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Theses of the PhD dissertation

**THE POSSIBILITY OF THE USE OF IN VITRO EMBRYO PRODUCTION AND PREIMPLANTATION
GENETIC DIAGNOSIS IN CATTLE BREEDING**

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AIMS OF RESEARCH

The dissertation reviews some applied reproduction techniques, which could play an important role in modern cattle breeding. A laboratory protocol of *in vitro* embryo production and an embryo biopsy technique are presented, which was developed for preimplantation genetic diagnosis for cattle embryos applied before embryo transfer. The results of investigation of development of biopsied embryos in embryonic and foetal stage in mouse model are also included.

1. Establishment of a reliable *in vitro* cattle embryo-production technology

Our purpose was to create a technological protocol for production of cattle embryos *in vitro* in different application fields.

- *In vitro* produced embryos could become objects of basic research, in studies of embryo development, embryo cryopreservation or cloning experiments.
- It is possible to use the technology as an element of a MOET system: we can produce transferable embryos originated from oocytes of valuable donor females.
- The *in vitro* embryo production is to serve gene protection. With the application of *in vitro* embryo production, there is a chance to produce embryos from culled animals post mortem. The embryos can be frozen and stored *ex situ* in embryo bank. The genetic heritage of females can also be conserved and it is possible to sustain the genetic variability of a closed, small gene reservoir population *in situ* with the use of embryos against gene erosion.
- In the practice of artificial insemination, the *in vitro* embryo production system can be used for functional characterisation of a sperm sample to determine the *in vitro* fertilisation ability.

The experimental goals are presented with the application fields.

Use of *in vitro* embryo production in gene conservation

- The efficiency of oocyte recovery from ovaries of culled Hungarian Grey heifers and cows were studied.
- In order to increase the efficiency of embryo production, the effect of changing of the culture medium on blastocyst ratio was investigated.

Use of techniques of *in vitro* fertilisation in functional sperm testing

- The positive selection influence of swim up on the motility of sperm cells was studied using an automated motility analyser.
- It was tried to obtain evidence for the reduced motility of a sperm fraction stained specially after Kovács-Foote staining. It was hypothesised that the living sperm cells with the intact acrosome and stained tail are immotile.
- A possible connection between the motility of sperm cells and the *in vitro* fertility was evaluated.

Application of *in vitro* embryo production for applied research

- The *in vitro* development of micromanipulated cattle embryos produced *in vitro* was studied.

Producing embryos *in vitro* for practical use

- Valuable embryos were produced *in vitro* from oocytes obtained by ovum pick up from oocyte donor heifers in a MOET nucleus breeding system. The female embryos after sexing were transferred into recipient females.

2. Developing biopsy methods for preimplantation genetic diagnosis

There is a methodological possibility to analyse embryos genetically before transfer. By analysing the blastomeres obtained from embryos by micromanipulation using PCR or FISH, the sex of embryos can be detected and the embryos can be screened for inherited congenital diseases and genotyped for genes which determine valuable qualities.

Biopsy of cattle embryos *in vitro*

- We were charged with invention of a micromanipulation method for biopsy of *in vitro* produced embryos in precompaction developmental stage.
- The anomalies of their *in vitro* development were compared with the results of mouse model.

Experiments in mouse model

- A new, effective, fast and simple biopsy method was developed for mouse or human precompacted embryos.
- The study of development of biopsied mouse embryos *in vitro*, and *in utero* has also been planned.

