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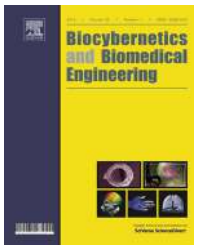

Original Research Article

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

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.

2. Materials and methods

2.1. In vitro *ischemia-reperfusion model*

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.

2.2. *Tissue harvest*

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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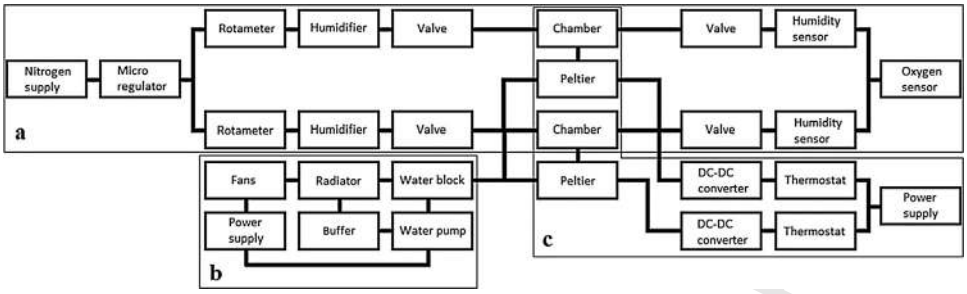

Fig. 1 – Block diagram of the *in vitro* ischemia system. The structure can be divided into the following parts: a, pneumatics and gas flow b, water cooling system c, temperature control and monitoring.

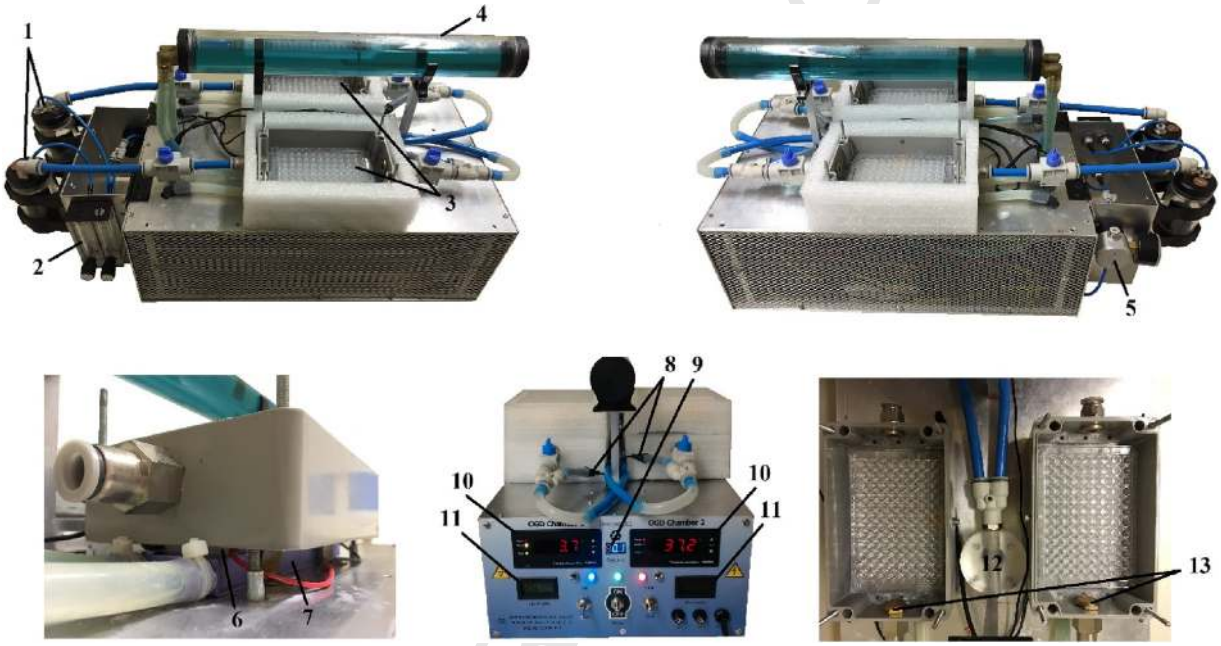

Fig. 2 – Main parts of the device. 1, humidifier bottles 2, flowmeter 3, samples in 96 well plates 4, buffer of the water cooling system 5, micro pressure regulator 6, Peltier module 7, water block 8, humidity sensors 9, oxygen display 10, termostats 11, humidity displays 12, oxygen sensor housing 13, mufflers.

days. The investigation was approved by the local Animal Research Committee according to the guidelines for animal experimentation.

