1995). Current methods of HSPC isolation focus on the immunological binding characteristics of antibodies to specific surface markers, such as CD34 or CD133, followed by separation using magnetic beads or cell sorting (Wojciechowski *et al*., 2008).

In studies of human PB, the fraction of MNCs positive for CD34 was typically less than 0.5% (Tong *et al*., 1994). Some studies has shown that populations of CD34+ cells exhibit a rolling phenotype that is quite different from that of leukocytes or other CD34- (Charles *et al*., 2007).

As already said EPCs have a fundamental role in vasculogenesis and angiogenesis and its identification is based on the cell surface expressions of various protein markers. There is no straightforward definition of an EPC marker because these cells seem to be a heterogeneous group associated with different cell surface antigen expression profiles. The most commonly described molecules that serve as biomarkers for recognition of an EPC population include CD34, CD133, and vascular endothelial growth factor 2 (VEGFR2) (Figure 8). The pionees study performed by Asahara *et al*. recognized EPCs as CD34-positive MNCs. HSCs that serve as a source of EPCs express CD34 (Fina *et al*., 1990).

Another *in vivo* study confirmed the enhanced regenerative capacity of a CD34+, late-adhering population of muscle-derived stem cells compared with the CD34- cell population (Jankowski *et al*., 2002). Thus it appears that CD34 plays an important role in identifying myogenic progenitors. However, the identification of a subpopulation of mouse satellite cells expressing CD34 suggests that CD34 antigen is also expressed in myogenic cells and

therefore highlights the need for multiple markers for identification of muscle-derived stem cells other than satellite cells (Beauchamp *et al*., 2000).

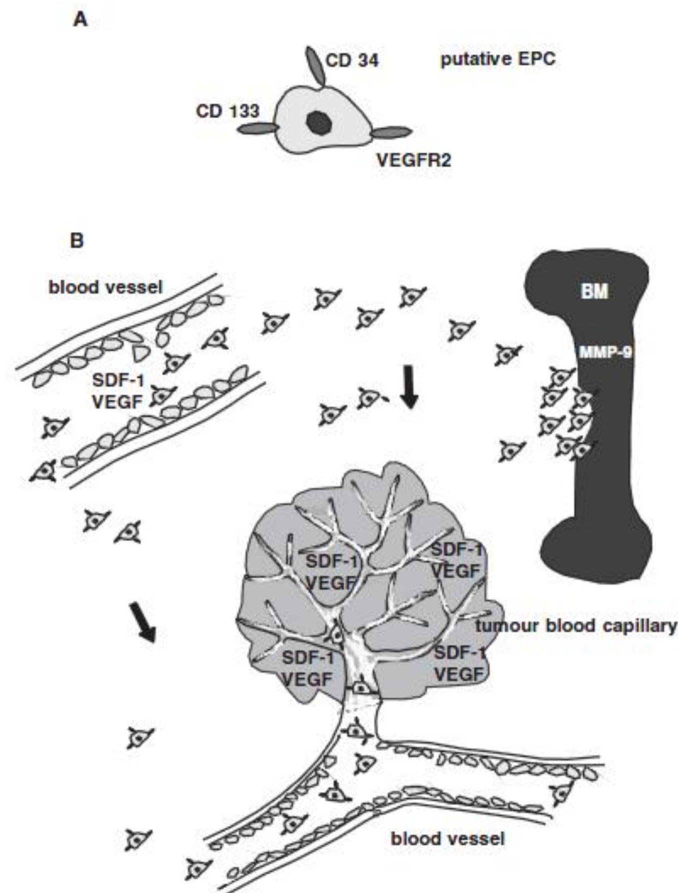


Figure 8. Endothelial progenitor cells (EPCs) are involved in blood vessel repair and tumor angiogenesis. A – CD34, CD133, and vascular endothelial growth factor receptor 2 (VEGFR2) are considered common markers of EPCs. **B** – increases in the expressions of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF) and stromal-derived factor-1 (SDF-1) are responsible for the mobilization and attraction of EPCs from the bone marrow (BM) into the peripheral blood (PB). PB EPCs are incorporated into injured vessel walls during the repair of distinct tissue vasculature. Under pathological conditions, tumor hypoxia leads to the up-regulation of VEGF and SDF expression, which attract EPCs and augment the formation of tumor vasculature.

Tumor VEGF and SDF-1 are also able to stimulate the proliferation of endothelial cells in a paracrine manner (Miller-Kasprzak *et al*., 2007).

About 1-3% of mononuclear BM cells expressed the CD34 antigen which is a highly glycosylated mucin-like structure. A part from the endothelial progenitors and stromal cells, the majority of the CD34+ cells are hematopoietic stem cells and progenitors capable of reconstituting long-term, multilineage hematopoiesis. Their presence in low number in the PB from normal unmobilized subjects demonstrates their continuous circulation under physiological conditions (Barosi *et al*., 2001).

Different antibodies recognize distinct epitopes of the same antigen. CD34 antigen expression is associated with concomitant expression of several other markers that can be classified as lineage non-specific markers (Thy1, CD38, HLA-DR, CD45RA, CD71) and lineage specific markers, including T-lymphoid (CD7), B-lymphoid (CD19), myeloid (CD33) and megakaryocytic (CD61) markers. The expression of lineage non-specific markers allows the heterogeneous CD34+ population be divided into two distinct subpopulations characterized, respectively, by low or high expression of Thy1, CD38, HLA-DR, CD45RA, CD71. These two cell subpopulations contain with early and late hematopoietic progenitor cells, respectively (Stella *et al*., 1995).

2. Aims

Hypobaric hypoxia exposure can contribute to a faster repair of damaged muscle caused by eccentric exercise stimulating secondary messengers and cytokines like EPO and VEGF. These molecules could be involved directly in tissue repair or by stimulating the release or differentiation of progenitor cells responsible for an efficient tissue regeneration. Once the group had recently found in a study with humans an increasing of hematopoietic stem cells (CD34+) inclusively several days after only three sessions of hypobaric hypoxia exposition with muscular electrostimulation, it seems that the hypoxic stimulus combined with muscular activation is efficient to increments the presence of circulating progenitor cells. Therefore the present study is aimed to establish the possible efficiency of intermittent hypobaric hypoxia exposure in the recovery of induced muscle damage in rats; for that we want to quantify the answer in amount of stem cells in peripheral blood to hypobaric hypoxia environment and establish the temporal dynamic of the changes in these cells concentration in the blood flow of all animals' groups.

3. Materials and Methods

3.1. Animals

A total of 30 male Sprague-Dawley rats (Janvier, France) aged 6 weeks, and with average body weight of 312.8±4.6 g at the beginning of the experiment, were kept in environmentally controlled rooms (17-25°C; 30-70% relative humidity) with 12-hour light/dark cycle. Rats were given filtered water feed and bedding supply provided by the Service Center for Laboratory Animals of the University of Barcelona. The animals were killed according to protocols for animal use, following the NIH guidelines for the care and use of experimental animals.

3.1.1. Experimental Design

The animals were randomly divided into three groups. Control (Ctrl), that includes the Positive Control (Ctrl+) and the Negative Control (Ctrl-); Hypoxia alone (HIP) and Hypoxia plus exercise (EHIP). With the exception of the Ctrl- group all the rats were submitted to the same diary training exercise, after it they were caused the damage and in the recuperation period the rats of Ctrl+ group were maintained in rest conditions in normoxia, the rats of HIP group were submitted to a diary program of exposition to intermittent hypobaric hypoxia for four hours and finally the rats of EHIP group, besides the hypobaric hypoxic stimulation, they were given a standard exercise training.

In order to have a clear observation of evolution after the induced injury animals were sacrificed and consequently analyzed in different times after the damage. Therefore we have one more variable in addition to treatment, i.e.. number of the days after damage (T). There were rats sacrificed in the day before damage (T0), one day after damage (T1), three days after damage (T3), seven days after damage (T7) and fourteen days after damage (T14). The animals needed to be sacrificed on sampling day.

3.1.2. Reception of the Rats

Before each lot of rats enters in the department they need to pass through quarantine of minimum 7 days. They were received at the service center in cages, along with the bedding supply and feed supply. Once arrived to the department the registration and the labeling take place.

3.1.3.Rat's Manipulation and Procedure

The rats were housed in cages. There were always two rats individually housed, they were called "in isolation" to serve as reference. These were maintained under the same conditions as the other animals but every day they were weighted, and the food and the water intake were measured and registered in specific documents. From time to time rats in isolation were changed.

Three days a week it was necessary to add food to all rats as well as the water and bedding was changed. Once a week it was cleaned the cages with soup and alcohol and the rats needed to be checked-up (nails, teeth, hair, etc.).

3.1.4.Rat Preconditioning for Treadmill

The treadmill has five cubicles, which mean that has a capacity for five rats, and a current board that makes the rats run. The parameters measured by the treadmill are the number of shocks (Number-S); the distance ran (Dist.) and the total time of shock (Time-S). Also the velocity and the current intensity could be controlled.

The Rat Preconditioning for Treadmill Protocol, document no. 101.055.001 (annex 1) took 10 days. The first 4 days had only one session per day (called session A) and the rest of the days two sessions per day, where the former one should be performed in the morning, session A, and the latter one in the afternoon, session B, with a minimum of 6h rest

between the end of session A and the beginning of session B. The exercise schedule should be followed precisely with changes in the intensity level of the current depending on "the fitness level" of the worst performer. The range of allowed intensity changes over the entire preconditioning process, and the initial intensity (I_i) should never be lower than minimal intensity $_{min}$ (can vary from 0.2 mA to 0.4 mA) nor higher than maximal intensity I_{max} (can vary from 0.6 mA to 1 mA), but shouldn't either be lower than the last change in the intensity from the previous session ($I_{u,\ prev}$). Also the velocity (from 0cm.s^{-1} to 40cm.s^{-1}) and the time of running (from 10 min to 43 min) were changed along the days. All sorts of registration and observation must appear on this protocol's corresponding document, no. 101.056.001 (annex 2). The rats were rewarded with a special food reward (chocolate) whilst in the treadmill cubicles and linked the reward with the sound of the specified bell.

