DEVELOPMENT OF EXPERIMENTAL DEVICES AND METHODS TO STUDY HYPOXIA AND ITS EFFECTS ON SPORT

Abstract of PhD Thesis

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Introduction

The causes of death from blood supply disorders are studied in a wide range of cellular, tissue and animal models due to their high occurrence. Indeed, during 2016, more than 1.8 million people in the European Union died of a heart attack or due to ischemic brain injury. Ischemia means reduced or lack of blood supply to the tissues that occurs for some reason. Even after a few minutes it can cause permanent damage in the case of highly aerobic tissues, like the brain or the heart. Reperfusion may further increase the extent of injury after the ischemic condition has resolved. The severity of the damage can be reduced by cooling the cells below body temperature, as this will significantly slow down the metabolic processes.

Oxygen-glucose deprivation (OGD) is a widely used *in vitro* method suitable for cellular ischemia and hypoxia assays. This model is widely used at body temperature, while the effects of cold on ischemic tissues represent a less explored scientific area. In addition to *in vitro* models, *in vivo* animal experiments also investigate the effects of ischemia and the hypoxic environment on the body. One of the most commonly used procedures is the Middle Cerebral Artery Occlusion (MCAO) in rats and mice.

However, there are non-invasive experimental settings that are also suitable for studying brain exposure to hypoxia. Some reports suggest that sleep apnea is one of the major independent risk factors for cardiovascular diseases and stroke. Obstructive Sleep Apnea (OSA) is a chronic, widely underdiagnosed condition characterized by intermittent hypoxia due to the interruption of sleep phases and shortness of breath. To better understand the relationship between sleep apnea and various clinical conditions, an animal model is needed. This should be able to simulate the sleep apnea conditions as accurately as possible to elucidate both the consequences and possible therapeutic strategies.

The presented experiments on human subjects mainly examined the hypoxic state occurring during anaerobic sports activity. There are many hypoxic methods involving both exercises and devices available to add to the trainings, but how to optimally choose their characteristics (stage of preparation in which to insert them, duration, form, etc.) is still an open question. However, these queries should be addressed to the field of training methodology. In the presented research, we were more interested in which physiological parameters determine the hypoxia tolerance.

Objectives

1. Development of an OGD experimental chamber with the following characteristics:
   a. adjustable temperature between 0 and 37 °C;
   b. ensuring an O_2 concentration lower than 0.5% throughout the experiment;
   c. adjustable gas flow to optimize the consumption;
   d. 90% humidity in order to prevent the samples from desiccation;
   e. modular design, capable of accommodating at least two sample plates. Experiments can be run at independent temperatures and times;
   f. validation of the device and protocol using *in vitro* cells and tissue samples,

2. Development of an intermittent hypoxic chamber suitable for modelling obstructive sleep apnea on mice. The requirements of the device are the following:
   a. it should be designed by modifying the original storage cage of the animals to avoid neophobic reactions;
   b. the O_2 concentration should rapidly change, according to the following protocol: 90s 21% O_2 / 90s 5.7% O_2 continuously alternating for 8 hours;
   c. the length of the time periods and O_2 concentrations should be adjustable;
   d. the humidity of the flowing gas must be in the range 50±10%;
   e. the gas has to circulate homogeneously in the closed box;
   f. it has to demonstrate the biological effect of the model.

3. Investigation of differences between trained freedivers and untrained control individuals during hypoxia. Analysing the physiological parameters that affect hypoxia tolerance.

   The following values were monitored during the measurements:

   blood saturation; spleen size, volumetric and elastography changes; FVC (Forced Vital Capacity).

   We were looking for answers to the following questions:

   a. Is there a significant performance difference between the two groups?
   b. Is there a significant difference in the physiological parameters mentioned above between the groups?

In summary, one of the aims of this work is to provide developments that can be used to study *in vitro* ischemia at different temperatures as well as to animal modelling of sleep apnea.

On the other hand, further experiments examine the human aspects of hypoxic training, in which the study of adaptation processes is the main aim.

Methods

Tissue culture experiments were performed on calvarial bone grafts isolated from rats. Samples were harvested from 3 to 4 months old, male Wistar rats, weighing ~ 350 g. Samples were incubated in 37 °C stem cell medium (DMEM, 10% FBS, 5% L-glutamine, 1% penicillin-streptomycin, Lonza) for 3 days. Subsequently, cold (4 °C) and warm (37 °C) OGD experiments were performed in parallel on n=12 samples per group in glucose-free medium. Following OGD, samples were placed in fresh stem cell medium and the reperfusion period was modelled by incubation for 3 days (37 ° C, 5% CO2). Cell viability was measured by colorimetric XTT (yellow tetrazolium) assay according to the manufacturer's instructions. The metabolic activity of the cells was expressed as absorbance, normalized to dry weight. A newly developed model was used for the examinations, which was able to meet the requirements set out in the objectives.

