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

STUDII PRIVIND PROPRIETĂŢILE CRIOPROTECTOARE ALE MEDIILOR DE VITRIFICARE CU DMSO, SUCROZĂ, FICOLL70 SI GALACTOZA UTILIZATE ÎN CRIOCONSERVAREA EMBRIONILOR

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The aim of our paper was to make a series of experiments in order to determine the concentration at which four cryoprotectants (DMSO, sucrose, Ficoll 70 and galactose) singly and in pairs would vitrify on plunging into liquid nitrogen and remain vitreous when thawed in water bath. As penetrating cryoprotector we used DMSO (MW=78.13 Da, Sigma D5879) and as nonpenetrating 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 DMSO there were tested concentrations from 1M to 6.5M, with concentrations step of 0.5M. For the nonpenetrating cryoprotectors there were tested concentrations of 5%, 10%, 15% and 20%. There were a total number of 168 solutions tested. The solutions vitrification ability on freezing was tested by direct plunging into liquid nitrogen at -196°C. Three thawing temperatures were tested 20°C, 25°C and 37°C. The concentration at which DMSO solutions passed into vitreous state was 5M, but at thawing none of them remained vitreous at thawing. When pairs of cryoprotectors were tested 67 solutions vitrified at freezing (23 for DMSO-sucrose, 23 for DMSO-Ficoll 70 and 21 for DMSO-galactose) and 26 of them remained vitreous at thawing. The DMSO and galacose pair give the best results on thawing (11 solution remained vitreous on warming) at 37°C.

Key words: crioprotectors, vitrification, DMSO, sucrose, Ficoll 70, galactose

Introduction

In farm practice the most usual procedure for freezing the embryos is controlled freezing. It necessitates expensive equipment and highly qualified personnel. Compared to the controlled freezing, vitrification presents the advantage that is simpler, faster and dose not necessitates expensive equipment.

Vitrification is a simple method of freezing that is used more and more. It's principle consist of the fact that some solutions become solids without forming crystals, when are excessively cooled.

At vitrification, during cooling the solutions become supercooled, because of the high concentration of crioprotectors, they remain liquids but as the temperature drops there is a continuous rise in viscosity, until it doesn't flow on a measurable time scale. This is the moment that the highly viscous solutions become solid's without forming ice crystals. The solidification without crystallization takes place because of the excessive rise in viscosity caused by ultra rapid cooling rates, between 15.000 30.000 °C/min (Liebermann and co. 2003)

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Materials and Methods

As penetrating cryoprotector we used DMSO (MW=78.13 Da, Sigma D5879), 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 DMSO 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 168 solutions were prepared from which:

- 12 solutions with DMSO (1M-6.5M)
- 4 solutions with sucrose (5%, 10%, 15% and 20%);
- 4 solutions with Ficoll 70 (5%, 10%, 15% and 20%);
- 4 solutions with galactose (5%, 10%, 15% and 20%);
- 48 solutions of DMSO with sucrose;
- 48 solutions of DMSO with Ficoll 70;
- 48 solutions with DMSO 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 Discussions

None of the non-penetrating cryoprotector (sucrose, Ficoll 70, and galactose) solutions vitrify on freezing.

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

When combinations of two cryoprotectors were used a great number of solutions vitrify on freezing.

In table 1, are presented the results from thawing the solutions from DMSO-sucrose combinations that vitrify during freezing:

Table 1

The behavior at thawing of the solutions with DMSO and sucrose

Crt. No	DMSO concentration (mol/liter)	DMSO molecular weight (Da)	Sucrose concentration (%)	Thawing temperature		
				20°C	25°C	37°C
1	3.5	78.13	15	R	R	R
2	3.5	78.13	20	R	R	R
3	4	78.13	15	R	R	R
4	4	78.13	20	R	R	R
5	4.5	78.13	10	R	R	R
6	4.5	78.13	15	R	R	R
7	4.5	78.13	20	R	R	R
8	5	78.13	5	R	R	R
9	5	78.13	10	R	R	R
10	5	78.13	15	R	R	R
11	5	78.13	20	R	R	R
12	5.5	78.13	5	R	R	R
13	5.5	78.13	10	R	R	R
14	5.5	78.13	15	R	R	R
15	5.5	78.13	20	R	R	R
16	6	78.13	5	R	**V**	**V**
17	6	78.13	10	R	**V**	**V**
18	6	78.13	15	R	**V**	**V**
19	6	78.13	20	R	**V**	**V**
20	6.5	78.13	5	R	**V**	**V**
21	6.5	78.13	10	R	**V**	**V**
22	6.5	78.13	15	**V**	**V**	**V**
23	6.5	78.13	20	**V**	**V**	**V**

Note: Da=Daltons *R – solutions that formed ice crystals at thawing*

V- solutions that remained vitreous at thawing

From table 1, it can be noticed that in combination with 5%sucrose DMSO is vitrifying at 5M. at a 10% sucrose concentration DMSO vitrifies at 4.5M. When mixed with 15% or 20% sucrose DMSO vitrifies at 3.5M. From the 23 solutions

that vitrified at freezing, at thawing, at 20°C, 2 did not formed ice crystals (DMSO 6,5 M + 15% sucrose; DMSO 6,5M + 20% sucrose). When thawing was performed at 25°C, 8 of the solutions did not form ice crystals (DMSO 6M + 5% sucrose; DMSO 6M + 10% sucrose; DMSO 6M + 15% sucrose; DMSO 6M + 20% sucrose; DMSO 6,5 M + 5% sucrose; DMSO 6,5 M + 10% sucrose; DMSO 6,5 M + 15% sucrose DMSO 6,5 M + 20% sucrose). At 37°C the results were the same as for 25°C.

In table 2, are presented the results from thawing the solutions from DMSO-Ficoll 70 combinations that vitrify during freezing:

Table 2

The behavior at thawing of the solutions with DMSO and sucrose

Crt. No	DMSO concentration (mol/liter)	DMSO molecular weight (Da)	Ficoll 70 concentration (%)	Thawing temperature		
				20°C	25°C	37°C
1	3.5	78.13	20	R	R	R
2	4	78.13	15	R	R	R
3	4	78.13	20	R	R	R
4	4.5	78.13	5	R	R	R
5	4.5	78.13	10	R	R	R
6	4.5	78.13	15	R	R	R
7	4.5	78.13	20	R	R	R
8	5	78.13	5	R	R	R
9	5	78.13	10	R	R	R
10	5	78.13	15	R	R	R
11	5	78.13	20	R	R	R
12	5.5	78.13	5	R	R	R
13	5.5	78.13	10	R	R	R
14	5.5	78.13	15	R	R	**V**
15	5.5	78.13	20	R	R	**V**
16	6	78.13	5	R	R	R
17	6	78.13	10	R	R	R
18	6	78.13	15	**V**	**V**	**V**
19	6	78.13	20	**V**	**V**	**V**
20	6.5	78.13	5	R	R	R
21	6.5	78.13	10	**V**	**V**	**V**
22	6.5	78.13	15	**V**	**V**	**V**
23	6.5	78.13	20	**V**	**V**	**V**

From table 2, it can be noticed that in combination with 5 and 10% Ficoll 70, DMSO is vitrifying at 4.5M. When the concentration of Ficoll 70 is 15% DMSO is vitrifying at 4M. At 20 % Ficoll 70 DMSO vitrifies at 3.5M. From the 23 solutions that vitrified on freezing, at thawing, at 20°C or 25°C, five solutions did not formed ice crystals (DMSO 6M + 15% Ficoll; DMSO 6,5M + 20% Ficoll; DMSO 6,5M + 10% Ficoll; DMSO 6,5M + 15% Ficoll, DMSO 6,5M+ 20% Ficoll). At thaing at 37°C 7 solutions did not formed ice crystals (DMSO 5,5M + 15% Ficoll; DMSO 5,5 M + 20% Ficoll; DMSO 6M + 15% Ficoll; DMSO 6,5M +

20% Ficoll; DMSO 6,5M + 10% Ficoll; DMSO 6,5M + 15% Ficoll, DMSO 6,5M+ 20% Ficoll).

