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The Effect of Intermittent Hypoxia on Bodyweight, Serum Glucose and Cholesterol in Obesity Mice

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Abstract: This article tests mice's indicators of body nutritional metabolism under tolerable hypoxic conditions, in order to explore the effects of moderate intermittent hypoxia on the bodyweight, blood sugar and blood cholesterol of obese mice and to identify the role of leptin in these effects; this study applies high-fat diet to establish Mice Obesity Models and observes the intervention effects of intermittent hypoxic training in this Model. Small healthy mice are classified in 4 groups at random, that is, Group A (Normal), Group B (Normal Hypoxia) fed with normal foods and undergoing Intermittent Hypoxic Training (IHT), Group C (Fatty-diet) fed with High-Fat and High-Sugar (HFHS) foods without IHT and Group D (Fatty-diet and Hypoxia) fed with HFHS foods with IHT. After 40 days of feeding and hypoxic training, weigh the mice, measure the levels of blood sugar and blood cholesterol with a full automatic biochemical analyzer, measure serum leptin concentration by enzyme-linked immunosorbent assay (ELISA) technique, inspect liver leptin receptor expression and liver fat slice by immunohistochemistry. It is found that compared to control group, after experiment, the average bodyweight, blood sugar, blood cholesterol and serum leptin concentration in Group C is increased significantly and numerous fat cells are distributed in the liver, which indicates that hyperlipemia model has been successfully established; after intermittent hypoxic training, the average bodyweight, blood sugar, blood cholesterol and liver fat cells distribution density and scope in Group B and D are lower than those in Group A and C, while serum leptin concentration is increased significantly; liver leptin receptor expression in Group D is higher than that in Group C. And hypoxia groups have no trauma conclusion. Moderate intermittent hypoxia can reduce bodyweight by increasing leptin concentration and enhancing liver leptin expression and it can also reduce the level of blood sugar and blood cholesterol and meanwhile prevent steatosis in liver cells effectively.

Key words: Hypoxia, serum glucose, cholesterol, leptin, leptin receptor, mice

INTRODUCTION

It is noted that average bodyweight of plateau inhabitants is lighter than plain inhabitants whose bodyweight will also reduce after removal to plateau, indicating the connection with hypoxic conditions on plateau. But in general, long time exposure to high altitude, low pressure and hypoxia will cause adverse reactions to human brains, lungs, digestive system and circulatory system (Westerterp-Plantenga *et al*., 1999; Bartsch, 1999). In case that a moderate artificial hypoxic environment could be created to promote nutritional metabolism and avoid trauma by longtime exposure to hypoxia, it would offer new possibility of prevention and cure of metabolic diseases. This study adopting IHT is directly based on this idea. IHT is to expose body to a certain extent of hypoxic stimulation in a discontinuous short time in simulated plateau hypoxic environment and most of the time the body is in normal oxygen environment, so as to result in healthy effects after repeated exposure to hypoxic stimulations. Is IHT as efficacious as the plateau effect in respect to weight loss as well as prevention and cure of hyperlipemia? If so, what is the mechanism of its function?

Leptin, as a kind of secretory protein comprising of 167 amino acids, is coded by human obesity (OB) gene (located on human chromosome 7q32) (Friedman and Halaas, 1998; Zhang *et al*., 1994). In previous studies, leptin was proven to be a kind of peptide hormone regulating food intake and energy metabolism (Morton *et al*., 2006; Scot, 1996), it is mainly composed and secreted by white adipose tissue, signaling energy

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metabolism in human body and acts on the metabolic control center of hypothalamus to control lipidosis by inhibiting food intake, reducing energy intake, increasing energy consumption and inhibiting fat composition (Klein *et al*., 2000). Leptin acts by the medium of its receptor. Leptin receptors are distributed on the surface of choroid, hypothalamus, as well as liver, kidneys, heart, adipose tissues and pancreatic islet cells. Receptor achieves this through the Janus tyrosine kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Huo *et al*., 2004). At present, studies on the metabolism promoting effect of leptin is mainly concentrated in its receptors distributed on the center, reporting less on the effects of receptors distributed on the periphery. Since its receptors are widely distributed on peripheral tissues, peripheral effects must exist.