3.1.5.Rat Exercise Training for Treadmill

This phase takes part in the next 10 days after preconditioning. The parameters measured are the same but the training is constant every day. The protocol of Rat Exercise Training for Treadmill, document no. 101.055.002 (annex 3) dictates an acceleration time of 5 minutes, from 25cm.s^{-1} to 45cm.s^{-1}; then it follows an exercise training time of stable velocity (45cm.s^{-1}), during 30 minutes. The exercise training takes place twice a day, session A in the mornings and session B in the afternoons, but the animals have to rest at least 6 hours between the same-day-sessions. The exercise schedule should be followed precisely where changes in the intensity level if the current are the only ones allowed. The initial intensity (I_i) should be the same as the last change applied ($I_{u,\ prev}$) in the previous session. Neither the initial intensity nor any other changes in the intensity during each session can be lower than I_{min} nor higher than I_{max} (with reference to the exercise schedule). The registration and observation were made on this protocol's corresponding document, no. 101.056.002 (annex 4). The rats were also rewarded exactly the same way as before.

3.1.6. Rat Induced Muscle Damage

After twenty days of running in the treadmill, the rats are supposed to be already condition to the treadmill and ready to the main point of the study – the muscular damage.

The Rat Induced Muscle Damage Protocol document no. 101.055.003 (annex 5) describes the process to induce a muscle damage via eccentric exercise in a single session but should be performed twice a day, one session in the morning , session A, and one session in the afternoon, session B, with a minimum of 4h rest between the end of the first session and the beginning of the last. The exercise schedule should be followed precisely with changes in the intensity level of the current depending on "the fitness level" of the worst performer. The protocol points for a velocity of 50 $cm.s^{-1}$ with a declination of 15°. Depending on the overall "fitness level" of the rats trained together, the velocity could be increased to the range of 55 $cm.s^{-1}$ to 70 $cm.s^{-1}$ during the course a session. Furthermore, if a session had reached about 2 hours, and the rats still didn't show any sign of muscle damage, the velocity could be changed constantly between 45 $cm.s^{-1}$ and 80 $cm.s^{-1}$, oscillating between the two extremes. The duration of the session depended on the rats individually. Once each rat (in its own time) was not able to continue running due to muscle damage it should be removed from the treadmill as quickly as possible.

Each rat needed to be weighed before the session, placed in the cubicles and registered the time when the exercise starts, the room temperature and the relative humidity. The registration and observation were made on the corresponding document, no. 101.056.003 (annex 6).

The damage markers for evaluation were troponin-I, myoglobin and creatine kinase (CK) analyzed with ELISA kits (Life Diagnosis, Inc.).

3.1.7. Intermittent Hypobaric Hypoxia Exposition

3.1.7.1. Hypobaric Chamber

A hypobaric chamber was used to submit the rats to the hypobaric hypoxia program. The total volume of the hypobaric chamber was approximately 450L, which allowed the housing of three rat cages. The chamber walls were made of polymethyl methacrylate plastic, which facilitated observation of animal behavior during the protocol. Relative vacuum was developed by a rotational vaccum pump (TRIVAC D4B, Leybold, Köln, Germany) by regulating the airflow rate at the inlet with a micrometic valve. Inner pressure was controlled by two differential pressure sensors (ID 2000, Leybold, Köln, Germany) connected to a vaccum (Combivac IT23, Leybold, Köln, Germany) driving a diaphragm pressure regulator (MR16, Leybold, Köln, Germany). Depending on the simulated altitude required, a low-pressure set point was established in a control system. After the desired level was reached, the internal barometric pressure of the chamber was regulated and maintained by the control system.

3.1.7.2. Intermittent Hypobaric Hypoxia Exposition Protocol

The Hypobaric Hypoxia Exposition Protocol, document no. 101.055.004 (annex 7) designates a single intermittent hypoxia treatment session of 4h in a hypobaric chamber, simulating the altitude of a 4000m (Pb=462 Torr; Po2= 97 Torr), and should be performed once a day until sampling. The day count started the day after the induced muscle damage, only counting the days when the protocol was used. The change from normal pressure to 462 Torr should be done according to the Delta Pressure Plan. This plan took 15 min, however the allowed range for the time duration is from 12 to 18 min, i.e.., 15 (±3) min. The duration of the session was 15 min + 4h + 15 min = 4.5 h in total, although the 15-minutes can vary. Rat cages with control rats of the same lot should be placed on top of the hypobaric chamber whilst the session is ongoing. The respective registrations are made in a specific document numbered 101.056.004 (annex 8).

3.1.8. Rat Rehabilitation Exercise after Hypoxia

This protocol is submitted to rats belonging to hypoxia plus exercise group (EHIP) (document no. 101.055.005, annex 9) and it describes rehabilitation in the form of low impact concentric exercise, with an inclination, for a single session which should be applied immediately after intermittent hypoxia session. The day count follows the schedule of the intermittent hypoxia sessions. The exercise schedule should be followed precisely where changes in the intensity level of the current are the only ones allowed. The initial intensity (I_i) should be the same as the last change applied ($I_{u, prev}$) in the previous session. Neither the initial intensity nor any other changes in the intensity during each session can be lower than I_{min} nor higher than I_{max} (with reference to the exercise schedule). According to the exercise schedule there is a phase of 1 min with no velocity or inclination, followed by 5 min of running starting with a velocity of 10 $cm.s^{-1}$ increasing until 30 $cm.s^{-1}$ and with an inclination of 5°; then it is more 15 min at constant velocity (30 $cm.s^{-1}$) and inclination (5°); ending with 1 minute of rest with no inclination. The registrations are made in the document no. 101.056.005 (annex 10).

3.2. Blood Sampling Procedure

Blood samples were collected by cardiac puncture. Prior to collection, animals were anesthetized with urethane. Sodium heparine was used as an anticoagulant. A fraction of each blood was separated for immediate hematological analyses, which were always completed within 10 min of blood withdrawal. Blood cell count was assessed by use of an automatic cell counter (Celtac α; Nihon Kohden Corp., Tokyo, Japan).

3.3. Flow Cytometry

CD45 and CD34 expression was analyzed by flow cytometry in rat's blood. The cells were incubated for 20 min at 4°C with monoclonal antibodies against rat antigens, including direct phycoerythrin- or fluorescein isothiocyanate- conjugated mouse anti-rat monoclonal

antibodies recognizing CD34 (Santa Cruz Biotechnology, Inc.) and CD45 (Santa Cruz Biotechnology, Inc.), respectively. After washing and resuspended in FACS Buffer (2%FBS in 1xPBS) (Sigma-Aldrich), samples were analyzed by collecting ±100,000 events, using BD-FACScan™ System (Becton Dickinson) and Cell-Quest software (Beckman Coulter).

3.3.1.Flow Cytometry Protocol

For each rat it was necessary 500 µl of blood, but in order to make the controls, we had to take plus 1500 µl of one of the rats sacrificed. It is taken 500 µl of heparinized blood to a 1.5 ml eppendorf vial. First it was centrifuged and the plasma was separated. The red blood cells were lysed with 1 ml of 1xFCM Lysing Solution (Santa Cruz Biotechnology, Inc.), during 5 minutes in the rotator, then it was centrifuged and the supernatant was taken. This step was repeated three times. Meanwhile it was made the cocktail (30 µl of CD45-FITC antibody, 30 µl of CD34-PE antibody, 540 µl of FACS Buffer) and the controls FITC (5 µl of CD45-FITC + 95 µl of FACS Buffer), PE (5 µl of CD4-PE + 95 µl of FACS Buffer) and the White (100 µl of FACS Buffer). The samples were kept in the dark, at 4°C during 20 minutes and then it was added 500 µl of FACS Buffer. The samples suffered a vortex followed by a centrifugation. The supernatant was taken and it was added 500 µl of 4% paraphormaldehyde to fix them. At this time the samples are ready to acquisition in the flow cytometer.

3.4. Statistical Analysis

Data for all parameters are expressed as the sample mean ± standard deviation of the mean. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, San Diego, CA). Once the number of samples for group and subgroup are not enough (N≥3) the strategy utilized was to compare positive control group (Ctrl+) with hypoxia group (HIP) by Student *t*-test; and to infer about the changes with the time after damage, in the positive

control group (Ctrl+), compare T0, T3, and T14 by one-way ANOVA test; at a level of significance $p < 0.05$.

4. Results

Normal growth was not affected by hypobaric hypoxia exposure, as reflected by body weight changes during the protocol.

4.1. Schematic representation of the experimental design and distribution of the animals for experimental and control group

Once we wanted to evaluate about the effect of hypoxia in the regeneration of tissue damage it was important to have a clear observation of the evolution period after the induced injury. Therefore it was fundamental to create homogeneous characteristics in groups of animals submitted to different conditions of recuperation. It is known already the regeneration is a long process involving the participation of different populations of cells in different stages.

The threshold of this study is the moment when damage is caused. But to have a coherent study it is essential to have a Ctrl- group, whose rats doesn't suffer the damage neither exercise training; the effects of exercise are evaluated in Ctrl+ group. Besides we have a group that after exercise training and tissue injury is submitted to a treatment of intermittent hypobaric hypoxia (HIP); and also another group that differs from the one before because after hypoxia treatment are given an exercise training again (EHIP). Since we want to know about the recuperation after induced damage, rats were sacrificed on sampling day. Samples were taken in T0 (the day before causing the damage), T1 (one day after the induced damage), T3 (three days after the induced damage), T7 (seven days after the induced damage) and T14 (fourteen days after the induced damage) (Figure 9).