2.3. *Viability measurement*

Colorimetric cell viability measurement was performed by XTT (yellow tetrazolium) assay according to the manufacturer's instructions [13]. The bone tissue pieces were placed in 96 well plates and kept in the incubator (37 °C, 5% CO_2) for 4 h. After the incubation the samples were removed and placed in a drying chamber for 1 day and then dry weight is measured. Optical density measurement of the supernatant was performed by spectrophotometer (BioTek PowerWave XS) at 460/620 nm wavelength, using the Gen5 software. Cell metabolic activity is expressed as absorbance per dry weight in arbitrary units.

3. Results

Four measurements were performed to characterize the device. Nitrogen consumption was calculated based on the amount gas flow measured by a flowmeter. The minimum flow rate is 1 l/min/chamber to keep the oxygen level of the required low level so the nitrogen consumption of the whole system is about 120 l/h. The decrease in oxygen levels is roughly linear and it takes about 2 min to reach an oxygen level of 0.5% at a steady flow rate (Fig. 3). The cooling and heating characteristics of the chamber follows a similar pattern, however, the time to reach the required temperature is much slower than that of oxygen. It takes approximately 20 min to reach 4 °C (Fig. 4). At this time point the samples are placed in the chamber according to the protocol. The samples only need a few minutes to equalize the temperature in the

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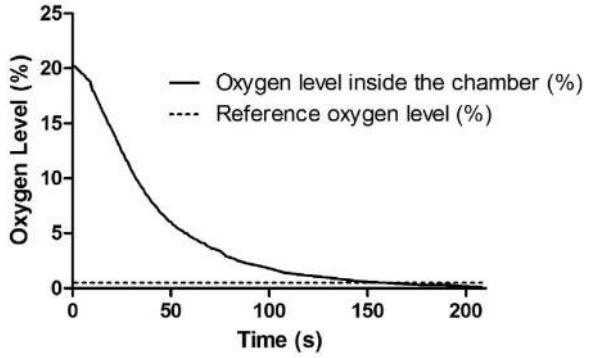

Fig. 3 – Oxygen level inside the chambers. Nitrogen flow is set to 1 l/min and it takes around 120 s to reach the reference 1% hypoxia level in both chambers. Then the system stabilizes at a 0.5 O_2 level.

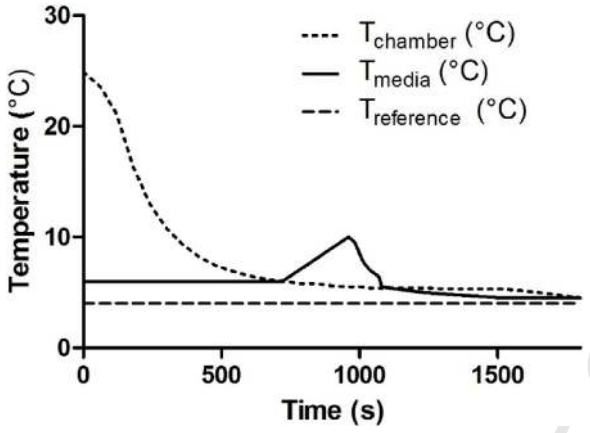

Fig. 4 – Cooling characteristics of the chamber and the media. It takes around 600 s to cool down the chamber to 6 °C. At this point cold media (6 °C) is added to the samples. The samples with the media are around 10 °C when they are placed into the chamber and it takes another 600 s to reach the reference temperature point (4 °C).