This study explores the connection of obesity and hyperlipemia to leptin and its receptor expression by high-fat diet induced obese mice; observe the IHT effects on the bodyweight, blood sugar and blood cholesterol of obese mice.

MATERIALS AND METHODS

Animal subjects: Eighty healthy, female (female mice react more sensitively to leptin and have high serum leptin concentration then male mice to be more easily measured) Kunming mice, 14-15 g in weight (purchased from Department of Laboratory Animal Science, Central South University).

Establishment and basis of hypoxic environment

Principles: Have a vacuum hypoxic box made up of steel plates and organic glass, connected to vacuum pump, pressure gauge and air inlet valve, then open the vacuum pump and adjust the air inlet valve, to maintain the air pressure within the box at necessary horizontal line and meanwhile to maintain the air circulating in and out of the box, so as to establish moderate constant hypoxic experimental environment.

Methods: It is measured that the partial pressure of oxygen (PaO_2) on plateau 3,000 m above sea level is about 70% of that at sea level and in general, 3,000 m above sea level is medically deemed plateau. The atmospheric pressure on plain is 0.1 MPa, of which, the PaO_2 accounts for about 20%, that is, 0.02 MPa. And it is observed by pre-experiment that mice behave normally in food, water intake and other daily activities under 7/10 atmospheric pressure, without any reactions to trauma. Therefore, the atmospheric pressure in the vacuum hypoxic box shall be adjusted to 0.07 MPa.

Animal models preparation: Mice are classified into 4 groups at random by drawing of lots, that is, Group A (Control) fed with normal foods, Group B (Hypoxia) fed with normal foods with IHT (undergoing 8 hypoxic trainings per day in the above-mentioned device, each of which lasts 15 min at 5 min interval); Group C (Fatty-diet) fed with HFHS foods (10% lard, 10% eggs, 5% white sugar and 75% base feedstuff) without IHT; Group D (Fatty-diet and Hypoxia) fed with high sugar foods with IHT. Feed two times at morning and evening every day (day and night ratio = 12:12) and ensure each group of small mice take unrestricted diet. Weigh every 10 days.

Specimen collecting and processing: Feed for 40 days and then water limitlessly and fast for 12 h, then inject 10% chloral hydrate intraperitoneally after anesthesia, then behead and sample blood, separate serum by 2000 r/min centrifuge 5 min and test. Select 10 mice from each group at random, perfuse 4% paraformaldehyde in left ventricle, then extract liver and secure it in paraformaldehyde promptly, dewater conventionally, wax and embed, keep dry for timely use. At the same time, extract brain and lung tissues to observe trauma conditions.

Testing process: Measure blood sugar and blood cholesterol levels with a full automatic biochemical analyzer by oxydasis of glucose and enzyme shade selection separately.

Measure serum leptin by ELISA technique, with reagent kits purchased from Wuhan Boster Bioengineering Company Limited.

Detect leptin receptor OB2Rb expression immunohistochemically in mice's liver tissues, with reagent kits purchased from Wuhan Boster Bioengineering Company Limited and make semi-quantitative analysis on immunohistochemical images by image analysis system (from Chengdu Technology and Market Company Limited): take mice liver images under 5 visual fields (coverage: 112 mm^2) and measure average optical density values of all immunohistochemical positive signals in the images.

Stain with Sudan III, observe mice's liver fat cells and compare their distribution and density. Degrees of liver steatosis: according to Sudan III stained specimen, under optical microscope, steatosis coverage in liver cells is classified in 4 degrees, that is, Normal Liver with steatosis coverage <10%; Degree 1 (Slight Fatty Liver) with steatosis coverage 10-33%; Degree 2 (Mild Fatty Liver) with steatosis coverage 34-50%; Degree 3 (Moderate Fatty Liver) with steatosis coverage 51-66%; Degree 4 (Heavy Fatty Liver) with steatosis coverage above 66% (Brunt *et al*., 1999).

