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Effects of cryoprotectant and plunging temperature in liquid nitrogen on the *in vitro* and *in vivo* development of murine morulae

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Efeitos do crioprotetor e da temperatura de imersão em nitrogênio líquido sobre o desenvolvimento *in vivo* e *in vitro* de mórulas de camundongos

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SUMMARY

The effects of plunging temperature in liquid nitrogen and cryoprotectant dilution methods were evaluated for compacted mouse morulae frozen in 1.5 M ethylene glycol (E), 1.5M propylene glycol (P) or 1.4 M glycerol (G). Morulae were equilibrated for 10 minutes in cryoprotectant solution and loaded into 0.25 ml straws with cryoprotectant solution in 3 columns (groups E1, P1, G1) or cryoprotectant in the center and PBS in the lateral columns (E2, P2). Straws were cooled at 0.5°C/min to -25 or -30°C and plunged into liquid nitrogen. Straws were thawed in water at 22°C for 20 seconds. Cryoprotectant was diluted in 3 steps for group G1 and in one step for groups E1 and P1 (direct transfer to PBS + 10% FCS) and E2 and P2 (shaken to mix the 3 columns before transferring to PBS+ 10% FCS). Plunging temperature had no significant effect on the proportion of morulae developed to blastocysts *in vitro*; this proportion was higher ($p < 0.0001$) in E1 (69.2%) than in E2 (60.3%), G1 (51.9%) and combined for P1 and P2 (46.9%). In second experiment, the proportion of transferred morulae that developed to viable fetuses was lower ($p < 0.07$) for E1-25 than for E1-30, G1-30, E2-25 or unfrozen (control) embryos (8.7, 20.0, 20.0, 17.4 and 19.8%, respectively). In conclusion, the ethylene glycol diluted directly in PBS (E1) exhibit the highest rate of *in vitro* embryos development, but based on *in vivo* embryos development was more efficacious in plunging temperature at -30°C (E1-30).

UNITERMS: Embryos; Cryopreservation; Glycerol; Ethylene glycol; Propanediols.

INTRODUCTION

Since the first report of the successful freezing embryos was published[45], various cryopreservation methods have been developed[25,30]. Currently, controlled freezing using glycerol as a cryoprotectant and dilution of the glycerol in one or many steps after thawing[2,6,16,31,35,38,39] is the most frequently employed method in the field[14,23,25,31,41,42].

Cryoprotectant dilution inside the straw using glycerol and sucrose[9,18] known as the "one step method", initially was a very attractive technique because it reduced the time spent in cryoprotectant dilution and, consequently, in the transfer of frozen embryos. Additionally, it reduced the equipment necessary for the process, rendering this technique similar to artificial insemination[9,10,41,42]. However, failures in field results and complexity of protocols rendered this technique was not commercially accepted[10,42]. One alternative for this is the use of high permeative cryoprotectants allowing direct transfer to the recipient without previous cryoprotectant dilution[10,42].

The emergent use of ethylene glycol and propylene glycol in the late 1980's stimulated the comparison of these cryoprotectants with glycerol and DMSO in embryo freezing using the step-wise dilution method[6,17,22,24,29]. In the majority of the papers, the ethylene glycol or propylene glycol provided superior results when compared with glycerol or DMSO. In the following years, studies were made to verify the efficiency of ethylene glycol[14,23,41,42] or propylene glycol[35,38] in the direct transfer method as well as the method of loading straws[42].

The use of ethylene glycol and propylene glycol in the direct transfer of embryos has stimulated comparisons with glycerol. Two experiments were conducted to determine the effects of these cryoprotectants, the plunging temperature in

liquid nitrogen and method of post-thaw cryoprotectant dilution on *in vitro* and *in vivo* development of frozen murine morulae.

MATERIAL AND METHOD

Experiment 1

Embryo recovery

Swiss albino females mice in age ranging from 2 to 6 month were superovulated intraperitonially with 5 IU eCG - (Intergonan) and 46-48 hours later, with 5 IU hCG - (Ekluton) and then caged with a Swiss albino male for mating. The next day (day 1), plug positive females were separated and on day 3 in the afternoon sacrificed. The oviduct was flushed with PBS + 1% fetal calf serum (FCS). All compacted morulae, when assessed with a stereomicroscope at 40x magnification, were pooled, washed four times in PBS plus 10% FCS and randomly assigned to 11 groups (Tab. 1).

Table 1
Experimental Freezing Groups. São Paulo, 1997.

