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Standardization of the Cryopreservation Process for Parathyroid Glands

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ABSTRACT

The treatment of secondary Hyperparathyroidism may require cryopreservation of parathyroid glands to allow autotransplant. So far, cryopreservation and cryostorage has been done by the clinicians themselves. In some cases, investment in necessary equipment was not effective due to the small number of cases.
In this paper, we evaluate a new combination of cryopreservation and transportation processes. The intention was to facilitate cryopreservation in the hospital and to store the tissue in a facility conforming to GMP. The tissue is stored in the gas phase of liquid nitrogen (LIN) in a cryostorage vessel. Tissue viability and function can be kept high to optimise the overall results. The high cryopreservation standards and the necessary equipment allows hospitals to use Good Storing Practice (GSP) and to reduce process costs. At the central facility, a new standard of storing tissue has been introduced, which enables a closed cooling chain and automated data collection as well as the storage of the tissue.

1. INTRODUCTION

Secondary hyperparathyroidism (SHPT) may occur in patients with renal failure leading to abnormally high serum phosphate. Also, SHPT is a side effect in patients with decreased calcium uptake caused by diseases such as Morbus Crohn, celiac disease or cirrhosis of the liver. Continuously high phosphate levels induce overproduction of parathormone (PTH) in the parathyroid glands and its secretion[1]. This leads to strongly reduced serum calcium levels which are then compensated by resorption of calcium out of bone matter. Persistent PTH production therefore leads to bone diseases like different kinds of osteodystrophy. The abnormal calcium and phosphate levels may also cause cardiovascular diseases in patients with SHPT leading to increased mortality[2].
Treatments includes improving calcium uptake with calcium, vitamin D, calcitriol or calcimimetic agents and decreasing phosphate levels with calciumcarbonate, calciumacetate or aluminiumhydroxide. Reduction of parathormone levels can be achieved by cinacalcet treatment [3,4,5].
Permanent synthesis of parathormone causes, in almost all cases, hyperplasia of all parathyroid glands. Therefore, surgical excision of the parathyroid glands often improves the health of the patient very quickly[6]. In some cases, patients develop a permanent hypoparathyroidism[7]. Cryopreservation and -storage of parathyroid tissue immediately after removal is recommended for all patients with SHPT to allow autotransplantation (Deutsche Gesellschaft f. Chirurgie). Cryopreservation of parathyroid glands has been performed in a variety of ways. The German guideline for surgical treatment of SHPT suggests a freezing rate of about -1°C / min and the use of a cell culture medium combined with DMSO (dimethyl sulphoxide) and autologous serum as cryopreservation medium[8].
Often, hospitals have no cryostorage facilities or staff trained in such techniques. The combination of an easy-to-use protocol; materials for cryopreservation and external storage facilities might be a potential alternative for these hospitals.
Our aim was, therefore, to find a method for successful alcohol-free cryopreservation of tissue and a transportation process using dry ice.

2. METHODS AND RESULTS

A freezing rate of about -1° C/ min can be achieved without a programmable liquid nitrogen freezing device. An alcohol-based device like Mr. Frosty (Nalgene, Thermo Fisher Scientific) works well for the freezing of cells[9]. Transportation, however, is critical. 2-propanol, which is used as the heat transfer agent, is volatile. Contact of this alcohol with the cryovials during transportation cannot be avoided. Furthermore, biological

specimens must be packed in accord with official instructions, UN3373 Cat B, which include the use of an absorbing material sufficient for the complete liquid content. In the case of the Mr. Frosty device, this means about 450 ml of isopropanol plus the volume of the specimen. Since 2-propanol is highly flammable, transport is only allowed in completely leak proof packaging, a condition not fulfilled by the alcohol-based freezing device.
We tested an alcohol-free freezing device. As recommended by the manufacturer, the device was not pre-cooled. 10 cryovials each with a volume of 2 ml and filled with 1 ml cell culture medium were placed inside the device. Two additional cryovials were each fitted with two type T thermocouples, which were connected to a multichannel temperature control unit. (Agilent, USA). Temperature measurement started at the moment the freezing device was placed within a secondary packaging with absorbent (BioPouch, Bio-packaging Ltd, Great Britain) in a transportation box with dry ice as used for shipments (ThermoGBOX650 S, Alex Breuer GmbH, Germany) (fig.1 A).
Temperature was measured for about 250 minutes. The mean temperature values are shown in fig. 1B. We observed a freezing rate of 0.86 K/min in the temperature range from 0°C to -60°C. The temperature approximated then asymptotically from -60°C to -80°C.