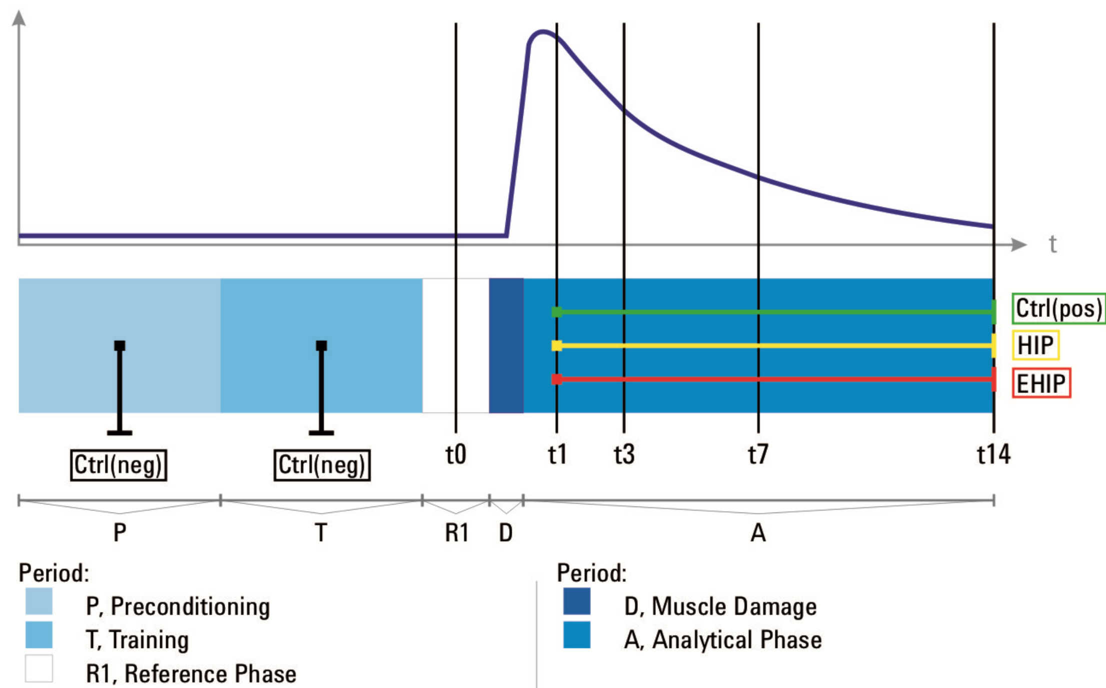


Figure 9. Schematic representation of the experimental design of the study. The first phase is called preconditioning (P) and it takes ten days; the second phase is the training phase (T), and it takes more ten days; the reference phase (R1) is one day of rest; after it is the day when the muscle damage is caused (D); and then is the analytical phase (A) that is divided in t1, t3, t7 and t14. In order to have a complete study the rats are killed and analyzed in different days after damage; t0 corresponds to the day before the damage, only possible with animals from Ctrl(pos) and Ctrl(neg) group; t1 corresponds to the day after damage; t3 is three days after damage; t14 is fourteen days after damage. The curve is representative of the damage markers detected by ELISA; it has a peak in t1 and decreases until t14. The project previews a study where not only the treatment changes, treatment with intermittent hypobaric hypoxia (HIP), hypoxia plus exercise (EHIP) and no treatment (Ctrl+ and Ctrl-) but also the number of days after damage (© 2012 J. G. Rios-Kristjansson).

As we can see it is a longstanding project methodology and how it is only starting, not only the number of animals for experimental group is small but also it varies a lot within each group (Table 1) so it is not easy to take consistent conclusions of the results.

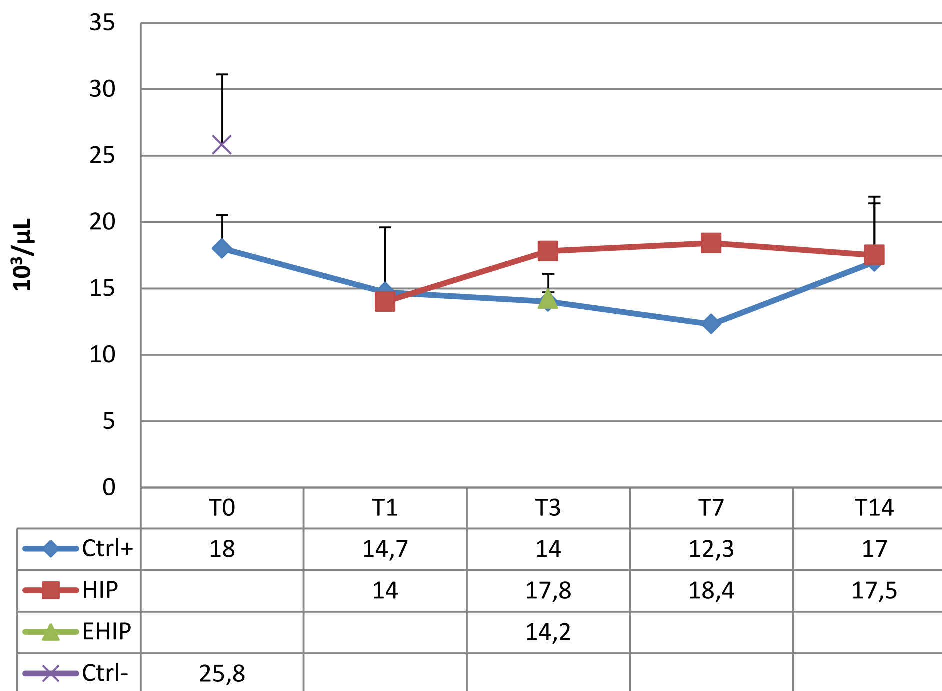
Table 1. Number of animals for experimental group in the different times after damage

	Ctrl +	HIP	EHIP	Ctrl -
T0	7			3
T1	2	1		
T3	4	2	2	
T7	1	1		
T14	3	3		
	17	7	2	3

4.2. Hematological parameters

The blood cell counter is a functional hematology analyzer with capabilities featuring proven histogram differential technology features. The blood is taken from the rat, treated with the anti-coagulant heparin and placed directly in this equipment which describes its characteristics. Hematological parameters for the experimental and control groups in different times after damage are given in the figures 10, 11 and 12. There were no significant differences between the values of diverse days after damage (T0, T3, and T14) in Ctrl+ group, for the three parameters measured.

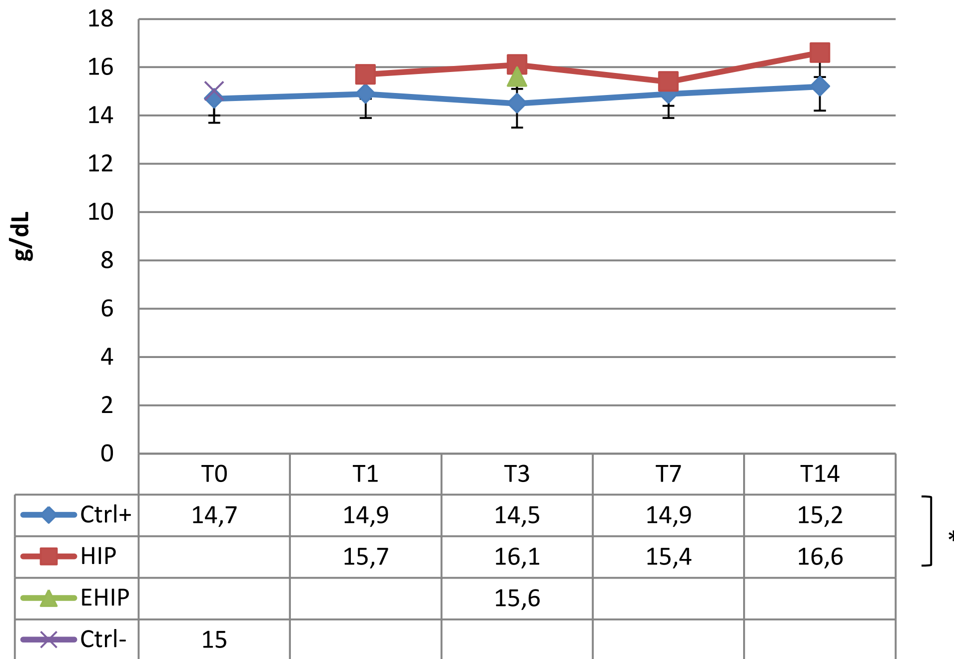
Figure 10 shows the curve of white blood cells (WBC) levels in different times after damage and for the different experimental and control groups. It is possible to see that the number of WBC in Ctrl- group is higher than in the other groups, despite it wasn't supposed, because the Ctrl- rats had no treatment. In HIP/T1 group WBC increases and in the opposite WBC of Ctrl+ group decreases. According to the equipment definitions programmed for the animal model, the normal range is between $5x10^3/\mu L$ and $14x10^3/\mu L$; $WBC>14x10^3/\mu L$ are considered already high levels.



	T0	T1	T3	T7	T14
Ctrl+	18	14,7	14	12,3	17
HIP		14	17,8	18,4	17,5
EHIP			14,2		
Ctrl-	25,8				

Figure 10. Number of white blood cells per μL of blood in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. There is no significant differences shown in this hematological parameter between HIP and Ctrl+ group.

Hemoglobin (Hb) is the blood carries oxygen from the respiratory organs to the tissues where it releases the oxygen to burn nutrients to provide energy to power the functions of the organism, and collects the resultant carbon dioxide to bring it back to the respiratory organs to be dispensed from the organism (Connie *et al*., 1998). Figure 11 shows the levels of Hb in each blood samples. Although the results doesn't vary with the time after damage, HIP group have always higher values of hemoglobin compared to the other groups. Agreeing with the blood cell counter only above 17 g/dl is considered high; the normal range goes from 12 g/dl to 16 g/dl.



	T0	T1	T3	T7	T14
Ctrl+	14,7	14,9	14,5	14,9	15,2
HIP		15,7	16,1	15,4	16,6
EHIP			15,6		
Ctrl-	15				

Figure 11. Levels of hemoglobin in grams per dL of blood in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. Between Ctrl+ and HIP group there are significant differences. Levels of significance are indicated by asterisks as:* $p < 0.05$.