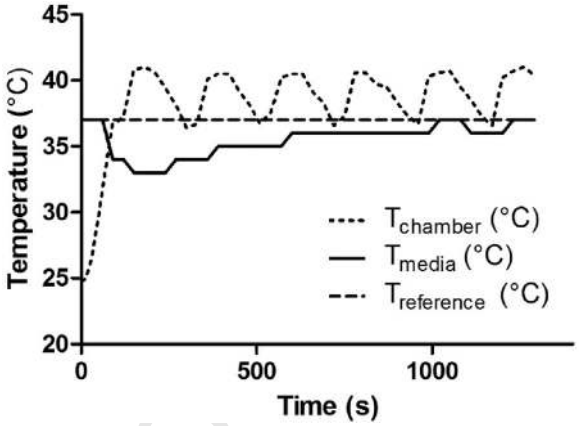

Fig. 5 – Warming characteristics of the chamber and the media. When the chamber reached 37 °C, the samples are placed in the chamber with cell culture media pre-warmed to 37 °C in a water bath. The initial temperature of the samples and the media is around 34 °C and it takes 600–900 s to reach the reference body temperature of 37 °C inside the chamber. The set temperature, humidity and O_2 levels remained constant up to 24 h.

chamber since we use cold media directly from the refrigerator (6 °C). In contrast, the heating characteristics have a different shape due to the different cooling and heating power properties of the Peltier module. The higher heating power and the thermal delay of the temperature sensor cause an oscillation around the desired value (Fig. 5). The pre-warmed samples are placed in the chamber after it reached the operation temperature. It takes only a few minutes to equalize the temperature difference between the samples and the chamber. Also, temperature oscillations are dampened in case the sample is already pre-warmed. Cold and warm oxygen-glucose deprivation experiments were performed with different durations (1, 7, 12, and 24 h, respectively) to observe the characteristics of tolerance of bone tissues against the ischemic condition. Fig. 6 presents the ratios between the groups and the corresponding control values at the measured time points. The warm group shows an unexpectedly high peak at 1 h then the viability starts to decrease and at the 7 h time point the viability of the tissue is already zero. The cold group shows a slowly descending tendency of viability, at 7 h it is still at about 60%, and we only observed a complete loss of viability at 24 h of OGD time.

4. Discussion

The present study showed that a cold-warm OGD chamber can be reliably used to model ischemia in live tissue samples. The gas exchange happens rapidly, which is not in sync with the much slower cooling times so pre-cooling of both the media and the chambers are necessary. In case of warming the speed difference is less of a problem, but fluctuations in temperature occur, which can be eliminated by the same precaution, *i.e.* pre-warming both the chamber and the media before starting the experiments. We used freshly harvested bone tissues to test the biological applicability of the system and observed that cold and warm OGD has markedly different effects on the viability of the tissues, indicating that the system is capable of reliable and reproducible modeling of the ischemic condition. The technical challenge of setting up an *in vitro* hypoxia chamber is often underestimated. Purging the air with nitrogen and assuming that a complete anoxia is achieved is a typical misconception, as even with well-insulated instruments it is hard to push the oxygen level below 1% – though at this level the mitochondrial oxidative phosphorylation is still functional. Using small dead-volumes and constant feedback of oxygen sensors allowed us to reach a consistent 0.5% O_2, which is adequate for modeling ischemia in a biological sense. Temperature control provided further technical challenges, both at the cooling and the warming directions. The optimal solution is to cool or heat the chamber

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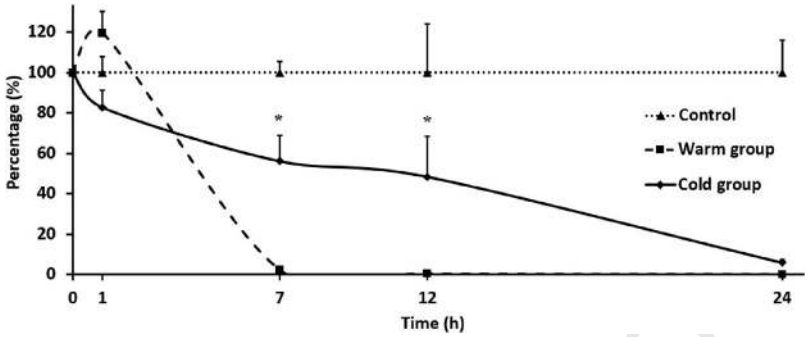

Fig. 6 – Ischemia tolerance of bone tissue with standard error bars at 37 and 4 °C. The measurements were performed at 1, 7, 12 and 24 h of OGD. After an additional 72 h of reperfusion, the cell number was measured with an XTT assay and the absorbance values were correlated with the cell number. The results were normalized to the control group which represents 100%. (mean ± SEM, one-way ANOVA, $^{*}p < 0.05$).

