PhD THESES

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EXPERIMENTS FOR PIG OOCYTES IN VITRO MATURATION, FERTILIZATION AND PIG EMBRYO IN VITRO DEVELOPMENT

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1. INTRODUCTION

There is a revolution of biotechnology in animal breeding nowadays. The first successful implantation of pig embryo was in 1986 by Becze at al. The experiments on the area of pig in vitro fertilization continued leading by Koppány at the end of the 80s. Rátky deals with pig embryo gathering and implantation recently by laparoscopic technique at ÁTK Institute. Our research team at the University of West Hungary deals with pig in vitro maturation, fertilization and embryo development co-operation with ÁTK researchers.

Well known that in spite of the philological distance between human and pig, the pig is similar to human considering the anatomic and physiologic constitution. This fact was confirmed by comparative genome mapping. This is the reason why the pig can be the source for xenotransplantation (implantation of organs from different species to human). The most important problem hindering the xenotransplantation is the hyperacute rejection. There are transgenic animals whose organs suitable for diseased human organs (heart, liver, kidney) substitution. It is possible to produce transgenic pigs which can be used for modelling human diseases (arteriosclerosis, sclerosis multiplex, Parkinson disease). Since the introduction of animal protection law in Hungary the importance of in vitro technique is increasing. With the maturation of pig in vitro oocytes we can get morphologically apparently mature oocytes, but in consequence of the insufficient nucleus and cytoplasmic maturation the fertilization efficiency is low, the polispermic oocytes rate is very high and the development of embryos is insufficient. It is justified, those different agents accumulate in the follicular fluid, which are produced by somatic follicular cells and they influence the maturation of oocytes. One of the most exciting fields of research nowadays is examining the effects of different growth factors.

Aims

- My ambition is to produce secunder oocytes or embryos for nuclear transfer and other in vitro manipulation experiments. For this reason it is necessary to create an in vitro maturation, fertilization and embryo cultivation system. The first step is to examine the resumption of meiosis in different maturation mediums.

- The effect of using different sperm concentrations for fertilization has to be examined.

- The effect of pig oocytes maturation in different maturation mediums for in vitro fertilization parameters has to be defined.

- The effect of fertilization with different frozen/thawed boar semen for the fertilization parameters.

- The matured oocytes fertilization in different fertilization mediums, or cocultivation with oviduct monolayer is to be compared.

- The role of different growth factors in meiosis resumption, and particularly the role of EGF (Epidermal Growth Factor) and NGF (Nerve Growth Factor) are to be examined.

- The cocultivation of FSP (Follicular Shell Pieces) or EGF supplement during maturation is to be defined.

2. MATERIAL AND METHODS

2.1. The scene and time of research

The examinations took place at Mosonmagyaróvár, Pannon University of Agricultural Sciences, Laboratory of Animal Husbandry between January 1998 and November 1999, and at the University of Missouri Columbia Department of Animal Sciences in the USA between 19th April and 14th May 1999.

2.2 Gathering and maturation of oocytes

The pig ovaries derived from large white meat swine species group from the local slaughterhouse and they were stored at 25 °C in 0.9% NaCl solution with 75 µg/ml penicillin G and 50 µg/ml streptomycin sulphate supplementation. The oocytes, follicular shell pieces and follicular fluid were sucked with a 10 ml plastic syringe connected to a 18 G marked needle. 30-40 cumulus oocyte complexes, which had uniform density cytoplasm, were placed in 400 µl maturation medium supplemented with 10 NE PMSG and 10 NE HCG. The mediums used were modified TCM 199, NCSU 23 and Waymouth. The 10% follicular fluid supplementation was evaluated at every maturation mediums. During the cocultivation with FSP, next to oocytes 6-700 µm somatic shell pieces were placed. The maturation continued in CO_2 thermostat, at 38.5°C, for 44 hours.

