30 Sessions of Intermittent Hypoxic Training causes Lymphocytosis in Wistar Rats

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ABSTRACT

The hypoxia, which occurs in high altitude, promotes adaptive changes which can be studied by use of intermittent hypoxic training (IHT). The IHT is also used to treatment of several diseases and in sports. Therefore, the objective of this study was to analyze the effect of 30 sessions normobaric intermittent hypoxic training on hematologic parameters in Wistar rats. Ten rats were submitted to IHT and ten to normoxia (N) for 30 days (15 minutes hypoxia, 14-11% O_2, 5 minutes re-oxygenation). Control group was exposed to 21% O_2. On day 31 the blood was sampled. The variables hematological that showed significative changes were total leukocytes and lymphocytes, increasing in 47,8% and 22,5%, respectively, in IHT group when compared to N group. The protein electrophoresis on cellulose acetate results no change among groups IHT and N. These results suggest that IHT stimulated immunity in Wistar rats and more studies are necessaries to clarify if IHT could be used for immunotherapy.

*Keyword*s: Hematopoiesis, White cells, Blood, Immunology

RESUMO

A hipóxia, que ocorre em alta altitude, promove mudanças adaptativas que podem ser estudadas com o uso de treinamento hipóxico intermitente (THI). O THI também é usado para o tratamento de várias doenças e nos esportes. Em vista disso, o objetivo deste estudo foi analisar o efeito de 30 sessões de treinamento hipóxico intermitente normobárico em parâmetros hematológicos de ratos Wistar. Dez ratos foram submetidos ao THI e dez à normóxia (N) por 30 dias (15 minutos de hipóxia, 14-11% O_2, 5 minutos re-oxigenação). O grupo controle foi exposto a 21% de O_2. No dia 31 o sangue foi coletado. As variáveis hematológicas que mostraram mudanças significativas foram leucócitos totais e linfócitos, aumentando em 47,8% e 22,5%, respectivamente, no grupo IHT quando comparado ao grupo N. Os resultados da eletroforese de proteínas em acetato de celulose não mostraram nenhuma mudança entre os grupos THI e N. Estes resultados sugerem que THI estimula a imunidade em ratos Wistar e mais estudos são necessários para esclarecer se THI poderia ser usado para a imunoterapia.

Palavras chave: Hematopoiese, Células Brancas, Sangue, Imunologia

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INTRODUCTION

Nowadays, it is well known that high altitude exposure (hypobaric hypoxia) induces changes in erythropoiesis mainly characterized by increase in red blood cells and haemoglobin concentration (Humberstone-Gough *et al.*, 2013; Serebrovskaya *et al.*, 2011; Hochachka, Gunga & Kirch , 1998). These changes are criteria for high altitude acclimatization since they partially restore normal blood oxygen (Schmidt & Prommer, 2010; Ward, Milledge & West, 1995) also being one contributor to increased work capacity at altitude (Hortsman, Weiskopf & Jackson, 1980).

To simulate high altitude we can use the intermittent hypoxic training (IHT) that consists of breath with low concentration of oxygen intercalated with periods of normal breath (21% O_2) (Bernardi *et al.,* 2001). This method comprises two forms: normobarotherapy, which relies on inhalation of air with low O_2 concentration under standard conditions, i.e. at the sea level (Wilber, 2001) and the hipobarotherapy (inhaled air with low O_2 concentrations, but under low pressure, as found by mountain and climbers) (Sirotinin, 1964). The IHT is used for acclimatization to the altitudes (Serebrovskaya, 2002) and is becoming a popular modality for clinical medicine and athletic training (Serebrovskaya *et al.*, 2011; Serebrovskaya *et al.*, 2008; Rybnikova *et al.*, 2008; Hamlin & Hellemans 2007; Serebrovskaya *et al.*, 2003).

The few studies found in literature on normobaric IHT on the blood white cells profile were carried out on humans. Considering the lack of studies evaluating the effect of normobaric intermittent hypoxia training on hematological parameters in animals, this study aimed evaluate the effect of 30 sessions of normobaric IHT on the blood profile of Wistar rats.

