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Review

SEVERAL KEY ASPECTS OF THE RESEARCH ON BOVINE PREIMPLANTATION EMBRYOS AT THE RESEARCH INSTITUTE FOR ANIMAL PRODUCTION (RIAP) IN NITRA

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ABSTRACT

This short review attempts to describe several important aspects of research at the RIAP Nitra in the area of embryo manipulation with *in vivo*-derived or *in vitro* produced cattle embryos performed from the beginning of an embryo transfer era and up to date. The paper summarizes results of more important studies including: manipulations on embryos (embryo transfer, embryo bisection for creation of identical calf twins, embryo cryopreservation), *in vitro* embryo production (IVP), the assessment of embryo quality according to morphology, nucleic acid synthetic activity in the blastomeres or the fine ultrastructure of embryo organelles, the use of bovine embryos as a model for testing bovine virus transmission, gamma-irradiation at the Chernobyl´s disaster and other influences, with referring to the articles, written by the employees of the RIAP during this period and published in the national and international literature. All obtained results as well as accumulated knowledge and methodical experience in this area provide a strong basis, which can be used at creating the gene bank of animal genetic resources.

Key words: cattle; embryo transfer; manipulation; *in vitro* fertilization

INTRODUCTION

Over a period of approximately forty years, commercial bovine embryo transfer and its associated technologies have become a large international business. The bovine embryo transfer industry, as it is known today, arose in North America in the early 1970s, and the dissemination of modern reproductive technologies over the world was significantly influenced by the fact, that the intercontinental transportation of live animals was very expensive and time-consuming. Reproductive technologies have gradually expanded around the word also to other continents, and in several countries workplaces within research institutes, universities or centres were established, which began to focus on research in the field of preimplantation embryo manipulations.

Also, in the former Czechoslovakia, the research in the area of collection and transfer of bovine embryos began intensively since 1973, when the International Embryo Transfer Research Team from the former Eastern bloc countries (Czechoslovakia, German Democratic Republic, Poland, USSR, Romania, Bulgaria, Hungary) and Mongolia was formed by the scientists from institutions of Academies of the Sciences, research institutes, high schools, universities and breeding services with the purpose to elaborate validated approaches of superovulation and synchronization of donor animals, embryo recovery and embryo transfer.

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On the basis of experimental research the implementation outputs were subsequently processed. In 1978 - the manual for surgical embryo transfer, and in 1982 - the completed protocol of non-surgical isolation and transfer of cattle embryos were implemented to practice. Also, biotechnical approaches for superovulation and synchronization of the sexual cycle in farm animals were elaborated.

After the validation of non-surgical isolation and transfer of cattle embryos in 1980-1984 in Slovakia the realization teams with centres in Nitra, Bernolakovo, Banská Bystrica and Košice were established. Sixty experts, involved into this activity, were trained at the RIAP Nitra and then acted according to the instruction under the RIAP Nitra coordination. Since 1985 the members of realization teams began to apply embryo transfer into practice under systematic and planned schedule. The aim was to obtain offspring from mothers of bulls and mothers with high milk production, as well as to produce calves for fattening. In the former Czechoslovakia, in 1987 about 16000 embryo transfers were performed with 55 % success rate, and in 1988 - even 22000 embryo transfers with 60 % success rate, of which about 5000 transfers were done in Slovakia. About a third transfer was designed to produce fattening calves. On the basis of the management and success in embryo transfer, the former Czechoslovakia was ranked among the most advanced countries using these biotechniques.

The work in field of assisted reproduction in cattle with a focus on bovine preimplantation embryos, which was initiated at the RIAP in Nitra already in 80-s years of the last century, continues to date but with the more emphasis on production of bovine embryos *in vitro* using *in vitro* fertilization (IVF) technique.

***In vivo* recovery of embryos and their quality**

Initial investigations on isolation and transfer of embryos at the RIAP Nitra were carried out on laboratory animals and rabbits. In 1970-s, pigs as first farm animal model were used for the study of oestrus synchronization in donors and recipients, superovulation, surgical recovery and transfer of embryos, their evaluation and culture *in vitro* (Pivko *et al*., 1973). First offspring after surgical isolation and transfer of porcine embryos was obtained in 1975.

Embryo quality and fertility rate mostly depends on quality of oocytes, therefore, this task was studied by autoradiography at the level of light and electron microscopy. It was found that during oocyte maturation at the germinal vesicle stage, viable oocytes, being either connected with cumulus cells or after finishing this connection, intensively synthesize RNA (Motlík *et al.*, 1978) and glycoproteins (Pivko *et al*., 1982a; Fléchon *et al.*, 1986). Newly synthesized proteins and RNA are localized in the germinal vesicle. Synthesized material, accumulated under the *zona pellucida*, is represented by hyaluronic acid containing glycosaminoglycans formed by cumulus cell secretion activity under influence of gonadotropins. Therefore, in the peri-ovulation period synthesis of proteoglycans by oocytes and cumulus cells takes place, and extracellular envelopes are formed with relative tight binding to the *zona pellucida*. Such a proper synthesis of RNA, specific proteins and glycosaminoglycans significantly affects processes of fertilization and early embryo development.

