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FACULTY OF AGRICULTURAL AND ENVIROMENTAL SCIENCE

PRODUCTION OF RABBIT EMBRYOS PRODUCED *IN VITRO* AND BY MICROMANIPULATION AND DEVELOPMENT OF EMBRYO CRYOPRESERVATION TO SUPPORT THESE METHODS

Thesis

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1. INTRODUTION AND GOALS

1.1. Introduction

The rabbit is an important animal model, which is widely used for studying various human diseases. Because of physio-pathological similarities, it is often used as an animal model for cardiovascular, respiratory and infectious diseases and cancer examination. It has long had a key role in the study of early human embryonic development as well, as in this respect, the rabbit is closer to primates than to rodents. For examination of human diseases, where the genetic cause is known, genetically modified (transgenic) animals can be produced, which are suitable for modeling the disease and testing drugs.

In transgenic animals the genetic material is changed, by means of transferring a new or altered gene or knocking in or out a present gene. This is usually achieved by injecting a gene construct into the pronucleus of the zygote (microinjection), however, this technique does not allow controlled gene integration. A more subitable method is the embryonic stem cell (ESC) technology, where genetically modified animals can be created by homologous recombination. This method has been used for twenty years in mouse, neverthless in rabbits it is not ready yet, and the lack of ESC is a serious obstacle of full utilization of this animal model. Other species such as pigs, sheep or cattle, where the embryonic stem cell technology does not work either, nuclear transfer of genetically modified somatic cell can provide a technical solution to this problem. Thus, the somatic cell cloning, as a technological system, can be an effective tool to create transgenic rabbit in the future.

The efficiency of cloning is quite low and the process requires a large number of oocytes, therefore, the 3R rules should be kept in mind, where the use of *in vitro* maturated oocytes produced from slaughterhouse ovaries are preferred. The practical utilization of *in vitro* maturation may have several significant aspects. For example, if a young woman has cancer, prior to the long-term chemotherapy and radiation, the ovarian tissue can be cryopreserved and stored till the end of therapy, and after recovery, the tissue can be thawed and the oocytes can be matured *in vitro*. After maturation, the fertilized ovum is capable to implant in the mother and develop to term. Oocyte maturation has an important role in animals as well. If the ovaries are removed on time from a dead female of an endangered species, they can be

stored frozen, or extract the oocyte to create gene bank (ex situ gene conservation). Later, these oocytes can be used after thawing and *in vitro* maturation, and if same species recipients and sufficient amounts of sperm are also available, it is possible to create a progeny, which may result in an increase in the number of individuals of the threatened species.

The cryopreservation procedures are generally more efficient on matured, multi-cell embryos. The larger cell number and the structure improve the survival of these embryos. The early rabbit embryo development is uniqe; a mucin coat is accumulated during oviductal transport on the developing embryo, which helps the implantation into the uterus. The *in vitro* produced embryos lack this mucin layer, which cause further complication in transfer and implantation of these preimplantation stage embryos (blastocysts). Thus, early stages of embryos (zygote, 2-4 cell embryos) are transferred into the oviduct for the better conception. Therefore, the cryopreservation of these early rabbit embryos are more more appropriate.

1.2. Objectives of the thesis

• To adapt the somatic cell cloning technology in Hungary and to increase the efficiency of this method.

• The aim of the maturation studies were to develop an effective and successful *in vitro* maturation system in rabbit.

•To test two cryopreservation procedures and to compare their effects on early-stage rabbit embryos.

2. MATERIALS AND METHODS

2.1. The applied rabbit strain

The ovaries which were used in the in vitro maturation experiments were originated from the rabbit slaughterhouse of Lajosmizse runed by Olivia Ltd. For the further experiment, Hycole hybrid rabbits were applied and kept in the animal house of Agricultural Biotechnology Center with 12-hour light program and were placed in individual cages with artificial ventilation. The animal studies had XXVIII/1998 Law § 25 and 243/1998 (December 31) and 36/1999 (April 2) the provisions of Government Decree tooken into account took place.

2.2. The *In Vitro* Maturation (IVM) system

The ovaries from the slaughterhouse to the laboratory were delivered in PBS supplemented with antibiotics and in two different temperatures (32°C and 37°C). After ovary punctions the oocytes were incubated at two different temperatures such as 37 °C or 38.5°C, 98% humidity and 5% CO_2 for the next 16 hours.