Hematocrit (HCT) is the volume percentage of red blood cells in blood, the ones which contain Hb. Figure 12 shows HCT percentage for the animals analyzed. Between 30%-50% it is considered normal levels of hematocrit, HCT < 50% it is high percentage, and HCT < 30% is low percentage. All of the results are in the normal range but hypoxia groups show supremacy compared to control groups.

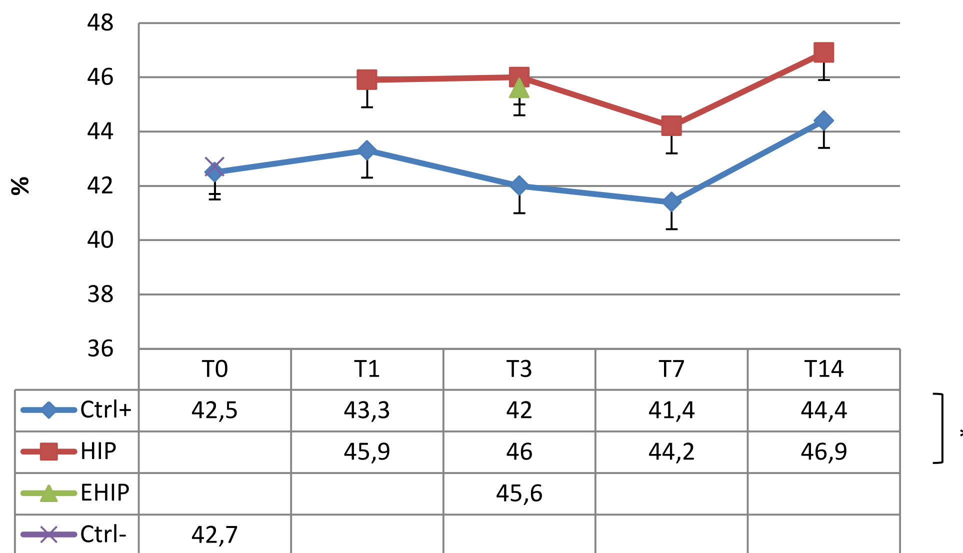


Figure 12. Percentage of hematocrit in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. There is significant differences between HIP and Ctrl+ group. Levels of significance are indicated by asterisks as:* $p < 0.05$.

4.3. Analysis of CD34+ and CD45+ rat stem cells in experimental and control group by flow cytometry

Experiments designed to identify stem cells from lysed whole rat blood employed CD34+ and CD45+ cells antibodies conjugated with PE and FITC fluorochromes, respectively. The gating strategy combining positive and negative selection employed to identify stem cells for this sort is described in Figure 13. Unlysed erythroid cells (e.g. reticulocytes) were easily distinguished by laser light scatter and excluded.

Despite the study is not complete yet, we have already some data. Flow cytometry is a powerful technique for the analysis of multiple parameters of individual cells within heterogeneous populations. The data gathered can be analyzed statistically by flow cytometry software to report cellular characteristics.

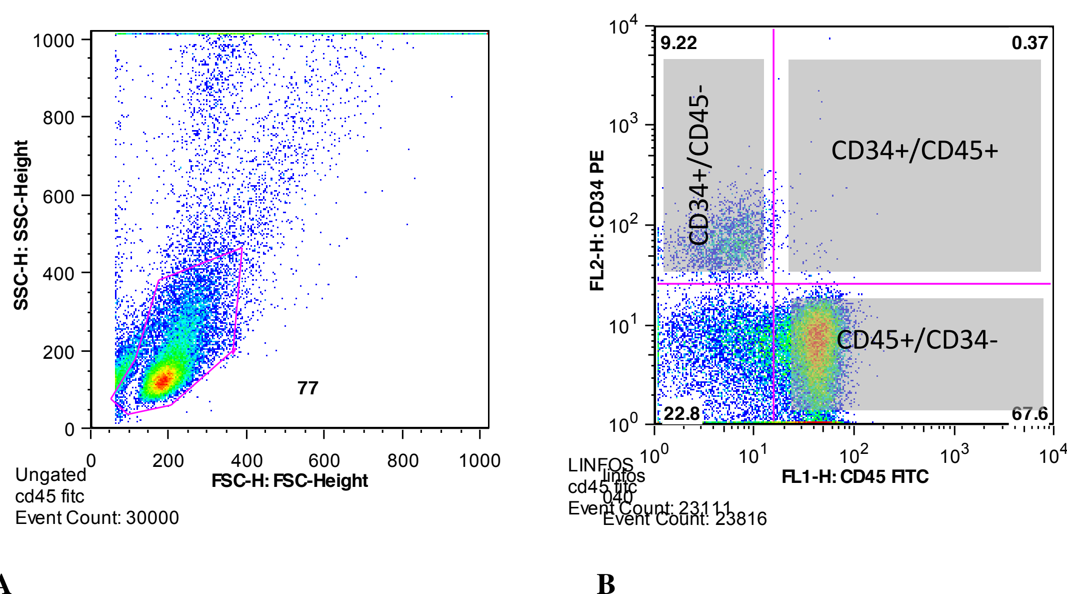
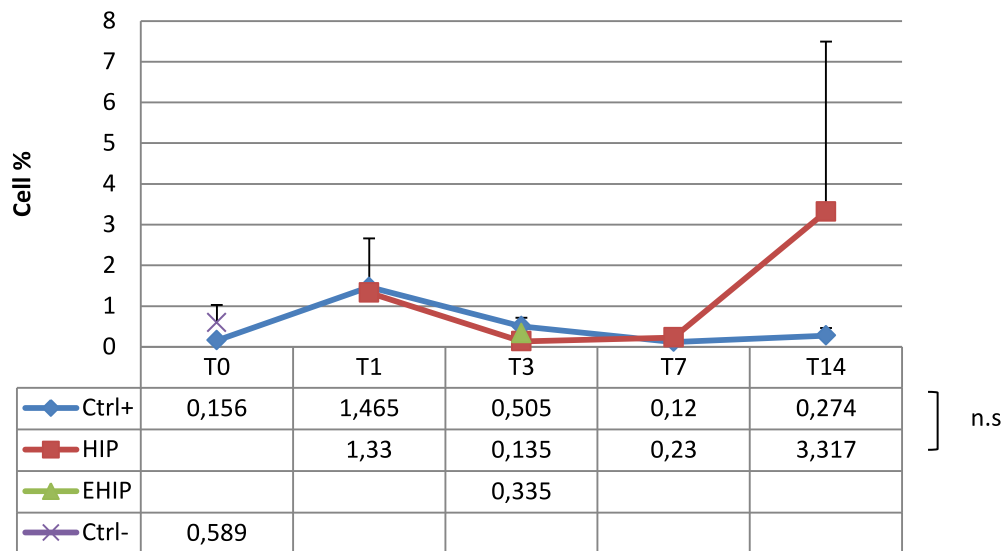


Figure 13. Gating strategy for flow cytometric identification and sorting of CD34+ and CD45+ rat stem cells. A. Selection of morphologically lymphocyte-like population of cells, excluding cellular debris and free platelets; percentage of cells selected (77) after a 30000 events, which means the number of cells which crossed the laser. **B.** Example of a result from one of the samples (HIP/T14); the numbers in the corners are the percentage of CD34+ cells (superior left); CD45+ cells (inferior right) and double positive (superior right).

LINFOS
cd45 fitc
Event Count: 23111

With FITC and PE, which have different wavelengths it was possible to evaluate about population of cells with CD34 markers, with CD45 markers and lastly, with both markers. There were no significant differences between Ctrl+ and HIP group for the three populations, but comparing the times after damage (T0, T3, and T14) in Ctrl+ group by one-way ANOVA test, there were significant differences in CD34+ cells.

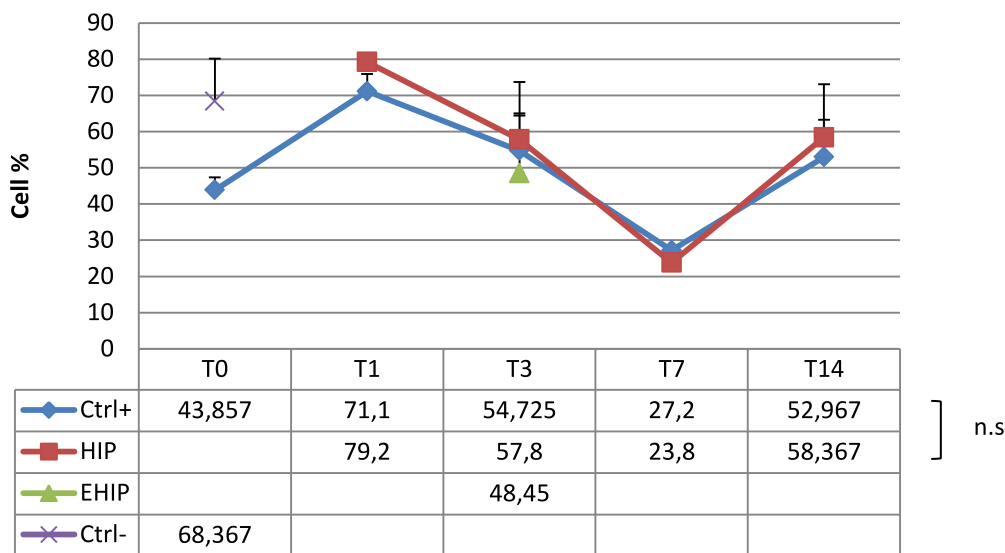
The percentage of CD34+ cells (Figure 14) appear to be more in Ctrl- compared with Ctrl+/T0. In T1 the percentage increase, but with small difference between Ctrl+ and HIP group. It decreases in T3 and again in T7. In T14 while Ctrl+ doesn't change too much, HIP group shows a considerable growth. About EHIP group no significant changes are seen, its values are similar to Ctrl+ and HIP values.