First experiments (beginning in 1980-s) on application of cattle embryos in embryo transfer at the RIAP were focused on the generation of calf twins. It was induced by the fact that such techniques as selection of animals for superovulation and exogenous application of gonadotropins did not bring satisfactory results. On the other hand, generation of twins by embryo transfer showed to be promising idea. In the experiment by Pivko *et al.* (1982b), 74 embryos were transferred to 37 recipients and 70 % of cows were pregnant (26), of them 65 % (17) gave birth to twins.

The next research on bovine embryos about non-surgical isolation of bovine embryos from superovulated cows by flushing of uterine horns at 7-9 days following insemination was reported by Kubovičova *et al.* (1991). This study was focused on the quality evaluation of *in vivo* -derived embryos on the basis of morphological criteria. Totally, 3337 preimplantation embryos were collected within the period of 1986-1988, of them 56.7 % (1892 embryos) were suitable for embryo transfer or cryopreservation and about 40 % (1327 embryos, including infertile eggs) were classified as inappropriate ones from the standpoint of their quality.

Quality of embryos recovered from cows on farms is associated with strict selection of donor cows with special focus on general health status of sexual organs accompanied with a temporal vitamin supplementation and nutrition improvement. Among the causes of high occurrence of improper embryos and unfertilized eggs collected at flushing there are an environment (animal housing, nutrition, zoohygienic conditions, donor cows itself), disturbed homeostasis of the organism, spermiotoxicity of vaginal mucus, inadequate response to the hormonal treatment, endometritides as well as low viability and motility of spermatozoa. However, technical and material facilities at the embryo transfer (embryotoxicity of flushing solutions, catheters and culture media) should not also be ignored.

Since embryo flushings in our experiments were performed on the 7th - 9th days after inseminations, the collected embryos were at different stages of development: compact morula, early blastocyst, blastocyst and expanded blastocyst. For embryo transfer more suitable embryos were morula, blastocyst and

expanded blastocyst stage, whilst early blastocysts were also suitable for micromanipulations, and in this research they were used for the generation of identical twins.

At the definition of qualitative criteria for embryos the main emphasis was put on regular formation of embryoblastic and trophoblastic cells, regular-shaped and intact *zona pellucida*, the perivitelline (PV) space and blastocoele cavity. Destruction, fragmentation and disintegration of embryonal cells were evaluated under a stereomicroscope using the following scale:

Excellent – an ideal embryo, round-shaped, symmetrical, with the cells homogenous in size, colour and structure;

Good – the rare occurrence of blastomere fragments in the PV space, irregular shape of blastomeres, and slight incidence of vesicles;

Fair – slight occurrence of scraps of excluded blastomeres and a few degenerated cells in the PV space, the vesicle formation progresses;

Poor – serious developmental disorders: numerous blastomeres excluded into the PV space, degenerated and differently-sized cells, numerous large vesicles, fragment formation.

According to this classification, from total number of flushed embryos (3337) 45.55 % was of excellent, 11.15 - good, 3.54 - fair and 39.77 % of poor quality. After flushing of superovulated cows a high number of fragmented embryos were yielded. This high rate of unfertilized eggs and fragmented embryos may be caused by abnormalities related to superovulation treatment. In practical conditions it is not possible to define exactly a margin between embryos of fair or poor quality.

Since morphological criteria of embryo evaluation are often insufficient, techniques of vital staining using various fluorochromes, for instance FDA (fluorescein diacetate) acquire special importance. Pivko *et al.* (1986) after FDA staining observed clear fluorescence in bovine embryos of preimplantation stages, in particular, fragmented or abnormal embryos accumulated less FDA signal than normal viable embryos. The association between integration/disintegration of blastomeres and FDA accumulating ability was supported also by the experiment with the intended destruction of the embryo by deep freezing. Following thawing it was possible to determine the extent of disintegration using FDA test. It was found that FDA-negative blastomeres have extensive degenerative alterations in the cytoplasm and no longer synthesize RNA (Pivko *et al*., 1986).

For evaluation of early embryo quality the processes related to initiation of the embryonal transcription and running of nucleogenesis were investigated. Definition of nucleologenesis stages enables using the morphology of nucleus and nucleolus as relevant morpho-functional marker of embryo viability evaluation. Using autoradiographical and immunocytochemical analyses of ultrastructural morphology and dynamics of nucleic acids Pivko *et al.* (1996) studied differential and functional status of early cattle embryos.

Manipulation on bovine embryos

At the beginning of the era of embryo transfer a main application of ET was generation of twin calves. Three common approaches to produce calf twins with the use of embryo transfer were known:

a) Transfer of two embryos into one recipient cow,
b) Transfer of two embryo halves after micromanipulations into one recipient cow (Holy *et al*., 1985),
c) Transfer of one embryo into contra-lateral uterine horn of previously inseminated recipient (Říha *et al*., 1986).

Using embryo transfer of fresh and frozen-thawed embryos into contra-lateral uterine horn of recipient cows Grafenau *et al.* (1992) obtained 59.2 and 55.8 % pregnancy rates, resp., and the birth of 1.44 calves per one pregnant recipient.