MATERIALS AND METHODS

Animals and IHT conditioning

Male Wistar rats (236±34g, n=8) were maintained under controlled temperature (21–23°C) at 12/12h light-dark photoperiod with lights on at 07:00 a.m. All experimental protocols were conducted in accordance with guidelines of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Research of the Federal University of Santa Maria (Protocol-55/2009. Process: 23081.009917/2009-69). Rats were placed to the intermittent hypoxic training in a custom-made 4-room acrylic chamber in which the atmosphere could be adjusted quickly and precisely using compressed gas (GO$_2$ Altitude Hypocator equipment). Rats

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were randomly divided into two groups that were submitted to IHT or normoxia (N) sessions. The IHT program was administered for 30 days and consisted of 15 minutes of hypoxic exposure with 5 minutes re-oxygenation and a total duration of two consecutive hours per day. The O_2 concentrations varied among 14% (week 1), 13% (week 2), 12% (week 3) and 11% (week 4). The normoxia group was subjected to the same chamber but with normal sea-level O_2 concentrations.

Hematologic analysis

The parameters analyzed were: red cell volume (RCV), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), total protein plasma (TPP), total leukocytes (TL), segmented neutrophyls, lymphocytes, eosinophils and monocytes. The counting of RCV, TL and the quantification of Hb were made in automatic accountant Mindray BC 2800 Vet®. The hematocrit determination was obtained in micro-hematocrit centrifugal machine with 19720G rotation. The MCV and MCHC were made by indirect calculations. The differential counting of leukocytes was realized in slide sanguineous by Panótico Rápido® using light microscopy.

Electrophoresis protocol

The total serum protein were determined by the biuret method, using commercial reagent Labtest® and the analysis was performed in semi-automatic spectrophotometer BioPlus-Bio-2000®, according to the instructions of the manufacturer. The fractionation of serum proteins was determined by electrophoresis on cellulose acetate strips celugell Labex®. The samples were layered on the strip and, after closing the horizontal plane, it was applied a constant voltage of 180 volts for 25 min. After the electrophoretic running, the strips were colored with Ponceau colorant for 15 min. The strips discoloration was carried out with 5% discoloring acetic acid, until samples became completely clear. After discoloration, strips were divided into methanol for 30 s to fix the material. Finally, the strips were divided into clearing solution for 1 min. Strips were dried at 60ºC for 15 min. The reading of bands was performed using the computer program Denscan® by scanning the strip.

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Statistical analysis

The Shapiro-Wilk test was carried out for normality. After finding that the values did not follow a normal curve, the nonparametric tests Mann-Whitney and Kruskal Wallis were performed. The Dunn test was used to evaluate the significances. The level of significance used for all tests was 5%. Statistica 7, SAS 9.1 and Bioestat 5. were the software used.

RESULTS

The hematological variables RCV, Hb, Ht, MCV, MCHC, TPP, segmented neutrophils, eosinophils and monocytes were not significantly different when comparing hypoxia with normoxia (Table 1). The total leukocytes and lymphocytes increased significantly (p<0.05) in animals submitted to the normobaric IHT (47,8% and 22,5%, respectively) compared with those in the normoxia group. The electrophoretic plasma analysis of animals subjected to hypoxia or normoxia showed no change in the pattern of plasma proteins (Table 2).

Table 1: Effects of intermittent hypoxic training on hematological parameters in Wistar rats

Variable	Normoxia	Hypoxia
RCV (x10^{6})	7.3±0.3[a]	7.2±0.6[a]
Hb (g/dL)	12.97±0.5[a]	13.1±0.9[a]
Ht (%)	45.1±2.7[a]	47.0±1.4[a]
MCV (fL)	63.4±2.6[a]	62.6±1.8[a]
MCHC (%)	30.5±0.6[a]	30.5±0.8[a]
TPP (g/dL)	8.1±0.4[a]	7.8±0.2[a]
Total Leukocytes (uL)	8.357±2631[a]	12.356±1887[b]
Seg. Neutrop. (uL)	2404±352[a]	2.532±598[a]
Lymphocytes (uL)	8212±2455[a]	10060±1257[b]
Eosinophils (uL)	7.6±20[a]	58±107[a]
Monocytes (uL)	92.8±64.7[a]	99.8±111[a]

Hematological parameters of Wistar rats exposed to 30 sessions of normoxia or hypoxia.
Values represent the mean ± SEM (p<0.05). Equal letters did not differ statistically.