	T0	T1	T3	T7	T14
Ctrl+	0,156	1,465	0,505	0,12	0,274
HIP		1,33	0,135	0,23	3,317
EHIP			0,335		
Ctrl-	0,589				

Figure 14. Percentage of CD34+/CD45- population of cells in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. There was no significant difference between Ctrl+ and HIP group (n.s. – no significant difference).A significant difference was detected comparing T0, T3, and T14 in the Ctrl+ group ($p < 0.05$).

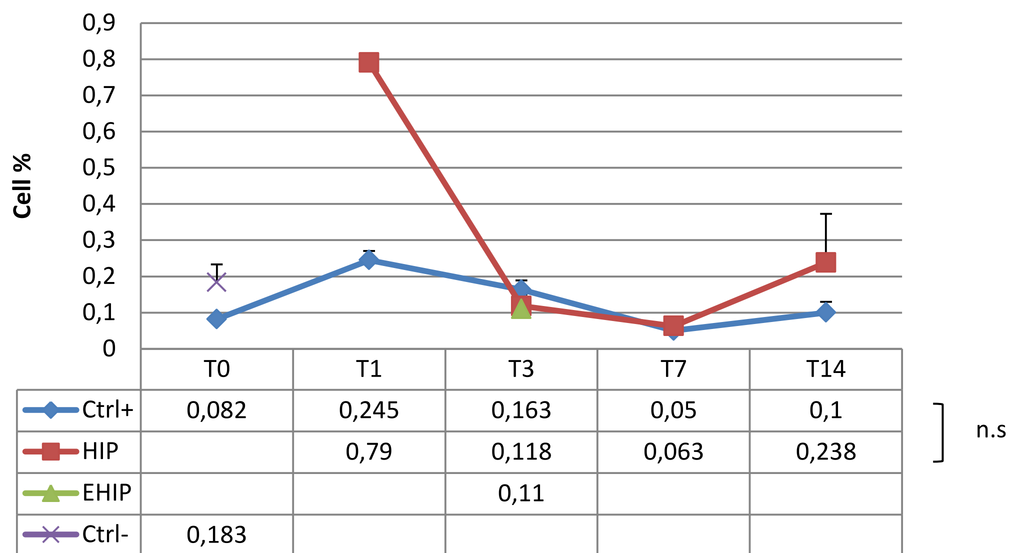
The percentage of CD45+ cells (Figure 15) also seems to be more in Ctrl- compared with Ctrl+/T0, despite it has a big standard deviation. In Ctrl+/T1 group the percentage is bigger than in T0, but even though, HIP group has higher values. In T3 we can see a decrease and again in T7, for both Ctrl+ and HIP group. In T14 the percentage increases for both groups, and the numbers are similar to what they were in T3.



	T0	T1	T3	T7	T14
Ctrl+	43,857	71,1	54,725	27,2	52,967
HIP		79,2	57,8	23,8	58,367
EHIP			48,45		
Ctrl-	68,367				

Figure 15. Percentage of CD45+/CD34- population of cells in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. There was no significant difference between Ctrl+ and HIP group (n.s. – no significant difference) neither between the days after damage in Ctrl+ group.

Finally, double positive population of cells (CD34+/CD45+) (Figure 16) is the rarest one. Still the cells from Ctrl- are more than in Ctrl+ in T0. In T1 of Ctrl+ group the percentage of cells is bigger than in T0 but HIP group have a considerable higher percentage of double positive cells. The percentage decreases in T3, where the three groups (HIP, EHIP and Ctrl+) have similar amount of cells; and again in T7 both in HIP and Ctrl+ group. In T14 we see an increasing of the levels, bigger in HIP group.



	T0	T1	T3	T7	T14
Ctrl+	0,082	0,245	0,163	0,05	0,1
HIP		0,79	0,118	0,063	0,238
EHIP			0,11		
Ctrl-	0,183				

Figure 16. Percentage of CD34+/CD45+ population of cells in experimental and control groups in different times after damage. The blue line with diamonds corresponds to control positive group (Ctrl+); the red line with squares corresponds to hypoxia group (HIP); the triangle, with only one point because the samples were all measured three days after damage; and the violet cross is relative to control negative group (Ctrl-) with also only one point. There was no significant differences between Ctrl+ and HIP group (n.s. – no significant difference) nor between the days after damage in Ctrl+ group.

In fact, we have already some data we can start from, but for example Ctrl- and EHIP group didn't help in the results. About EHIP group we just didn't see any differences from the HIP group but about Ctrl-, the results were against what they should be.

5. Discussion and Conclusion

Intermittent hypobaric hypoxia exposure showed to improve human's physical condition working as a stimulus for normal developmental and physiological processes. Oxygen level regulates HIF-1 pathway and therefore the expression of a number of growth factors and cytokines (Semenza, 2000). Also the physical exercise revealed to be beneficial to health, activating and mobilizing stem and progenitor cells fundamental to tissue regeneration (Laufs *et al*., 2004). This study gathering all this information proposed to see if hypobaric hypoxia exposure has, in fact, effects on specific populations of stem cells in order to infer about tissue regeneration.

Many studies have reported that chronic hypoxia induces deleterious effects on body mass (Boyer and Blume, 1984; Rose *et al*., 1988). An experiment study of chronic intermittent hypobaric hypoxia exposition in rats with a 4 by 4 and 2 by 2 alternating daily schedule of sea level and simulated 4600 m altitude demonstrated a severe body weight reduction and compromised survival rate (Siques *et al*., 2006). However, possibly due to the lower degree of hypoxia exposure, we detected no negative effects on normal growth rate. This indicates that the hypoxia exposure regime applied offers good compatibility with the standard living conditions of these experimental animals.

The hematological parameters illustrate some adaptations to the acclimatization program to hypoxia. The elevated leukocyte counts, all above the normal level of WBC per µL of blood, could be considered as a consequence of induced muscle damage and the higher values in HIP group due to a possible sign of an underlying inflammatory response to the hypoxic stress. But the fact of Ctrl+/T0 value is even higher than HIP goes against it.

Despite the differences are not significant also due to the low amount of samples, the increases in hemoglobin and hematocrit in HIP group are clearly associated with an enhancement of blood oxygen capacity. The results also demonstrate that the acclimatization is a longstanding process once in T14 both Hb percentage and HCT levels reach their maximum. The decrease in T7 can be explained by the erythropoiesis time

course. It is also possible to conclude that the intermittent ($4h.d^{-1}$) hypobaric hypoxia (460 Torr) in a hypobaric chamber activates the erythropoietic response of the laboratory rats.

Following adequate stimulation, stem and progenitor cells may leave the cell niche and circulate in the PB. EPCs enumeration has gained attention as a potential biomarker of vascular injury and endothelial damage/dysfunction (Gehling *et al*., 2007). Implementation of sensitive and specific EPC enumeration methods has been hampered by disagreement regarding selection of the most appropriate primary immunophenotypic identifier(s) and detection methods (Thomas *et al*., 2009). In the present study, EPC enumeration methods from lysed whole rat blood were performed using flow cytometry. Flow cytometry is an attractive technique since it allows analysis of large number of cells quickly, with low subjectivity, and is suitable for rare cell detection (Khan *et al*., 2005). It is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics. The fluidics system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream; the most commonly used is the argon laser because the 488 nm light that it emits excites more than one fluorochrome; the combination of FITC and PE can be used simultaneously because each is excited at 488 nm and its peak emission wavelengths are not extremely close to each other (FITC – 530 nm; PE – 570 nm) so each signal can be detected by a separate detector; the amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle; therefore also optical filters make part of optics system to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that can be processed by the computer; light signals are generated as particles pass through the laser beam in a fluid stream; a voltage pulse is created when a particle enters the laser

beam and starts to scatter light or fluorescence. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles. A subset of data can be defined through a gate. A gate is a numerical or graphical boundary that can be used to define the characteristics of particles to include for further analysis (Givan, 1992).

Stem cells are an extremely debated subject due to its importance in our organism's maintenance and regeneration because of their ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation. In most tissues stem cells are rare (Reya *et al*., 2001). About the mesenchymal stem cells (MSC) it is known they are extremely important in tissue repair once they can differentiate into a variety of cells and they are inclusively regulated by HIF-1 (Liu *et al*., 2011). Endothelial progenitor cells (EPC) are involved in different processes for new blood vessel formation, playing a significant role in the repair of injured endothelium (Urbich *et al*.,2004). Hematopoietic stem cells besides originates the most part of the cell types of our blood they also give rise to non-hematopoietic tissues, suggesting that these cells may have a greater differentiation potential (Reya *et al*., 2001). The lifespan of various blood cells can range from years for some lymphocytes, to months for erythrocytes, to mere hours for neutrophils, making continuous production of blood cells (hematopoiesis) necessary for even a short term homeostasis (Gunsilius *et al*., 2001).

CD34 antigen is present on immature hematopoietic precursor cells and all hematopoietic colony-forming cells in bone marrow and blood, including unipotent and pluripotent progenitor cells. CD34 antigen is stage but not lineage specific, independently of the differentiative lineage it is expressed only by ontogenetically early cells (4). There are evidences that CD34 can be expressed in HSC (Bender *et al*., 1991¸ Wojciechowski *et al*., 2008;), EPC (Miller-Kasprzak *et al*., 2007), satellite cells (Beauchamp *et al*., 2000) and also in fibrocytes (Bucala *et al*., 1994; Zammit *et al*., 2006).

C45 is a member of leukocyte common antigen family. It is present in all leukocytes and is weakly expressed on hematopoietic progenitor cells (Bender *et al*., 1991; Charbonneau *et*

al., 1992). Some data shows that EPC also express CD45 leukocyte marker (Rehaman *et al*., 2003) and there are some studies about CD45 expression in fibrocytes (Bucala *et al*., 1994).