TLR2-luc-GFP transgenic mice were used as an *in vivo* model of hypoxia-induced TLR2 activation (Intermittent hypoxia group n=7; control group n=7). The day-night cycle consisted of 12 hours of both light and darkness. Water and food were provided *ad libitum* (also during the experiment). The temperature and humidity of the storage rooms were controlled. Two groups of male 85±20-days-old mice were randomly selected (IH n=7, CTRL n=7). Animals in the hypoxic group were treated 8 hours daily for 21 days. The experiment was conducted using a self-developed chamber with a unique control system. The inner oxygen concentration varied every 90s (5.7% / 21%) during the experiment. The rate of nitrogen flow was set at 50 l / min.

A live brain imaging technique was used to visualize the stress-induced TLR2 transcriptional activity by means of a bioluminescent molecular imaging camera (Xenogen IVIS-200, Caliper LifeSciences). Mice were injected intraperitoneal with D-luciferin 25 minutes before imaging (150 mg/kg, 20 mg/ml D-luciferin in 0,9% saline solution). The animals were then anesthetized with a mixture of 2% isoflurane and 100% oxygen at a flow rate of 1.5 l / min.

In addition to cellular and histological observations, we were also interested in the adaptation processes to hypoxic conditions. Human subjects participated into breath-holding experiments, in which the physiological response of certain organs was measured. Freedivers who perform regular hypoxia workouts were involved. The control group consisted of individuals without previous freediving experience.

We looked for a suitable method for detecting hypoxia tolerance in terms of differences in physiological indicators between the control and freediving group. Measured parameters: blood oxygen level, lung capacity. In addition, splenic reactions to hypoxia were examined by various imaging procedures (Ultrasound, MR).

Results

Four measurements were performed for the technical characterization of the OGD chamber. The minimum nitrogen flow was set to 1 litre / min / chamber to steadily maintain the required 0.5% oxygen concentration (after a settling time of ~150 s). The time to cool the chamber to 4 °C was about 20 minutes. The time to heat the chamber to 37 ° C was shorter due to the different heating and cooling performance of the Peltier module. Humidity ranged from 92 to 98% for the hot chamber and from 10 to 15% for the cold one. There was no significant fluid loss in any of the cases.

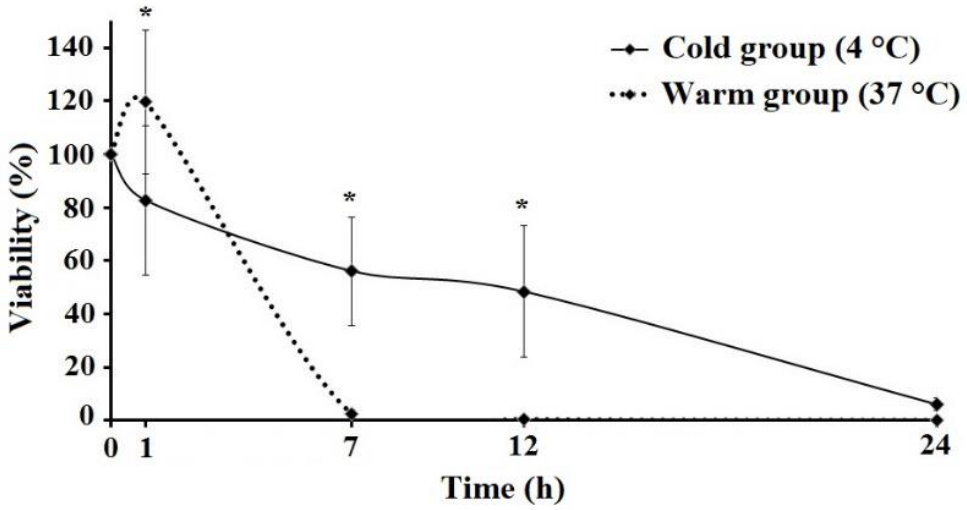

Figure 1. *The ischemic tolerance of bone tissue at 37 and 4 °C temperatures.* The experiment was performed with 1, 7, 12 and 24 hours of duration. Unexpected peak was visible in the warm group at 1h exposure. Thereafter, a sudden decrease was observed compared to the values of the cold group. There was no measurable viability in the warm group after 7h exposure. The results show the ratio compared to the control group (100%) of the given experiment (n=12) (Bago et al. 2018).

