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STUDY OF THE MORPHOLOGY OF OVARY AND CYTOLOGY OF OOCYTE AS BASIS FOR ESTABLISHING METHODS IVM, IVF AND EMBRYO TRANSFER**

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Abstract: In three types of domestic animals: cattle, pigs and sheep, morphology of ovary was studied. Results such as differences in shape, size within and between species, number of follicles in maturation, changes in sex cycle, connection of follicles in maturation are presented in figures. By method of aspiration, oocytes were isolated from ovaries and their cytology analyzed as the first step in methods of *in vitro* maturation (IVM), followed by *in vitro* fertilization (IVF) and embryo transfer. These methods are wide spread in modern livestock production (cattle, horses) since they have many advantages of which the most important is to obtain more offspring from high quality female heads. Method has disadvantage: potential diminishing of biodiversity, therefore special attention is necessary in order not to endanger biodiversity and still get more offspring with high production abilities and traits. IVM, IVF and embryo transfer represent new approach to selection, fast and with similar effect like in conventional artificial insemination, therefore they should be applied in practice also in our country.

Key words: ovary, follicle, oocyte, *in vitro* maturation of oocyte (IVM)

Introduction

Methods of *in vitro* maturation of oocytes, *in vitro* fertilization and embryo transfer, were established in developed countries in the eighties of

the last century. However, although lot has been done on improvement and optimization of the method, final success of *in vitro* fertilization is approx. 80%, and of embryo transfer 40%, and number of live born offspring around 10-20%. This means that there is still possibility for improvement and increase of success of this method. In this paper, a review of morphology of ovary is given, as an organ in which oocytes mature *in vivo*, and of oocytes themselves, which are different from the aspect of cytology during *in vitro* (and *in vivo*) maturation, as well as short review of the most important, studied so far, biochemical characteristics of oocytes in domestic animals (*Tomek et al.,* 2002, 2005, 2006*, Smiljakovic* 2003, 2006*)*.

Objective of these modern methods is to increase the number of offspring of high quality heads of cattle especially in those species which have small number of offspring (cattle, horses, sheep), i.e. another form of artificial selection. Artificial selection is applied since the beginning of agriculture, as growing of cultivated plants and cultures, as well as animal species for different purposes, primarily for human nutrition. Domestification of plants and animals brought on the method of artificial selection and improvement/breeding. Embryo transfer is the attempt to improve artificial selection based on biological knowledge of animal reproduction. There is large number of embryo transfers performed in the world each year (540.000 in 2002) (*Wetscher et al,* 2005). Methods relating to embryo transfer are practiced as routine, and on the other hand there are large teams of experts engaged in finding better methods for collecting and storing oocytes and spermatozoids, *in vitro* fertilization and growing of an early embryo, as well as implantation of embryo into recipient dams.

Material and methods

In this paper, the method of observation was mainly used (observation and noticing) of morphological and cytological traits of ovaries and oocytes, primarily of pigs, since this material was the most accessible in the Institute for Animal Husbandry, Belgrade-Zemun, Serbia, whereas biochemical characteristics of oocytes in cattle were determined in Research Institute of Biology of Livestock - FBN, Dummerstorf, Germany (*Tomek et al.,* 2002, 2005*; Smiljakovic,* 2006*)* and in pigs (*Kubelka,* 2002*, Ellederova,* 2006).

Ovaries were taken on the slaughter line, in protein medium 0,5% PBS transferred to the laboratory and categorized according to the sex cycle stage. From immature and maturing follicles, by aspiration, oocytes were

taken and placed into protein medium 1%PBS, Gentamycin 0,25%, 1% glucose +EDTA, before microscoping on binocular microscope with magnification 2x and 4x. All the time oocytes were maintained on temperature of 37^0 Celsius.

Oocytes which were used for biochemical study were frozen on -80^0 Celsius, thawed and SDS-PAGE and Western blotting were carried out with incubation with antibodies for proteins and especially kinase enzyme whose presence, amount and phosphorilation (activity) were determined by chemiluminesence.

