Cryopreservation of late preimplantation stage mouse embryos, with a special emphasis on introducing hydrostatic pressure into the freezing protocol

Thesis of the PhD dissertation

Dr. Csaba Pribenszky

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Szent István University
Postgraduate School of Veterinary Science

Supervisor and Advisory Committee:

Prof. Dr. Cseh Sándor
Department and Clinic of Obstetrics and Reproduction
Szent István University, Faculty of Veterinary Science

Prof. Dr. Solti László
Department and Clinic of Obstetrics and Reproduction
Szent István University, Faculty of Veterinary Science

Prof. Dr. Huszenicza Gyula
Department and Clinic of Obstetrics and Reproduction
Szent István University, Faculty of Veterinary Science

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1. INTRODUCTION

Embryo cryopreservation provides permanent preservation technique for germplasm, breeding line regeneration or proliferation, global genetic transport, increased selection pressure of herd genetics, and methodology for genetic resource rescue.

The aim of the presented experiments were to improve the efficacy of the cryopreservation of embryos being in late preimplantation stages such as expanded, hatching or hatched blastocysts.

The conclusions obtained with mouse embryos are of importance in the future experiments to be conducted with other species as well.

Our experimentations are divided into two separate fields of research:

I. Examination of in vitro survival and in vivo developmental capacity of zona pellucida free, hatched blastocysts cryopreserved by rapid freezing with different cryoprotectants using different freezing and thawing protocols.

II. Study the possible beneficial effects of high hydrostatic pressure (HHP) in the process of embryo-(cryo)preservation based on the following two separate hypothesises:

1. HHP reduces substantially the freezing point of the pressure medium. Based on this phenomenon, we tried to build up a protocol,

through which we would be able to keep and preserve embryos in their optimal culture medium, under pressure, unfrozen even around – 21 °C.

2. HHP induces the production of shock proteins in biological systems. Based on the alleged principle of cross-protection we tried different HHP treatments prior to the procedure of freezing of blastocysts in order to improve their ability to resist the “shock” of freezing.

2. SURVIVAL OF RAPIDLY FROZEN HATCHED MOUSE BLASTOCYSTS

Recently, more attention is paid to the cryobiology of embryos being in later developmental stages such as hatching and hatched blastocyst. In human in vitro fertilization (IVF) to lower the incidence of multiple gestations and improve implantation rates, the embryos are cultured to the blastocyst stage, and transferred at the hatching or hatched stages. Moreover, it is known, that cryopreserved embryos undergo physicochemical changes that could inhibit hatching, thus contributing to unsuccessful conceiving. Consequently in the last years the interest is increasing in freezing of hatched blastocysts.

The objective of our study was to examine the effect of rapid freezing with ethylene glycol (EG) or glicerol (GLY) on the in vitro and in vivo survival of zona-free mouse blastocysts. The influence of different equilibration time prior to freezing and re-hydration methods after thawing was also studied.

Females were superovulated by intraperitoneal injection of 10 IU of PMSG followed by 10 IU of hCG 46 h later. After 6 hours of hCG administration, females were mated with fertile males in monogamous pairs. One to two cell stage embryos (Day 0 and Day 1) were harvested by flushing the oviduct. Embryos were cultured at 37 ^{o}C with 5 % CO_2 and maximal humidity in air in G 1.2-2.2 media under mineral oil until the hatching/hatched blastocyst stage.

Hatching and/or hatched blastocysts on Day 5 (140 -142 h after hCG injection; Day 0 = plug formation) were randomly allocated into different treatment groups, including control group. Embryos were equilibrated in DPBS + 10 % FCS + 0.25 M sucrose containing either 1.5 or 3.0 M EG (Group I.) or 1.5 or 3.0 M glycerol (Group II) for 2, 10, 20 and 30 min at RT. Then, the embryos were loaded in freeze medium into 0.25 ml straws (7-8 embryos/straw) and held 1 cm above the surface of liquid nitrogen in LN_2 vapour for 2 min. Finally, the embryos were immersed into LN_2.

For embryo thawing, the straws were air-thawed for 20 seconds, and then dipped into 22-24 ^{o}C water bath for 2 min. After thawing, the embryos were transferred from the freeze medium to re-hydration medium (DPBS + 10 % FCS + 0.5 M sucrose). After 10 min of re-hydration, embryos were rinsed three times in DPBS supplemented with 10 % FCS (Washing Medium) at RT. Half of the embryos were re-hydrated directly in WM (10 min) at RT. Finally, the embryos were transferred and cultured in G2.2, as above, for 24 hours.