In fact, from the results it is possible to see the increasing of all the populations of cells from the day before the damage to the day after, showing the immediate response from the body. Despite the differences are not statistically significant it is possible to see the effect of hypoxia, mostly in double positive population of cells and it can be explained by the inflammatory response right after the induced damage (T1) given by the stimulation of lymphohematopoietic cells (CD34+/CD45+). The results from CD34+ cells in T14, are an evidence that the hypobaric hypoxia exposure was efficient as a stimuli of stem cells multiplication, while the levels of Ctrl+ doesn't change too much with the time, the levels of HIP increases; also in the population of CD34+/CD45+ it is possible to see the same, but with less difference between the groups. Only CD45+/CD34- which marks all lymphocytes have the increasing in T14 both in HIP and Ctrl+ group, in accordance with the two-phase regeneration process, a first phase of degeneration and a second of regeneration.

The values of double positive group are evidently much higher than the other two because theoretically we were analyzing only the lymphocytes or morphologically lympho-like cells.

About the Ctrl- group no conclusions at all can be taken because, for some inexplicable reason, the samples showed levels of damaged markers in ELISA and they weren't submitted to any exercise training. But it can explain the controversial values of its results.

Regarding EHIP group the results were very similar to the ones of HIP group, showing no effect of exercise in the recuperation time; this evidence doesn't match with what was expected because exercise not only provokes hypoxic stimuli but also showed to induce the release of growth factors, cytokines and hormones (Laufs *et al*., 2004). Therefore the results were likely to be better than in HIP group, what didn't happen. First it would be necessary to have more samples and in different days after damage, for now it can be

explained as a too weak exercise train session, with need of more running time and more frequently.

Nevertheless it is important to highlight the fact of this study is in a preliminary phase and the number of samples for group and subgroup are too low. Some groups had so few samples that were impossible to compare them statistically. Since the rats enter the process it took 1 to 1.5 month to complete the experimental phase, the exercise training is time extensive and due to space availability it was only possible to have ten rats at the time. Also the technical part was developed from the zero, which led to use the first lots of rats for the optimization of the protocol both for muscle injury induction and flow cytometry and discard them from the results.

For better conclusions it would be recommended the use of more (CD133 for example) and more specific antibodies because stem cells are a very heterogenic group with a hole of markers expressed, also varying from early to late stages. Another thing was to analyze not only the lymphocyte population in the cytometer, because it can be much more stem cells with no lymphocyte-like morphology in the sample. Culture of muscle fibers cells (myocytes) would also facilitate the study and it would be possible to repeat the staining and acquisition in the cytometer.

Finally, there is absolutely no doubt about the benefits of hypobaric hypoxia exposure not only to athletes but also to patients of a variety of illnesses. In sport the injuries of muscle-tendon system are very common with big impacts at different levels, inclusively economic. If our proposal is correct it would be an amazingly innovative strategy to fight that question. Moreover this knowledge can give rises to other therapeutics strategies that may alleviate some of the pathological conditions associated with poor muscle regenerative capacity, such as the one observed in muscular dystrophy patients and in the course of normal aging.

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Annexes

Procedure: QGR/101.051.002

Parameters: d, day (d); a, acceleration (cm s^{-1} min^{-1}); t, time (min); υ, velocity (cm s^{-1}); I, current (mA); T, temperature (ºC).

General Notes

A All sort of registration and observation must appear on this protocol's corresponding document, no. QGR/101.056.001

B This protocol describes the process of getting the rats used to the environment of the treadmill, then to comprehend the function of the treadmill, and lat 45 cm s^{-1}.

C The first 4 days have only one session per day (called session A) and the rest of the days two sessions per day, where the former one should be performed in the morning, session A, and the latter one in the afternoon, session B, with a minimum of 6 h rest between the end of session A and the beginning of session B.

D The exercise schedule should be followed precisely with changes in the intensity level of the current depending on 'the fitness level' of the worst performer. The range of allowed intensity changes over the entire preconditioning process, and the initial intensity (I_i) should never be lower than I_{min} nor higher than I_{max}, but shouldn't either be lower than the last change in the intensity from the previous session ($I_{u,prev}$).

Exercise Schedule

	phase 0		phase I			phase II			phase III		phase IV		Summary				Cubicle				
Day	t_0	$υ_0$	$(t_a)_1$	a_1	υ	$(t_a)_2$	a_2	υ	$(t_υ)_3$	υ	t_4	υ	$t_{exerc.}$	t_{total}	I_{min}	I_{max}	1	2	3	4	5
No.	min	cm s^{-1}	min	cm s^{-1} min^{-1}	cm s^{-1}	min	cm s^{-1} min^{-1}	cm s^{-1}	min	cm s^{-1}	min	cm s^{-1}	min	min	mA	mA	Rat's position				
1	10	0 (5)	0	0	0	0	0	0	0	0	0	0	0	10	0.2	0.6	i	ii	iii	iv	v
2	10	0 (5)	0	0	0	0	0	0	0	0	0	0	0	10	0.2	0.6	v	i	ii	iii	iv
3	3.5	0 (5)	5	~4	10-30	0	0	0	10	30	2	0	15	20.5	0.2	0.6	iv	v	i	ii	iii
4	3.0	0 (5)	5	~4	10-30	0	0	0	15	30	2	0	20	25	0.2	0.6	iii	iv	v	i	ii
5A	2.0	0 (5)	5	~3	15-30	2	2	30-34	25	34	2	0	32	36	0.2	0.6	ii	iii	iv	v	i
5B	2.0	0 (5)	5	~3	15-30	2	2	30-34	25	34	2	0	32	36	0.2	0.6	i	ii	iii	iv	v
6A	2.0	0 (5)	5	~3	15-30	2	~2	30-35	30	35	2	0	37	41	0.2	0.8	v	i	ii	iii	iv
6B	2.0	0 (5)	5	~3	15-30	2	~2	30-35	30	35	2	0	37	41	0.2	0.8	iv	v	i	ii	iii
7A	1.0	0 (5)	5	~3	15-30	3	~3	30-40	32	40	2	0	40	43	0.2	0.8	iii	iv	v	i	ii
7B	1.0	0 (5)	5	~3	15-30	3	~3	30-40	32	40	2	0	40	43	0.2	0.8	ii	iii	iv	v	i
8A	1.0	0 (5)	5	~3	15-30	3	~3	30-40	32	40	2	0	40	43	0.4	0.8	i	ii	iii	iv	v
8B	1.0	0 (5)	5	~3	15-30	3	~3	30-40	32	40	2	0	40	43	0.4	0.8	v	i	ii	iii	iv
9A	1.0	0 (5)	5	~3	15-30	3	~3	30-40	32	40	2	0	40	43	0.4	1	iv	v	i	ii	iii
9B	1.0	0 (5)	5	~4	15-35	3	~3	35-45	32	45	2	0	40	43	0.4	1	iii	iv	v	i	ii
10A	1.0	0 (5)	5	~4	15-35	3	~3	35-45	32	45	2	0	40	43	0.4	1	ii	iii	iv	v	i
10B	1.0	0 (5)	5	~4	15-35	2	5	35-45	30	45	2	0	37	40	0.4	1	i	ii	iii	iv	v

Guide

1 Register all settings, conditions, and initial parameters along with the Rat ID number for each cubicle.

2 Place the rats in the cubicles, register the time when the exercise starts.

3 During each session, the number of shock (Number-S) for each rat should be registered after each phase (according to the exercise schedule) and about every 5 minutes during phase III (according to the register table below).

4 Make notes on the running style of each rat during the session according protocol QGR/101.055.010.

5 At the end of phase III, after lowering the velocity, unplug the motor and register the Distance (Dist.) and then the number of shock (Number-S).

6 After phase IV, press the red stop button on the treadmill monitor and register the number of shock (Number-S), the total time of shock (Time-S), the room temperature (T_f) and the relative humidity (RH_f), and register the intensity level as it is left after the session ($I_{u,new}$).

7 Reward the rats that performed accordingly with a special food reward whilst in the treadmill cubicles and link the reward with the sound of the specified bell.

8 Register how the rats reacted to the reward.

9 Place each rat in its cage and clean the treadmill.

10 Make sure all parameters necessary for the registration have been accounted for on the registration sheet and sign for the registration.

Registration Table

Day No.	1A	2A	3A	4A	5A/B	6A/B	7A/B, 8A/B, 9A/B, 10A	10B
phase 0	10	10	3.5	3	2	2	1	1
phase I	-	-	8.5	8	7	7	6	6
phase II	-	-	-	-	9	9	9	8
phase III	-	-	14	10	15	15	15	15
	-	-	18.5	15	20	20	20	20
	-	-	-	23	25	25	25	25
	-	-	-	-	30	30	30	30
	-	-	-	-	34	35	35	35
	-	-	-	-	-	39	41	38
phase IV	10	10	20.5	25	36	41	43	40

i The Registration Table explains at what minute a registration should be performed during each session. Note that that some patterns with respect to minutes is the same between some days – This does not mean that these time changes between certain days correspond to changes in, e.g., in velocity, acceleration, etc, in the Exercise Schedule, and the setup should be viewed individually.

□

Sheet Start Date:
Sheet End Date:
Sheet Number:

Procedure: QGR/101.055.001
Parameters: The same as in protocol.

Prtc version: ________ $I_{u,prev}$: ______ mA I_i: ______ mA **Signature:**

Prtc Day: ________ $I_{u,new}$: ______ mA **u(Ph III):** ______ $cm\ s^{-1}$

Session: ________ T_i: ______ °C

Hour: ________ RH_i: ______ %

Lot No.: ________ T_f: ______ °C

RH_f: ______ %

General Observations:

(a) Time-S increasing, faeces stuck on the current board
(b) Time-S increasing, w/o an obvious reason
(c) Number-S increasing, w/o an obvious reason
(d) Number-S doesn't increase

Number-S

Phases		t, min	Rat ID	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run
Ph 0	No Run																	
Ph I	Accel																	
Ph II	Accel																	
Ph III	Run																	
	Distance																	
Ph IV	Rest																	
	Time-S																	

Observations:

Procedure: QGR/101.051.002

Parameters: d, day (d); a, acceleration (cm s^{-1} min^{-1}); t, time (min); υ, velocity (cm s^{-1}); I, current (mA); T, temperature (ºC).