Results

Morphology of ovary. Ovaries of sheep, pigs and cattle were compared. Proportional to size of the animal is the ovary size, from the smallest in sheep to the largest in cattle. Also, considering that only one or two lams or calves are born by dam in single partus, on ovaries of these animals single follicle in maturation or post-ovulatory follicle is noticed. In pigs, since sows give birth to 10-12 piglets, number of follicles in maturation or ovulatory or post-ovulatory follicles, depending on the sex cycle stage during which the animal was sacrificed, more activated follicles at the same time are observed (Figure 1).

Figure 1. Comparative morphology of sheep, pig and cow ovaries

On figure 2, the difference in pig ovaries during sex cycle is obvious, which in pigs last for 24 days, from ovulatory follicles which are noticed at the beginning of cycle, post-ovulatory follicles with distinct vascularization, followed by creation of yellow bodies which secrete hormone progesterone, to white bodies if there was no fertilization, than ovaries with immature follicles, ovaries with maturing follicles, to mature follicles which burst releasing into the oviduct oocytes in metaphase of the second meiotic division, in the stage when oocytes can be fertilized by spermatozoids in oviduct.

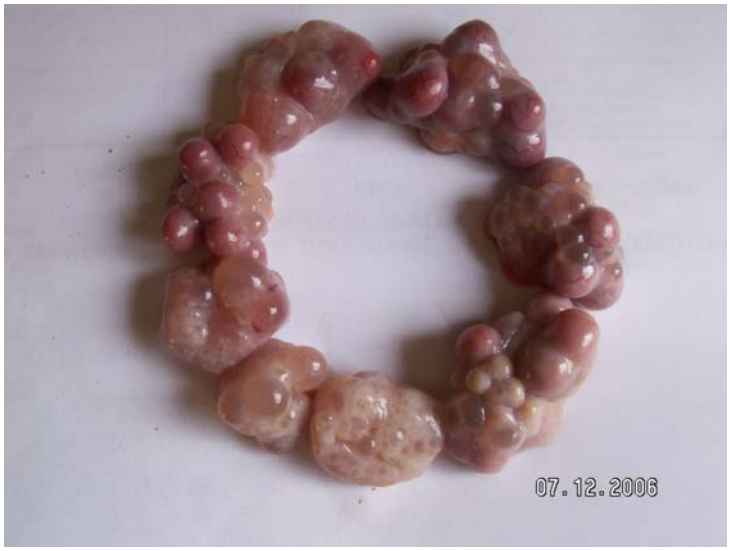

Figure 2. Pig ovaries during one sex cycle (emphasis on differences in morphology of follicles)

Beside usual physiological difference in morphology of ovaries and their follicles during sex cycle, also difference in size of ovaries which are in the same stage of sex cycle was registered (Figure 3), in pigs from the same fattening stage, same origin (Landrace), and same breeding conditions. Are these differences of genetic or epigenetic character, or caused by different hormonal status of individual animals, remains to be studied in the future.

It is possible even in single animal that two ovaries are in different maturity stage and size, which is shown on figure 4. One of the ovaries from the same pig contains immature follicles, whereas the other ovary contains

immature follicles but also large number of maturing follicles, and ovary is considerably larger. Also, it is usual that ovaries in regard to their maturation stage and size are bilaterally symmetrical in one animal (as shown on the Figure 3, left – two pairs of ovaries with immature follicles).

