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Cryopreservation of mouse morulae in glycerol, sucrose and honeybee royal jelly *

Criopreservação de mórulas de camundongos em glicerol, sacarose e geléia real

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SUMMARY

Compacted mouse morulae were frozen at 0.3°C/minute or 0.5°C/minute from –6°C to –24°C or –32°C in 10% of glycerol plus different sucrose concentrations with or without 0.1% of honeybee royal jelly. Embryos were thawed in water bath at 22°C for 20 seconds and cryoprotectant dilution was done in three steps. Embryos were cultured in Whitten's medium for 24, 48 and 72 hours at 37°C, 5% of CO_2 and 100% of humidity. The *in vitro* development ranged from 56.6% to 100% after 72 hours. Expanded blastocysts were transferred to pseudopregnant recipients on the third day of the estrous cycle. Viable fetuses rates for embryos frozen to –24 or –32°C at 0.3°C/minute in 10% glycerol + 10% sucrose, 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly, 10% glycerol + 0.1% honeybee royal jelly or 10% glycerol were respectively: 28.1% and 13.6%, 48.7% and 31.9%, 28.6% and 13.2%, 20.0% and 42.4%. Viable fetuses for embryos frozen to –24°C or –32°C at 0.5°C/minute in 10% glycerol + 10% sucrose or 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly were respectively 29.0% and 15.3%, 48.8% and 32.0%. We can conclude that addition of 10% sucrose to 10% glycerol was efficient for embryo freezing at 0.3 or 0.5°C/minute and plunged in liquid nitrogen at –24°C. The honeybee royal jelly addition provided higher viable fetuses rates when embryos were cooled at 0.3 or 0.5°C/minute and plunged in liquid nitrogen at –24°C.

UNITERMS: Mice; Embryo; Cryopreservation; Sucrose; Royal jelly.

INTRODUCTION

The embryo freezing techniques in liquid nitrogen allowed the widespread practice of embryo transferring and trading between distant regions, diminishing the cost and sanitary risk[5].

Jackowski *et al.*[6] noted that a high survival rate for most mammalian cells frozen at below zero temperatures needs the presence of cryoprotectants and that the influx kinetics of these substances can be influenced by the stage of embryo development and the temperature[10]. The cryoprotectant decreases the damage of the solution effect at low temperatures, by diminishing the intracellular solutes and cell dehydration.

Many factors must be analyzed when the embryos are in different cryoprotectant solutions before freezing, like the time and temperature of equilibrium, the toxicity and concentration of the cryoprotectant and the stage of embryo development[8].

Bürkle *et al.*[3] studied the effect of glycerol associated with honeybee royal jelly in freezing mice embryos. They verified that embryonic development, implantation and fetuses rates larger in the group with 0.1% of honeybee royal jelly and 10% of glycerol, due to cellular membrane stabilization.

Visintin *et al.*[13] froze mouse morulae in four solutions containing glycerol plus sucrose and honeybee royal jelly. They verified that the addition of 0.1% of honeybee royal jelly in the cryoprotectant solution containing 10% of glycerol or 10% of glycerol plus 10% of sucrose resulted in better blastocysts and fetuses rates. But no potential effect of the honeybee royal jelly was noted on the freezing solution containing 10% of glycerol and 10% of sucrose.

Dobrinski[4] observed that pregnancy rates of frozen embryos are not equivalent with non frozen embryos. This difference is related to the destruction of embryonary cell

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architecture and its cytoskeleton during the freezing process, due to intracellular ice formation, damaging the plasmatic membrane.

According with Leibo[7], embryos have high concentration of cryoprotectant during thawing, which must be removed to prevent the excessive water influx that causes osmotic shock.

Then this removal may be done stepwise with decreasing cryoprotectant concentration or addition of sucrose in the medium, which decreases the number of steps[9], increasing post-thawing embryonic survival rates.

The sucrose allows good diffusion of the cryoprotectant and controls the increase of cellular volume during cryoprotectant removal, keeping the equilibrium of the initial extracellular osmolarity[12].

The association between glycerol and sucrose was employed by Williams; Johnson[14], which indicated that 2.0M of glycerol plus 0.5M of sucrose presented 84% of embryo development.

The objective of this research was to find an association of different cryoprotectants that allows the increase of freezing speeds, achieving high pregnancy rates and simplifying embryo transfer technology in the field.