A total of 457 embryos rapidly frozen with EG were recovered after thawing. Embryos frozen in 1.5 M EG showed significantly reduced survival compared to the control ones (57% and 43% vs. 97%; $p<0.01$) and to the embryos frozen with 3M EG (57% and 43% vs. 94%, 84%, 85%, 95% and 98%; $p<0.01$). There was no significant difference amongst the groups where the concentration of the cryoprotectant was 3M and 1.5M. The equilibration and rehidration times only showed significant effect on the survival in combination with the concentration of the cryoprotectant, but not on their own. No significant difference was noticed between the survival rates at thawing, and 12 and 24 hours after thawing at each of the groups.

230 embryos were transferred to 25 recipients, 17 of which became pregnant. At the in vivo studies no groups showed significant difference from the control.

A total of 503 embryos frozen with GLY were recovered after thawing. The survival in all of the groups was significantly reduced compared to the control ($p<0.01$). The cryoprotectant concentration of 1.5 M proved to reduce significantly the survival rate, while the re-hydration time and equilibration time alone had no significant effect.

At the in vivo studies, 190 embryos were transferred to 21 recipients, 11 became pregnant. No result was significantly different from that of the control group; there was no significant difference within the treated groups, either.

Comparing the in vitro survival rates, there was a significant difference between EG and GLY.

There was no significant difference between the different groups frozen with EG and GLY in the in vivo survival rates From the present study, it can be concluded, that zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium, using as short as 2 min equilibration time (that is conveniently enough to load the blastocysts into straws and seal them) at RT. The results

also indicate that the mechanical protection of the zona pellucida (ZP) is no longer needed during freezing in these stages.

3. MODEL EXPERIMENTS ON PRESERVING MOUSE BLASTOCYSTS BY USING HIGH HIDROSTATIC PRESSURE

Cryoinjuries are almost inevitable during the freezing of embryos. The present study examines the possibility of using high hydrostatic pressure to reduce substantially the freezing point of the embryo holding solution, in order to preserve embryos at subzero temperatures, thus avoiding all the disadvantages of freezing. 210 MPa pressure lowers the phase transition temperature of water to -21 °C.

The purpose of the second set of experiments was to study the effect of high hydrostatic pressure treatment prior to freezing to the survival of expanded mouse blastocysts. High hydrostatic pressure has been reported to induce the production of “shock proteins” in bacteria, which can provide a possibility of cross-protection to other environmental stresses. The possible beneficial effects of this alleged principle was examined on embryo freezing: pressure treatment was combined with a cryopreservation protocol.

Blastocysts (produced and cultured as it was described above) were loaded into plastic straws without air-bubbles (7-9 embryos / straw), with M2, then straws were heat-sealed. Straws were placed into the pressure-chamber filled with water as pressure medium. The custom-made pressurizing device, which was capable of providing precisely controlled pressure in the range of 1 to 150 MPa was made of stainless steel with the inner diameter of 2 cm, and was connected to a pressure-gauge. Hydrostatic pressure was

generated by pushing a piston into the pressure chamber through the manual control of screws. Achieving the desired amount of pressure took from 20 seconds to 5 min (10 MPa to 150 MPa, respectively); the duration of pressure release was 3 seconds. At the experiments where the effects of gradual decompression were investigated, release time was between 30-210 min. At experiments conducted at 0°C, the pressure chamber was simmered in the cooling bath of Bio-cool (FTS-Systems, NY, USA).

Embryos were randomly allocated to three groups. Blastocysts of Group I. were cryopreserved as mentioned below, in a vitrification solution containing 7 M EG according to Nowshari and Brem (1998). Embryos of Group II. were treated with 60 MPa pressure for 30 min, then were frozen in the same way. Group III. served as untreated control. After thawing, embryos were cultured in vitro for 24 hours.

Embryos were equilibrated for 5 min in a solution containing 1.5 M EG and 0.25 M sucrose in M2, supplemented with 10 % Fetal Calf Serum (FCS), then transferred into a vitrification solution (7 M EG, 0.5 M sucrose in M2 with 10 % FCS) pre-loaded in a 0.25 ml plastic straw (7-9 embryos / straw). Finally, straws were heat-sealed. After 1 min exposure to the vitrification solution, the straw was slowly immersed in liquid nitrogen. Straws were thawed by transfer into 30 °C water for 30 sec and then the embryos were recovered and placed in rehydration medium (0.5M sucrose in M2 supplemented with 10% FCS) for 5 min. Embryos then were cultured in medium G 2.2 as described above.

In the first set of experiments embryos were exposed to different hydrostatic pressures from 10 to 150 MPa (by 10 MPa increments) for various times (1s, 5 min, 15 min and 30 min to 300 min by 30min), at room temperature. In each group 14-16 embryos were used and each experiment was repeated 3 times.