In the first series of experiments in one of the groups growth factors (IGF-I (50ng/ml) and EGF (10ng/ml)) and hormones (hCG (5 IU/ml) and PMSG (5 IU/ml)) was added to the TCM-199-based IVM medium, while in another group of the gametes was not used this supplement. In the second series of the experiments 10% and 20% fetal calf serum-containing medium IVM groups were examined with the previous ones. After 16 hours maturation the cumulus cells were removed by use of 0.1% solution of hyaluronidase. For the further physiological experiments (parthenogenetic activation) the oocytes were examined with stereomicroscope, and those were considered matured, where the first polar body was detected. *In vivo* matured oocytes were used as a developmental control. In order to determine the meiotic status, the remaining oocytes were fixed (1:3 mixture of ethyl alcohol and acetic acid, for 24 hours) and stained with 1% Orcein (solve in 45% acetic acid, 3min); than phase contrast microscopy was used to observe the oocyte maturation status.

In the experiments where the dynamics of the maturation process was investigated, the samples were collected every 3 hours during incubation and were fixed and evaluated by the aforementioned method.

2.3. The nuclear tranfer (NT) system

Mature (20-22 weeks old) Hycole hybrid female rabbits were superovulated by the injection of 120 IU PMSG intramuscularly and 180 IU hCG was injected intravenously 72 hours later. Mature oocytes were flushed from the oviducts 13–14 hours after the hCG injection with PBS supplemented with 10% FCS. The oocytes were denuded in 5 mg/ml hyaluronidase M199. The oocytes were incubated in 5μl/ml Hoechst 33342 in culure medium for 20 minutes before the manipulation, than the enucleation were in 7.5 mg/ml cytochalasin-B solution at RT.

The donor cells of the somatic cell nuclear transfer were provided by the surrounding cumulus cells of the oocytes, which were inserted in the perivitelline space of the enucleated oocyte using micropipette. The cytoplast-cell construct were induced to fuse in appropriate orientation by electric pulses. 1 hour later the fused embryos were activated applying the previously electrical pulses and treated for 1 hour with 2 mM 6-DMAP and 5 μg/ml CHX in EBSS-complete medium. The cloned embryos were then subsequently cultured in EBSS-complete medium with or without 5nM TSA for 10 hours long. The parthenogenetic (PGA) control embryos were generated with the activation process of NT.

Two- to four-cell stage nuclear transfer cloned embryos were transferred alone or co-transferred with 3-4 parthenogenetic embryos on the next morning of NT day. 10 to 16 embryos were transferred through the infundibulum into each of the oviduct of the recipients using a laparoscopic technique. The recipients which were induced into pseudopregnancy by i.m. injection of 0.2 ml GnRH analog, 22 hours later following the hCG injection of the oocyte donor females. The pregnancy was detected by palpation on 10th day after the ET. One recipient was allowed to give birth naturally, but the others were operated by caesarean sections, than the live pups were fostered. The pups were measured daily, before and after lactation, and recorded the daily body weight changes.

2.4. Production of *in vivo* and *in vitro* rabbit embryo

The *in vivo* embryos were produced by the naturally mating of superovulated does. The zygotes were obtained as previously described. The in vivo zygotes were culture in EBSS till the cryopreservation experiments.

The *in vitro* embryos were produced by useinf of oocytes of superovulated does and sperm of buks. The semen was collected with IMV artificial vagina. The sperm was centrifuged, and the pellet was resuspending in 10 ml HIS solution and placed into the incubator for 15 minutes. After some washing steps and swim up the sperm was capacitated for 12 hours. The fertilization was occurred in four well dishes (Nunc) in 500 µl sperm and 5 to 6 COC were

co-incubate, for 6-8 hours at 38.5°C, 5% CO_2 and 98% humidity. The zygotes were cleaned after fertilization from the sperm and the remaining cumulus cells. The *in vitro* zygotes were culture in EBSS till the cryopreservation experiments.

2.5. Solid surface vitrification (SSV) method

The SSV methods was used which was originally described by Dinnyes et al. (2000). The vitrification solution consisting 35% EG, 5% polyvinyl-pyrrolidone and 0.4 M trehalose in CZB-H medium. The zygotes were equilibrated in medium consisting of 4% EG in base medium at room temperature for 5-10 min. Following equilibration, groups of 5 to 10 embryos are rinsed three times in small drops of vitrification solution for 25–30 s. They are then either placed into a trehalose solution (Toxicity test) or dropped on the dry surface of a steel cube which was cooled to around -150 to -180°C by partial immersion into LN_2. The drops varying in size between 1 and 2 µl were vitrified. The vitrified droplets were collected in a cryovial. For warming the the droplets were dropped into a 37°C 0.3 M trehalose solution for 1 min. Further steps of rehydration and elution of cryoprotectants consisted of 2 min in each of 0.15 M trehalose, 0.075 M trehalose, and base medium at room temperature, before five washes. Finally the zygotes were cultured. The survival, the developmental and the blastocyst rate was recorded, the embryos were stainded with Hoechst 33342 and the cell nuclei were counted using reverse microscope applying UV excitation.