One of embryo manipulations commonly used on bovine embryos during 80s years of last century was microsurgical embryo bisection in order to generate identical calf twins. At the Research Institute of Animal Production two genetically identical calves were generated in 1987, and chimerical mice were created in 1989 by the team of Dr. P. Babušík (unpublished results).

The survivability of bovine embryo halves two hours after bisection is relatively high (65-90 %; Picard *et al*., 1986). Microsurgical bisection of the embryo leads to mechanical damage of about 1/5 of cells from total number of blastocyst cells. Embryo halves are retarded in the development but have a great regenerating ability resulting in the compaction of halves and the formation of blastocoelic cavity (Picard *et al*., 1986).

Pivko *et al*. (1995) analysed proliferating activity of early embryos recovered non-surgically (flushing of the uterine horn) from cows of Slovak Pinzgau breed. Early embryos at the blastocyst stage were bisected into two equal halves, cultured in TCM medium with 20 % fetal calf serum for two hours and then analyzed autoradiographically with the use of 3H-labelled thymidine (Pivko *et al*., 1995). Blastocyst cells actively proliferated (especially inner cells and those undamaged) already two hours following bisection, what was proved by the DNA synthetic activity indicating on running cell cycles.

Microsurgical bisection of bovine embryos in combination with embryo transfer techniques was applied to obtain non-identical calf twins for improving

the efficiency of beef cattle production (Sreenan and Danagh, 1984; Říha and Polášek, 1987; Holy *et al*., 1985).

Among embryo manipulations, the team headed by prof. J. Pivko performed intracytoplasmic sperm injection (ICSI) (Pivko *et al.*, 2003) and parthenogenetic activation by electric AC/DC pulse (Pivko *et al*., 2004) to produce bovine embryos *in vitro*. The volume of the Golgi apparatus was significantly increased in the ICSI-derived embryos compared to parthenogenetic embryos. Expansion of the Golgi apparatus was probably caused by the ICSI technique used. In early 8-blastomere embryos following AC/DC activation and ICSI a high relative volume of vacuoles and lipids was observed what led to decrease in the volume of organelles participating in the proteosynthesis.

Bovine embryos as a model for testing bovine viral infections

Epidemiological aspects of embryo transfer in farm animals remain currently highly actual task. Exchange of genetic material (oocytes, embryos, and sperm) within the country and between countries necessitated devoting more attention to security and reduction of health risk at the embryo transfer. Health risk of viral infection associated with embryo transfer is not dependent only from animals - donors of sperm and embryos, but also from environmental conditions to which gametes and embryos are exposed. More often of them are flushing and culture media and conditions of recovery, manipulations, storage and transfer.

We studied development and viability characteristics of bovine embryos after their experimental infection with bovine herpesvirus-1 (BHV-1) (Makarevich *et al*., 2007). In this study we examined whether: (1) the *in vitro* exposure of embryos to the BHV-1 virus can compromise their further development and alter the ultrastructural morphology of cellular organelles; (2) whether the *zona pellucida* can be a barrier protecting embryos against infection; and (3) whether trypsin washing after viral exposure can prevent virus penetration inside the embryo and subsequent virus induced damages. It was found that BHV-1 exposure impairs embryo development independent of the presence of ZP or the trypsin treatment step, since most of experimentally infected embryos were arrested at the morula stage. Therefore, *zona pellucida* itself may not be an enough barrier to prevent virus-induced damages, unless it is accompanied by trypsin washing (Makarevich *et al*., 2007).

In the further study, Kubovicova *et al.* (2008) performed experimental infection of bovine *in vivo* isolated embryos with bovine viral diarrhoea virus (BVDV). BVDV viral suspension was introduced under the ZP by the microinjection procedure and ultrastructural morphology of cell organelles was examined. About 83 % of embryos were arrested at the morula stage and this arrest was associated with irreversible alterations in the ultrastructure of organelles like disintegrated nuclei, the loss of the nucleoli, intercellular gap junctions and dilatation of intercellular space.

Several studies proved that *in vivo* and *in vitro* produced embryos are different. Also health risk using *in vitro* culture may be related to contamination of serum-supplemented medium, bovine serum albumin and other biological additives. Viral contamination at the stage of *in vitro* fertilization can be caused by the BVDV virus adhered to the membrane of sperm, which enters into the oocyte following the penetration of the *zona pellucida*. Effective sanitary procedures preventing transmission of infections by embryos of farm animals were elaborated by the International Embryo Transfer Society (IETS) and International Office of Epizootology (OIE). These procedures represent methodical instruction for washing of embryos before the transfer in order to eliminate pathogens, eventually cells or cellular fragment adhered to the *zona pellucida*. Application of these simple washing steps on embryos before their *in vitro* culture or transfer diminishes the risk of infection transmission to minimum, if not completely.

