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Cryopreservation of cells using defined serum-free cryoprotective agents

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Abstract: For regenerative purposes, there is a high demand for viable and active cells. A big issue is to have enough viable cells available at any given time. One solution is cryopreservation. In this context, DMSO is used as cryoprotective agent (CPA) along with fetal bovine serum for nutrient supply and stress shielding effects. To use these cells for human clinical studies, it is important to eliminate the serum to prevent foreign immune reactions and virus transmittance and DMSO for its toxic effect. In this study a serum free cryopreservation solution and protocol has been established. The combination of methylcellulose and poloxamer 188 provide the basis for the new CPA. Other additves are α-tocopherol, ectoine, prolin and ascorbic acid. The CPAs were examined with 3T3-cells and multipotent stromal cells from the common marmoset monkey (*Callithrix jacchus)*. The cells were preserved with various CPA concentrations, incubation times and different cooling rates. To enable a higher throughput of encouraging conditions a fluorescence microscopy analysis was used. The use of methylcellulose, poloxamer 188 and α-tocopherol enables the reduction of DMSO [up to 2.5% (v/v)] and the elimination of serum without viability losses compared to control.

Keywords: antioxidants; cryopreservation; fluorescence microscopy; methylcellulose; multipotent stromal cells; poloxamer 188; serum free.

1 Introduction

The cryopreservation of human cells opens new options for medical therapies. The physicians get new opportunities to cure their patients with new methods.

The cells are stored at temperatures below −150°C. The metabolic activity stops at these temperatures [1].

The biggest issue is to ensure the viability and the functionality of the cells after thawing. The use of cryoprotective agents (CPA) reduces the required cooling rate and enhances the cryopreservation outcome. The most common used CPA is a combination of 5–10% (v/v) dimethylsulfoxid (DMSO) and up to 90% (v/v) foetal bovine serum (FBS) in combination with a cooling rate of 1 K/min [1]. DMSO is toxic in high concentrations [e.g. 10% (v/v)] and cause denaturation of cells' proteins [2–4] and leads to differentiation of stem cells.

FBS is an undefined mixture of proteins and growth factors that nourishes the cells. Moreover the high protein content stabilizes and protects the membrane against shear stresses [5].

For clinical applications it is important to eliminate the animal serum to prevent immune responses. Furthermore FBS is an undefined substance which has different concentrations in every charge [6–8].

This study shows that there is a possibility to eliminate the FBS and reduce the concentration of DMSO with the usage of alternative CPAs.

2 Material and methods

2.1 Material

Unless otherwise stated, chemicals were purchased from Sigma Aldrich (Germany).

2.2 Cell lines

The pre-tests were conducted with 3T3-cells. To validate the results, bone marrow multipotent stromal cells (MSC) of the common marmoset monkey *(Callithitix jacchus)* were used.

2.3 Cultivation of 3T3-cells and MSCs

3T3-cells were cultured in 75-cm^2 flasks with Dulbecco's Modified Eagle's Medium (DMEM, Merck Millipore,

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Table 1: Additives of the basic cell freezing solution.

Name	Pro (mM)	E (mM)	AA (µM)	α-toc (µM)	FBS [% (v/v)]
A	–	–	–	–	–
B	10	100	–	–	–
C	10	100	500	–	–
D	–	–	–	200	–
Control	–	–	–	–	15 (3T3) 22.5 (MSC)

Germany) with 10% (v/v) FBS and 1% (v/v) Penicillin / Streptomycin (Pen / Strep; Biochrome, Germany). MSCs were cultured in a 160 mm^2 culture dish using a culture medium (MSC-medium) containing DMEM, 15% (v/v) FBS, 1% (v/v) Pen / Strep and 50 µM ascorbic acid (AA). Cells were cultured under 37°C and 5% CO_2 in a humified incubator (Binder GmbH, Germany) until 85% confluence.

2.4 Serumfree CPAs

The potential CPAs were based on 0.1% (v/v) methylcellulose (M) (4000 cP) and 1% (v/v) poloxamer 188 (P). Additional compunds were proline (Pro, Carl Roth, Germany), ectoine (E; Biomol, Germany), AA and α-tocopherol (α-toc) in the concentrations shown in Table 1.