We ran cold and warm OGD experiments with different durations (1, 7, 12, and 24 h, respectively) to observe the tolerance of bone tissue to ischemia. Figure 1 shows the ratios between the groups as a function of the controls of the given measurement.

The following parameters were monitored during the testing of the self-developed OSA chamber: latency time between O_2 levels, accuracy of the controller, extent of gas consumption, humidity of the chamber. Based on the results, the oxygen concentration stabilizes with a ± 0.5% error in each phase after about 22s due to the system delay. Approximately 3200L of nitrogen (80 bar, 40L bottle) were used for the 8-hours experiment. Humidity ranged from 60 to 70%.

The physiological effect of the model was investigated using bioluminescent imaging. Figure 2 shows the changes in the degree of cerebral stress during the experiment. The bioluminescent activity increases sharply during the first weeks and then it slows down during the rest of the experiment.

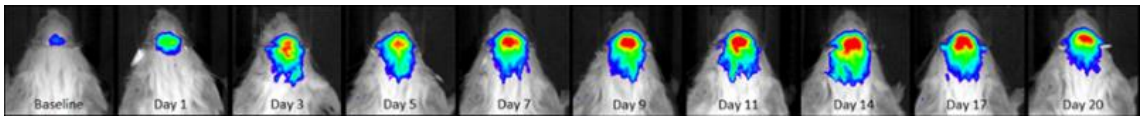

Figure 2. *The bioluminescent assessment of OSA experiment.* Bioluminescent images taken during the obstructive sleep apnea experiment. Recordings were made before the experiment and on day 1, 3, 5, 7, 9, 11, 14, 17 and 20. Colours indicate the location and extent of brain stress caused by hypoxia (IH, n=8; CTRL, n=7) (Polsek et al. 2017).

Human data revealed a significant difference in static and dynamic apnea performance between the two groups (freedivers, control). However, this difference does not clearly appear in the examined physiological parameters. The control subjects also showed a measurable variation in saturation in case of appropriate preparation, so this value did not show a significant difference. Spirometry measurements did not reveal any significant difference between the groups. However, we measured a significantly higher value for the freedivers who were able to collect air using the so called "packing" technique (+1±0,3 L).

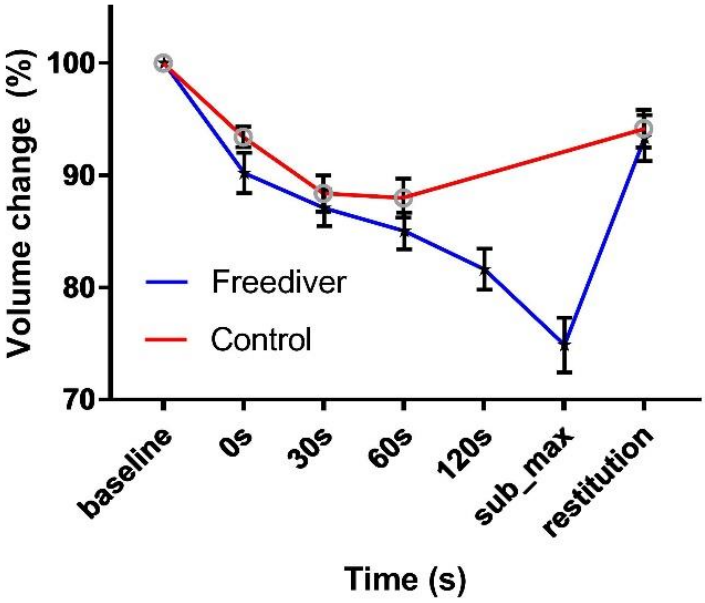

Figure 3. *Volume change data measured in the freedivers and control groups*. ANOVA tests revealed no significant differences at 0, 30, and 60s. Nevertheless, freedivers showed a tendency to reach larger volume changes thanks to longer apnea performance. Furthermore, freedivers showed a greater change during the 2-minute rest period (n = 10 freedivers, n = 13 controls).

Spleen volumes did not fall outside the normal range in any of the cases. MRI proved to be the most suitable technic for monitoring spleen volume changes due to apnea. Although the results presented in Figure 3 do not show a significant difference between the 0-60 s measurement points, it’s worth noting that the freedivers withstood the apnea test for a significantly longer time and thus reached larger volume changes.