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Table 2: Effects of intermittent hypoxic training on electrophoretic analysis in Wistar rats

Variable	Normoxia	Hypoxia
total protein (g/dL)	6.09±0.31[a]	5.68±0.19[a]
Albumin (g/dL)	3.66±0.48[a]	3.45±0.17[a]
Alfaglobulins (g/dL)	0.72±0.14[a]	0.67±0.07[a]
Betaglobulins (g/dL)	0.88±0.12[a]	0.79±0.16[a]
Gamaglobulins (g/dL)	0.84±0.19[a]	0.78±0.06[a]
A:G Ratio	1.54±0.34[a]	1.55±0.14[a]

Serum protein determined by electrophoresis on cellulose acetate strips celugell Labex®. Values represent the mean ± SEM ($p < 0.05$). Equal letters did not differ statistically.

DISCUSSION

The significant increase in total leukocytes and lymphocytes in rats submitted to normobaric IHT has never been described in the literature. The 30 days program of normobaric cyclic, 15 minutes exposures to 14-11% O_2 with 5 minutes re-oxygenation and a total duration of two consecutive hours per day, increased in 47,8% total leukocytes and 22,5% lymphocytes numbers, respectively, when compared with normoxia. To further investigate these immunological responses we performed an electrophoretic analysis and the results showed no difference among groups (Table 2). The electrophoresis is a separation technique of plasmatic proteins based on the migration of charged molecules in a solution, depending on the application of an electric field. We assess the albumin and globulin (alpha, beta and gamma) fractions. Decreased albumin concentration is observed in situations that promote your loss, low or high protein intake catabolism. The fractions alpha globulins exhibit increased levels in inflammatory, infectious and immune diseases. The increase in beta globulin is observed in cases of disturbance of lipid metabolism or iron deficiency anemia. The absence or reduction of gamma band indicates congenital or acquired immunodeficiencies. Its increase suggests elevation of polyclonal immunoglobulins associated with inflammatory conditions, infectious or neoplastic (McPherson, 1999). However, in our study there was not change in these parameters which suggests that lymphocytosis promoted by hypoxia is not related to the lineage of B cells, which give rise to the fraction of gamma globulins.

The change in total leukocytes and lymphocytes was recently observed in study with humans (Serebrovskaya *et al*., 2011), which showed that the use of acute IHT (1 session) increases transiently, leukocytes and lymphocytes, but the effect lasts for only 30 min post-IHT. However, when the normobaric IHT was administered for 14 days, there was no change in leukocyte and

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lymphocyte counts 24 hours after the program, though lymphocytes have increased by one week later.

It can be suggested that the increase of total leukocytes and lymphocytes in our study are related to the fact that hypoxia is known by directly affects various cellular processes through effects on the gene expression which is performed by the hypoxia-inducible factor (HIF) (Bunn & Poyton 1996; Semenza, 2000). Under hypoxic conditions, the HIF-1α decomposition is inhibited, leading to nuclear accumulation of protein, which is associate with HIF-1β and it binds to the hypoxia responsive elements (HRE) (Fukuda *et al*., 2007; Semenza, 2003). However, HIF is also activated via nuclear factor κB (NF-κB) a transcription factor also involved in controlling the expression of several genes related to inflammatory response, mainly in lymphoid response (Jiang *et al*., 2010). Transcription factors activated by HIF-1 and NF-kB have been identified as a critical component of the cellular and systemic response to hypoxic stress. HIF1 activity is generally associated with hypoxia, and NF-kB is associated with reoxygenation–reperfusion (Witt, Mark & Huber*,* 2005). Conversely, upon activation by inflammatory signals or pro-oxidant stresses, as in the case of IHT being a light stressor, NF-kB activates genes particularly involved in the inflammatory response, as well as modulating the cellular response to oxidative injury. In addition, HIF1 and NF-kB activation leads to up-regulation of many genes involved in the recruitment, adhesion and activation of leucocytes (Witt, Mark & Huber, 2005). Thus, the effects through various cellular processes mediated by HIF-1 and NF-kB activation could explain our results on increase of leukocytes and lymphocytes.

CONCLUSION

Our results showed that IHT caused immune response, which is not related to the stimulation of B lymphocytes, as results of electrophoresis. Additional studies should be performed to verify the duration of lymphocytosis in rats after IHT sessions, as well as the viability of these cells and to clarify the origin of leukocytosis and lymphocytosis. New finding could be help to suggest the potential use of IHT for immunotherapy.

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