BASIS OF THE RESEARCH

Technology of *in vitro* cattle embryo production

The first calf was born in 1981 produced by *in vitro* fertilization. In the experiment, the oocytes, obtained by flushing from oviducts of live animals, were fertilized with fresh bull sperm. After fertilisation, the zygotes were transferred into oviducts of recipient animals surgically. Twenty years ago, the invasive technique was not a reliable alternative possibility beside of the simple, cheap MOET technology. A cheap, effective *in vitro* embryo production system became comparable just after the simplification of oocyte recovery, increasing the quantity of oocytes, and rising of efficiency of fertilization. In the last decade, the purpose of the technological improvement was to produce cheap, but good quality embryos (in quality equal to flushed embryos) *in vitro*. The efficiency of the embryo production system could be improved by enhancing the productivity of each technological steps. The oocytes can be obtained from ovaries of live and culled animals either. Immature oocytes can be collected, which are surrounded with cumulus cells from the follicular fluid, which was aspirated from preantral follicles. After a day length of *in vitro* maturation the oocytes reach the same developmental stage as the oocytes obtained by natural ovulation. Only the oocytes blocked in the second metaphase of meiosis and containing a polar body are capable for propagation. Before fertilisation, the sperm obtained from a thawed straw is pre-treated with different methods (sperm washing, swim up, centrifugation on Percoll gradient) to make free the sperm cells from the extender. The cumulus cells have to be removed from the fertilized oocytes after coincubation of gametes for 22-24 hours. The embryos reach the blastocyst stage in a week of *in vitro* culture.

Preimplantation genetic diagnosis

Mammalian embryos are able to regenerate after embryo-slicing (bisecting, or making quadrates) in the early preimplantation developmental stage, and produce healthy fetuses and progeny after the transfer of demi-embryos. Removing some embryonic cells

(blastomeres) from embryos before compaction does not cause a deleterious effect on development. The totipotent embryonic cells are able to substitute the removed cells, and develop further. It is possible to use the removed cells for genetic analysis. There are different micromanipulation techniques invented to remove the blastomeres. The slicing of a blastocyst is made by microblade or a narrow glass needle. After bisecting the zona pellucida, some trophoblastic cells could be separated in amount 10-30% of the volume of the whole embryo. During micromanipulation, the inner cell mass is untouched and remained intact. After the separation of the trophoblastic cells the rest of the embryo can be transferred into recipient females.

By the technique of microaspiration it is possible to remove one-three blastomeres with a micromanipulator-guided aspirator capillary from a precompacted embryo through a hole (with approximately 30 μm diameter) made on the zona pellucida.

The blastomeres could be analysed genetically by FISH or PCR for sexing the embryos, detect inherited disorders (i.e. BLAD, DUMPS, citrullinaemia, miophosphorilase deficiency, alfa-mannosidosys) or genotyping important major genes (i.e. kappa-kazein).

METHODS OF RESEARCH

1. Methods of in vitro embryo production

Oocyte recovery

Following the slaughtering of animals, the ovaries are collected individually at the slaughterhouse. The organs are washed with physiological salt solution, placed in PBS and kept at body temperature. The transfer to the laboratory lasted three hours. The preantral follicles (size: 2-6 mm) were punctured and the follicular fluid was aspirated by a 18 G Luer needle joined to a 10 ml syringe. The size of the ovaries was measured. The oocytes were collected and counted separately in Petri dishes (3.5 cm diameter) in TALP-HEPES washing medium.

In vitro maturation of oocytes

The oocytes covered by cumulus cells were matured overnight in TCM-199 maturation medium supplemented with 2 $\mu\text{g}/\text{ml}$ FSH, 1 $\mu\text{g}/\text{ml}$ E₂, 10 ng/ml EGF, and 10%FCS (30-40 oocytes/400 μl) in 5%CO₂, 98% relative humidity at 38.5 °C.

Fertilization of matured oocytes

The straws were thawed in a minute in 37°C water bath. The sperm cells were freed from the extender with sperm washing or swim up.

Sperm washing

The content of a straw was diluted with Sperm-TALP to 4 ml at 38.5 °C. The sample in a centrifugation tube was centrifuged for ten minutes with 300g. The sperm cells were sedimented to the bottom of the tube. After removing the supernatant, the cells were diluted with fresh Sperm-TALP again and the centrifugation was repeated. After removing the supernatant the whole sperm fraction concentrated at the bottom of the tube.

Sperm pretreatment - Swim up method

For improving the quality of the substance used for fertilization beside of removing the extender it is possible to select for motile sperm cells. During the swim up method the content of a thawed straw is layered under 1 ml Sperm-TALP medium. The sample is incubated for 60 minutes, while the motile sperm cells swim up into the Sperm-TALP medium. The extender and the dead cells remain at the bottom of the tube. 770 µl of supernatant is removed and diluted with 3 ml Sperm-TALP medium. The tubes are centrifuged for 10 minutes in 300 g. Following that, the supernatant is removed and the motile sperm cells are mixed in 50-100 µl remained Sperm-TALP medium.