In table 3, are presented the results from thawing the solutions from DMSO-sucrose combinations that vitrify during freezing:

Table 3

The behavior at thawing of the solutions with DMSO and galactose

Crt. No	DMSO concentration (mol/liter)	DMSO molecular weight (Da)	Galactose concentration (%)	Thawing temperature		
				20°C	25°C	37°C
1	4	78.13	15	R	R	R
2	4	78.13	20	R	R	R
3	4.5	78.13	10	R	R	R
4	4.5	78.13	15	R	R	R
5	4.5	78.13	20	R	R	R
6	5	78.13	5	R	R	R
7	5	78.13	10	R	R	R
8	5	78.13	15	R	R	R
9	5	78.13	20	R	R	**V**
10	5.5	78.13	5	R	R	R
11	5.5	78.13	10	R	R	R
12	5.5	78.13	15	R	R	**V**
13	5.5	78.13	20	**V**	**V**	**V**
14	6	78.13	5	R	R	**V**
15	6	78.13	10	**V**	**V**	**V**
16	6	78.13	15	**V**	**V**	**V**
17	6	78.13	20	**V**	**V**	**V**
18	6.5	78.13	5	**V**	**V**	**V**
19	6.5	78.13	10	**V**	**V**	**V**
20	6.5	78.13	15	**V**	**V**	**V**
21	6.5	78.13	20	**V**	**V**	**V**

From table 3, it can be noticed that in combination with 5% galactose DMSO vitrifies at a concentration of 5M. at a concentration of 10% galactose, DMSO vitrifies at 4.5M. When combined with 15% or 20% galactose a 4M concentration of DMSO is sufficient for vitrification. At thawing, at 20°C, from the 21 solutions that vitrified, 8 did not form ice crystals (DMSO 5,5M + 20% galactose; DMSO 6 M + 10% galactose; DMSO 6M + 15% galactose; DMSO 6 M + 20% galactose; DMSO 6,5M + 5% galactose; DMSO 6,5M + 10% galactose; DMSO 6,5M + 15% galactose; DMSO 6,5M + 20% galactose). At 25°C thawing temperature the results were the same as for 20°C. When thawed at 37°C, 11 solutions didn't form ice crystals (DMSO 5M+ 20% galactose; DMSO 5,5M + 15% galactose; DMSO 5,5M + 20% galactose; DMSO 6 M + 5% galactose; DMSO 6 M + 10% galactose; DMSO 6M + 15% galactose; DMSO 6 M + 20%

galactose; DMSO 6,5M + 5% galactose; DMSO 6,5M + 10% galactose; DMSO 6,5M + 15% galactose; DMSO 6,5M + 20% galactose).

From analyzing the tables 1, 2 and 3, it can be noticed that the mixtures of penetrating (DMSO) and nonpenetrating cryoprotectors (sucrose, Ficoll 70 and galactose) lead to a decrease of the concentration of penetrating crioprotector necessary for vitrifying the freezing media. From the nonpenetration cryoprotectors used, sucrose proved to be the most efficient in decreasing the concentration of DMSO. When sucrose was used in a concentration of 15% the media vitrified at 3.5M DMSO, in case of Ficoll 70 it was necessary a concentration o 20% for vitrifying at freezing the media with 3.5M DMSO. In case of galactose, none of the tested concentrations led to vitrify, at freezing, the media with 3.5M DMSO.

Also from the tables 1, 2 and 3 there can be seen the importance of thawing temperature, in preventing the formation of ice crystals. At 20°C, thawing temperature from the 144 solutions with DMSO and nonpenetrating crioprotector tested, 15 solutions didn't form ice crystals. At 25°C, 21 combinations didn't form ice crystals. When thawing temperature was 37°C we obtained 26 combinations that didn't form ice crystals. It can be noticed that once the thawing temperature is raised the DMSO concentration, at which there are no ice crystals, lowers. At 20°C and 25°C thawing temperature the DMSO concentration necessary for preventing media recrystallization of the media is 5.5 M (see table 3) and at 37°C is 5M.