The treatment exceeding a certain amount of pressure and time (e.g. embryos challenged by 90 MPa for 30 min or 30 MPa for 3h) caused reversible morphological changes. The expanded blastocysts compacted inside the zona pellucida: the blastocoel disappeared, the size of the blastomeres reduced but their structural integrity showed no alteration. After 4-5 hours of in vitro culture these blastocysts re-expanded and hatched from the zona pellucida in 24 hours as controls ($p<0.05$) (a). Embryos receiving less impact (e.g. 90 MPa for 1 s or 30 MPa for 2 h) showed no morphological change and hatched within 24 hours of in vitro culture such as controls ($p<0.05$) (b), while embryos challenged with a greater impact (e.g. embryos challenged by 90 MPa for 2 h or 30 MPa for 5 h) did not re-expand from the compacted stage and disintegrated within 2 hours, or were already disintegrated after decompression (c) The survival rates of the in vitro control groups were between 99 and 97 % (hatching rate: 93-94%) (n=10-12 / group).

For in vivo evaluation, challenged embryos were judged “survived” (a&b) and “dead” (c) after 2 hours of in vitro culture after decompression and were transferred into recipients separately. Out of 170 transferred “a” and “b” embryos, 145 healthy pups were born (85%), but 0 were born from 49

“c” embryos (0%). 36 untreated embryos were transferred as controls, 30 embryos were born (83%).

There were no significant differences between the survival rate, hatching rate (in vitro) and birth rate (in vivo) of the non pressurized control, the compacted and the non-compacted pressurized “a” and “b” embryos ($p<0.05$).

In the second experiment we investigated whether the survival rate of the pressurized embryos could be improved by gradual decompression. Expanded blastocysts were kept at 90 MPa for 30, 60 and 120 min, (where the survival rate at room temperature with instant decompression was 50%, 0% and 0%, respectively) then the pressure was gradually released in 9 steps for 30, 60, 90, 120 and 150 min. The results showed that survival significantly improved by gradual decompression, which had an optimal range depending on the time the embryos had spent under pressure. The optimal come-up time rose the longer the time embryos spent under pressure. The maximal survival rate, achievable by decompression, reduced as the time of the pressurization increased. At in vivo evaluation 54 “survived” and 35 “dead” embryos were transferred to 9 recipients. 47 embryos (87%) implanted out of 54 “survived”, and 0 embryos implanted out of the 35 “dead” embryos at the 18 days dissection. The implantation rate of the “survived” group was not different from the control ($p<0.05$).

In the third part of the experiment the role of temperature was investigated on the survival capacity of the pressurized embryos: 30, 60 and 90 MPa pressure was applied to embryos for 1 sec, 5, 10, 15, 30 and 60 min at low temperature (0 °C). While non-pressurized embryos can live at 0 °C for a substantial amount of time without any significant change in their survival, simultaneous pressure treatment with 30, 60, 90 MPa was lethal for 100 % of the embryos after 45, 10, 5 min, respectively. A significantly reduced survival rate was observed with the embryos pressurized at low temperature compared to the groups treated at room temperature ($P<0.01\%$).

At the in vivo evaluation 40 “survived” and 28 “dead” embryos were transferred to 7 recipients. 34 embryos (85%) implanted out of the 40 “survived”, and 0 embryos implanted out of the 28 “dead” embryos at the 18 days count. The implantation rate of the “survived” group was not different from the control ($p<0.05$).

Embryos kept at 0 °C under 90 MPa pressure for 30 min were also decompressed gradually. No embryos survived at any of the retrieval times we used (30 60, 90, 120, 150, 180 min). 8-12 embryos were used in each group, experiments were repeated for three times.

In the fourth study we explored whether the survival rate of cryopreserved expanded mouse blastocysts could be improved by pressure treatment before the freezing procedure. 30 -39 embryos were used in each experimental group, experiments were repeated 3 times. Significant differences were observed in the survival rate between the pressurized and

non-pressurized groups ($p<0.01$). The re-expansion was faster (4-6 hours vs. 20 hours) and the survival rate was higher (98% vs. 46%) in those embryos that received pressure treatment before cryopreservation. There was no significant difference between the control and the pressure treated group in the survival and hatching rate.

We conclude that gradual decompression of the pressurized embryos significantly increases the survival rate. Pressurization at low temperature (0 °C) significantly reduces the survival capacity of the embryos, and gradual decompression has no effect at this stage. Based on the present experiments the storage of embryos at subzero temperatures under high pressure is not applicable, since the two effects (pressure and low temperature) together significantly reduces survival even at 0°C. These experiments can serve as examples in the application of this phenomena to other biological material.