Influence of gamma-irradiation on quality of bovine embryos

After the accident at the Chernobyl´ nuclear power station we determined autoradiographically the RNA synthetic activity in early embryos derived from cows reared in the irradiated zones, which had chronic exposure to gamma-irradiation. The nuclei and nucleoli of such embryos have revealed no deviations from physiologically normal nucleogenesis (Pivko *et al.*, 1997). On the contrary, in the embryos which were experimentally exposed to gama-irradiation at certain doses (1, 2 and 4 Gy gamma) using a cobalt bomb 60Co, at dose of 4 Gy we have found termination of RNA synthesis and increased incidence of various forms of chromatin segregation in blastomere nuclei, particularly in the form of marginalization of chromatin (Pivko *et al*., 2002).

***In vitro* production of bovine embryos for research purposes**

In vivo isolated embryos, derived from cows of defined breeds and genotypes, are intensively used in practice for breeding purposes, e.g. in embryo transfer, cryopreservation for the purpose of storage of animal gene resource a.o. Oppositely, biological material for creation of *in vitro* produced embryos (oocytes) is mostly available from local slaughterhouses (excepting ovum pick-up technique), their origin is rather undefined and such embryos cannot be used for breeding purposes.

Nevertheless, the application of *in vitro* produced cattle embryos represents good model for their use to test different external effects or conditions. *In vitro* fertilization expands possibilities of cattle reproduction by the creation of transferable embryos originated from cows *post-mortem*. This technique also provides a source of embryos for research in assisted reproduction and biotechnology.

During 1980-1990s embryo transfer in cattle in Slovakia had 50-60 % success rate. Following transfer of 5-6 transferable (1st and 2nd quality grades) embryos at average 2-3 calves were born. This fact induced more intensive application of *in vitro* embryo production (IVP) technique involving maturation of oocytes (*in vitro* maturation, IVM), their fertilization (*in vitro* fertilization, IVF) and culture of embryos up to higher preimplantation stages (*in vitro* culture, IVC). Sustainable results over the world indicate that average success rate was about 80-90 % for IVM, 75-85 % for IVF and 30-40 % for IVC, of them about 10-12 liveborn calves per 100 oocytes can be born. However, problems of variable success rate are often occurred, what is caused by genetic origin of gametes (oocytes, sperm) and by using different baths of serum or serum derivatives, which contain a number of unknown growth-stimulating substances. It was also showed that prolonged *in vitro* culture period may have unfavourable long-term consequences, like prolonged pregnancy length, large size of foetus and certain morphological abnormalities. It is assumed that these alterations may be caused by changes in the control of the gene expression.

RIAP started dealing with bovine *in vitro* embryo production in the end of 1990s. First studies were done in cooperation with Agricultural centre of Finland and the reports described about proliferation potential and apoptosis of bovine *in vitro* produced embryos (Makarevich and Markkula, 2002), which were cultured in SOF– medium in the atmosphere of incubator with suppressed oxygen tension. During the 2000s the experiments on bovine *in vitro* embryo production at the RIAP were intensively continued. Kubovičová *et al*. (2003) reported about bovine *in vitro* fertilization using ICSI, where 42.2 % of oocytes developed to embryos compared to 24.5 % obtained in classic IVF procedure. However, ICSI-derived embryos showed altered ultrastructural morphology of organelles (Pivko *et al.*, 2003).

The experiments on comparison of *in vivo* or *in vitro* produced embryos were focused on the ultrastructure of pre-compacted embryos at 1 to 8-cell stage (Pivko *et al*., 2003; 2004). Comparison of *in vivo* and *in vitro* embryos showed that IVP embryos had an altered ultrastructural morphology. Early embryos produced *in vitro*, either by AC/DC pulse activation or using ICSI, had a decreased relative volume of the cytoplasm in comparison with *in vivo* embryos, which can be explained by a higher occurrence of vacuoles and lipids in the blastomere cytoplasm.

Limiting factor at these techniques is a low success of embryo production of good quality and developmental capacity. The quality of IVP embryos depends on conditions of cultural milieu, particularly temperature, light regime, composition and properties of a culture medium, gas composition in the incubator, sperm quality and others.

As an *in vitro* maturation medium, TCM 199 medium added with fetal calf serum and gonadotropic hormones more often is used. There are known several embryo culture media commonly used for bovine *in vitro* produced embryos: TCM199 (Shamsuddin *et al*., 1994), KSOM (Liu and Foote, 1995), CR1α (Rosenkranz *et al*., 1990), SOF (synthetic oviduct fluid; Tervit *et al*., 1972), ISM1, B2 INRA (Camous *et al*., 1984), G1.2/G2.2 (Lane *et al*., 2003), etc.

Olexikova *et al.* (2009) tested three culture systems for production of bovine embryos *in vitro*: 1) SOF- medium without O_2 suppression, 2) SOF-medium with O_2 suppression in the atmosphere to 5 %, and 3) B2-INRA medium in co-culture with BRL-(Buffalo Rat Liver) cell monolayer. Embryo development was assessed on the basis of embryo cleavage rate and blastocyst rate. It was found that more successful culture system in given laboratory conditions was a combination of B2-INRA medium and BRL cell co-culture, which allowed obtaining higher cleavage and blastocyst rates compared to other systems tested in this study.