Statistical analysis: Express experimental data in mean±SD (x±s), apply SPSS software to analyze the results, where intergroup comparison applies variance analysis and pair comparison applies pairing t-test.

RESULTS

General conditions of animals after hypoxia: Mice in Hypoxia Group act agilely, with normal breath and body temperature, eat and drink actively, without gasp, dullness and fever, etc.

Effects of intermittent hypoxia on bodyweight of mice: Before experiment, average bodyweight of any group of mice approximates to each other, with less difference than 0.05 g. After experiment, the average bodyweight of each group increases, where that of Group C is the heaviest, representing the highest growth rate compared to that before experiment; bodyweight of Group A has insignificant difference with that of Group B and the two groups have approximate growth rates compared to those before experiment; bodyweight of Group D is the lightest, representing the lowest growth rate (Table 1).

Effects of intermittent hypoxia on blood sugar of mice: After experiment, the blood sugar concentration in Group C is the highest, reaching 12.61 mmol L^{-1}; that in Group D is 11.32 mmol L^{-1}, lower than that in Group C ($p<0.05$); that in Group A and B is even lower than that in former two groups, where, that in Group B is slightly lower than that in Group A (Table 2).

Effects of intermittent hypoxia on blood cholesterol concentration in mice: Blood cholesterol concentration in Group C is the highest, reaching 3.04 mmol L^{-1}; that in Group D is 2.76 mmol L^{-1}, lower than that in Group C ($p<0.05$); that in Group A and B is even lower than in former two groups, where that in Group A is slightly lower than that in Group B (Table 3).

Effect of intermittent hypoxia on the serum leptin concentration: IHT and high-fat diet will increase serum leptin concentration, showing statistical difference from Control Group. And Fatty-diet and Hypoxia Group has higher serum concentration than purely Fatty-diet Group (Table 4).

Effects of intermittent hypoxia on the expression of liver leptin receptor: It is indicated in immunohistochemistry that normal liver of small mice has a few expression of leptin reception, purely hypoxic training has no significant impact on the expression of liver leptin receptor, while high-fat diet can reduce it, but high-fat diet with hypoxic training will increase leptin receptor (Fig. 1, Table 5).

Effects of intermittent hypoxia on liver fat distribution: Take slices of liver tissues of small white mice, stain them with Sudan III, then observe it under microscope, thus tangerine round droplets are formed in cytoplasm, which are called lipid droplets in liver. Compare fat cells distribution in four groups of pictures (Fig. 2).

Group A (Normal) has insignificant fat cells distribution, where 9 cases have normal livers (steatosis

Table 1: Effect of intermittent moderate hypoxia on bodyweight of mice

Bodyweight	Control (A)	Hypoxia (B)	Fatty-diet (C)	Fatty-diet+Hypoxia (D)
Before (g)	14.38±1.59	14.43±1.76	14.35±1.76	14.32±1.88
After (g)	25.30±3.22	25.84±1.78	28.06±2.96	23.29±5.14
Increase rate (%)	78.03±22.40	79.07±12.38	95.54±20.65	62.64±35.92*

(x±s, n = 20; *p<0.05 vs C)

Table 2: Effect of intermittent moderate hypoxia on serum sugar of mice

Blood sugar	Control (A)	Hypoxia (B)	Fatty-diet (C)	Fatty-diet+Hypoxia (D)
Mean (mmol L^{-1})	10.82±1.45	10.03±1.79	12.61±1.78	11.32±1.97*

(x±s, n = 20; *p<0.05 vs C)

Table 3: Effect of intermittent moderate hypoxia on serum cholesterol of mice

Blood cholesterol	Control (A)	Hypoxia (B)	Fatty-diet (C)	Fatty-diet+Hypoxia (D)
Mean (mmol L^{-1})	2.16±0.36	2.61±0.65	3.04±0.67	2.76±0.27*

(x±s, n = 20; *p<0.05 vs C)