The cryopreservation of blastocyst stage mouse embryos had been well-established. Our aim was not to invent another protocol for the cryopreservation of mouse embryos, but to put down a model experiment based on novel bases, which can serve as an example to other cryobiological studies where success rates need to be improved. We showed that embryos can survive a substantial amount of pressure; that the survival of embryos can be improved by gradual decompression, and that 0 °C together with the pressure treatment considerably reduced survival. We also showed, that the applied pressure treatment prior to freezing could improve the in vitro developmental speed, survival and hatching rates of the embryos. Knowledge of this experiment can be useful in improving success rates in embryo-cryopreservation (and embryo-manipulation) of other mammalian species including the human.

Further studies are required to support the deeper understanding of pressure assisted metabolic and structural changes of the pressurized embryos and its role in cross-protection.

4. RESUME OF THE DISSERTATION

From the present study, it can be concluded, that zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium, using as short as 2 min equilibration time (that is conveniently enough to load the blastocysts into straws and seal them) at room temperature. The results also indicate that the mechanical protection of the zona pellucida is not vital during freezing in these stages.

Though our results with EG seem optimal, further experiments should be conducted using larger concentrations of non-permeating cryoprotectants applied either directly or step by step to dehydrate the embryos more before plunging. Pre-freeze equilibration of the embryos in the cryoprotectant solution at 0 °C instead of room temperature may also improve survival. At transfer, numerous embryos have been lost; main reason was that they attached to the wall of the glass pipette. Their shape also changed during moving them in between the drops (culture mediums), and in the straws and transfer pipettes. In my opinion, developing a standardised procedure for the transfer of zona-free blastocysts would improve in vivo success rates.

The cryopreservation of blastocyst-stage mouse embryos is well established. However we chose this developmental stage as a model to study the possible beneficial effects of high hydrostatic pressure (HHP) in the process of embryo-(cryo)preservation. Our investigations in this subject were based on the following two separate hypothesises:

1 HHP reduces substantially the freezing point of the pressure medium. Based on this phenomenon, we tried to build up a protocol, through which we would be able to keep and preserve embryos in their optimal culture medium, under pressure, unfrozen even around – 21 °C.

2 HHP induces the production of shock proteins in biological systems. Based on the alleged principle of cross-protection we tried to treat embryos with HHP prior to the procedure of freezing in order to improve their ability to resist the “shock” of freezing.

The phase change temperature of water decreases with pressure from 0°C at 0.1 MPa to -21°C at 210 MPa and the opposite effect is observed above this pressure level. In the first set of experiments, similarly, we intended to reduce the phase transition temperature of the embryo holding solution through the application of HHP with the aim to preserve embryos in their culture medium at temperatures below 0 °C, for a substantial time without freezing. We calculated that the freezing point of the embryo-holding medium at 200 MPa would be around minus 30°C – 35°C. We postulated that this range of temperature, without all the deleterious effects of the freezing process, would successfully preserve embryos provided, that they tolerate pressure of this magnitude well.

In order to find out if embryos survive at altered pressure conditions we studied the behaviour of embryos under pressure at room temperature. We

found that embryos could survive a substantial amount of pressure without any visible change in their morphology (e.g. 90 MPa for 1 s or 30 MPa for 2 h). The maximum time the embryos survived under pressure reduced (>6 hours to 1 sec) with increasing pressure (from 10 MPa to 150 MPa). We also experienced a reversible morphological change after retrieving the embryos from the pressure chamber: embryos compacted depending on the magnitude and the duration of the applied pressure treatment. As for the next stage, we tried to improve the survival of the pressurized embryos, postulating that the pressure itself is not detrimental, but the sudden change of biophysical and biochemical features during the coming-up. We also intended to observe the effects of the two impacts, namely low temperature and high-pressure environment, jointly.

In the second series of trials, we tried to improve the survival rate of the pressurized embryos by gradual decompression. Our studies showed that the survival rate of the pressurized embryos increased strikingly if they were retrieved gradually. While 60 min at 90 MPa was lethal for all of the embryos, 80 % survived when 120 min gradual decompression was used. These findings convinced us that high pressure itself was not lethal. On the other hand, the survival of embryos pressurized at 0 °C reduced significantly. While at room temperature embryos had an average survival rate of 90% at 30 MPa for 45 min, none of the embryos survived the same impact at 0 °C. After 10 min or 5 min at 0 °C, none of the embryos survived at 60 MPa and at 90 MPa respectively, though at room temperature the survival rate was around 90 % in both cases.