The ability of BRL cells to promote preimplantation embryo development is perhaps based on the secretion of different growth factors by these cells, like insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), leukaemia inhibitory factor (LIF) and others (Duszewska *et al*., 2000). Makarevich and Markkula (2002) have found that the addition of insulin-like growth factor I into maturation (IVM) and/or culture (IVC) medium (SOF-medium) stimulates cell proliferation (PCNA) and reduces apoptosis in fresh bovine IVP embryos, therefore, improving the quality of blastocysts.

Later, Makarevich *et al.* (2012a) tested the addition of IGF-I into post-thaw medium on bovine *in vivo* isolated embryos following long-term cryostorage. Survival effect of IGF-I was confirmed, as the addition of IGF-I during post-thaw culture (48 h) improved the quality of bovine cryopreserved embryos. In particular, IGF-I advanced development of thawed embryos to the blastocyst stage, elevated total cell number, decreased percentage of apoptosis and improved actin cytoskeleton quality.

Quality of IVP embryos may be estimated also on the basis of tolerance against cryopreservation. IVP

embryos are more sensitive to cryopreservation than their *in vivo* counterparts. Thus, Markkula *et al*. (2001) analyzed proliferating cell nuclear antigen (PCNA) in fresh and vitrified IVP blastocysts and have found that the blastomeres with missing PCNA reaction indicated on the disorders that were not observed under morphological evaluation. Generally, Day 7 blastocysts tolerated the cryopreservation better than Day 8 blastocysts.

Pivko *et al*. (2003b) analysed ultrastructure of bovine IVP embryos following cryopreservation in open pull straws (OPS vitrification, Vajta *et al*., 1997). They observed that vitrification and warming of IVP embryos resulted in immediate injuries at the cellular and sub-cellular levels, however the most of them were normalised following 24 hours of post-thaw culture.

Embryo yield in relation to the cow´ body condition and the season

In recent years experiments on bovine IVP embryos were focused on interrelations of cow body condition and quality of embryos. Bovine oocytes were recovered from cows of BCS 1, 2 or 3, then *in vitro* fertilized with bull semen and cultured until preimplantation stage embryos. Body condition of cows affected initial quality of oocytes, but did not affect embryo cleavage, blastocyst rate and actin quality of subsequent IVP embryos (Kubovičová *et al*., 2012; Chrenek *et al*., 2015).

Different situation was observed on embryos *in vivo* recovered from superovulated cows (Kubovičová *et al*., 2013; Makarevich *et al*., 2015). The cow´s body condition affected the overall embryo recovery rate (proportion of collected embryos to palpated corpora lutea). The significantly higher number of embryos was collected from cows with average body condition (BCS3 - 65.81 % embryo recovery rate) compared to the cows with low (BCS2 - 50.6 %) or high (BCS4 - 21.43 %) condition. Also the season significantly affected embryo recovery rate. The significantly higher percentage of embryos was recovered during spring months (59.6 % recovery rate) compared to summer months (37.0 %) and slightly increased again during the autumn (48.3 %). On the contrary, the quality (yield of transferable embryos) was better during the autumn months (78.9 %) compared to spring (58.4 %) or summer (60.0 %) months.

The use of bovine IVF for analysis of sperm penetrating/fertilizing ability

In recent several years bovine IVF system is intensively used at the RIAP for evaluation of sperm penetrating or fertilizing ability *in vitro*. Before using a male for breeding purposes, is it important to know that he is fertile. Testing individual male fertility by artificial insemination is expensive and labour intensive procedure. The most adequate method to assess the sperm fertility may be in vitro fertilization, since this procedure evaluates the spermatozoa-oocyte interactions occurring during fertilization process, allowing determination of different endpoints in early stages of the embryo development. In our research we used bovine pre-matured oocytes in heterologous system to examining ram sperm penetrating/fertilizing ability. Fertilizing ability of ram spermatozoa was tested following 48 h of cooling storage in the presence of growth factors, either EGF (Makarevich *et al.*, 2011) or IGF-I (Makarevich *et al.*, 2012b). Penetration ability was measured basing on the number of oocytes with at least one sperm inside the *zona pellucida*, and fertilizing ability was measured by counting the number of divided embryos. In these experiments it was found that IGF-I improved the penetration and cleavage rate of embryos compared to control, whilst EGF did not improve these characteristics. However, these studies validated the use of bovine IVF system for evaluation of fertilizing capacity of ram sperm. At present at the RIAP, the examinations of fertilizing ability of bovine sperm doses from Pinzgau bulls are intensively carried out using bovine IVF system.