MATERIAL AND METHOD

Cross-bred mice from CB6F1/Han lineage were superovulated with 5IU of PMSG (15PM) and 5IU of hCG 46 hours later (13PM). The mice were matted just after hCG injection and observed the vaginal plug in the following day (D.1) at 7AM.

On the third day (D.3) embryos were recovered and classified based in Agrawala[1].

In the Experiment I, to test the *in vitro* development, the excellent compacted morulae were frozen in 8 different groups at 0.3°C/minute from –6°C to –24°C (I = 10% glycerol; II = 10% glycerol + 0.1% honeybee royal jelly; III = 10% glycerol + 2% sucrose; IV = 10% glycerol + 4% sucrose; V = 10% glycerol + 6% sucrose; VI = 10% glycerol + 8% sucrose; VII = 10% glycerol + 10% sucrose and VIII = Control). In the Experiment II, to test the *in vivo* development, the embryos were frozen in different groups: 3 at 0.3°C/minute and 3 at 0.5°C/minute from –6°C to –24°C or 3 at 0.3°C/minute and 3 at 0.5°C/minute from –6°C to –32°C (XI = 10% glycerol + 10% sucrose; XII = 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly; XIII = 10% glycerol + 0.1% honeybee royal jelly; XIV = 10% glycerol; XV = 10% glycerol + 10% sucrose and XVI = 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly).

For freezing, embryos were held in medium from 10-15 minutes, loaded in straws, plunged in alcohol at –6°C and kept in this temperature for 5 minutes for equilibrium. After seeding, temperature was decreased at 0.3°C/minute or 0.5°C/minute from –6°C to –24°C or –32°C, when straws where plunged in liquid nitrogen.

The embryos were thawed in water bath (22°C) for 20 seconds and the cryoprotectants removed in three decreasing glycerol concentration solutions (6.6%; 3.3% and 0.0%) with sucrose (10%).

The embryos were cultured in Whitten medium at 37°C, 5% of CO_2 and 100% of humidity for 24, 48 and 72 hours (Experiment I) and for 24 (Experiment II).

The *in vitro* development was considered when the embryos arrived the early blastocyst, blastocyst, expanded blastocyst and hatching blastocyst stages (Experiment I).

Expanded blastocysts were transferred to recipients to evaluate implantation, resorption, and fetuses rates (Experiment II). The recipients were sacrificed at 11 days after embryos transfer.

The statistical analysis was done by Chi-square[2] when comparing two different treatments and analysis of variance (ANOVA) and Tukey's test[11] when comparing three or more treatments (α = 0.05).

RESULTS

The results of morphological evaluation of compacted morulae frozen at 0.3°C/minute from –6°C to –24°C in different

Table 1

Development of embryos frozen at 0.3°C/minute from –6°C to –24°C in different cryoprotectant solutions after 24, 48 and 72 hours in culture, São Paulo, 1999.

In vitro development		I n=378	II n=396	III n=318	IV n=388	V n=497	VI n=397	VII n=430	VIII n=500
24 hours	n	288	310	210	300	326	367	415	395
	%	76.2^c	78.3^b	66.0^e	77.3^c	69.8^d	92.4^a	96.5^a	79.0^b
48 hours	n	320	343	210	335	415	367	430	468
	%	84.7^f	86.6^e	66.0^g	86.3^e	88.9^d	92.4^c	100.0^a	93.2^b
72 hours	n	325	340	180	335	425	367	430	475
	%	86.0^d	85.9^d	56.6^e	86.3^d	91.0^c	92.4^c	100.0^a	95.0^b

I – 10% glycerol; II – 10% glycerol + 0.1% honeybee royal jelly; III – 10% glycerol + 2% sucrose; IV – 10% glycerol + 4% sucrose; V – 10% glycerol + 6% sucrose; VI – 10% glycerol + 8% sucrose; VII – 10% glycerol + 10% sucrose; VIII – Control. Different letters in the same column means statistical difference (α = 0.05).

Table 2
Implantation, resorption and fetuses rates of embryos frozen at 0.3°C/minute from –6°C to –24°C, São Paulo, 1999.

Group	Embryos	Implantation		Resorption		Fetuses	
	N	n	%	N	%	n	%
XI	185	111	60.0[a]	59	31.9	52	28.1[b]
XII	150	97	64.7[a]	24	16.0	73	48.7[a]
XIII	170	65	38.2[b]	30	17.6	35	28.6[b]
XIV	190	75	39.5[b]	37	19.5	38	20.0[b]

XI – 10% glycerol + 10% sucrose; XII – 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly; XIII – 10% glycerol + 0.1% honeybee royal jelly; XIV – 10% glycerol. Different letters in the same column means statistical difference (α = 0.05).