Conclusions

1. Un-penetrating cryoprotectors (sucrose, Ficoll 70 and galactose) used singly can not accomplish the vitrification during freezing;
2. Although DMSO can, singly, vitrify during freezing starting at 5M, at thawing the solution devitrified and can not be recommended as proper vitrification media;
3. The DMSO-sucrose and DMSO-Ficoll 70 combinations have a better vitrification capacity compared to the DMSO-galactose combinations. For the DMSO-sucrose and DMSO-Ficoll 70 we obtained 23 combinations that vitrify at freezing, while for DMSO-galactose we obtained 21 combinations.
4. Unpenetrating cryoprotectors added to vitrification media, reduce the concentration of DMSO required for vitrification of the media, the best results were obtained with sucrose 15%, the DMSO concentration required for vitrification was 3.5M;
5. At thawing, the best results were obtained for the DMSO-galactose combinations. At 37°C 11 combinations (DMSO 5M+ 20% galactose; DMSO 5.5M + 15% galactose; DMSO 5.5M + 20% galactose; DMSO 6 M + 5% galactose; DMSO 6 M + 10% galactose; DMSO 6M + 15% galactose; DMSO 6 M + 20% galactose; DMSO 6.5M + 5% galactose; DMSO 6.5M + 10% galactose; DMSO 6.5M + 15% galactose; DMSO 6.5M + 20% galactose) didn't formed ice crystals. For the combination DMSO-sucrose there were 8 combinations that didn't formed ice crystals (DMSO 6M + 5% sucrose; DMSO 6M + 10% sucrose; DMSO 6M + 15% sucrose; DMSO 6M + 20% sucrose; DMSO 6.5 M + 5% sucrose;

DMSO 6.5 M + 10% sucrose; DMSO 6.5 M + 15% sucrose DMSO 6.5 M + 20% sucrose) and for DMSO-Ficoll 70 there were 7 combinations (DMSO 5.5M + 15% Ficoll 70; DMSO 5.5 M + 20% Ficoll70; DMSO 6M + 15% Ficoll70; DMSO 6.5M + 20% Ficoll70; DMSO 6.5M + 10% Ficoll70; DMSO 6.5M + 15% Ficoll70, DMSO 6.5M+ 20% Ficoll 70);

6. For thawing the DMSO media the 37°C was the best temperature. From the 144 media tested, at thawing at 37°C, 26 of the media didn't formed ice crystals at thawing. At 25°C, 21 of the mixtures didn't formed ice crystals and for 20°C thawing temperature 15 mixtures.

7. Fallowing the freezing/thawing experiments with DMSO as nonpenetrating cryoprotector we obtained 26 solutions that can be used as vitrification media for mammalian embryos (DMSO 5M+ 20% galactose; DMSO 5,5M + 15% galactose; DMSO 5,5M + 20% galactose; DMSO 6 M + 5% galactose; DMSO 6 M + 10% galactose; DMSO 6M + 15% galactose; DMSO 6 M + 20% galactose; DMSO 6,5M + 5% galactose; DMSO 6,5M + 10% galactose; DMSO 6,5M + 15% galactose; DMSO 6,5M + 20% galactose; DMSO 6M + 5% sucrose; DMSO 6M + 10% sucrose; DMSO 6M + 15% sucrose; DMSO 6M + 20% sucrose; DMSO 6,5 M + 5% sucrose; DMSO 6,5 M + 10% sucrose; DMSO 6,5M +15% sucrose DMSO 6,5 M + 20% sucrose; DMSO 5,5M + 15% Ficoll 70; DMSO 5,5 M + 20% Ficoll 70; DMSO 6M + 15% Ficoll 70; DMSO 6,5M + 20% Ficoll 70; DMSO 6,5M + 10% Ficoll 70; DMSO 6,5M + 15% Ficoll 70, DMSO 6,5M+ 20% Ficoll 70)

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