2.5 Cryopreservation

The samples were frozen in 0.2 ml PCR strips (Carl Roth, Germany). For freezing, two different freezing devices (WB 230 Askion C-Line® and CM-2000 Air Products GmbH, Germany) were used. For 3T3-cells a cell-concentration of 2×10^5 and for MSCs of 10^6 cells were used per sample. The control-solution for 3T3-cells was composed of DMEM mixed up with 10% (v/v) DMSO and 15% (v/v) FBS. The cooling rate was 1 K/min. The MSC freezing medium consisted of DMEM with 5% (v/v) DMSO and 22.5% (v/v) FBS. MSCs were frozen with a cooling rate of 7.5 K/min to −30°C followed by 3 K/min to −80°C [9].

Cells were frozen using either 1 or 5 K/min (3T3) and the above mentioned cooling rate for MSCs. Both cell types were frozen using different parameters (DMSO concentrations for 3T3: 0–5%/MSCs: up to 2.5%) and different incubation times (10, 30 and 60 min). No FBS was added in groups except for controls.

Cells were stored at −150°C for at least 24 h and then thawed and analysed. For thawing, the PCR strips were swivelled in a 37°C water bath until only a small clump of ice was visible. Afterwards they were centrifuged in a centrifuge (Biozym Scientific, Germany) with 6000 rpm for 10 s, supernatant removed, re-suspended in culture medium and transferred to a 12-well plate and cultured for 24 h at 37°C.

2.6 Fluorescence microscopy

After incubation, the cells were washed and then fixed with 4% paraformaldehyd for 10 min. Thereafter the cell nuclei were stained with the fluorescence dye Hoechst 33,342 for 1 h. To count the cells in a sector of every well, 36 pictures of the centre of each well were taken with the fluorescence microscopy Axiovert 200 (Carl Zeiss, Germany). Images were analysed with ImageJ (Fiji, open source).

2.7 Re-cultivation rates

To determine the efficiency of re-cultivation after thawing, only MSCs were used.

One milliliter, containing 10^6 cells, were frozen in 2 ml cryovials (Biochrome, Germany) using the above mentioned CPAs.

After thawing, the cells were transferred into falcon tubes (Sarstedt, Germany) and centrifuged at 4°C and 900 rpm for 8 min.

After removing the supernatant, the cells were re-suspended in 1 ml MSC-medium and cultured for 24 h at 37°C.

After incubation, the cells were detached with Trypsin EDTA (PAA, Germany) and counted by an automatic cell-counter ViCell™ XR (Beckmann Coulter GmbH, Germany) by Trypan blue dye exclusion. Efficiency of re-cultivation is represented by the ratio of total number of adherent cells to initial number of cells during seeding and was considered as cell viability after cryopreservation.

3 Results

3.1 Cryopreservation

At first, the new process for low volume cryopreservation was examined and validated. With this setup, it was possible to get a high throughput with several parameters like cooling rates, incubation time or different concentrations of DMSO for each run.

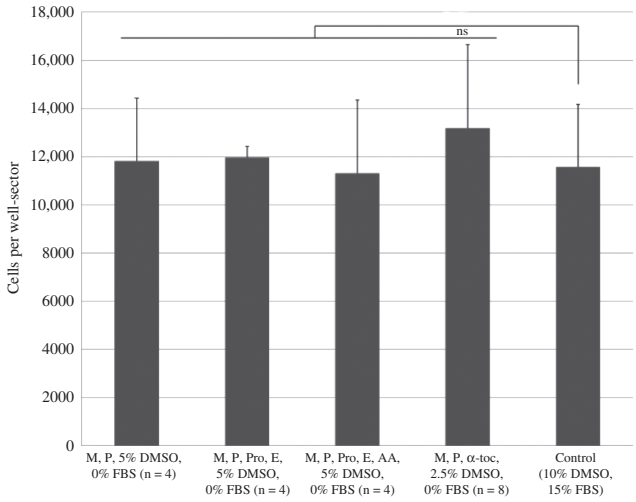

Figure 1: Cryopreservation outcomes of 3T3 with a cooling rate of 1 K/min and a incubation time of 10 min.

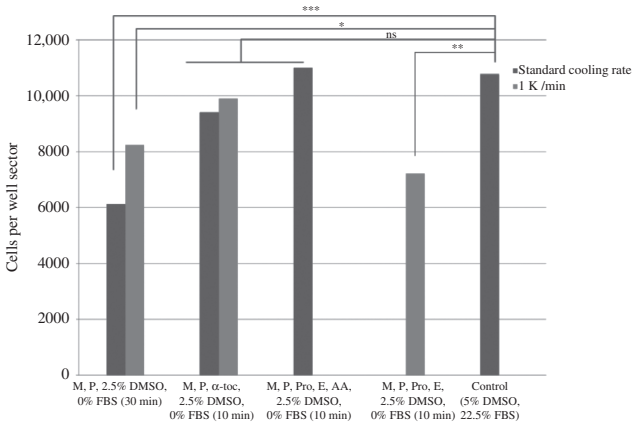

Figure 2: Survival of MSCs after cryopreservation using different cooling rates and incubation times. The number in brackets are the used incubation time.

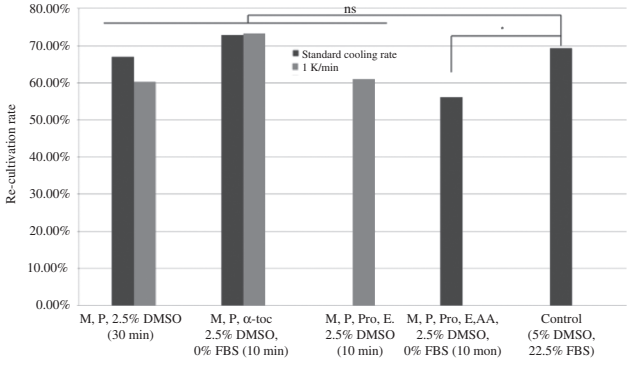

Figure 3: Re-cultivation rates of cryopreserved MSCs. Comparison between two cooling rates.

3.1.1 3T3

The investigations showed, that the most promising results were achieved while using 1 K/min, no FBS and 10 min incubation time (Figure 1).

As it is shown, the base solution (M, P) with addition of 5% (v/v) DMSO, yields nearly the same cell count as the control with 10% (v/v) DMSO and FBS. The highest amount of viable cells was yielded by the basis solution with addition of α-toc and 2.5% (v/v) DMSO. Moreover all other solutions delivered similar results compared to the control. All the cell counts were in the range between 11,000 and 13,000 cells per well-sector.

3.1.2 MSC

Afterwards, the results were validated by using MSCs. Figure 2 shows an extract of these outcomes. A comparison between the two used cooling rates are shown. Furthermore different incubations times were used (number in brackets).

The highest cell count was found while using the standard cooling rate (7.5 K/min to −30°C and 3 K/min to −80°C) and the basis solution (M, P) with addition of Pro, E, AA and 2.5% (v/v) DMSO. There is no significant different between this condition and the control which contented 5% (v/v) DMSO and 22.5% (v/v) FBS.

The highest cell count with the cooling rate of 1 K/min, which is the most important for the common laboratories, was delivered by the use of the basis solution, α-toc and 2.5% (v/v) DMSO. There was also no significance in the deviation of cell count in comparison with the control.

3.2 Re-cultivation rates

Finally the previous results were confirmed by investigating the efficiency of re-cultivation for the best results of the MSC studies. The results are shown in Figure 3.

No significant differences were found for all groups except M, P, E and AA.

There are some differences in comparison with the results from above (3.1.2.). Even though no differences were found, the CPA with α-toc seems the best choice for the serum free cryopreservation of stem cells.

4 Conclusion

In standard freezing protocol FBS is used in different concentrations (5–95%) but for clinical applications animal derived undefined substances are forbidden.

This study shows that it is possible to reduce the DMSO concentration and completely exclude serum. This should be the foundation to preserve human stem cells with this new CPA. But it is important to further investigate if the CPA protects not only the viability but also the functionality of cells.

Furthermore the process, to investigate cell numbers after cryopreservation by fluorescence microscopy enables a higher quantity of trials in shorter time, which also means lower costs. If the results could be validated with a small amount of additional studies, it would be a low cost method to improve the cryopreservation protocol for regenerative approaches.

Complementing methods could be the use of controlled induced ice formation. With different nucleation temperatures it is might be possible to preserve the cells without toxic substances like DMSO.

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Author's Statement

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