2.3. The identification of the phases of cell division

To identify the phases of cell division the cumulus cells were removed with 0.1% hyaluronidase enzyme after it they were mounted on a slide, and fixed

in 1:3 acetic acid:ethanol. The fixed oocytes were stained with 0.1% (v/v) orcein in 45% acetic acid (v/v) and examined under invert microscope at 200x and 400x magnification.

2.4. The in vitro fertilization of oocytes

After finishing the maturation, and the hyaluronidase treating, 30 denuded oocytes were placed in 50 µl mTBM fertilization medium or in 2 ml TCM 199 medium. Both of these mediums were supplemented with 1 mM coffeine and 0.1% BSA (mTBM) or 7 mM Calcium-lactate (TCM 199). The thawed, centrifuged (with 3x1900 g for 4 minutes) and PBS washed pellet were resuspended in in vitro fertilization medium. The setting of sperm concentration were made with Bürker chamber defining, while the final fertilization volume was kept in view (100 µl in mTBM, 2 ml in mTCM).

2.5. Production of oviduct monolayer

For the production of oviduct monolayer the cells were gathered after 45 minutes trypsin EDTA washing of the oviduct. The neutralization of the excessive trypsin was carried out with foetal calf serum. After 30 minutes 1500 g centrifugation the gained pellet was resuspended in TCM 199 supplemented with 10% foetal calf serum. After the setting of cell number (110 cell/ml) the cells were cultivated in 2 ml volume, in 2 cm diameter Petri dishes, at 38.5°C, in CO_2 thermostat. The cultivation medium was replaced in every second day. The uniform oviduct monolayer were formed 7-9 days after inoculation.

2.6. The check of fertilization parameters

The oocytes were placed into sperm free medium 6 hours after beginning of in vitro fertilization. 12 hours after the fertilization the oocytes or zygotes were mounted on a slide, and fixed in 1:3 acetic acid:ethanol for 48-72 hours. The fixed oocytes were stained with 1% (v/v) orcein in 45% acetic acid (v/v) and examined under invert microscope at 200x and 400x magnification. The oocytes were considered to be penetrated (PEN), when they had one or more [polispermic (POL)] swollen sperm head(s) and/or male pronuclei with their corresponding sperm tails. The average number of spermium/oocyte=S/O what penetrated into oocytes and the rate of male pronuclei (MPN) were defined.

2.7. In vitro embryo development

For the realization of in vitro embryo development 30 zygotes were placed in 400 µl NCSU 23 medium supplemented with 0.4% BSA. The rate of formation of morulas or blastocysts were examined for 48-144 hours after fertilization at 100x, 200x inverse microscope magnification. Orcein staining was used casually for define the cell number of blastocysts.

2.8. Statistical evaluation

Experiments were repeated three times. Data (mean ± SEM) were pooled and analysed by Excel program. ANOVA/ NANOVA part of Statistica program was applied for the one way variance analysis.

3. RESULTS

3.1. Comparison of different in vitro mediums

My aim is to create an in vitro maturation, fertilization and embryo cultivation system. The first step was to look for mediums, proper for in vitro maturation. The experiments were carried out with three different cultivation mediums: NCSU 23, TCM 199, Waymouth which features were compared. These maturation mediums were also examined with 10% follicular fluid supplementation. The expansion of cumulus cells were complete in Waymouth and TCM 199 mediums , but in NCSU 23 the outer layer of cumulus cells is loosened without 10% follicular fluid supplementation. . In case of follicular fluid supplementation, 90% of the oocytes reached the MII: stage in each examined maturation mediums, it was verified by orcein staining of maturated oocytes. 52.0% (NCSU23), 57.0% (Waymouth) and 59.8% (TCM 199) of the oocytes reached the MII. stage without follicular fluid supplementation, and 30% of the oocytes remain in GV stage in NCSU 23 medium.