Table 4: Effect of intermittent moderate hypoxia on concentration of leptin in mice blood serum

Leptin	Control (A)	Hypoxia (B)	Fatty-diet (C)	Fatty-diet+Hypoxia (D)
Mean (ng mL^{-1})	42.938±11.0142	88.563±14.7672*	67.540±23.2091*	43.700±11.2684$^{\#}$

(x±s, n = 20; *p<0.05 vs A; $^{\#}$p<0.05 C)

Table 5: Mean optical density analysis of leptin receptor immunopositive signals in mice liver

Density	Control (A)	Hypoxia (B)	Fatty-diet (C)	Fatty-diet+Hypoxia (D)
Mean (×100)	153.31±22.7	154.27±21.5	123.57±9.91*	178.83±9.79$^{\#}$

(x±s, n = 20; *p<0.05 vs A; $^{\#}$p<0.05 C)

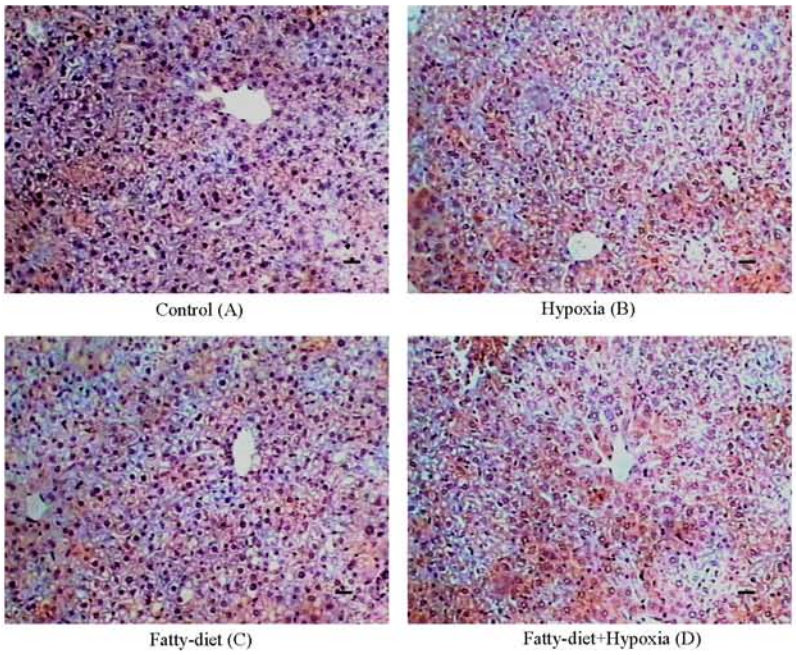

Fig. 1: Effect of intermittent moderate hypoxia on the level of leptin receptor immunoreactivity in mice liver (x50, bar = 100 μm). The result showed that there existed the fewest expression of leptin receptor in the liver of Group C and the most expression of leptin receptor in the liver of Group D