The higher the pressure at a low temperature the less time the embryos survived; consequently we hypothesized that the metabolism of the embryos plays a more significant role in the adaptation to high hydrostatic pressure as the pressure rises.

Embryos pressurized at 0 °C were also decompressed gradually. Assuming that a slowed metabolism (due to low temperature) needs more time to restore the normal atmospheric equilibrium we also tried to lengthen the retrieval times, used successfully in the second experiment. The application of gradual decompression at low temperature did not have a beneficial effect on the embryo-survival.

Based on the present experiments the storage of embryos at subzero temperatures under high pressure is not applicable, since the two effects (pressure and low temperature) together significantly reduces survival even at 0°C.

In the second set of experiments, our aim was to explore the effect of a simple sublethal shock -hydrostatic pressure- to the survival capacity of the expanded mouse blastocysts to another “shock”: cryopreservation. Based on the results of previous publications conducted with microorganisms we postulated that HHP induces the production of shock proteins in the embryos, too. We also postulated, that similarly to the studies with bacteria, these changes would “cross-protect” the embryos at the “shock” of freezing.

Since pressure cannot be directly responsible for squeezing the water out of the blastocysts, we assumed, based on literature data that the compaction of the embryos was due to the consequences of pressure-induced production of different proteins (CSPs, HSPs), reversible alterations in protein structure and metabolic processes. For this reasons only blastocysts, which compacted after pressurization, were used in the experiments. We applied a pressure impact of 60 MPa for 30 min – where approximately 90% of the embryos became compacted and survival was not different from the untreated control. According to the results the applied pressure treatment stunningly improved the in vitro development of the embryos after freezing. After 6 hours 98 % of the pressure treated blastocysts were morphologically (diameter, structural integrity, and general morphology) exactly identical to the control embryos and 95% of the blastocysts fully hatched within 20 hours, together with the controls. Embryos frozen without pressure treatment reexpanded only 20 hours after thawing. The proportion of the re-expanded blastocysts was significantly inferior to those receiving pressure treatment (only 29 % was at least 2/3 expanded). In addition, no embryos hatched from the non-treated group.

(Comment: we also cryopreserved embryos that were not compacted after the pressurization. The developmental speed, survival and hatching rates were reduced compared to the compacted ones. Due to the low number of embryos in this experiment, data is not presented.)

Our results showed that the applied pressure treatment prior to freezing could improve the in vitro developmental speed, survival and hatching rates of the embryos. Knowledge of this experiment can be useful in improving success rates in embryo-cryopreservation (and embryo-manipulation) of other mammalian species including the human. It can also be a model in improving the success rates with other type of cells, such as sperm, oocytes, stem cells, etc.

Further studies are required to support the deeper understanding of pressure assisted metabolic and structural changes of the pressurized embryos. The possible production of shock proteins and their role in cross-protection shall also be proved and studied, since our possibilities unfortunately did not include the tools of molecular biology. Similarly, more experiments are needed with different developmental stages of embryos, different cell types, different pressure/time parameters. Also, experiments should be conducted with expanded blastocysts which are non-compacted after the pressure treatment, because the compaction can also be a result of pressure-altered permeation and diffusion properties: the production of shock proteins may also be independent from this feature (in this case compaction can be a sign of the “sub-lethal zone”).

5. RESEARCH ACHIEVEMENTS

1 Zona-hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3M EG based freezing medium. As short as 2 min equilibration time provides sufficient time for the ethylene glycol to penetrate the cells because of the increased permeability of the hatched blastocyst stage.

2 Introduction of high hydrostatic pressure in the process of cryopreservation.

3 Embryos can survive a substantial amount of pressure without any visible change in their morphology. The maximum time the embryos survive under pressure reduces with increasing pressure. Transferring the survived pressurized embryos, they develop into healthy pups as well as controls.

4 Embryos go through a reversible morphological change after the retrieval from the pressure chamber: they become compacted depending on the magnitude and the duration of the applied pressure treatment.

5 The survival rate of the pressurized embryos increases strikingly if they are retrieved gradually.

6 The survival of embryos pressurized at 0 °C reduces significantly. The higher is the pressure at a low temperature the less time the embryos survive.

7 We showed that pressure treatment prior to freezing stunningly improves in vitro the speed of reexpandation, survival and hatching rates of the embryos.

6. LIST OF PUBLICATIONS

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CS. PRIBENSZKY, S. CSEH, ZS. ABONYI-TÓTH AND L. SOLTI: SURVIVAL OF RAPIDLY FROZEN HATCHED MOUSE BLASTOCYSTS. ZYGOTE 2003. 11. PP. 361-366.

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