Groups	Plunging Temperature in Liquid Nitrogen	Medium in Central Column with the	Medium in Lateral Columns
E1*-30	-30°C	E	E
E2**-30	-30°C	E	PBS
E1-25	-25°C	E	E
E2-25	-25°C	E	PBS
P1-30	-30°C	P	P
P2-30	-30°C	P	PBS
P1-25	-25°C	P	P
P2-25	-25°C	P	PBS
G1-30	-30°C	G	G
G1-25	-25°C	G	G
Nonfrozen			

E: Ethylene glycol; P: Propylene glycol; G: Glycerol; PBS: Phosphate Buffered Saline; * cryoprotectant in the columns; ** cryoprotectant in center and PBS in lateral columns.

Freezing Procedure

The embryos were transferred into cryoprotectant solution, equilibrated for 10 minutes at room temperature (20-26°C) and loaded in 0.25 ml plastic straws (IMV). Straws were placed in a controlled-rate freezing machine (HAACKE) at -5°C, seeded after 5 minutes, decreased the temperature at 0.5°C/min to either -25 or -30°C and five minutes later the straws plunged into liquid nitrogen.

Thawing Procedure and Cryoprotectant Dilution

After a minimum storage period of 2 days, straws were thawed in a water bath at 22°C for 20 seconds.

The embryos frozen in glycerol were expelled, located and the cryoprotectant diluted in step-wise procedure (6.6% glycerol + 10% sucrose, 3.3% glycerol + 10% sucrose) followed by PBS and Whitten's medium, 10 minutes each.

The embryos frozen in ethylene glycol or propylene glycol in the three columns were expelled, located, transferred to PBS and after 10 minutes washed in Whitten's medium. But the straws containing only PBS in their lateral columns were shaken to mix the three columns and the embryos were kept in these solutions for 10 minutes before being transferred to PBS and to Whitten's medium.

Development Embryo Evaluation

After cryoprotectant dilution the morulae were cultured in 10 ml microdrops of Whitten's medium plus 0.4% BSA, which were covered with paraffin oil and placed in an incubator at 37°C, 5% CO_2 and high humidity atmosphere.

The embryos were assessed with a stereomicroscope at 40x magnification at 24, 48 and 72 hours of culture and identified into one of four categories: not found after thawing (NFT); degenerated after cryoprotectant dilution (DCD); degenerated after culture (DAC) or developed to blastocysts stage (expanded or hatched) after culture (BAC). The number of embryos with fracture of zona pelucida was recorded in the DCD category.

Experiment 2

Superovulation of females and embryo collection, freezing and thawing was performed as described in Experiment 1. Five groups were used, which were included 3 groups with the highest percentage of viable embryos in Experiment 1 (E1-25; E1-30 and E2-25), G1-30 as a control freezing group and fresh embryos (as nonfrozen group). Recipients (CAF1 females) were synchronized with IP injections of 2.5 IU eCG, followed 46 to 48 hours later by 2.5 IU of hCG and mated with vasectomized males. On the next day, the females plug positive were selected and used two days later, as recipients. Thawing and cryoprotectant dilution of embryos were performed in Experiment 1. A total of 5 to 12 embryos were transferred into the uterine horns of recipient (3 to 6 per horn). Eleven days after transfer the recipients were sacrificed, the uterine horns were recovered and incised and the number of viable fetuses and resorptions were determined.

Statistical Analysis

Pearson's chi-square homogeneity tests were performed to test the null hypothesis that there were no significant differences in the proportion of morulae that formed blastocysts among the 10 groups of frozen embryos or among all 11 groups

of embryos (including the nonfrozen group control). The probability for each of these tests was $p < 0.0001$ (both null hypothesis were rejected). The conventional approach would be to subsequently conduct pair-wise comparisons. However, with the 10 groups of frozen embryos, there are 45 pairs. Consequently, a specialized analysis appropriate for this situation (Automatic Interaction Detector, AID[3,5]) was used to locate differences among the groups and to identify interactions among the 3 factors (cryoprotectant, plunging temperature in liquid nitrogen and method of cryoprotectant dilution) and the random variable, that is a categorical variable with four possible categories: NFT, DCD, DAC and BAC.

The rates of viable fetuses, resorptions and implantations were calculated and chi-square analyses used to determine if there was an effect of group.

RESULTS

Experiment 1

The distribution of embryos after thawing is shown in Tab. 2. As previously indicated, there were significant differences among groups in the proportion of morulae that developed to blastocysts, for the 10 groups of frozen embryos or for the 11 groups ($p < 0.0001$). For nonfrozen and frozen embryos, 97.6% and 55.5%, respectively, developed to blastocysts (the frozen embryos ranged from 72.2% to 40.6%). The AID analysis indicated that both cryoprotectant and method of cryoprotectant dilution were factors which influenced embryo survival while the two plunging temperatures had no influence. Furthermore, there was no significant difference between Groups P1 and P2 in the proportion of morulae that developed to blastocysts. Therefore, it was possible to combine groups, ultimately resulting in four combined groups, as shown in Tab. 3. The proportion of morulae that developed to blastocysts in treatment E1-25 and -30 (69.2%) was higher ($p < 0.0001$) than in any another combined group.