directly instead of using the gas flow for this purpose since the gas consumption is another important feature of the system. Furthermore when the gas flow is used to cool or warm the samples the thermal power decreasing even more and the system will be slower. It is important to note that the optimal number and power of the Peltier devices are critical. If the power of the modules is too low the desired temperature cannot be reached. On the other hand the dissipated heat will be huge if the power is too much. Rapid cooling without the threat of freezing is not feasible, as our present study showed there is a significantly slower cooling time than either warming or the changes in O_2 supply that must be kept in mind in parallel experimental designs. Pre-cooling the media on wet ice and the chamber system by an early equilibration phase without samples can solve this problem as we have shown. Although warming with a Peltier system can be much faster, it is still advised to follow a similar routine than with cooling for two reasons. 1, direct comparisons are best done with parallel experiments under cold and warm conditions simultaneously, and 2, rapid warming may induce heat shock or other pathophysiological changes that are complicating the interpretation of the results. Nonetheless, the system design described in the present study can adequately handle these issues and provide a simple and reliable tool to investigate *in vitro* ischemia between temperature levels of 4 and 37 °C. The applied ischemic times in the case of certain cell types are usually determined by empirical ways. The goal of an OGD session is to achieve cell death levels of minimum 50%, preferably 70–80%. If the death rate is higher, investigation of reperfusion is not realistic as the damage is too severe. If the death rate is lower, there is very limited room for improvement and the effects of the investigated materials may be overlooked. The accepted ischemic times for each system have to be established individually through a series of preliminary experiments even when using similar equipment. In case temperature is also taken into consideration it adds another degree of freedom to the system and the limited published technical details of cold OGD provide little starting points for such experiments. Therefore, we compared cold and warm conditions, *i.e.* 4 °C and 37 °C and used the same OGD times in order to see if the conditions make a difference. It was expected that about 2–3 h are needed to reach a 50% loss in viability even in warm conditions. However, it took much longer, about 7 h under warm conditions to reach this level. This is probably due to the choice of tissue. The vast majority of OGD literature is on monolayer cells of high oxygen-consumption, *i.e.* neurons or cardiomyocytes [14,15]. A fresh bone tissue, where the integrity of the 3D structure is preserved is probably more resilient than artificially sustained monolayers. Moreover, in our case the metabolic activity of the tissue is mainly due to the marrow content, which is known to be more ischemia resistant than terminally differentiated cells such as neurons. The current study was designed to be of a mainly technical nature, and we only used animal tissues as test substances for the system. Bone was chosen for this purpose because it is easy to harvest uniform sizes and because preliminary experiments were already available. Indeed, the generally expected differences at longer time points, *i.e.* that cells survive longer under cold ischemia was observed in the present study. However, during the cold-warm ischemia comparisons we stumbled upon an interesting finding: that warm ischemia increases the viability of the tissues if the OGD lasts for only 1 h but not longer. It must be noted that the tissue viability measurements were done 3 days later than the end of the ischemic period so it really measures post-ischemic recovery. In this context, the increased metabolism after a limited non-lethal OGD is probably due to ischemic preconditioning, a well-known phenomenon that explains the robust protection of a preceding minor ischemic episode from a later lethal one [16]. The exact mechanism and the lack of such findings under cold conditions require further studies, however, this also highlights the scientific value of a cold-warm parallel hypoxia chamber design described in the present study.

5. Conclusion

We conclude that the *in vitro* ischemia model of oxygen and glucose deprivation can be extended to cold and warm ischemic conditions, thus enabling the investigation of cooling effects on the pathophysiology of ischemia. Indeed, as our test tissue of bone showed, there is a marked difference of cell survival between cold and warm ischemia times. Interestingly,

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warm OGD increases the cell metabolism over baseline in the early time point of 1 h and then the survival rapidly drops, while cooling the samples to 4 °C eliminates this early increase but can extend the survival of the tissues up to 12 h or even more. In case of pre-cooled or pre-warmed media are placed in the chambers, the gas exchange can reach the required low level of oxygen in a few minutes allowing a reliable measurement system that models cold and warm ischemia *in vitro*.

Conflict of interest

Q3 The authors report no conflicts of interest.

Acknowledgements

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