CONCLUSION

This review demonstrates that the RIAP was and to date is still a key institution in cattle assisted reproduction, which has a long-term tradition of research with bovine embryos both *in vivo* and *in vitro*. However, in recent decades interest in embryo transfer and other assisted reproduction techniques in cattle was rapidly dropped. It is a result of the current agricultural policy and situation in the branch of animal production in Slovakia. During last decade, cattle population in Slovakia rapidly decreased. Moreover, interest of breeding centres or individual farmers in embryo transfer and other assisted techniques dropped to minimum, and the main reason of this situation is an absence of state support in this field of animal production. Nevertheless, for more than 40 years of this research activity, in the laboratories and farms of the RIAP several key techniques and methods of animal superovulation, embryo recovery, embryo manipulations and transfer, quality evaluation, cryopreservation and *in vitro* embryo production were elaborated and implemented to practice. Important findings were published in international and domestic literature. Moreover, the RIAP during many years was a holder of a special accreditation (registered under the code ETTSR01) permitting embryo transfer procedures for whole the Slovak Republic. In order to restore

activities of the embryo transfer workplace at the RIAP with the competence to export embryos to the Europe Union it would be necessary to equip additionally the workplace with material and personnel facilities in order to meet requirements not only for experimentation but also for trading (exchange) with embryos of genetically significant and endangered animal species within the EU.

At present, investigations on bovine embryos at the RIAP are focused mostly on *in vitro* embryo production, optimization of cryopreservation techniques for long-term storage of embryos, oocytes and ovarian tissues in order to elaborate methodological approaches for creating embryos with improved cryotolerance. Therefore, altogether these events demonstrate that the RIAP possess with a proper basis for implementation of methodical, equipment and personal facilities for the establishing gene bank of animal genetic resources in Slovakia.

REFERENCES

CAMOUS, S. – HEYMAN, Y. – MEZIOU, W. – MENEZO, Y. 1984. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *Journal of Reproduction and Fertility*, vol. 72, 1984, p. 479–485.

DUSZEWSKA, A. M. – REKLEWSKI, Z. – PIENKOWSKI, M. – KARASIEWICZ, J. – MODLINSKI, J. A. 2000. Development of bovine embryos on Vero/BRL cell monolayers (mixed co-culture). *Theriogenology*, vol. 54, 2000, p. 1239–1247.

FLECHON, J. E. – MOTLIK, J. – HUNTER, R. H. F. – FLECHON, B. – PIVKO, J. – FULKA, J. 1986. Cumulus oophorus mucication during resumption of meiosis in the pig. A scanning electron microscope study. *Reproduction, Nutrition, Developpement*, vol. 26 (4), 1986, p. 989–998.

GRAFENAU, P. – OBERFRANC, M. – KUBOVIČOVÁ, E. – PIVKO, J. – ANTALOVÁ, H. – LAURINČIK, J. – LEVČENKO, E. – BULLA, J. 1992. Controlled production of twins by embryo transfer in cattle of meat type. *Journal of Farm Animal Science*, vol. 25, 1992, p. 67–74.

HOLÝ, L. – BABUŠÍK, P. – KADEŘA, J. – LOPATÁŘOVÁ, M. – CAHEL, E. 1985. Mikorchirurgické dělení embryí a jeho význam pro zvyšovaní počtu potomstva a tvorbu identických dvojčat. *Veterinářství*, vol. 35, 1985, p. 510–511.

CHRENEK, P. – KUBOVIČOVÁ, E. – OLEXÍKOVÁ, L. – MAKAREVICH, A. V. – TOPORCEROVÁ, S. – OSTRÓ, A. 2015. Effect of body condition and season on yield and quality of *in vitro* produced bovine embryos . *Zygote*, vol. 23 (6), 2015, p. 893–899.

KUBOVIČOVÁ, E. – ZELEŇÁKOVÁ, J. – PIVKO, J. 1991. Biologická kvalita embryí. *Vedecké práce VÚŽV*, vol. 24, 1991, p. 33–41.

KUBOVIČOVÁ, E. – LANDA, V. – PIVKO, J. – ŠUPOVÁ, A. – ŘÍHA, Ľ. – GRAFENAU, P. 2003. Production of cattle embryos in conditions *in vitro*. *Journal of Farm Animal Science*, vol. 36, 2003, p. 41–45.

KUBOVIČOVÁ, E. – MAKAREVICH, A. V. – PIVKO, J. – CHRENEK, P. – GRAFENAU, P. – ŘÍHA, L. – SIROTKIN, A. V. – LOUDA, F. 2008. Alteration in ultrastructural morphology of bovine embryo following subzonal microinjection of bovine viral diarrhoea virus (BVDV). *Zygote*, vol. 16, 2008, p. 187–193.

KUBOVIČOVÁ, E. – MAKAREVIČ, A. V. – HEGEDUŠOVÁ, Z. – SLEZÁKOVÁ, M. – BEZDÍČEK, J. 2012. Effect of body condition score on oocyte yield and *in vitro* embryo development. *Výzkum v chovu skotu (Cattle Research)*, vol. LIV, no. 1 (197), 2012, p. 17–22.

KUBOVIČOVÁ, E. – MAKAREVIČ, A. – STÁDNIK, L. – HOLÁSEK, R. – HEGEDUŠOVÁ, Z. 2013. Effect of body condition and season on the yield and quality of cattle embryos. *Journal of Microbiology, Biotechnology and Food Science*, vol. 2, 2013, p. 1426–1435.

LANE, M. – GARDNER, D. K. – HASLER, M. J. – HASLER, J. F. 2003. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology*, vol. 60, 2003, p. 407–419.