General Notes

A All sort of registration and observation must appear on this protocol's corresponding document, no. QGR/101.056.002

B This protocol describes the exercise schedule for a single session but should be performed twice a day, one session in the morning, session A, and one session in the afternoon, session B, with a minimum of 6 h rest between the end of session A and the beginning of session B.

C The day count continues from the last day of the preconditioning period (*protocol QGR/101.055.001*), only counting the days when the protocol is used.

D The exercise schedule should be followed precisely where changes in the intensity level of the current are the only ones allowed. The initial intensity (I_i) should be the same as the last change applied ($I_{u,prev}$) in the previous session. Neither the initial intensity nor any other changes in the intensity during each session can be lower than I_{min} nor higher than I_{max} (with reference to the exercise schedule).

Exercise Schedule

	phase I			phase III		phase IV		Summary		Current	
	$(t_a)_1$	a_1	υ	$(t_u)_3$	υ	t_4	υ	$t_{exerc.}$	t_{total}	I_{min}	I_{max}
Session	min	cm s^{-1} min^{-1}	cm s^{-1}	min	cm s^{-1}	min	cm s^{-1}	min	min	mA	mA
A & B	5	≈ 5	25-45	30	45	1	0	35	36	0.8	1.8

Guide

1 Register all settings, conditions, and initial parameters along with the Rat ID number for each cubicle.

2 Place the rats in the cubicles, register the time when the exercise starts.

3 Make notes on the running style of each rat during the session according protocol QGR/101.055.010.

4 Whilst performing according to the exercise schedule, do the following in phase I:

[0 → 1] min: [5 → 25 → 30] cm s^{-1}
[1 → 2] min: 30 cm s^{-1}
[2 → 3] min: [30 → 35] cm s^{-1}
[3 → 4] min: [35 → 40] cm s^{-1}
[4 → 5] min: [40 → 45] cm s^{-1} ← 5th minute, End of phase I
[5 → 35] min: 45 cm s^{-1}
[35 → 36] min: 0 cm s^{-1} ← manually turn the velocity down before stopping it completely

5 After phase I and every 5 minutes during phase III, register the number of shocks (Number-S) for every rat.

6 At the end of phase III (35th minute), after lowering the velocity, unplug the motor, register the Distance (Dist.) and then the number of shock (Number-S).

7 After phase IV (36th minute), press the red stop button on the treadmill monitor and register the number of shock (Number-S), the total time of shock (Time-S), the room temperature (T_f) and relative humidity (RH_f), and register the intensity level as it is left after the session ($I_{u,new}$).

8 Reward the rats that performed accordingly with the special food reward whilst in the treadmill cubicles and link the reward with the sound of the specified bell.

9 Register how the rats reacted to the reward.

10 Place each rat in its cage and clean the treadmill.

11 Make sure all parameters necessary for the registration have been accounted for on the registration sheet and sign for the registration.

□

Sheet Start Date:

Procedure: QGR/101.055.002 | Sheet End Date:

Parameters: The same as in protocol. | Sheet Number:

Prtc version: ________ $I_{u,prev}$: ______ mA I_i: ______ mA **Signature:**

Prtc Day: ________ $I_{u,new}$: ______ mA u(Ph III): ______ cm s^{-1}

Session: ________ T_i: ______ °C

Hour: ________ RH_i: ______ %

Lot No.: ________ T_f: ______ °C

RH_f: ______ %

General Observations:

(a) Time-S increasing, faeces stuck on the current board
(b) Time-S increasing, w/o an obvious reason
(c) Number-S increasing, w/o an obvious reason
(d) Number-S doesn't increase

Number-S

Phases		t, min	Rat ID	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run
Ph I	Accel	5																
Ph III	Run	10																
		15																
		20																
		25																
		30																
		35																
	Distance	35																
Ph IV	Rest	36																
	Time-S	36																

Observations:

Procedure: QGR/101.051.002

Parameters: d, day (d); a, acceleration (cm s^{-1} min^{-1}); t, time (min); υ, velocity (cm s^{-1}); I, current (mA); T, temperature (ºC).

General Notes

A All sort of registration and observation must appear on this protocol's corresponding document, no. QGR/101.056.003

B This protocol describes the process to induce a muscle damage vie eccentric exercise in a single session but should be performed twice a day, one session in the morning, session A, and one session in the afternoon, session B, with a minimum of 4 h rest between the end of session A and the beginning of session B.

D The exercise schedule should be followed precisely with changes in the intensity level of the current depending on 'the fitness level' of the worst performer.

E Depending on the overall 'fitness level' of the rats trained together, the velocity can be increased to the range of 55 cm s^{-1} to 70 cm s^{-1} during the course a session. Furthermore, if a session has reached about 2 hours, and the rats still do not show any sign of muscle damage, the velocity can be change constantly between 45 cm s^{-1} and 80 cm s^{-1}, oscillating between the two extremes. Both alternations need to be registered on the corresponding registration sheet if applied.

F The duration of the session depends on the rats individually. Once each rat (in its own time) is not able to continue running due to muscle damage it should be removed from the treadmill as quickly as possible.

Exercise Schedule

phase 0			phase I				phase III			Current	
t_0	$υ_0$	$Φ_{dec}$	$(t_a)_1$	a_1	υ	$Φ_{dec}$	$(t_υ)_3$	υ	$Φ_{dec}$	I_{min}	I_{max}
min	cm s^{-1}	º	min	cm s^{-1} min^{-1}	cm s^{-1}	º	min	cm s^{-1}	º	mA	mA
1	0	0	5	5	20-50	0-(15)	x	50	15	1.2	1.8

Guide

1 Register all settings, conditions, and initial parameters along with the Rat ID number for each cubicle.

2 Each rat needs to be weighed before the session.

3 Place the rats in the cubicles, register the time when the exercise starts, and the room temperature (T_i) and the relative humidity (RH_i).

4 Make notes on the running style of each rat during the session according protocol QGR/101.055.010.

5 Whilst performing according to the exercise schedule, do the following in phase I:

t	υ	$Φ_{dec}$	
[0 → 1] min:	0 cm s^{-1}	0º	← 1st minute, End of phase 0
[1 → 2] min:	[5 → 25 → 30] cm s^{-1}	0º	
[2 → 3] min:	[30 → 35 → 40] cm s^{-1}	0-5º	
[3 → 4] min:	[40 → 45] cm s^{-1}	5-10º	
[4 → 5] min:	[45 → 50] cm s^{-1}	10-15º	
[5 → x] min:	50 cm s^{-1}	15º	← 6th minute, End of phase I

6 After phase I and every 10 minutes during phase III, register the number of shocks (Number-S) for each rat. It is absolutely necessary to force the rats to run with every trick possible, until muscle damage is clearly visible (and if not that, until absolutely complete exhaustion).

7 When each rat (on its own time) cannot run anymore, quickly remove it from the treadmill, register the running time, the Distance (Dist.) and then the number of shock (Number-S).

8 When each rat has finished its session, it needs to be weighed again.

9 Reward the rats that performed accordingly several minutes after they have finished (or until they do not show sign of tiredness) with the special food reward whilst in their cages and link the reward with the sound of the specified bell.

10 Register how the rats reacted to the reward.

11 Register the room temperature (T_f) and the relative humidity (RH_f).

12 After 'the last rat standing/running' has finished, clean the treadmill.

13 Make sure all parameters necessary for the registration have been accounted for on the registration sheet and sign for the registration.

□

Sheet Start Date:

Sheet End Date:

Sheet Number:

Procedure: QGR/101.055.003

Parameters: The same as in protocol.

Prtc version: ________ I_i: ______ mA

Prtc Day: ________ u(Ph III): ______ cm s^{-1}

Session: ________ T_i: ______ ºC

Hour: ________ RH_i: ______ %

Lot No.: ________ T_f: ______ ºC

RH_f: ______ %

Signature:

General Observations:

(a) Time-S increasing, faeces stuck on the current board
(b) Time-S increasing, w/o an obvious reason
(c) Number-S increasing, w/o an obvious reason
(d) Number-S doesn't increase

Number-S

Phases		t, min	Rat ID	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run	Cubicle No.	Obs.	Run
Ph 0	No Run	1																
Ph I	Accel	6																
Ph III	Run	10																
		20																
		30																
		40																
		50																
		60																
		70																
		80																
		90																
		100																
Stop Time																		
Distance																		
Time-S																		

Observations:

Procedure:	QGR/101.051.002
Parameters:	

General Notes

A All sort of registration and observation must appear on this protocol's corresponding document, no. QGR/101.056.004

B This protocol describes a single intermittent hypoxia treatment session of 4 hours in a hypobaric chamber, simulating the altitude of 4000 m (462 torr), and should be performed once a day until sampling.

C The day count starts the day after the induced muscle damage (*protocol QGR/101.055.003*) with H1, then H2, H3, etc., only counting the days when the protocol is used.

D The change from normal pressure to 462 torr should done according to the Delta Pressure Plan. This plan takes 15 min, however the allowed range for the time duration is from 12 to 18 min, ie, 15 (±3) min.

E The duration of the session is 15 min + 4 h + 15 min = 4.5 h in total, although the 15-minutes can vary according to General Note D.

F Rat cages with control rats of the same lot should be placed on top of the hypobaric chamber whilst the session is ongoing.

Guide

1 Remove water bottle from the cage/cages and replace it with small bowl of water.

2 If more than one cage needs to be in the chamber, they each have their separate registration sheet.

3 Weigh the feed before the treatment and register.

4 Place the cage/cages inside the chamber.

5 The general operational procedure for a hypobaric session is described in protocol QGR/101.035.013, with the addition of the delta pressure plan for this particular protocol.

6 Follow the Delta Pressure Plan I to perform pressure changes that simulate 0 m to 4000 m.

7 Register the initial time/hour in the end time for this pressure plan.

8 When the pressure of 462 torr has been reached, the 4 h session can begin.

9 Remember to place the cage/cages with the control rats of the same lot on top of the chamber.

10 When 4 h of the treatment have passed, turn off the pump and follow the Delta Pressure Plan II to perform pressure changes that simulate 4000 m to 0 m. (Use the ventilator valve to affect the pressure change)

11 Register the initial time/hour in the end time for this pressure plan.

12 Meanwhile the process of the Delta Pressure Plan II is taking place, the cages on top of the chamber can be removed and the straps that hold the lid can be loosened.