2.6. Vitrification in straw (VS3a) method

The other vitrification method occurred in straw. The used vitrification protocol was adopted from Kasai et al. (1990) with minor modification. The vitrification solution was consisting 40% EG, 5% polyvinyl-pyrrolidone and 0.4 M trehalose in CZB-H medium (VS3a). The zygotes were equilibrated in medium of 4% EG at room temperature for 5-10 min, than the embryos were loaded into 100% VS3a in a 0.25-ml plastic straws wich were prepared prior to vitrification. The straw was heat sealed and left at room temperature for 1 minute, then held in liquid nitrogen (LN_2) vapor (−150 to −180°C) for 3 minutes and then placed directly into LN_2. Straws were stored in LN_2 (−196°C) until warming. The warming was accomplished by holding the frozen straw for 10 seconds in air; 15-20 seconds in a 37°C water bath, then shaking vigorously to mix the contents within the straw. The sealed end of the straw was cut and its contents emptied into a Petri dish and embryos were recovered and transferred into 0,3 M trehalose and than washed in lower concentration of trechalose for dehydrating. Finally the zygotes were cultured and evaluate as describe above.

2.7. Evaluation of results

GraphPad Instat software was used for statistical analyses. The data were analyzed by χ^2, Welch-corrected in a unique t-test, Mann-Whitney test, Fisher's test and ANOVA analysis. Significance level was considered at $p<0.05$.

3. RESULTS

3.1. The combined effects of the different factors on *in vitro* maturation

During my work *in vitro* matured oocytes were produced from slaughterhouse ovaries. The effect of the *in vitro* maturation system parameters such as **temperature of transport**, **composition of the *in vitro* maturation medium** and **temperature of incubation** were investigated. The ovaries were delivered at 32 °C and 37 °C, and in view of the maturation rate value, the lower temperature of ovary transportation proved to be more effective. The rate of the matured oocytes from different incubation temperatures only showed significant difference when the ovaries were transported at 37°C, in this case the 38.5°C incubation temperature was more successful. The maturation rate of the oocyte from the two different IVM medium resulted no significant difference.

3.2. The effects of the different maturation media on *in vitro* maturation and embryo development

During *in vitro* maturation the usage of four different IVM media did not showed significant different maturation rate among the tested groups. After parthenogenetic activation the oocytes wich were matured in 20% FCS supplement containing maturation medium were less activated than the 10%FCS, and hormone-growth factor containing media matured oocyte groups and even these groups were less activated than the supplement-free group. All IVM oocytes activation rate was lower than in vivo matured control group.

Regard to early embryo development the supplement-free group showed the significantly lowest blastocyst rate. The other blastocyst ratios of the *in vitro* matured groups were higher than this, but none of them reach the value of the *in vivo* group.

3.3. Dynamics of the *in vitro* maturation

In our maturation study under the first three hours of the maturation no matured, MII stage oocytes were found. Between the two experimental groups (10 and 20% FCS) till the six hours of maturation no different was observed in the proportion of matured oocytes

concerned. However between the 9th and 12th hours of maturation, significantly more matured eggs were present in 10% FCS solution containing IVM group than in 20% FCS one. In the subsequent maturation period no other differences were observed in the maturation rate.

3.4. *In vitro* and *in vivo* development of TSA treated SCNT embryos

In our nuclear-transfer experiments cloned rabbit embryos were treated or not with a histone deacetylase inhibition such as TSA, and followed up the *in vitro* and *in vivo* development. *In vitro* results showed that TSA treatment did not affect the SCNT blastocyst rate and cell number either. Similar results were also indicated during *in vivo* observations; no differences were found in the gestation and birth rates respectively. However the cloned offspring birth weight and placenta size was significantly higher in the TSA-untreated group.

3.5. Post natal development of SCNT pups

The rate of postnatal development of the two groups did not differ from each other, but the TSA treatment had an effect on longevity, because none of the TSA-treated animals survived to the age of puberty, while in the control cloned group (non TSA treated) four of six rabbits survived at least 11 months.

3.5. Effect of the parthenogenetic co-transfer

In case of embryo transfer 3-4 parthenogenetic embryos were co-transferred with 25 to 30 cloned ones. Better results were obtained in all of the measured indicators (fertility rate, percentage of all newborns, live offspring rates) when parthenogenetic embryos have also been added to the cloned ones. Live offsprings were obtained in both of the groups, and the later development of individuals was not effected by the fact of co-transfer. Better results were observed in cases of co-transers, but this positive effect was not statistically supported because of the low number of individuals.