Table 2

Effects of cryoprotectants, plunging temperatures in liquid nitrogen and cryoprotectant dilutions on the frozen and nonfrozen embryos that developed to blastocists during culture for 72 hours. São Paulo, 1997.

Frozen and Nonfrozen Embryos Groups	Nº of Embryos	NFT (%)	DCD (%)		DAC (%)	BAC (%)
E1-30	126	8.7	11.9	(0.8*)	7.1	72.2
E2-30	115	1.7	5.2	(0.9*)	26.1	66.9
E1-25	124	11.3	7.3	(4.8*)	15.3	66.1
E2-25	127	2.4	3.2	(2.4*)	40.2	54.3
P1-30	107	4.7	14.0	(13.1*)	32.7	48.6
P2-30	133	3.8	19.6	(6.1*)	36.1	40.6
P1-25	110	4.5	15.5	(15.5*)	32.7	47.3
P2-25	104	1.9	18.3	(15.4*)	26.9	52.9
G1-30	103	4.9	10.7	(0*)	35.9	48.5
G1-25	105	13.3	2.9	(2.9*)	28.6	55.2
Nonfrozen	124				2.4	97.6

* Percentage of embryos with fracture of zona pellucida is included in DCR categorie; NFT percentage of compacted mouse morulae not found after thawing; DCD percentage of degenerated embryos after cryoprotectant dilution; DAC percentage of degenerated embryos after culture; BAC percentage of embryos that developed into blastocysts after culture.

Table 3

Separation of Frozen Groups after application of AID technique. São Paulo, 1997.

Combined Groups	Nº	NFT (%)	DCD (%)	DAC (%)	BAC (%)
E1-25 and -30	250	10.0^{b}	9.6^{b}	11.2^{b}	69.2^{c}
E2-25 and -30	242	2.1^{a}	4.1^{cb}	33.5^{a}	60.3^{b}
G1-25 and -30	208	9.1^{b}	6.7^{b}	32.2^{a}	51.9^{ab}
P1, P2 both -25 and -30	454	3.7^{a}	16.9^{a}	32.4^{a}	46.9^{a}

^{a,b,c} = within each column, means with different superscripts are different ($c^2 = 98.56$, d.f.= 9, $p < 0.0001$); NFT = percentage of compacted mouse morulae not found after thawing; DCD = percentage of degenerated embryos after cryoprotectant dilution; DAC = percentage of degenerated embryos after culture; BAC = percentage of embryos that developed into blastocysts after culture.

Experiment 2

The distribution of frozen morulae and the rates of implantation, resorption and fetuses 11 days after transfer is shown in Tab. 4. Implantation and viability rates for Group E1-25 were lower ($p < 0.10$ and $p < 0.07$, respectively) than for the other groups.

Table 4

Number of transferred frozen morulae and mean rates (%) of implantation, resorption and fetuses.São Paulo, 1997.

Groups	Frozen Morulae Transferred (n)	Implantation n (%)	Resorptions n (%)	Fetuses n (%)
Control	91	$32(35.2)^{a}$	14(15.4)	$18(19.8)^{c}$
G1-30	95	$28(29.5)^{a}$	9 (9.5)	$19(20.0)^{c}$
E1-30	105	$31(29.5)^{a}$	10 (9.5)	$21(20.0)^{c}$
E1-25	103	$16(15.5)^{b}$	7 (6.8)	$9\ (8.7)^{d}$
E2-25	109	$27(24.8)^{a}$	8 (7.3)	$19(17.4)^{c}$

^{a,b,c} Within a column, means with different superscripts are different (a, b $p < 0.1$ and c,d $p < 0.07$).

DISCUSSION

Based on development to the blastocyst stage following *in vitro* culture, the best cryoprotectant was ethylene glycol, followed by glycerol and propylene glycol, consistent with many previous studies[6,15,24,38,41,42] but different

from the others[17,32,33]. Although these three cryoprotectants are all permeable and have similar mechanisms of action[8,14], their permeability coefficients differ[4,21,37].

There was no significant effect of the 2 plunging temperatures in liquid nitrogen (-25 and -30ºC) in Experiment 1. However, in Experiment 2, the group E1-25ºC presented inferior fetus rates when compared with the nonfrozen group, G1-30, E1-30 and E2-25 ($p < 0.07$). These results are in accordance with some authors that, using glycerol, observed that the critical temperature for plunging embryos in liquid nitrogen is up to -20ºC provided that a rapid thawing procedure is used[1,8,16,28,39,44]. Lehn-Jensen; Greve[8], using cryomicroscopy and slow cooling of bovine embryos in glycerol, concluded that between -20ºC and -25ºC occurs the most important dehydration in the embryo. Perhaps the high permeability of the embryos to ethylene glycol increases the embryo dehydration allowing plunge at higher temperatures in liquid nitrogen.