Conclusions

Based on the results, the developments presented in the dissertation are suitable for modelling of *in vitro* ischemia and *in vivo* hypoxia. The designed protocols provide reproducible and reliable results.

Validation tests during the development of the OGD model confirmed that the device meets the desired requirements. It is able to maintain the 4 and 37 °C temperatures, and it has adequate insulation to ensure that the oxygen concentration remains below 0.5% during all the trials. The nitrogen consumption of the system was optimized with the help of pneumatic elements. The humidity was kept at an optimal level for tissue cultures throughout the tests. The validation tests confirmed that the chamber is similarly functioning to other hypoxic chambers reported in the literature and available on the market, thus proving the use for experimental tissue models. Tissue survival is affected by two parameters in OGD experiments: temperature and duration of the exposure to ischemia. The two-chamber design allowed to run parallel studies at different temperatures. Repeated experiments show that the model can induce reproducible biological effects on the samples.

As we hypothesized, the results of the study suggest that temperature and time of ischemia significantly influence cell survival rates. The system can be used for ischemia tolerance enhancement studies, during which an optimized procedure can be developed for the conservation of any tissue type. This can be a useful tool for transplant research and also allows for a better understanding how to processes that take place during many diseases and acute injuries.

The technical validation of the sleep apnea model was implemented empirically. The settings ensured the achievement of the desired flux rate and oxygen concentrations during the test. The menu allowed free parameter selection, so the system became suitable for testing other protocols as well. The data obtained during the technical measurements are in line with the values reported in other studies, confirming that the chamber is reliable for modelling the sleep apnea. The chamber, the applied flow rate and the humidity were adequate for the animals since no abnormal behaviours were observed. Serial exposure to hypoxia triggers an inflammatory process in the animal brains during the biological validation. The process was highlighted via bioluminescent imaging, and the lesions could be confirmed by histological examination after the

experiment. The results suggest, that sleep apnea can be modelled *in vivo* in intermittent hypoxia chamber. The device may also be suitable for monitoring adaptive mechanisms induced by hypoxia. This provides an opportunity to obtain more detailed information about the role of each organ during both hypoxia and anoxia.

The developments proved successful in both cases. We provided methods that could constituted basic knowledge for other researches. This is supported by the results of the experiments conducted independently of the present work since its beginning, which led to the realization of one BSc diploma, one PhD dissertation and two scientific publications. Furthermore, the success of this work is further announced by the patent issued this year for the obstructive sleep apnea chamber.

The testes on human subjects revealed a clear-cut difference in performance between the control and the freedivers group. Based on the values measured in freedivers, it can be concluded that apnea tolerance can be improved by training. However, no differences were found in the physiological measurements that could clearly represent this adaptation. Nevertheless, lung capacity and spleen size may be indicators of apnea performance. Spleen reactions can be monitored by imaging techniques. We determined that ultrasound imaging is suitable for recording the spleen contraction, but not for determining the exact extent of size change. Our observations showed that MRI is the most accurate technique, and its use is suggested for future studies.

Three different approaches were followed in this work to study hypoxia, enabling the research from the cellular level to the human level. Based on the observations and the available literature, the experiments are well matched. The obstructive sleep apnea chamber is an excellent connection between OGD and apnea research. *In vivo* induced lesions can be further studied through an *in vitro* OGD experiment using the two chambers. In addition, there are similarities in the processes that occur during voluntary apnea and sleep apnea. Based on this, the OSA chamber is also suitable for apnea experiments, and the results can also be considered reliable for human studies.

List of own publications

Publications related to the dissertation:

Bago M, Horvathy D, Simon M, Marschall B, Pinto A, Kuten O, Polšek D, Hornyak I, Nehrer S, Lacza Zs. (2018) Temperature controlled dual hypoxic chamber design for in vitro ischemia experiments. Biocybern Biomed Eng, 38(3):498-503.

Polšek D, Bago M, Živaljić M, Rosenzweig I, Lacza Zs, Gajović S. (2017) A novel adjustable automated system for inducing chronic intermittent hypoxia in mice. PLoS ONE 12(3): e0174896.

Independent publications:

Horvathy D, Szanto P, Marschall B, Bago M, Csery M, Hornyak I, Doros A, Lacza Zs. (2020) Ketamine decreases cell viability of bone explants and impairs bone healing in rats. J Orthop Surg Res, 15(46):1-5.

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