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Original Research Article

Temperature controlled dual hypoxic chamber design for *in vitro* ischemia experiments

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ABSTRACT

In vitro ischemia models are designed to study various aspects of hypo-perfusion, focusing on the consequences of acute events under body temperature. Cold ischemia, on the other hand, is less investigated even though the beneficial effects of cooling is expected. The aim of the present work was to develop a device modeling cold and warm ischemia *in vitro*. We designed a dual hypoxic chamber suitable for cell culture plates. Oxygen-glucose deprivation was applied with continuous nitrogen flow and glucose-free cell culture media to mimic ischemia. Using Peltier units the temperature in both chambers were independently set between 4 and 37 °C. Once the chambers reached the target temperature, samples were placed inside for the ischemic period, followed by a reperfusion stage under standard cell culture conditions. We tested rat calvaria bone pieces undergoing 1, 7, 12 and 24 h of ischemia at 4 and 37 °C. After 24 h of reperfusion, cell number was measured with a tetrazolium cell viability assay. The shortest 1 h period of ischemia paradoxically increased the post-reperfusion cell count, while cold-ischemia had an opposite effect. After 7 h of warm ischemia the cells were already unable to recover, while under cold ischemia 60% of the cells were still functioning. After 12 h of cold ischemia 50% of the cells were still be able to recover, while at 24 h even the low temperature was unable to keep the cells alive. The markedly different effect of warm and cold ischemia suggests that this newly designed system is capable of reliable and reproducible modeling of ischemic conditions. Moreover, it also enables deeper investigations in the pathophysiology of cold ischemia at the cellular and tissue level.

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1. Introduction

20 The ischemic condition is caused by the lack of blood supply since tissues have constant oxygen- and nutrient demand. When this demand is not met tissue damage occurs with a severity largely depending on the duration of the ischemic. Significant damage appears even after the ischemia is resolved, either because ischemia created cellular injury that is irreversible even if nutrient supply is re-established or because the re-oxygenation itself causes further harm [1]. Ischemia under body temperature is a well-described phenomenon and it can be investigated in various animal and *in vitro* models to mimic tissue infarction [2]. Cold-ischemia, however, is a less investigated phenomenon which has significant importance in two medical fields: 1, ischemia-reperfusion therapy, where cooling is already used in newborns [3], and 2, transplanted tissues and organs that are kept in cold storage until implantation with the idea that they shall survive longer [4]. As both these fields of therapy are emerging, more scientific knowledge is required to understand the effects of low temperature on ischemic tissues.

Several scientific models are designed to investigate ischemia. The best model is to use animals where full organs can be subjected to ischemia that closely follows the human condition such as middle cerebral artery occlusion in rats and mice [5,6]. However, the *in vivo* models do not allow tissue or cellular level investigations neither monitoring the course of the disease, so *ex vivo* models are widely used to this end. The most well-known such model is oxygen-glucose deprivation (OGD) which was first developed for mimicking stroke on neural cell cultures [7]. The model is based on the assumption that the key feature of ischemia is the lack of oxygen and nutrients, both of which are easily controlled in a cell culture flask. Withdrawal of nutrients is achieved by a change of media, while oxygen is purged by nitrogen in a closed chamber. This latter feature is more problematic than it sounds as the solution can also contain dissolved oxygen. Thus, it is important to take special care for the sealing of the chamber to lower the actual oxygen level below 1%. And this 1% is the threshold for oxidative metabolism in mitochondria, so it is of crucial importance that oxygen is truly depleted and it is monitored in a meaningful way as close to the cells as possible otherwise the oxygen deprivation is really only partial hypoxia [8].

Most available scientific literature on cold ischemia relates to transportation of organs or tissues that are kept at 4 °C [9,10]. On one hand, the ischemic time can be extended by cooling since it reduces cellular metabolism and the requirements for oxygen. On the other hand, the low temperature has harmful effects on the tissues because low temperature can change mammalian cell properties like metabolic pathways and the Na^+/K^+ ATPase. The development of organ preservation protocols allowed the deliver, of functional organs of high quality [11]. The transplantation of tissues is less known but more practiced than organs under similar circumstances. Bone is the most transplanted tissue (after blood) with about 2 million procedures per year and there is increasing demand for fresh bone *e.g.* in dental or joint replacement procedures [12]. As demand increased further investigations are needed to design protocols for live tissue preservation and transport. Hence, the aim of the present work is to create and test a chamber that can be used to study *in vitro* ischemia at different temperatures, serving both scientific research goals as it allows the monitoring of ischemia and development of optimized protocols for organ and tissue transportation. 82

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2. Materials and methods

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2.1. In vitro *ischemia-reperfusion model*

85 A custom designed device (Figs. 1 and 2) was built to model *in vitro* ischemia conditions at different temperatures. It contains two independent aluminum enclosures with IP65 and IP67 certifications for sealing (Gainta Industries Ltd.). Two pneumatic connections were formed to let the nitrogen flow into the boxes. Mufflers were used to spread the incoming gas and purge the oxygen from all parts of the chambers. The enclosures are separable by the manual valves at the inlet and the outlet so the systems can be used independently from each other. Peltier modules (TEC-12710) are applied to set the temperatures of the chambers between 0 and 50 °C. The modules are controlled by a TMS-125 thermostat. The dissipated heat of the Peltier modules is removed by a closed water cooling system. Fine tuning of the nitrogen flow is performed by a micro pressure regulator and a rotameter, respectively. Two plastic containers with bubble stones were built in the gas flow to keep the humidity at 100% when body temperature is used. No humidifier is used when the temperature is set of 4 °C to prevent ice formation. Alphasense O2–A2 oxygen sensor with transmitter board was used with custom designed display electronics to measure the oxygen level at the output of the chambers. Humidity is measured constantly during the measurements with capacitive humidity sensors. Ischemia was performed by using glucose free cell culture medium (Lonza DMEM) and by switching the inflow gas to pure O_2. 110

2.2. *Tissue harvest* 111

112 Male Wistar rats weighing ~350 g were euthanized by CO_2 and decapitated by guillotine. A midline skin incision was performed to gently remove the skin and periosteum from the surface of the skull. The head was fixed in a stereotaxic frame under a standing drill. Calvaria pieces were removed by a 4 mm trephined burr at 850 rpm. Four bone pieces were harvested from the parietal bones and another two from the frontal bones. The isolated tissues were placed in Petri dishes in standard stem cell culture media (DMEM, 10% FBS, 5% L-glutamine, 1% penicillin–streptomycin, Lonza) and kept in the incubator at 37 °C, 5% CO_2 for 3 days. All oxygen-glucose deprivation (OGD) experiments were carried out afterwards in 96-well plates in the ischemia chamber described above. At the start of OGD, glucose free cell culture medium equilibrated with N_2 was added to the samples, gas flow was switched to N_2 and the temperature was set to 4 or 37 °C, respectively. During the reperfusion period the samples were placed in a fresh cell culture media and kept in the incubator (37 °C, 5% CO_2) for 3

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