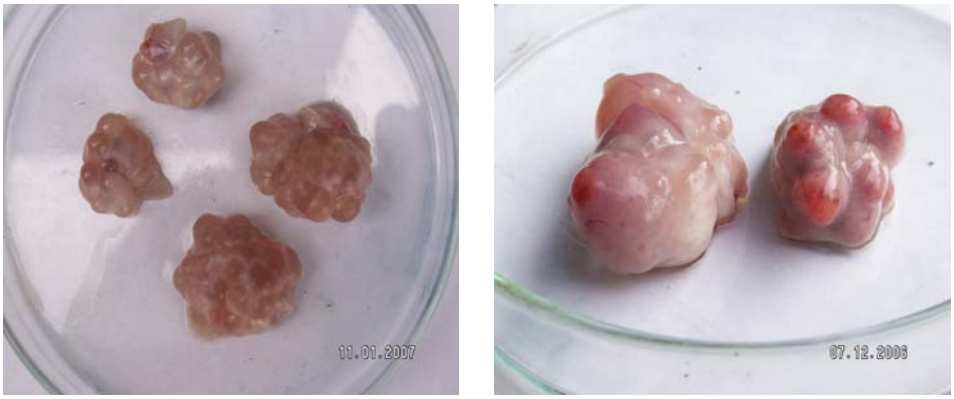

Figure 3. Differences in size of ovaries and follicles of pigs reared in same breeding conditions, of the same origin and in the same stage of sex cycle (left: immature follicles, right: ovulatory follicles)

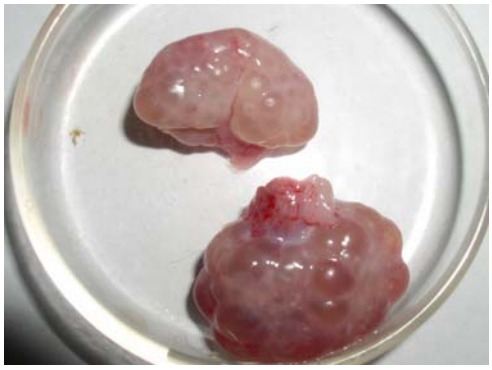

Figure 4. Differences between ovaries from single animal

The next figure shows the anatomy of ovary, which consists of two to three lobes (Figure 5), rarely four (Figure 6). By injecting stain in one

lobus/lobe, it gradually enters all follicles of single lobe, whereas the other lobe by its connective tissue or vascular specificity remains unstained.

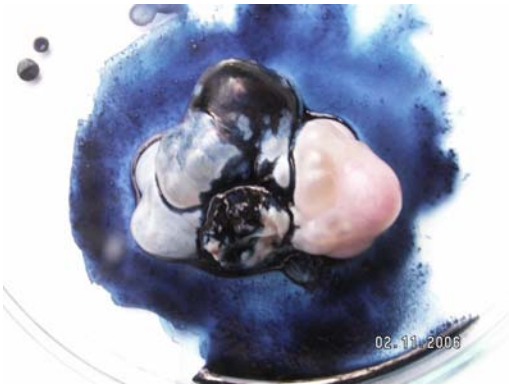

Figure 5. Ovary lobes visible after stain injecting

Figure 6. Mesenteres between lobes visible after application of stain

During aspiration of oocytes from ovaries to be used in embryo transfer, oocytes are collected from immature ovary follicles.

Cytology. Cells from immature follicles were in diplotene prophase of the first meiotic division and they mature *in vitro* to the metaphase of the second meiotic division, stage when they can be fertilized by spermatozoids.

Size of immature oocytes varies from 100-150μm. Even after aspiration they are surrounded by Zona pellucida and cumulus cells, which form Corona radiata. Ooocytes from pigs and cows are presented in following figures.