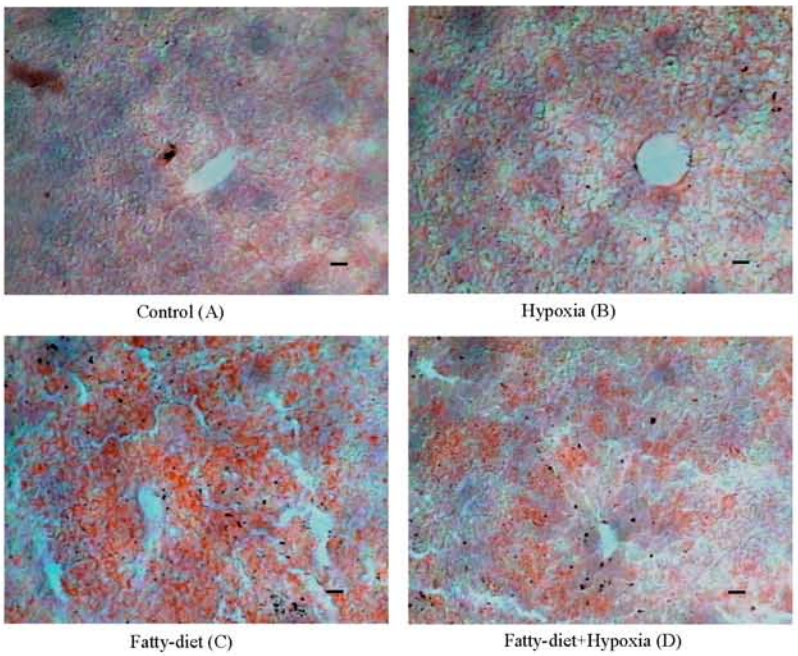

Fig. 2: Tissue section of mouse liver (x50, bar = 100 μm, dyeing with SudanIII). Red-orange circular droplets in cytoplasm were found under light microscope, which were the hepatic lipid droplets. There were a small amount of fat cells was distributed in Group A. In Group B, the distribution of fat cells was the sparsest. The distribution of a large number of fat cells was shown around the central vein in Group C. Compared with Group C, the scope and density of the distributed fat cells were decreased in Group D

coverage <10%), while one case has slight fatty liver (steatosis coverage: 10-33%).

Group B (Hypoxia) has the thinnest fat cells distribution, the lightest color and the least density and scope among the four groups of pictures. Total 10 cases have normal livers (liver steatosis coverage <10%).

Group C (Fatty-diet) shows significant fat cells distribution around central veins, in high density and dispersed distribution, where liver cells are scattered in rufous lipid droplets with relatively even sizes. One case has slight fatty liver (steatosis coverage: 10-33%); 7 cases have medium fatty livers (steatosis coverage: 51-66%) and 2 cases have heavy fatty livers with 66% or more liver cells suffering steatosis.

Group D (Fatty-diet and Hypoxia) has lower density and scope of liver cells distribution than Group C. Two cases have normal livers (steatosis coverage <10%); 5 cases have slight fatty livers (steatosis coverage: 10-33%); 3 cases have medium fatty livers (steatosis coverage: 51-66%).

Effect of intermittent hypoxia on liver, brain and lung in mice: Take respective slices of the liver, lung and brain tissues of mice, stain them with HE and observe, there is no significant difference between hypoxia groups and non-hypoxia groups and hypoxia groups do not conclude any trauma.

DISCUSSION

In this experiment, for obese mice induced by HFHS diet, it can be observed under optical microscope that their livers have suffered steatosis and there are lipid droplets in liver cell cytoplasm. After 40 days IHT to small mice, it is found that intermittent hypoxia can inhibit the increase of bodyweight, blood sugar and blood cholesterol and can also reduce the liver steatosis effectively and in particular has significant effects on small mice fed with HFHS foods. Its production mechanism may be related to the ability of intermittent hypoxia in promoting serum leptin concentration and the reactivity of liver leptin receptor.

In regard to thermal energy metabolism, in hypoxic conditions, consumption of energy increases significantly, water metabolism falls into disorder and the absorptive function of gastrointestinal tracts become weak, thus bodyweight falls. About 93% energy of a healthy body comes from aerobic metabolism and this proportion can fall to 84% in case of oxygen undersupply or lower than normal oxygen partial pressure, thus the utilization of capacity reduces. But IHT can increase the level of RBC2, 3-DPG (2, 3-diphosphatidylglyceric acid) level, increase the ability of blood in releasing oxygen to tissues, thus increasing the ability of aerobic metabolism. Under hypoxia, muscle cell mitochondrial enzymatic activity increases (Takahashi *et al*., 1996), glycolysis increases, fatty acid oxidation increases and cholesterol synthesis reduces. It is also demonstrated by studies that urgent hypoxia impairs the function of mitochondrial oxidative phosphorylation. ATP production decreases (Minezaki *et al*., 1994). Meanwhile, there are some evidences show that hypoxia can make glucose transporter on adipose cell increase selectively (Wood *et al*., 2007), such as glucose transport 1, 3 on arthrodial cartilage cells of mice (Ren *et al*., 2007). Therefore, intermittent hypoxia can reduce the level of bodyweight, blood sugar and blood cholesterol effectively.