3.2. The effect of application of different sperm concentrations on fertilization

For the fertilization of matured oocytes Hungarian large white meat swine frozen semen was used. The evaluation of sperm viability was carried out by Kovács –Foote sperm staining procedure. The best boar's semen was used in the experiments. One of the main problems of pig in vitro fertilization is the polispermy. To avoid polispermy experiments were made to define the appropriate sperm concentration. The effects of $2{,}5x10^5$, $5x10^5$, $1x10^6$ sperm

concentrations were examined to in vitro fertilization parameters. The polispermic rate was lower at lower sperm concentration (49.9, 58.2 81.8%), but the low sperm concentration caused low penetration rate. Evaluating the average sperm number penetrated to oocytes, it could be seen that using the lower sperm concentration one or two sperm penetrated each oocytes, while fertilising with $1x10^6$ sperm concentration generally 8 sperms were found in each oocytes. Finally the $5x10^5$ sperm concentration was chosen for fertilization.

3.3. The effect of maturation of oocytes in different maturation mediums on in vitro fertilization parameters

The influence of applying of 10% follicular-fluid supplementation of different maturation mediums (TCM 199, NCSU 23, Waymouth) to in vitro fertilization parameters was examined in the following experiments.

Making the one way variance analysis the results showed that there was no significant difference between penetration and polispermic rate, consequently the condition of citoplasmic maturation are the same all of the three mediums. The rate of male pronuclei were significantly ($P<0.05$) lower in Waymouth medium (55.5%), than in NCSU 23 (68.2%) or TCM 199 medium (66.3%). The explanation of this phenomenon could be that the starting cystein concentrations were the same in each mediums and the GSH content, which is important in the development of male pronucleus, was lower in Waymouth, than in NCSU 23 or TCM 199.

3.4. In vitro fertilization with different boar semen

Three boar semen were chosen for in vitro fertilization on the basis of Kovács-Foote semen staining evaluation. We concluded that the penetration rate of the first boar semen used at the most part of the examinations was high (80.4%) and beside this the male pronuclei development rate was also high. The penetration rate of the 2nd boar's semen (52%) was significantly lower ($P<0.05$), than the 1st and 3rd boars'. Fertilizing the oocytes with the 2nd boar's semen, fewer sperms penetrated, but from these fewer penetrated sperm formed male pronuclei nearly to the same extent (70.4%) with the 1st boar. The penetration rate of the 3rd boar's semen (76.2%) was the same as the 1st, but there were significantly ($P<0.05$) higher polispermic rate (80.2%), than using the 1st (61.4%) and the 2nd (60.2%) boar and the spermiums/oocyte (7.7) was much higher at the 3rd boar. There were differences between the effects on the fertilization parameters during the examinations of the three boars, but to justify the correlation of the in vitro and in vivo fertility results, some more experiments should be done.

3.5. The effect of fertilization in different fertilization mediums on the formation of fertilization parameters

According to the results of experiments both TCM 199 and mTBM mediums can be used for in vitro fertilization. There was significant difference at only the polispermic rate applying TCM 199 (76.2%, while in mTBM 56.3%). No differences were observed between penetration, polispermic rate and male pronucleus formation using mTBM and TCM 199 for in vitro fertilization mediums.

3.6. Cocultivation during in vitro fertilization

I acquired an oviduct cocultivation method in the scope of an international co-operation in Spain at the University of Murcia Department of Animal Biotechnology. With the help of this method I realized in the following experiments the fertilization on the oviduct monolayer and parallel to it the as the control the fertilization in TCM 199 medium without cocultivation. Evaluating the results it can be concluded that comparing control and oocyte cultured with Porcine Oviduct Epithelial Cells (POEC)= oviduct monolayer under maturation the results show higher penetration rate (82.3 vs. 90.9%) and significantly ($P < 0.05$) reduced polispermic rate (67.0 vs. 52.7%). The cocultivation did not influence the male pronucleus formation (71,0 vs 73.8%). The cocultivation is advantageous from the point of view of fertilization success, but it is hard to create and cultivate an oviduct monolayer.