3.6. Comparison of two differetnt cryopreservation method on rabbit zygote

In experiments *in vivo* rabbit embryos were cryopreserved with two different vitrification techniques (VS3a vs. SSV). In the first method (VS3a) single straw, while in the other (SSV) tiny drops of medium were used for the witrification. In examination of the toxic effects of cryo protective agents (CPA) of the two cryopreservation procedures, in case of VS3a solution the survival, cleavage and morula rate was lower than the controls, but in proportion to the blastocyst, this difference was not statistically justified. When the two vitrification process methodology (straw vs. small volume vitrification) was compared, the survival and cleavage rate was the same extent weaker compared to the control, however in the subsequent embryo development differences was found between the two procedures, because the blastocyst rate (9%) was much lower in VS3a group than in SSV (43%).

3.7. Comparison of cryopreservation of *in vitro* and *in vivo* rabbit zygotes

The gentler method from the previous experiments (SSV) was also tested on *in vitro* genereted zygotes and similarly good embryo development results was achieved (36%). Only one difference was found in the group of toxicity group of *in vitro* fertilized embryos, such as lower blastocyst cell number was observed what could be explained with the greater sensitivity of the CPA.

4. NEW SCIENTIFIC RESULTS

1. A defined *in vitro* maturation medium was developed which resulted in a 89% maturation rate (MII matured oocyte). After parthenogenetic activation, 82% of them started to develop and 22.7% of them reached blastocyst stage.

2. It was found that during their *in vitro* maturation, rabbit oocytes show different maturation dynamics in the presence of 10 or 20% (v/v) fetal calf serum, which had an effect on subsequent development *in vitro*.

3. The SSV and VS3a vitrification processes were used successfully in cryopreservation of rabbit zygotes. It was the first time these methods were used in rabbit.

4. It was found that rabbit zygotes obtained from *in vitro* fertilization can be cryopreserved with SSV technique with the same efficiency as those obtained from fertilization *in vivo*.

5. Cloned rabbit embryos from first Hungarian adult somatic cell nuclear transfer experiments were successfully transferred with laparoscopic technique and live offspring was produced.

5. DISCUSSIONS

Several publications reported use of *in vitro* matured rabbit oocytes, but often the circumstances are very vaguely described. In many cases, hormone-treated, laboratory animals are used as ovary donor; however, these ovaries are not equivalent with ovaries originated from slaughterhouse. Large percentages of animals are scrapped for slaughter due to reproductive problems, so the use their ovaries can be a challenge. In the first series of the experiments, the parameters of the *in vitro* maturation system were optimized. The transport temperature of ovaries was set to 32°C or 37°C, and the transport at the lower temperature proved to be more effective taking into consideration the rate of maturation. It can be concluded that transporting at body temperature causes greater damage to the oocytes than transporting at lower temperature, probably due to various destructive enzymatic processes in the ovaries facilitated by long (1-2 hours) storage at body temperature.

The oocyte maturation is a complex process; along the nuclear maturation, the cytoplasmic maturation must also be completed to provide the overall function of the cell. Therefore, in the second series of experiments, some of the oocytes were parthenogenetically activated; beside to the nuclear maturation their developmental ability was observed too. Parthenogenetic activation was chosen and not IVF, as to reduce the differences that may arise from sperm quality variation. In respect of activation, differences were found between different maturation media. In addition, embryo development showed weaker results in the non-supplemented IVM group compared to other IVM groups: although nuclear maturation has been completed, but the failed or partial cytoplasmic maturation of the oocyte caused difficulties in later embryo development. When IVM medium containing hormones and growth factors was used, results were similar with medium with FCS supplement. This is useful because using a well-defined medium; the same efficiency can be achieved as with the serum of unknown composition.

The activation of the oocytes is affected by many factors such as the timing of maturation. In the 10% FCS IVM group, most of the cells reached the MII stage in the 9th hour of maturation versus 20% FCS group where it was in the 15th hour only. The oocytes matured earlier had already started the aging process, when the cells were activated (16h), so they activated more easily compared to the oocytes in the 20% FCS group.