13 It is important to remove the ventilator valve, the filtration tube and the sensor after each session.

14 Remove the cage/cages form the inside of the chamber and clean the rubber floor with alcohol.

15 Replace the water bowl with the water bottle and clean the bowl.

16 Reweigh the feed for the rats that were inside the chamber and register.

17 Make sure all parameters necessary for the registration have been accounted for on the registration sheet and sign for the registration.

The Delta Pressure Plans

Next page ...

The Delta Pressure Plans

The subsequent table describes two plans to follow when controlling the pressure increase or decrease, where the decrease reflects a simulation of an altitude change from 0 m to 4000 m and is called the Delta Pressure Plan I (ΔP_I); and the increase reflects a simulation of an altitude change from 4000 m to 0 m and is called Delta Pressure Plan II (ΔP_{II}).

Δt	ΔP_I	Δt	ΔP_{II}
[0 → 1] min:	[750-740 → 724] torr	[0 → 1] min:	[462 → 477] torr
[1 → 2] min:	[724 → 705] torr	[1 → 2] min:	[477 → 496] torr
[2 → 3] min:	[705 → 686] torr	[2 → 3] min:	[496 → 515] torr
[3 → 4] min:	[686 → 667] torr	[3 → 4] min:	[515 → 534] torr
[4 → 5] min:	[667 → 648] torr	[4 → 5] min:	[534 → 553] torr
[5 → 6] min:	[648 → 629] torr	[5 → 6] min:	[553 → 572] torr
[6 → 7] min:	[629 → 610] torr	[6 → 7] min:	[572 → 591] torr
[7 → 8] min:	[610 → 591] torr	[7 → 8] min:	[591 → 610] torr
[8 → 9] min:	[591 → 572] torr	[8 → 9] min:	[610 → 629] torr
[9 → 10] min:	[572 → 553] torr	[9 → 10] min:	[629 → 648] torr
[10 → 11] min:	[553 → 534] torr	[10 → 11] min:	[648 → 667] torr
[11 → 12] min:	[534 → 515] torr	[11 → 12] min:	[667 → 686] torr
[12 → 13] min:	[515 → 496] torr	[12 → 13] min:	[686 → 705] torr
[13 → 14] min:	[496 → 477] torr	[13 → 14] min:	[705 → 724] torr
[14 → 15] min:	[477 → 462] torr	[14 → 15] min:	[724 → 740-750] torr

Δt: The time frame in min

ΔP_I: The Delta Pressure Plan I in torr

ΔP_{II}: The Delta Pressure Plan II in torr

□

Doc. Title:	**Rat Intermittent Hypoxia Session**			Project:	DEP2010-22205-C02-01
Doc. No.:	QGR/**101.056.004**	Doc. Type:	Registration	Sector:	332(40)
Doc. Version:	1.1	Revision Date:	2012.05.30	DEV-1:	Ginés Viscor
Version Date:	2012.05.30	Revision by:	J G Ríos	DEV-2:	J G Ríos
Editor:	J G Ríos	Next Revision:	2012.06.08	Page:	1 of 1

Sheet Start Date: ______

Procedure: QGR/101.055.004 — Sheet End Date: ______

Parameters: The same as in protocol. — Sheet Number: ______

Prtc version: ______ $t_{hypoxia}$: ______ h $t(\Delta P_I)$: ______ **Signature:**

Prtc Day: ______ P_{goal}: ______ torr $t(P_{goal})$: ______

Working P-range

Lot No.: ______ $w_{feed,before}$: ______ g $t(\Delta P_{II})$: ______

ideal: P_{goal} ±3 torr

Cage: ______ $w_{feed,after}$: ______ g $t(P_{end})$: ______

accept.: P_{goal} ±10 torr

Rat IDs: ______

		Awake								Sleeping			Knocking			O_2 flux				Measurable parameters							
time	rat ID	awake	stressed	slightly stressed	calmer	clam	eating	grooming	sleeping	half-sleeping	same position	different posit.	good response	small response	no response	good response	small response	no response	other observation	P ≤ 453	453 > P < 459	465 > P < 473	P ≥ 473	other observ.	T, °C	RH, %	P, torr
ΔP_I: 0-15 min																											
P_{goal}: 0-0.5 h																											
P_{goal}: 0.5-1 h																											
P_{goal}: 1-1.5 h																											
P_{goal}: 1.5-2 h																											
P_{goal}: 2-2.5 h																											
P_{goal}: 2.5-3 h																											
P_{goal}: 3-3.5 h																											
P_{goal}: 3.5-4 h																											
ΔP_{II}: 0-15 min																											

Observations ______

Procedure: QGR/101.051.002

Parameters: d, day (d); a, acceleration (cm s^{-1} min^{-1}); t, time (min); υ, velocity (cm s^{-1}); I, current (mA); T, temperature (ºC).

General Notes

A All sort of registration and observation must appear on this protocol's corresponding document, no. QGR/101.056.005

B This protocol describes rehabilitation in the form of low impact concentric exercise, with an inclination, for a single session which should be applied immediately after intermittent hypoxia session (*protocol QGR/101.055.004*). If more than 30 minutes pass between the end of the hypoxia session and the beginning of this rehabilitation a deviation report needs to be written.

C The day count follows the schedule of the intermittent hypoxia sessions.

D The exercise schedule should be followed precisely where changes in the intensity level of the current are the only ones allowed. The initial intensity (I_i) should be the same as the last change applied ($I_{u,prev}$) in the previous session. Neither the initial intensity nor any other changes in the intensity during each session can be lower than I_{min} nor higher than I_{max} (with reference to the exercise schedule).

Exercise Schedule

phase 0			phase I				phase III			phase IV			Current	
t_0	$υ_0$	$Φ_{inc}$	$(t_a)_1$	a_1	$υ$	$Φ_{inc}$	$(t_υ)_3$	$υ$	$Φ_{inc}$	$(t_υ)_4$	$υ$	$Φ_{inc}$	I_{min}	I_{max}
min	cm s^{-1}	º	min	cm s^{-1} min^{-1}	cm s^{-1}	º	min	cm s^{-1}	º	min	cm s^{-1}	º	mA	mA
1	0	0	5	≈5	10-30	0-5	15	30	5	1	0	0	1.0	1.6

Guide

1 Register all settings, conditions, and initial parameters along with the Rat ID number for each cubicle.

2 Place the rats in the cubicles; register the time when the exercise starts.

3 Make notes on the running style of each rat during the session according protocol QGR/101.055.010.

4 Whilst performing according to the exercise schedule, do the following:

t	υ	$Φ_{inc}$	
[0 → 1] min:	0 cm s^{-1}	0º	← 1st minute, End of phase 0
[1 → 2] min:	[5 → 10 → 20] cm s^{-1}	0º	
[2 → 3] min:	[20 → 25] cm s^{-1}	0º	
[3 → 4] min:	25 cm s^{-1}	0-5º	
[4 → 5] min:	[25 → 30] cm s^{-1}	5º	
[5 → 6] min:	30 cm s^{-1}	5º	← 6th minute, End of phase I
[6 → 21] min:	30 cm s^{-1}	5º	← 21st minute, End of phase III
[21 → 22] min:	0 cm s^{-1}	0º	← 22nd minute, End of phase IV

5 After each phase I and every 5 minutes during phase III, register the number of shocks (Number-S) for every rat.

6 At the end of phase III (21st minute), after lowering the velocity and unplugging the motor, register the Distance (Dist.) and then the number of shock (Number-S).

7 After phase IV (22nd minute), press the red stop button on the treadmill monitor and register the number of shock (Number-S), the total time of shock (Time-S), the room temperature (T_f) and relative humidity (RH_f), and register the intensity level as it is left after the session ($I_{u,new}$).

8 Reward the rats that performed accordingly with the special food reward whilst in the treadmill cubicles and link the reward with the sound of the specified bell.

9 Register how the rats reacted to the reward.

10 Place each rat in its cage and clean the treadmill.

11 Make sure all parameters necessary for the registration have been accounted for on the registration sheet and sign for the registration.

□

Doc. Title:	**Rat Rehabilitation on a 5-Channel Treadmill**			Project:	DEP2010-22205-C02-01
Doc. No.:	QGR/**101.056.005**	Doc. Type:	Registration	Sector:	333(41)
Doc. Version:	1.1	Revision Date:	2012.05.30	DEV-1:	Ginés Viscor
Version Date:	2012.05.30	Revision by:	J G Ríos	DEV-2:	J G Ríos
Editor:	J G Ríos	Next Revision:	2012.06.08	Page:	1 of 1

	Sheet Start Date:	
Procedure: QGR/101.055.005	Sheet End Date:	
Parameters: The same as in protocol.	Sheet Number:	

Prtc version: ________ $I_{u,prev}$: ______ mA I_i: ______ mA **Signature:**

Prtc Day: ________ $I_{u,new}$: ______ mA u(Ph III): ______ cm s^{-1}

Session: ________ T_i: ______ ºC

Hour: ________ RH_i: ______ %

Lot No.: ________ T_f: ______ ºC

RH_f: ______ %

General Observations:

(a) Time-S increasing, faeces stuck on the current board
(b) Time-S increasing, w/o an obvious reason
(c) Number-S increasing, w/o an obvious reason
(d) Number-S doesn't increase

Number-S

Phases		t, min	Rat ID	Cubicle No.			Cubicle No.			Cubicle No.			Cubicle No.			Cubicle No.		
					Obs.	Run		Obs.	Run		Obs.	Run		Obs.	Run		Obs.	Run
Ph 0	No Run	1																
Ph I	Accel	6																
Ph III	Run	11																
		16																
		21																
	Distance	21																
Ph IV	Rest	22																
	Time-S	22																

Observations: