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STUDII PRIVIND PROPRIETĂŢILE CRIOPROTECTOARE ALE MEDIILOR DE VITRIFICARE CU ETILEN GLICOL, SUCROZĂ, FICOLL70 ŞI GALACTOZĂ UTILIZATE ÎN CRIOCONSERVAREA EMBRIONILOR DE MAMIFERE

STUDIES REGARDING THE CRIOPROTECTIVE PROPRIETIES OF THE VITRIFICATION MEDIA, WITH ETHYLENE GLYCOL, SUCROSE, FICOLL 70 AND GALACTOSE USED IN MAMMALIAN EMBRYO CRYOPRESERVATION

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Crioprotectors are the main component of any vitrification media. The penetrant crioprotectors are essential for cell dehydration and for the decrease of the freezing point of the solution, allowing a longer time for dehydration to set in. The aim of our paper was to make a series of experiments in order to determine the concentration at which four cryoprotectants (ethylene glycol, sucrose, Ficoll 70 and galactose) singly and in pairs would vitrify on plunging into liquid nitrogen and remain vitreous when thawed in water bath. A total of 156 solutions were tested. During freezing, vitrification was evidenced by the formation of transparent glass when the unsealed straws were plunged into liquid nitrogen, at -196°C. Crystallization (ice formation) resulted in a milky appearance. Solutions that vitrify on freezing were tested if they remain vitreous on thawing. For thawing we tested three temperatures 20°C, 25°C and 37°C. During thawing, solutions that did not devitrified were transformed from solid clear state to the liquid state without evidence of a milky appearance. From the combinations of two cryoprotectors were tested a number of 51 solutions vitrify on freezing (19 solutions with ethylene glycol and galactose; 19 solutions with ethylene glycol and sucrose; 13 solutions with ethylene glycol and Ficoll). The ethylene glycol and galacose pair give the best results on thawing (3 combinations remained vitreous on thawing) at 37°C.

Keywords: crioconservation, vitrification, ethylene glycol, galactose, sucrose, Ficoll70

Introduction

Crioprotectors are the main component of any vitrification media. The penetrant crioprotectors are essential for cell dehydration and for the decrease of the freezing point of the solution, allowing a longer time for dehydration to set in.

the choosing of the cryoprotector should be performed with care the toxicity must be the first concern and permeability comes second. At high concentrations the crioprotectors are toxic, but this can be ameliorated by lowering the time and the temperature of contact (Aga 1998).

The aim of our paper was to make a series of experiments in order to determine the concentration at which four cryoprotectants (ethylene glycol, sucrose, Ficoll 70 and galactose) singly and in pairs would vitrify on plunging into liquid nitrogen and remain vitreous when thawed in water bath.

Materials and Methods

As penetrating cryoprotector we used ethylene glycol (MW= 62,07 Da; Sigma AL 293237(p.a.)), and as non-penetrating cryoprotectors we used Sucrose (MW=342.3 Da; Sigma S7903), Ficoll 70 (MW= 60,000-80,000 Da; Sigma F4375) and galactose (MW = 180,16 Da; Sigma G 6152).

For ethylene glycol there were tested concentrations from 1M to 6.5M, with concentration steps of 0.5M. For the non-penetrating cyoprotectors we tested concentrations of 5%, 10 %, 15% and 20%.

The vitrification solutions were made in PBS (Phosphate Buffered Saline, Sigma P3813) pH 7.4.

A total of 156 solutions were prepared from which:

- 12 solutions with ethylene glycol (1M-6.5M);
- 48 solutions of ethylene glycol with sucrose;
- 48 solutions of ethylene glycol with Ficoll 70;
- 48 solutions with ethylene glycol and galactose .

The solutions were tested for vitrification abilities using the method described by Ali J and Shelton J. N. (1993). The solutions to be tested were loaded into 0.25ml straws using a syringe. During freezing, vitrification was evidenced by the formation of transparent glass when the unsealed straws were plunged into liquid nitrogen, at -196°C. Crystallization (ice formation) resulted in a milky appearance.

Solutions that vitrify on freezing were tested if they remain vitreous on thawing. For thawing we tested three temperatures 20°C, 25°C and 37°C. During thawing, solutions that did not devitrified were transformed from solid clear state to the liquid state without evidence of a milky appearance. Devitrification (ice formation, re-crystallization) conferred a milky appearance during thawing.

Results and Discussion

The ethylene glycol (penetrating cryoprotector) solutions with concentration higher or equal to 5,5 M vitrify on thawing. But none of them (5.5M, 6 M and 6.5 M) remained vitreous at thawing.

When combinations of two cryoprotectors were used 51 solutions vitrify on freezing.

- 19 solutions with ethylene glycol and galactose;
- 19 solutions with ethylene glycol and sucrose;
- 13 solutions with ethylene glycol and Ficoll.

The results obtained at thawing the solutions with ethylene glycol that vitrify at freezing are presented in table 1.

Table 1

The behavior at thawing of the solutions with ethylene glycol and galactose

Nr crt.	Molar concentration of ethylene glycol (moles/liter)	Concentration of galactose (%)	Thawing temperature		
			20°C	25°C	37°C
1	3.5	20	R	R	R
2	4	20	R	R	R
3	4.5	15	R	R	R
4	4.5	20	R	R	R
5	5	10	R	R	R
6	5	15	R	R	R
7	5	20	R	R	R
8	5.5	5	R	R	R
9	5.5	10	R	R	R
10	5.5	15	R	R	R
11	5.5	20	R	R	R
12	6	5	R	R	R
13	6	10	R	R	R
14	6	15	R	R	R
15	6	20	**V**	**V**	**V**
16	6.5	5	R	R	R
17	6.5	10	R	R	R
18	6.5	15	R	R	**V**
19	6.5	20	**V**	**V**	**V**

R – solutions that formed ice crystals at thawing
V- solutions that remained vitreous at thawing

From table 1 it can be noticed that when mixed with 20% galactose, ethylene glycol is vitrifing at 3,5 M. When the non penetrating cryoprotector concentration is decreased at 15%, 10% and 5% the concentration of ethylene glycol necessary for vitrification is increased at 4,5 M, 5M and 5,5 M, respectively. In respect to the thawing, at 20°C, only two solutions didn't form ice crystals (ethylene glycol 6M + 20% galactose; ethylene glycol 6,5 M + 20% galactose). At 25°C thawing temperature the same results were registered. At 37°C three solutions didn't vitrify (ethylene glycol 6M + 20% galactose; ethylene glycol 6,5 M + 20% galactose and ethylene glycol 6,5 M + 15% galactose).

In respect to the combinations of glycerol and sucrose, at 5% or 10 % non penetrating crioprotector in solution ethylene glycol is vitrifing at 5 M concentration. When the non-penetrating crioprotector concentration is increased at 15% ethylene glycol solution is vitrifing at 4,5 M. From the 19th solutions with ethylene glycol and sucrose that vitrify on freezing none remained vitrified at thawing in 20°C, 25°C or 37°C water bath.

When Ficoll 70 was used as non penetrating crioprotector at 20% concentration ethylene glycol solution is vitrifying at 4,5%. At 10% and 15% and 5% Ficoll 70 in media ethylene glycol solution is vitrifying at 5.5 M.

The results obtained are comparable with the specialty literature that state that ethylene glycol has pore capacity of vitrification (Valdez 1992). The use of ethylene glycol in cryopreservation of the embryos is based on it's low toxicity. Szell et. al. (1989) have proved that ethylene glycol is the least toxic from the crioprotectors used and has the best penetration rate. In the research regarding the toxicity of crioprotectors Moore K. et. al. (2006) has established a toxicity scale on which ethylene glycol is the least toxic and acetamida is the most toxic.

Conclusions

1. The concentration at which ethylene glycol solution is vitrifying is 5,5M, but the solution devitrified and can not be recommended as proper vitrification media;
2. Non penetration crioprotectors, added to the vitrification media can reduce the concentration of ethylene glycol needed for vitrification. in media with 20% galactose the concentration of ethylene glycol necessary for vitrification was 3,5 M;
3. Galactose was the best non-penetrating crioprotector in combination with ethylene glycol. from the three non-penetrating crioprotectors it was the only one that prevent ice crystals formation;
4. 37°C is the best thawing temperature, from the one tested (20°C, 25° and 37°C), there were three solution that prevented ice crystal formation, compared with 2 obtained at the other temperatures tested;
1. Following the freezing/thawing experiments with ethylene glycol as nonpenetrating cryoprotector we obtained three solutions that can be used as vitrification media for mammalian embryos (ethylene glycol 6M+20% galactose; ethylene glycol 6,5M +15% galactose; ethylene glycol 6,5M +20% galactose).

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