It is indicated by studies that in urgent hypoxic conditions, human serum leptin level increases. On the other hand (Grosfeld *et al*., 2002; Meissner *et al*., 2005) studies suggest that hypoxia can increase the expression of hypoxia inducible factor-1 (HIF-1) in body, while leptin is a kind of hypoxia induced gene product. In this experiment, the changes of serum leptin level of small mice in hypoxia groups reflects a tendency of changes consistent with foregoing conclusions, thus demonstrates intermittent hypoxia can increase serum leptin concentration under certain circumstances.

Leptin cannot function until bind to its receptor. It is indicated by lots of studies that the majority of obese patients has increased plasma leptin concentration, but with leptin resistance phenomenon (Munzberg *et al*., 2005). This phenomenon is caused by the saturation of transport of leptin across blood-brain barrier and other reasons (Cinaz *et al*., 2005), where the decrease of leptin receptors facilitates the saturation of transport of leptin. Therefore, the compensatory decrease of expression of leptin receptor in the bodies of obese small mice is an important mechanism of leptin resistance of obese small mice. Even if the leptin level in body increases, it cannot exert its biological function. It is indicated by this study that the bodyweight and its growth of Fatty-diet and Hypoxia Group is significantly lower than that of Fatty-diet Group, where not only serum leptin concentration of Fatty-diet and Hypoxia Group is significantly higher than that of Fatty-diet Group, but also expression of liver leptin receptor of Fatty-diet and Hypoxia Group is significantly enhanced, indicating that intermittent hypoxia can enhance leptin receptor expression, enhance the bind of leptin to leptin receptor and thus reduce bodyweight.

Hyperleptinemia causes hyperinsulinism, when insulin level increases, fat cells take in more glucose and lipid, thus become mast fat cells (Tobe *et al*., 2001). Since

the signal transduction between insulin and leptin has a cross-pathway (Szanto and Kahn, 2000), insulin resistance results that peripheral fat mobilization enhances, serum dissociative fatty acids increase, liver takes in more; meanwhile, fatty acids in liver has less oxidation, esterification forms more triglycerides, liver cells have impaired ability of fat transport from liver, fats deposit in liver cells, which forms fatty liver. Leptin resistance interacts with insulin resistance, forming a vicious circle, together with facilitated liver steatosis of obese big mice. Meanwhile, leptin may directly resist steatosis from periphery (Unger, 2002). Leptin can activate AMP-activated protein kinase (adenosine monophosphate-activated protein kinase) to reduce lipidosis (Minokoshi *et al*., 2002) and also specifically inhibit liver stearoyl-CoA desaturase-1, thus reducing liver VLDL (very low density lipoprotein) production and lipidosis (Cohen *et al*., 2002). Therefore, the fact Fatty-diet and Hypoxia Group has significantly lower liver steatosis than Fatty-diet Group is probably collective result of obesity, leptin, leptin resistance and other factors.

Obesity is a disease due to imbalance of appetite and energy, which is related to genetics, environment dietary structure and other factors. Following the people's increasing living standards, the proportion of population with obesity is increasing year by year, thus obese people have higher risk of suffering from other severe diseases, including diabetes, shock, heart diseases or even cancer. At present, obesity has become the main killer that endangers human health, in place of diseases resulting from malnutrition and infection; although all countries of the world are positively seeking the treatment and efficacious medicine for losing fat, till now, the efforts are not satisfactorily fruitful. The experiment in this article proves that IHT has significant effectiveness for losing weight, reducing blood sugar and blood cholesterol and preventing liver steatosis and makes preliminary exploration to its mechanism and action means. In the hope of offering a new field for preventing and treating obesity, hyperlipemia and other diseases. Meanwhile, it is indicated that intermittent hypoxia causes no significant trauma to normal body, so it is promising to use it as a measure for healthcare or even treatment. But further studies are still wanted for making the mechanism clear, how intermittent hypoxia works.

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