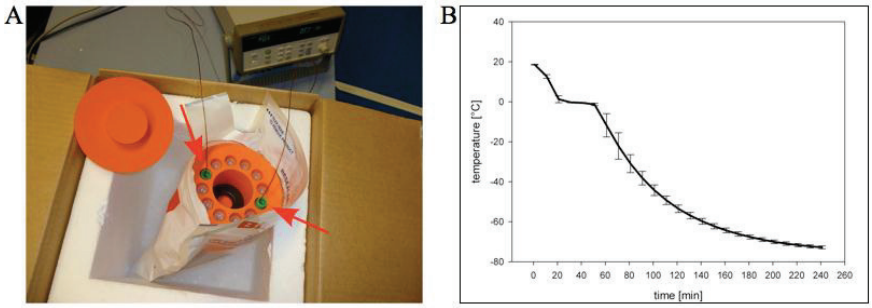

Figure 1: Freezing rate determination of an alcohol-free freezing device; A: experimental set-up, thermocouples in 2 ml cryovials were placed in the freezing-device in secondary packaging in a dry ice box. B: freezing rate of 2 ml cryovials within alcohol-free freezing device. Error bars indicate standard deviation.

To test cell viability in different freezing devices, human mesenchymal stem cells (hMSC-UC, Human MSCs from umbilical cord matrix, PromoCell GmbH, Germany) were chosen because of their reliability in growth and sensitivity to external factors[10]. Cells were grown to 80% confluence, then harvested using trypsin and aliquoted at a concentration of 500000/ml with cryopreservation medium in 2 ml cryovials. Cells were then frozen: in the non-alcohol freezing device; in an alcohol-containing freezing device (both in a -80°C freezer) or in a programmable liquid nitrogen-based device at – 1°C / min (Sy-Lab, Austria).
The viability of fresh, non-frozen cells served as a positive control and the influence of the cryopreservation medium was tested as well.
After thawing, the cryopreservation medium was removed and replaced by regular growth medium and cells were plated and incubated in a 96-well plate for 24 hours. Viability was then tested using the alamar blue assay (Invitrogen, USA) with fluorescence measurement in a plate reader (Tecan, Switzerland).
Best viability of MSCs was achieved with the programmable device (fig. 2). Cells survived the non-alcohol device slightly better (although not significantly) than the alcohol-containing device. The comparison of cells results from the alcohol-containing device with those from the programmable device shows a highly significant reduction of cell viability, whereas we could not observe a significant difference between the cell viability preserved with the non-alcohol device compared to the viability of cells using the programmable LIN-device.

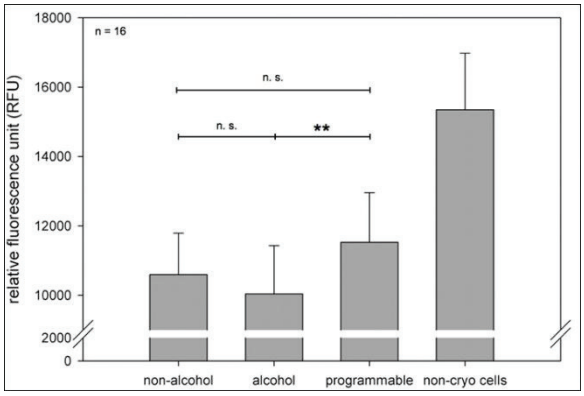

Figure 2: Cell viability of human mesenchymal stem cells after cryopreservation in different types of freezing device measured with alamar blue fluorescence. non-alcohol: Alcohol-free freezing device; alcohol: alcohol-containing freezing device, programmable: programmable LIN-based freezing device, rate -1°C/min; non-cryo cells: cells were not cryopreserved and used as a control Error bars indicate standard deviation.