Semen qualification

There is a possibility to determine the quality of a sperm sample using measurement of motility or with application of light microscopic staining methods.

The motility of sperm cells was studied by a HTM Motility Analyzer instrument. The motile sperm cells are divided into two classes: sperm of high motility - HMOT, $v > 50 \mu\text{s/s}$, and sperm of low motility - LMOT, $v < 50 \mu\text{s/s}$. With the summary of these groups the ratio of all motile sperm cells (sum motility - SMOT), and the static sperm cells (STAT) can be obtained. It is possible to determine the ratio of sperm cells expressing straight movement (straightness of sperm - STRT), and the velocity (mean progressive velocity -VEL) as well.

For the evaluation of sperm quality by light microscopy the Kovács-Foote staining was used. With the use of Trypan blue, the live/dead state of cells can be detected. The cells

can be classified according to the status of acrosome based on the staining pattern of Giemsa. Seven categories can be distinguished. The acrosome membrane of living cells can be intact, damaged, or the acrosoma can be loose. There is a special category, where the acrosoma is intact, but the tail is stained. It is possible to separate the dead cells according to the acrosoma state: there are cells with intact, damaged acrosoma, or some cells do not have acrosome.

A data input supporting software was developed (DOS and Windows versions) as an aid for the Kovacs-Foote staining. The software makes the cell counting easier with the use of the Num Pad keys for data input. During the input process, the computer gives a feedback for the operator: different staining categories are linked to different sounds. When counting is finished, the DOS version runs a simple statistical analysis of data. Both programs save the results into a txt file that can be converted to MS Excel format for further analysis. The help file contains the manual of use, and the description of the Kovacs Foote staining method. The bilingual (Hungarian/English) software is freeware and can be downloaded from the <http://sperm.abc.hu> information web site.

In vitro fertilisation

The matured oocytes are fertilised in $1 \cdot 10^6$ sperm cell/ml concentration in 22-24 hours in IVF-TALP medium supplemented with 20 $\mu\text{g/ml}$ heparin at 39 °C, in atmosphere of 5% CO_2 .

In vitro embryo culture

Followed by the coincubation of gametes, the fertilised oocytes with cumulus cells are placed in an Eppendorf tube containing 80 μl TALP-HEPES washing medium. The cells are vortexed for one and half minutes for removing of attached cumulus cell from the zona pellucida. The denuded embryos are washed three times in TALP-HEPES and once in the culture medium. The embryos are put into the equilibrated culture drops, and cultured for a week in vitro. In our laboratory, there are two media used for culture. The TCM-199 basic medium is supplemented with 10% FCS and a monolayer is established from cumulus cells on the bottom of the culturing dish for co-culture. Embryos are cultured in 5% CO_2 , at 98% relative humidity at 38.5 °C. The SOF basic medium is supplemented with essential and non-essential amino-acids, citric acid, myoinositol and 5% FCS. The culturing environment has reduced oxygen level (5% O_2) in the air. The result of fertilization was evaluated on Day 3 of

culture with cleavage ratio (cleaved embryos/fertilized oocytes), and the efficiency of culture (based on the ratio of developed blastocysts) was examined on day 7 or on day 8.

2. Methods of preimplantation genetic diagnosis

2.1. Biopsy of cattle embryos

A microaspiration biopsy technique was developed for 8-16 cell stage in vitro produced embryos. The zona is penetrated mechanically.

Making capillaries

The aspirator capillary was made with microgrinding of the pulled capillary and there was a spike made with microforge on the tip of it.

Preincubation of embryos

For eliminating of junctions between the embryonic cells the embryos were incubated in Ca^{2+} - and Mg^{2+} -free PBS+1mg/ml PVA for 20 minutes at 38.5°C.