LIU, Z. – FOOTE, R. H. 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twanty percent CO_2. *Biology of Reproduction*, vol.53, 1995, p. 786–790.

MAKAREVICH, A. V. – MARKKULA, M. 2002. Apoptosis and proliferation potential of bovine embryos stimulated with insulin-like growth factor I during *in vitro* maturation and culture. *Biology of Reproduction*, vol. 66, 2002, p. 386–392.

MAKAREVICH, A. V. – PIVKO, J. – KUBOVICOVA, E. – CHRENEK, P. – SLEZAKOVA, M. – LOUDA, F. 2007. Development and viability of bovine preimplantation embryos after the *in vitro* infection with bovine herpesvirus-1 (BHV-1): immunocytochemical and ultrastructural studies. *Zygote*, vol. 15, 2007, p. 307–315.

MAKAREVICH, A. V. – SPALEKOVA, E. – OLEXIKOVA, L. – LUKAC, N. – KUBOVICOVA, E. – HEGEDUSOVA, Z. 2011. Functional characteristics of ram cooling-stored spermatozoa under the influence of epidermal growth factor. *General Physiology and Biophysics*, Special Issue, vol. 30, 2011, S36–S43.

MAKAREVICH, A. V. – SPALEKOVA, E. – OLEXIKOVA, L. – KUBOVICOVA, E. – HEGEDUSOVA, Z. 2012a. Effect of insulin-like growth factor I on functional parameters of ram cooled-stored spermatozoa. *Zygote*, vol. 22, 2012a, p. 305–313.

MAKAREVIČ, A. – KUBOVIČOVÁ, E. – HEGEDŰŠOVÁ, Z. – PIVKO, J. – LOUDA, F. 2012b. Post-thaw culture in presence of insulin-like growth factor I improves the quality of cattle cryopreserved embryos. *Zygote*, vol. 20, 2012b, p. 97–102.

MAKAREVICH, A. V. – STÁDNÍK, Ľ. – KUBOVIČOVÁ, E. – HEGEDÜŠOVÁ, Z. – HOLÁSEK, R. – LOUDA, F. – BERAN, J. – NEJDLOVÁ, M. 2015. Quality of preimplantation embryos recovered *in vivo* from dairy cows in relation to their body condition. *Zygote*, 07/2015. DOI: http://dx.doi.org/10.1017/S0967199415000295.

MARKKULA, M. – RATY, M. – JAUHIAINEN, L. – PARANKO, J. – RAULA, J. – MAKAREVICH, A. 2001. Ratio of proliferating cell nuclear antigen-positive nuclei to total cell number is higher in Day 7 than in Day 8 vitrified *in vitro*-produced bovine embryos. *Biology of Reproduction*, vol. 65, 2001, p. 52–59.

MOTLÍK, J. – KOPEČNÝ, V. – PIVKO, J. 1978. The fate and role of macromolecules synthesized during mammalian oocyte meiotic maturation. I. Autoradiografic topography of newly syntetized RNA and protein in the germinal vesicle of the pig and rabbit. *Annales de Biologie Animale, Biochimie, Biophysique*, vol. 18, 1978, p. 735–746.

OLEXIKOVÁ, L. – KUBOVIČOVÁ, E. – MAKAREVIČ, A. V. – POPELKOVÁ, M. – PIVKO, J. 2009. Vplyv systému kultivácie na úspešnosť produkcie bovinných embryí *in vitro*. *Acta Fytotechnica et zootechnica*, vol. 3, 2009, p. 68–71.

PICARD, L. – GREVE, T. – KING, W. A. – BETTERIDGE, K. J. – JORGENSEN, P. H. 1986. Bisection of post-compaction bovine embryos: the evidence in viability between the two monozygotic calves. *Acta Veterinaria Scandinavica*, vol. 27, 1986, p. 33–48.

PIVKO, J. – ILLÉŠ, J. – ŠVELAN, J. – MAJERČIAK, P. 1973. Metodický postup a chirurgická technika pri výskume oplodňovacieho procesu prasníc. *Poľnohospodárstvo*, vol. 8, 1973, p. 652.

PIVKO, J. – MOTLÍK, J. – KOPEČNÝ, V. – FLÉCHON, J. E. 1982a. The fate and role of macromolecules synthesized during mammalian oocyte meiotic maturation. II. Autoradiografic topography of (3H)-fucose incorporation in pig oocytes cultured *in vitro*. *Reproduction, Nutrition, Development*, vol. 22, 1A, 1982a, p. 93–106.

PIVKO, J. – FULKA, J. – MOTLÍK, J. – PAVLOK, A. – TOMÁNEK, M. – MAJERČIAK, P. – GRAFENAU, P. – VANČIŠIN, J. 1982b. Prenos embryí hovädzieho dobytka pri riadenej produkcii dvojičiek. *Veterinární Medicína*, vol. 27, 7, 1982b, p. 395–404.