Rapid dilution of the cryoprotectant after thawing may be essential. Although many authors have not observed cryoprotectant toxicity at room temperature[29,41], the cryoprotectant becomes harmful to the embryos above 25ºC[9,14,25,36]. This toxicity may be one of the reasons for the *in vitro* differences. Probably the E1-30 and -25 *in vitro* groups had more cryoprotectant dilution than the groups (E2-30 and -25) that were submitted initially to a smaller amount of PBS. Nevertheless, this difference was not observed *in vivo* as G1-30, E1-30 and E2-25 presented similar results. The E1-25 had inferior results suggesting that cryoprotectant dilution inside the straw is advantageous when immersion at –25ºC in liquid nitrogen is adopted.

The percentage of morulae not found after thawing ranged from 1.7 to 13.3%, consistent to those from other authors that ranged from 2.2 to 10.5%[7,40]. These losses can be attributed to the morulae sticking to the straw despite repeated washings to dislodge them. The proportion of morulae degenerated after cryprotectant dilution ranged from 2.9% to 19.6%. The highest incidence occurred when propylene glycol was used, mainly due to a high incidence of fracture of the zona pellucida. Perhaps the rapid thaw (20 seconds in water at 22°C) did not give the morulae enough time to devitrify[11,12,13,19,20], resulting in a higher breakage of vitreous solution[27,32,33].

In this study, there was no difference among the fresh and freezing groups G1-30, E1-30 and E2-25. Similar pregnancy rates between control and freezing groups have already been observed by other authors using mouse embryos and glycerol[7], DMSO[43], ethylene or propylene glycol[22,32]. For bovine embryos, similar pregnancy rates have been observed using ethylene glycol and glycerol[23,41,42]. Nevertheless, some authors have observed superior results with ethylene glycol for this bovine[14] and for goat and sheep embryos[6,17]. The implantation, resorption and fetal development rates for the control group were, respectively, 35.2%, 15.4% e 19.8%. These results are similar to Leal[7] who, after transfer of fresh mouse blastocysts, obtained an implantation rate of 39.3%, resorption rate of 11.9% and fetus rates of 18.2% and lower than Ponzillius *et al.*[26], 34.5%, 31.8% and 19.8%. Stefani[34], following transfer of day 4 mouse blastocysts to recipients with one day asynchrony (day 3) obtained superior results regarding implantation (49%) and fetus rates (41%), while Whittingham[43] after culturing and selecting embryos before transfer, obtained an implantation rate of 92%, resorption rate of 25% and fetuses rates of 67%.

In conclusion, the ethylene glycol diluted directly in PBS resulted in the highest rate of *in vitro* embryos development. Based on *in vivo* embryos development, ethylene glycol diluted directly in PBS was efficacious in plunging temperature at -30ºC, but not at -25ºC.

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RESUMO

Os efeitos das temperaturas de imersão em nitrogênio líquido e dos métodos de remoção dos crioprotetores foram avaliados em mórulas de camundongos congeladas em etilenoglicol (E), propilenoglicol (P) e glicerol (G). Os embriões foram equilibrados em E (1,5M), P (1,5M) ou G (1,4M) por 10 minutos e envasados em palhetas de 0,25 ml com crioprotetor nas três colunas (E1, P1e G1) ou PBS nas colunas das extremidades (E2, P2). As palhetas foram resfriadas a 0,5ºC/minuto até -25 ou -30ºC e imersas em nitrogênio líquido. A descongelação dos embriões foi feita em água a 22ºC por 20 segundos. O crioprotetor dos embriões congelados em glicerol (Gl) foi removido em 3 etapas, dos congelados em etilenoglicol e propilenoglicol com crioprotetor nas 3 colunas (El e Pl) removido diretamente em PBS e dos congelados em etilenoglicol e propilenoglicol com PBS nas colunas das extremidades (E2, P2), após mistura das três colunas dentro da palheta, em PBS. Não houve influência da temperatura de imersão sobre o desenvolvimento embrionário *in vitro*, observando maior taxa ($p < 0,0001$) para El (69,2%) que para E2 (60,3%), Gl (51,9%) e a combinação P1 e P2 (46,9%). Para o desenvolvimento *in vivo*, a taxa de fetos foi menor ($p < 0,07$) para o grupo E1-25 do que para o Controle; Gl–30ºC; El–30ºC e E2–25ºC. Pode-se concluir que *in vitro* o melhor crioprotetor foi o etilenoglicol com remoção direta em PBS e que *in vivo* o etilenoglicol e o glicerol foram semelhantes a -30ºC.

UNITERMOS: Embriões; Criopreservação; Glicerol; Etilenoglicol; Propanodióis.

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