Steps of biopsy

The biopsy was performed in a micromanipulation chamber (same as the one used for the mouse model) with Leitz or Narishige micromanipulator arms set up on Leitz and Olympus inverted microscopes. The embryo was fixed with the holder capillary. The aspirator capillary penetrated the zona pellucida with the assistance of a spike on the tip, and was placed through the slit. The blastomere was sucked into the aspirator capillary with applied gentle vacuum in it.

In vitro culture of embryos

Following micromanipulation, the biopsied embryos were cultured individually in small SOFaaci +5% FCS medium droplets (10 μl) while the genetic analysis was made, or until the embryos reached the blastocyst stage.

2.2. Study of embryo biopsy on mouse model

Embryo production and in vitro culture

6-8 weeks old C57Bl6/CBA F1 female mice were superovulated by injection of 5 IU PMSG, and 5 IU hCG 46 hours after the first injection. The females were mated with C57Bl6/CBA F1 males, and killed by cervical dislocation on the second day of their pregnancy. The embryos, which has been flushed from the oviduct, were cultured in M16 medium for a day till reaching the 8-cell stage in a CO₂ incubator at 37°C and 98% relative humidity in air.

Method of a new embryo biopsy

In the newly invented biopsy, a hole is created by acidic digestion (zona drilling) on the zona pellucida of the embryo in pre-compaction developmental stage and a blastomere is removed through the hole with an aspirator capillary.

Making capillaries

The holder and drilling capillaries were produced from borosilicate glass tubes with a capillary puller. The holder capillary was broken with the glass bead of the microforge at 50 µm outer diameter and fire polished to 25 µm. The capillary was bended to a 20° angle at 100 µm length from the tip. The zona drilling capillary was broken at 10 µm outer diameter and melted to 6-7 µm inner diameter. The whole capillary was filled with acidic Tyrode solution (AT; pH=2.4).

Preincubation of embryos

For eliminating of junctions between the embryonic cells the embryos were incubated in Ca²⁺- and Mg²⁺-free PBS solution (pH 7.2) for 20 minutes at 37°C.

Steps of biopsy

The biopsy was done in a micromanipulation chamber, which was made from a lid of a plastic Petri dish (3.5 cm diameter). In the dish, 5-8 microdrops (10 µl M2) were covered with paraffin oil. Each droplet contained only one embryo. The micromanipulation was made on a heated stage of Olympus IMT-2 inverted microscope with assistance of hydraulically controlled Narishige micromanipulator arms. The embryo was fixed at 9 o'clock position with vacuum generated in the holder capillary. The zona drilling capillary was gently placed on the

zona pellucida where the membrane of a blastomere touched the largest area of the inner surface of the zona. The tip of the capillary was moved vertically in a 10 μm track to thin the zona pellucida evenly. The flushing AT produced an oval hole with a diameter of 25-30 μm . At this moment, the drilling capillary was removed from the embryo and the vacuum of the holder was interrupted to let the embryo loose. By means of the medium flushing out of the holding pipette and with the assistance of the drilling capillary, the embryo started to rotate. Negative pressure was generated again in the holding pipette, which, along with the aid of the drilling capillary turned the embryo around by 180 degrees. A weak negative pressure helped to aspirate a blastomere very gently, in order to maintain its membrane integrity, through the hole of the zona pellucida into the holder capillary. The drilling capillary was removed from the embryo and the microscope stage was moved to position the holder capillary with the embryo to the edge of the droplet. The stage was moved further and the oil layer separated the blastomere from the embryo. The separated blastomere was flushed from the holder capillary into the medium droplet. Following the successful biopsy, the capillaries were relocated into the next droplet without changing the vertical position of the capillaries using only the movement of the microscope stage. While the holder pipette was kept in fixed position, only the drilling pipette was moved in three dimensions during the whole process.

In vitro embryo culture

Control and biopsied embryos were cultured in M16 culture medium for a day in a humidified atmosphere of 5% CO_2 in air at 37°C. Part of the embryos were used to assess embryonic development on the second day of culture. It was performed by calculating the rate of embryos that developed to the blastocyst stage and determining the total number of nuclei stained with Hoechst 33342 fluorescent dye.