Table 3
Implantation, resorption and fetuses rates of embryos frozen at 0.5°C/minute from –6°C to –24°C, São Paulo, 1999.

Group	Embryos	Implantation		Resorption		Fetuses	
	n	n	%	N	%	n	%
XV	200	128	64.0[a]	70	35.0	58	29.0[b]
XVI	215	132	61.4[a]	27	12.6	105	48.8[a]

XV – 10% glycerol + 10% sucrose; XVI – 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly. Different letters in the same column means statistical difference (α = 0.05).

Table 4
Implantation, resorption and fetuses rates of embryos frozen at 0.3°C/minute from –6°C to –32°C, São Paulo, 1999.

Group	Embryos	Implantation		Resorption		Fetuses	
	n	n	%	N	%	n	%
XI	125	49	39.2[c]	32	25.6	17	13.6[b]
XII	135	97	71.8[a]	54	40.0	43	31.9[b]
XIII	175	64	36.5[c]	41	23.4	23	13.2[b]
XIV	170	97	57.1[b]	25	14.7	72	42.4[a]

XI – 10% glycerol + 10% sucrose honeybee royal jelly; XIII – 10% glycerol + 0.1% honeybee royal jelly; XIV – 10% glycerol. Different letters in the same column means statistical difference (α = 0.05).

Table 5
Implantation, resorption and fetuses rates of embryos frozen at 0.5°C/minute from –6°C to –32°C, São Paulo, 1999.

Group	Embryos	Implantation		Resorption		Fetuses	
	n	n	%	N	%	n	%
XV	190	83	43.7[b]	54	28.4	29	15.3[b]
XVI	200	133	66.5[a]	69	34.5	64	32.0[a]

XV – 10% glycerol + 10% sucrose; XVI – 10% glycerol + 10% sucrose + 0.1% honeybee royal jelly. Different letters in the same column means statistical difference (α = 0.05).

cryoprotectant solutions and cultured *in vitro* for 24, 48 and 72 hours can be observed in Tab. 1.

Tab. 2 and 3 show implantation, resorption and fetuses rates of embryos frozen at 0.3°C/minute and 0.5°C/minute from –6°C to –24°C in different cryoprotectant solutions, thawed and cultured *in vitro* during 24 hours.

Tab. 4 and 5 show implantation, resorption and fetuses rates of embryos frozen at 0.3°C/minute and 0.5°C/minute from –6°C to –32°C in different cryoprotectant solutions, thawed and cultured *in vitro* during 24 hours.

DISCUSSION

The permeability to the cryoprotectant influences the cellular response to the freezing and thawing process. This happens because embryos permeated with cryoprotectant can survive in faster freezing speeds.

The association between glycerol and sucrose was employed by Williams; Johnson[14], which indicated the solution of 2.0M of glycerol plus 0.5M of sucrose as being the best combination, presenting 84% of embryo development.

The interruption of temperature decrease around –30°C to –35°C presents a good equilibrium between cell dehydration and intracellular ice formation, resulting in high embryonic survival rates during thawing[8].

Different sucrose concentrations were added to the cryoprotectant solution with 10% of glycerol to cause embryo dehydration before freezing. This is necessary because at 0.3°C/minute or 0.5°C/minute some intracellular water remains, what causes intracellular ice formation and consequently embryo damage when plunged in liquid nitrogen at –24°C or –32°C.

The addition of 0.1% honeybee royal jelly to the cryoprotectant solution had the objective of enhancing the embryo survival rates. Bürkle *et al.*[3] mentioned that these substances reduce the non lethal damage to the embryos during the freezing process. This may be due to the stabilization of the cell membrane noticed in the implantation and fetuses rates at –24°C (Tab. 2 and 3).

The successfully frozen embryos may suffer irretrievable damage during cryoprotectant removal[7], in this study, the cryoprotectant solution was removed stepwise[3,9], at a 22°C temperature, using a 10% sucrose solution added to decreased glycerol concentration (6.6%; 3.3% and 0.0%). Excellent results were noted on embryo recovery and development rate after culture (Tab. 2 and 3).

The addition of sucrose in the solution containing decreased glycerol concentration or not[6,10] allows good diffusion of the cryoprotectant and controls the increase of cellular volume during cryoprotectant removal. The ideal concentration of sucrose for cryoprotectant removal is dependent on cryoprotectant concentration during freezing[12].

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