3.7. The effect of maturation medium supplementation with different growth factors on embryo development

Participating in the research at the University of Missouri Columbia, B. N. Day Laboratory I met the possibility of leaving out the cocultivation unlike to the foregoing laboratory practice. I examined the effects of maturation medium supplementations with different concentrations (10, 20, 30 ng/ml) of EGF to pig embryo development. The rate of formation of morulas was higher (53.9, 56.7, 45.4%) than in the control (35.5%) and the rate of blastocysts (5.5, 19.5 12.3%) were also significantly ($P<0.05$) higher than in the control (3.0%). Adding the different concentrations of EGF during IVM enhanced the maturation of oocytes and subsequent embryo development.

Our group was the first who examined the effects of maturation medium supplementation with different concentrations (1, 5, 10 ng/ml) of NGF to pig embryo development. The rate of formation of morulas were control: 37.3%; 1 ng/ml: 47.23%; 5 ng/ml: 41.2%; 10 ng/ml: 31.1% . The rate of blastocysts developing were: control: 3,0% ; 1 ng/ml: 11.2%; 5 ng/ml: 6.3%; 10 ng/ml: 4,1%. The results show that some NGF (1, 5 ng/ml) concentrations during maturation enhanced the oocyte maturation and embryo development, but the differences were not significant. It is necessary to make some more examinations after modifying the concentration-limits.

3.8. Comparison of follicular shell pieces cocultivation with EGF supplementation during maturation

I examined in the followings that whether the EGF supplementation can really substitute the cocultivation under maturation or not. The maturation medium was TCM 199 with follicular shell pieces (FSP) or 10ng/ml EGF supplementation. There was no significant difference between oocytes maturation using two maturation mediums (90%, 87%). I concluded during examining the fertilization parameters that the polispermic rate was 78% cocultivated with somatic cells and 65.2% with EGF supplementation. The polispermic rate was 45.3%, or 62.1% with EGF supplementation, this shows a significant difference ($P<0.05$). The male pronuclei development rate was 73.3% with cocultivation somatic cells, or 60.4% with EGF supplementation. Evaluating the results there was no significant difference in this value.

4. NEW SCIENTIFIC RESULTS

- Comparing the maturation of oocytes cocultivation with follicular shell pieces, or EGF supplementation the results shows that the EGF has an important role in the oocyte maturation and afterwards the embryo development, but the follicular somatic cells secrete other growth factors (IGF, NGF) and different material, important in the maturation, which make the maturation process complete.

- The effect of maturation with different growth factors on pig embryo development proved that the EGF supplementation helps the cytoplasm and nuclear maturation of oocytes.

- The NGF growth factor also helps the oocyte maturation on the basis of experimental results. Participation of the NGF in the ovulatory process appears to provide a unique example for the neuroendocrine integration and to its role in controlling living processes.

5. PROPOSALS

- The further study of pig oocyte maturation. The examination of factors participating in maturation.

- One way of avoiding polispermy is to inject the sperm to oocyte with micromanipulation. The sperm can be injected below the oocyte zona pellucida on the oocyte cell-membrane surface or directly into the cytoplasm. Our team have started its researches on this field.

- The other way to avoid polispermy is to decrease significantly the fertilization time-period. We plan further examinations to improve the efficiency of fertilization methods.

- The well functioning in vitro fertilization system is useful for in vitro estimation of different boars' fresh/frozen sperms in vivo fertilization ability.

- The in vitro produced secunder oocytes can serve as a source for enucleated oocytes needed in nuclear transfer experiments. We plan experiments to realise germ stem-cells nuclear transfer.

- To examine more extensively the role of NGF growth factor.

- The results of the experiments and the new scientific results offer a possibility of stock-breed improvement.

6. PUBLICATIONS AND PROCEEDINGS CONNECTED TO THE Ph.D. THESES

6.1.Publications

6.1.1. Foreign language publications

1. **Sz. Nagy – G Házas – Á Bali Papp – F. Szász – F. Szász Jr. – A. Kovács – R.H. Foote (1999):** Evaluation of sperm tail membrane integrity by light microscopy Theriogenology 52 1153-1159.

2. **Á. Bali Papp– J Iváncsics – J. Dohy (2000)**: Effect of follicular shell pieces or epidermal growth factor in a serum-free maturation medium on in vitro fertilisation parameters. Theriogenlogy (Abst.) 53 448.