Examination of foetuses

The other part of the embryos was transferred into pseudopregnant recipient C57Bl6/CBA F_1 females. The foetuses were investigated at three checking points of their development *in utero*. The pregnant females were killed on 9th, 13th and 19th day of the conception. The foetuses were 8.5 day old (early somite stage), 12.5 day old (beginning of organogenesis), and 18.5 day old (before birth). Their viability and state of development was evaluated morphologically. Five pregnant females allowed giving birth, and the viability and fertility of the offspring were examined in their adulthood.

MAIN CONCLUSIONS OF THE DISSERTATION

***In vitro* production of cattle embryos**

Study of oocyte retrieval

The size of ovaries obtained from cows was significantly bigger and the amount of follicles were approximately twice than the ovaries of heifers. This difference was not reflected in the amount of obtained oocytes per ovaries. Although, more follicles were found on the surface of cow ovaries, the aspiration of heifer ovaries was more efficient. Based on our data, it can be calculated that 9-10 oocytes can be retrieved from a culled animal, and 2-3 embryos can be produced from them. When embryo bank is to be established from *in vitro* produced embryos, it should be taken into consideration that with the transfer of thawed embryos, 4-7 female calves can be produced from ten culled oocyte donors.

Improvement of in vitro embryo production with application of a new culture medium

Productivity of two culture media was compared based on blastocyst ratio (blastocysts/fertilized oocytes) on the 7th day of *in vitro* culture. The TCM199+10% FCS with granulosa monolayer produced 13% blastocyst. The SOF medium supplemented with 10µl/ml MEM (100x), 30µl/ml BME (50x), 0.5 mg/ml sodium citrate, 0.1 mg/ml myoinositol and 5%FCS resulted in 23.5% blastocyst ratio.

According to the results, we introduced the SOF system in our laboratory for routine *in vitro* embryo culture. (The efficiency of embryo production could be increased using new sperm with better *in vitro* fertility and applying stricter selection of oocytes.)

Application of *in vitro* fertilization for functional analysis of sperm samples

Proving the selection effect of swim up according to motility analysis of sperm cells

According to motility analysis of six bulls before and after swim up, we can conclude that the motility parameters of sperm cells from supernatant differ significantly from the parameters of thawed and pre-treated sample. The ratio of high motility cells (HMOT) and the ratio of whole motile fraction (SMOT) increased. The average velocity of cells increased in each sample after the treatment in each bull as well (VEL). The ratio of cells moving straight was not changed significantly (STRT). The results have proven the positive selection

effect of swim up, because the quality of sperm fraction in the supernatant was significantly improved by the treatment.

Application of swim up treatment for proving the reduced motility of a sperm fraction determined by Kovács-Foote staining

Sperm samples of nine bulls were stained before and after swim up by Kovács-Foote staining method. According to the results, the ratio of live sperm cells with intact acrosoma but stained tail reduced in the supernatant after swim up, while the ratio of living cells with intact acrosoma and tail membrane significantly increased. It was proven that the Kovács-Foote staining is able to give information about the motility of living cells according to the staining pattern of the tail, and determine the motile fraction of a sperm sample.

Comparison of hierarchy of bulls determined by results of motility and staining with rank positions determined by the results of in vitro fertilization

The hierarchy of bulls set up by results of motility determined with a motility analyzer and the staining results were not equal with the hierarchy determined by the results (cleavage ratio) of the in vitro fertilization. Therefore, it can be concluded that neither the motility analysis, nor the Kovács-Foote staining is able to predict the in vitro fertility of a bull.

Elaboration of biopsy methods and examination of their effects on embryonic and foetal development

Method of microaspiration for cattle embryos

The biopsy takes only 0.5-1 minute. It is very effective, using embryos which only contain blastomeres with intact cell membrane, the biopsy is able to succeed with 90-95%. Using the method, it is possible to ensure the integrity of cell membranes of both the embryo and the removed blastomeres.

In vitro and in vivo developmental capability of biopsied cattle embryos

The developmental competence of biopsied embryos was similar to the control embryos *in vitro*, approximately 38% of the biopsied embryos reached the blastocyst stage. The biopsy method was applied on valuable, in vitro produced embryos, which developed from oocytes obtained from pregnant or non-pregnant heifers by ovum pick up technique.