PIVKO, J. – KOPECNÝ, V. – TOMÁNEK, M. – KANKA, J. 1986. Evaluation of cow and swine embryos. *Scientific Works of the RIAP in Nitra*, vol. 22, 1986, p. 69–75.

PIVKO, J. – GRAFENAU, P. – KOPEČNÝ, V. – KUBOVIČOVÁ, E. – KORŠLAK, P. 1995. Proliferation activity of early cow embryos after bisection. *Journal of Farm Animal Science*, vol. 28, 1995, p. 149–153.

PIVKO, J. – KOPEČNÝ, V. – GRAFENAU, P. 1996. Komparatívne aspekty morfologicko-funkčnej diferenciácie vývoja jadrových štruktúr raných embryí. *Slovenská gynekológia a pôrodníctvo*, vol. 3 (4), 1996, p. 130–137.

PIVKO, J. – BARAN, V. – GRAFENAU, P. – KOPEČNÝ, V. – PELECHATYJ, N. S. – BONDARČUK, V, N. – KOŽUCH, A. J. – KOVALČIK, L. M. 1997. A study of the dynamical ultramicromorphology of differentiating cow early embryo nucleoli in embryos collected in the area of chronic irradiation near Tchernobyl. *Veterinární Medicína (Prague)*, vol. 2, 1997, p. 33–38.

PIVKO, J. – KUBOVIČOVÁ, E. – GRAFENAU, P. – OBERFRANC, M. 1998. Ultraštrukturálna analýza včasných embryí kráv po zmrazení. Acta fytotechnica et zootechnica Nitra, *Slovaca Universitas Agriculturae Nitriae*, vol. 1, 1998, p. 22–26.

PIVKO, J. – KUBOVIČOVÁ, E. – GRAFENAU, P. – ZIBRÍN, M. – CHLEBOVSKÝ, O. 2002. Autoradiographically detected impairment of RNA synthesis pattern in 8-16 cell bovine embryos after irradiation. *General Physiology and Biophysics*, vol. 21, 2002, p. 55–64.

PIVKO, J. – LANDA, V. – KUBOVIČOVÁ, E. – ŠUPOVÁ, A. – GRAFENAU, P. – MAKAREVIČ, A. – ŘÍHA, Ľ. – ZIBRÍN, M. 2003a. Comparative morphometry of precompacted bovine embryos produced *in vivo* or *in vitro* after parthenogenetic activation and intracytoplasmic sperm injection (ICSI): ultrastructural analysis. *Zygote*, vol. 11, 2003a, p. 207–217.

PIVKO, J. – KUBOVIČOVÁ, E. – GRAFENAU, P. – ŘÍHA, Ľ. – ZIBRÍN, M. 2003b. Ultrastructural changes of *in vitro* produced bovine embryos after OPS vitrification followed by cultivation. *Journal of Farm Animal Science*, vol. 36, 2003b, p. 11–16.

PIVKO, J. – LANDA, V. – KUBOVIČOVÁ, E. – ŠUPOVÁ, A. – GRAFENAU, P. – MAKAREVIČ, A. – ZIBRÍN, M. 2004. Ultrastructural morphometry of precompacted bovine embryos produced *in vivo* and *in vitro* after activation by electric pulse ac/dc. *General Physiology and Biophysics*, vol. 23, 2004, p. 101–112.

ROZENKRANZ, T. F. – ZENG, G. Z. – SCHOFF, P. K. – FIRST, N. L. 1990. A simple medium for *in vitro* development of bovine embryos. *Journal of Animal Science*, vol. 68, 1990, suppl. 1, P.430.

ŘÍHA, J. – POLÁŠEK, M. – VĚRTELÁŘ, J. – JÍLEK, J. – BANÁŠ, P. 1986. Nechirurgický přenos embryí inseminovaným přijemkyním při produkci dvojčat u skotu. *Živočišná výroba*, vol. 31 (2), 1986, p. 107–110.

ŘÍHA, J. – POLÁŠEK, M. 1987. Produkce telat-dvojčat u skotu po přenosu embryí inseminovaným příjemkyním. *Živočišná výroba*, vol. 32 (4), 1987, p. 489–497.

SREENAN, J. M. – DANAGH, M. C. 1984. Embryo transfer in cattle is now a practical proposition. *Farm Food Research*, vol. 10 (2), 1984, p. 53–61.

SHAMSUDDIN, M. – LARSSON, B. – GUSTAFSSON, H. – RODRIGUEZ-MARTINEZ, H. 1994. A serum-free, cell-free culture system for development of bovine one-cell embryos up to blastocyst stage with improved viability. *Theriogenology*, vol. 41, 1994, p. 1033–1043.

TERVIT, H. R. – WHITTINGHAM, D. G. – ROWSON, L. E. A. 1972. Successful culture *in vitro* of sheep and cattle ova. *Journal of Reproduction and Fertility*, vol. 30, 1972, p. 493–497.

VAJTA, G. – BOOTH, P. J. – HOLM, P. – GREVE, T. – CALLESEN, H. 1997. Successful vitrification of early stage bovine *in vitro* produced embryos with the open pulled straw (ops) method. *Cryoletters*, vol. 18, 1997, p. 191–195.