3. **Á. Bali Papp – J. Iváncsics– J. Dohy (2000):** Production of genetically modified farm animals. Hungarian Agricultural Research 1. 9 9-11.

6.1.2. Hungarian language publications

1. **Bali Papp Á. – Iváncsics J. – Dohy J. (1999):** Sertésembriók in vitro előállításának lehetőségei. (The possibilities of in vitro pig embryo production - review). Magyar Állatorvosok Lapja. 9. 121 559-564.

2. **Somfai T. – Bali Papp Á. – Iváncsics J. (1999):** A sertés petesejtek aspirációja és in vitro maturáltatása.(Aspiration and in vitro maturation of pig oocytes) Acta Agronomica Óváriensis. 1. 41 101-112.

Proceedings

1. **Bali Papp Á. (1998):** Production of genetically modified farm animals. In: Tenk A., Szabó Z. (eds.) I.C.A. Summer School on „Agricultural Challenges and EU Enlargement". Pannon Agricultural University Faculty of Agricultural Sciences Mosonmagyaróvár. 247-253.

2 **Bali Papp Á. – Sótonyi L. – Iváncsics J. (1998):** Sertés petesejtek in vitro maturáltatása.(In vitro maturation of pig oocytes) XXVII. Óvári Tudományos

Napok: Új kihívások a mezőgazdaság számára az EU-csatlakozás tükrében. Mosonmagyaróvár, Állattenyésztési Szekció I. 7-12.

3 **Bakainé Kelemen L. – Nagy Sz. – Bali Papp Á. – Iváncsics J. (1998):** A tárolás hatása a kansperma életképességére és az akroszóma integritására. (The effect of storing on boar semen viability and acrosome integrity) XXVII. Óvári Tudományos Napok: Új kihívások a mezőgazdaság számára az EU-csatlakozás tükrében. Mosonmagyaróvár, Állat-tenyésztési Szekció. I. 208-212. (poszter)

4 **Bali Papp Á. - Sótonyi L. - Iváncsics J. (1998):** Sertés petesejtek in vitro érlelése és termékenyítése. (In vitro maturation and fertilization of pig oocytes) XIV. Biotechn. Kerekasztal Konferencia. 15-16 Oct. Hódmezővásárhely, Hungary.

5 **Bali Papp Á. – Iváncsics J. – Dohy J. (1999):** Biotechnológiai módszerek hasznosítási lehetőségei a sertésnemesítésben. (Application of biotechnological methods in pig breeding) IV. Magyar Genetikai Kongresszus. 11-14. Apr. Siófok, Hungary, 95-96.

6 **Bali Papp Á. – Iváncsics J. – DohyJ. (1999):** Különböző in vitro technikák az állatnemesítés szolgálatában. (Different in vitro methods in animal breeding) „Kitörési pontok a magyar állattenyésztésben" tudományos konferencia MTA, Budapest. Állattenyésztés és Takarmányozás. 6. 750-751.

7 **Bali Papp Á. – Iváncsics J. – Dohy J. (1999):** Különböző növekedési faktorok hatása a sertésembriók in vitro fejlődésére. (The effect of different growth factors on pig embryo in vitro development) 6. Szaporodásbiológiai találkozó. A fogamzás javításának lehetőségei háziállatokban. 25-26. Oct. Balatonfüred, Hungary, (megjelenés alatt)

8 **Sz. Nagy – A. Bali Papp – P. Sarlós – Gy. Gábor – J. Iváncsics - A. Kovács (1999):** The tale of the tail – IV. International Conference on Boar Semen Preservation, 8-11 August, Beltsville, MD, USA, 14. – (poster)

9 **Á. Bali Papp, Sz. Nagy, J. Iváncsics, A. Kovács, T. Pécsi and J. Dohy (1999):** Comparison of viability and acrosome status of boar spermatozoa frozen in mini or maxi straws - 50th Annual Meeting of EAAP, 22-26 August, Zurich, Switzerland, 126. –(poster)