Following sexing of the removed blastomeres, five female embryos were transferred into recipient cows. From the pregnancy, two female calves were born.

A new biopsy method for mouse embryos

Our new method simplifies the biopsy method because only two capillaries and micromanipulators are needed for the micromanipulation. For making capillaries, only a microforge is needed. The obtained blastomeres keep their cell membrane integrity, so they can be analysed genetically. The remained blastomeres also remain intact after the micromanipulation. The method can be used for human therapy as well.

In vitro development of biopsied embryos

The developmental competence of biopsied embryos was equal to the control ones during *in vitro* culture, the rate of hatching blastocyst was same in both groups. We found significant difference between biopsied and control blastocyst in term of cell number. The reduced cell number can be caused partly by losing cells which could be developed from the removed blastomeres. The micromanipulation can decrease the velocity of cleavages, and because of the retarding effect of the recompaction process on *in vitro* development. The early extrusion of trophoctoderm through the damage of the zona pellucida without the expansion of the blastocyst can set back the proliferation of the cells.

Development of biopsied embryos in utero

Compared to the development of control foetuses, the only difference in development of the biopsied embryos was found on the 9th day of conception (early somite developmental stage). Foetuses developed from the biopsied embryos delayed a 0.5-1 day in their development. There were no any abnormalities manifested during the further development and after birth. The offspring was healthy and no anomalies were found in their reproduction.

Comparison of developmental results of biopsied cattle embryos with observations in the mouse model

Because of the similarities in the *in vitro* development of mouse and cattle biopsied embryos, it is supposed that the early extrusion of trophoctoderm cells without the expansion of the blastocyst and without the tailing away of the zona pellucida may cause a delay in the early development of foetuses after the embryo transfer, as observed in mouse model.

NEW RESULTS

1. Application of swim up and in vitro fertilisation for functional analysis of semen samples

- A software was developed for the counting and evaluation of Kovacs-Foote stained cells. The software can be downloaded freely from the Internet. It enables the user to input staining data using a PC keyboard during counting, providing a continuous work.
- Using swim up, demonstrated to concentrate motile and live cells, it was evidenced that the motility of spermatozoa judged with Kovacs-Foote staining as live cell with intact acrosome and stained tail is decreased.

2. Elaboration of embryo biopsy techniques, study of the effects of the intervention on the embryonic and foetal development

- A new protocol was elaborated for biopsy of in vitro produced 8-16 cell cattle embryos that can also be used in the practice. Two heifer calves were born from the transfer of biopsied and sexed female embryos.
- A novel, simple and efficient embryo biopsy method was elaborated suitable for use in mouse and in human.
- The effect of the biopsy on in vitro embryonic and foetal development could be assessed in mouse. By blastocyst stage, cell number of biopsied embryos was smaller than that of control embryos. On 9th day of pregnancy, a 0.5-1 day lag was observed in the development of the biopsied embryos compared to the control ones that difference could not be detected in the later phase of the development. The lag can be attributed to the effect of the biopsy. This can be explained by the delay due to the recompaction of the embryos and the early extrusion of the trophectoderm through the damaged zona pellucida. The early hatching from the zona pellucida noticed in mouse could also be observed in cattle embryos biopsied by microaspiration and cultivated in vitro.

PRACTICAL USABILITY OF THE RESULTS

- A reliable *in vitro* embryo production system was elaborated that is unique in Hungary. It is capable to produce transferable embryos for basic as well as for applied science.
- Based on the evaluation of individual oocyte retrieval and embryo cultivation, our embryo production technology can be used in embryo bank set up for gene preservation.
- The use of swim up and *in vitro* fertilisation enables the functional evaluation of the semen samples.
- For educational purpose, two web sites were created to present the semen evaluation possibilities and *in vitro* embryo production.
- The micromanipulation protocol developed for *in vitro* produced cattle embryos was applied successfully in MOET nucleus breeding schemes, as part of the preimplantation genetic diagnosis of embryos produced from oocytes retrieved by ovum pick up and fertilised *in vitro*.
- A new micromanipulation technique was elaborated that significantly simplified the embryo biopsy in mouse and in human.

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