In the somatic nuclear transfer (SCNT) trials, *in vitro* and *in vivo* effects of various treatments were investigated on cloned rabbit embryos with a view to improving the

efficiency of the technology. In the first series of experiments, cloned rabbit embryos were treated with a histone deacetylase inhibitor, such as Trichostatin A (TSA) and *in vitro* and *in vivo* development were studied. The *in vitro* results showed that the TSA treatment did not increase the SCNT blastocyst rate and cell number of blastocyst compared to the untreated group. This observation is consistent with data published by other groups. However, results in the literature are contradictory, that can be explained by different concentrations of TSA and treatment time, as well as differences in the cloning protocol, the donor cell type and the differences among embryo culture conditions. Similarly, controversial observations were found in bovine. This suggests that the effect of TSA treatment on in vitro development of SCNT embryos is related to special experimental environment and further optimization of working conditions are required.

In vivo development of TSA-treated and untreated SCNT rabbit embryos was also studied. No difference was detected in the gestation and birth rates between the TSA-treated and untreated groups, and both groups had live offspring. Difference in longevity was observed between the two groups, since TSA-treated embryos from cloned offspring have lived up to 19 days, while the TSA-untreated group, four out of six pups survived and reached sexual maturity. To understand and explain the phenomenon, further experiments are required.

In our experiments, the birth weight and placental size of the cloned offspring was significantly higher in TSA-untreated group. Cloned animals often show the large offspring syndrome (LOS), which manifests in increased organs, water in brain or respiratory disorders. In many cases, the size of placenta is also increased and oedematous, which is the compensation of errors of cloning. In our case, the effect of TSA treatment could not be to established, because of the number of offspring born to one mother are very different from each other, which in itself can cause fetal body weight deviations.

Body weight changes were observed in TSA-treated and untreated groups of offsprings surviving at least 19 days. Apart from the weight loss of the first day, all the pup's weight increased over the time. In the first nine days, both groups had pups below the average of body weight. Based on these observations, the members of TSA treatment group did not suffer in their lack of weight gain and therefore is was not the reason of their death, and not the lower initial body weight was an obstacle of survival and further development.

In the second series of experiments, parthenogenetic (PGA) embryos were transferred (co-transfer) with cloned ones, and examined the effects of pregnancy maintainance and subsequent development. Previous publications showed that oc-transfer of PGA is useful for implantation and pregnancy pregnancy maintainance in cloned pig and mouse. The repair

mechanism with parthenogenetic embryos on in vivo development of the SCNT embryos and the background of beneficial effects of co-transfer is not fully known yet. In nature, during the *in vivo* development, embryo-endometrium interaction and signals are needed from the embryo and extra embryonic membranes to maintain the pregnancy. The parthenogenetic rabbit embryo can develop only for 10-11 days; therefore their usage is beneficial to avoid the post-implantation situation of competing with the weaker clones. Our results suggested that all the investigated parameters, including pregnancy, number of fetus formed, and live birth, were higher in the co-transfer group than in the SCNT embryo transfer control group, but the difference due to the low number of pups could not be confirmed statistically.

In the embryo cryopreservation trials, two vitrification techniques were compared first. As a first step, toxic effects of the cryo protective agents (CPA) were compared. When VS3a solutionwas used, the survival, the proliferation rate and the percentage of morula was lower than the controls, but the observed difference of blastocyst ratio was not statistically different. It can be concluded that the concentration of CPA of VS3a had a slightly negative effect on the early embryo development, but this negative effect in the later stage of embryo was no longer prevailed, so the long-term effects of the two vitrification solutions was not significantly different. When the two vitrification procedures was compared (straw vs. small volume), both the survival and the developmental rate were weaker compared to the control, indicating that both cryopreservation procedure damaged the embryos, resulting in about 50% cleavage. Differences were found between the two vitrification techniques in later embryo development: the blastocyst rate of VS3a group was significantly lower in comparison to those seen in the SSV group. This suggests that the VS3a technique, where straws was used during the cooling, is less suitable for rabbit zygotes cryopreservation than the SSV vitrification with small volume of CPA. The publications of rabbit zygote vitrification in the litterature are scarce and with one exception, fairly low *in vitro* growth data were reported. We can conclude that the procedure applied is an effective cryopreservation technique. Our method was tested on embryos produced *in vitro* (no reference in literature), and similarly good results have been achieved. The only difference was observed in blastocyst cell numbers when the cells numbers of the toxicity group of *in vitro* fertilized embryos were similarly reduced as those of the frozen-thawed embryos. This difference did not exist in the *in vivo* embryos, where the mean cell number of toxicity group embryos was similar to those in the control group. We can conclude that the *in vitro* produced embryos are more sensitive to the CPA than their counterparts *in vivo*, which was shown in lower blastocyst cell number of the *in vitro* group.

6. PUBLICATIONS

Book chapter

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7. ACKNOWLEDGEMENT