MSC cells were also used for testing the transportation process during the cryopreservation time. Cells were again grown under standard conditions until 80% confluence. After harvesting with trypsin, the cells were aliquoted at a concentration of 500000/ml with cryopreservation medium in 2 ml cryovials. The aliquots were then placed into the non-alcohol freezing device. This was then packed in a secondary packaging with absorbing material and placed in a transportation box filled with 5 kg dry ice. This box was then closed according to the manufacturers instructions and transported by car for about 30 km and left overnight in the car. On the next day, the aliquots were stored in the gas phase of LIN in a cryostorage vessel at a temperature of about -150°C. After about one week of storage, the cells were thawed, plated in a 96-well plate, grown overnight and an alamar blue viability assay was performed.

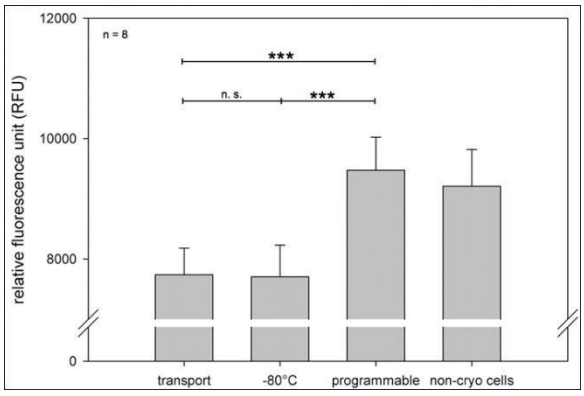

Figure 3: Cell viability of human mesenchymal stem cells after cryopreservation with different cryopreservation methods measured with alamar blue fluorescence assay. transport: Cryopreserved in alcohol-free freezing device packed in dry ice and transported; -80°C: cryopreserved in alcohol-free freezing device in -80°C freezer, programmable: cryopreserved in programmable LIN-dependent freezing device, rate -1°C/min; non-cryo cells: cells were not cryopreserved and used as a control. Error bars indicate standard deviation.

Transportation during the cryopreservation process had no negative effect on the viability of the hMSCs when compared to the cryopreservation in a -80°C freezer, both with the alcohol-free device. Cell viability was similar in both situations (fig. 3). In this test, the difference between the cryopreservation with the alcohol-free device and the programmable device was slightly higher than in the test before (fig. 2), so the difference between these methods could here be shown as extremely statistically significant (fig. 3).

3. DISCUSSION

Cryopreservation of cells or tissue in a non-programmable freezing device is a widely used method in the laboratory. Simultaneous cryopreservation and transportation has not been shown before.

For the surgical treatment of secondary hyperparathyroidism SHPT, hospitals need highly expensive cryostorage capacities and trained staff. The combination of effective and easy cryopreservation with the external storage of the transplant tissue would be a cost effective method for more hospitals to offer the surgical removal of the parathyroid glands and their cryopreservation.
Our tests show that the use of a non-alcohol freezing device is a suitable method for freezing highly sensitive biological material when no programmable LIN-based freezing device is available (as is the case in most hospitals). The freezing rate and the temperature curve given by the manufacturer was verified in our experiments[11].
Compared to the programmable method of freezing, cryopreservation in the alcohol-free device reduced cell viability by less then 20 %. For parathyroid glands this is acceptable.
Transportation in packaging conforming to UN3373 Cat. B in dry ice during freezing does not at all impair the viability of the highly sensitive cells when compared to freezing in an expensive -80°C freezer.
Simultaneous non-alcohol freezing and transportation to the storage facility is a cheap, easy and reproducible method for the cryopreservation of transplant tissues. Transportation shortly after excision lowers the risk of the specimen getting lost or forgotten within the hospital.
Further experiments will be performed to test the influence of this whole clinical process on the structure of parathyroid glands as well as on the viability and functionality of parathyroid cell culture.

4. REFERENCES

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