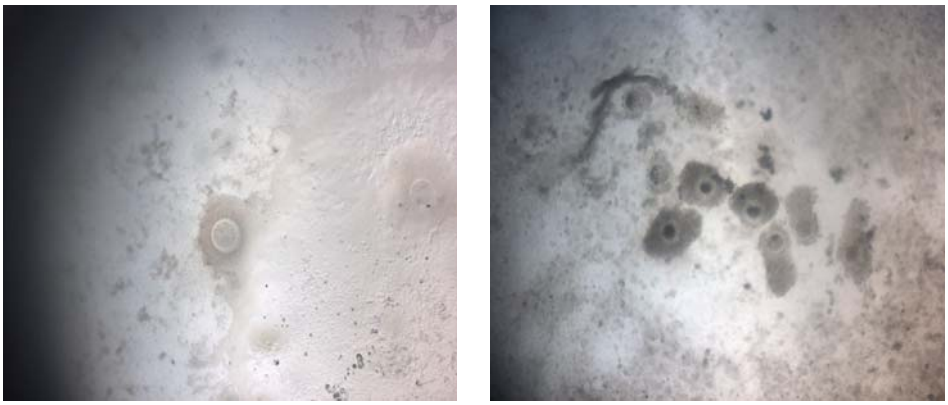

Figure 7. Oocytes of cow (top) and pig

On top micrograph cow oocyte is presented (two cells, one central and one to the right side of the figure), and on the bottom figure pig oocytes are presented, mainly apoptotic oocytes which remain structurally in COC (cummulus-oocyte-complex). So, oocytes are noticed (cow oocytes are bigger than pig oocytes – both micrographics were taken under same microscope magnification, 4x), surrounded by white circle – glycocalix Zona pelucida, and than cumulus cells which form Corona radiata of irregular shape. During *in vitro* maturation of oocytes, cumulus cells are multiplied so in work before in vitro fertilization this cell layer is removed, in order for oocyte to be more accessible for fertilization, and also these cells are thought to have inhibitory effect on oocyte maturation.

Discussion

As results show, variability in morphology of ovary in pigs is very high. Also, it is important to emphasize that physiological variability, changes in morphology and anatomy of ovaries during sex cycle, as well as dependence of sex maturity of sows are expressed (*Smiljakovic, unpublished results*).

Other authors (*Galli. and Lazzari,* 2003*, Tomek et al,* 2002), stated that for *in vitro* maturation of oocytes of domestic animals the most productive is taking of oocytes from immature follicles in order to achieve synchronization of *in vitro* techniques and embryo transfer, and from our results (unpublished) it is noticed that prior to collecting of oocyte samples on slaughter line, approx. 505 of ovaries contained mainly immature follicles. The quality of collecting of immature oocytes from immature follicles is of essential importance for the quality and number of blastocysts which are then implanted by embryo transfer into recipient dams (*Alm and Torner,* 2003), and quality of immature cells can be investigated, according to these authors, by staining of oocytes prior to placing them in the medium for *in vitro* maturation.

In order to increase the success of the method, the most suitable media for cultivation of oocytes and early embryos are studied, and biochemically, or more precisely proteomically, the effect of different additives and toxins (pharmacoproteomics) to standard medias for cultivation are investigated, by monitoring the protein status of the oocyte (*Tomek et al,* 2002, 2005, *Kubelka et al,* 2002). Os essential importance is analysis of MAP kinase, which provide during maturation always the specific profile of bands on SDS-PAGE, as well as Akt kinase, and 4E-BP1 protein (*Tomek et al.,* 2002, 2005*, Smiljakovic,* 2006; *Ellederova et al.,* 2006). The genetic basis of these proteins should be further analyzed (genotyping and gene's regulation) and this is much easier now with sequencing of cattle genome which is of size 3000Mb, distributed in 29 pairs of autosomes and pair of sex chromosomes.

Conclusion

Methods of *in vitro* maturation, *in vitro* fertilization and embryo transfer represent significant progress in artificial selection and breeding of domestic animals, by favouring heads of high quality and increasing of their posterity. Oocytes of high quality female heads of cattle and horses are collected mainly by the method of ovum-pick-up by laser guided probe in live cows and mares, and on the other hand, in large slaughterhouses, immediately after slaughtering. In this way, the posterity of high quality heads of cattle and horses has been considerably increased.

Although the biodiversity has been partially endangered like in any artificial selection, there should be room for these methods since biodiversity is not more endangered by these methods than with for example artificial insemination, which is already in practice in our country. First results in

development of the method in our conditions are positive and open many new fields of engagement, starting with work on optimization of the method and its application in livestock production, to biological analyses of the quality of oocytes and spermatozoids.

IZUČAVANJE MORFOLOGIJE JAJNIKA I CITOLOGIJE JAJNE CELIJE KAO OSNOVA ZA USPOSTAVLJANJE METODA IVM, IVF I EMBRIOTRANSFERA

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Rezime

Kod tri vrste domacih zivotinja: goveda, svinja i ovaca, izucavana je morfologija jajnika. Rezultati: razlika u obliku, velicini inter i intraspecies, broj folikula u zrenju, promene tokom polnog ciklusa, povezanost folikula u zrenju; prikazani su na slikama. Iz jajnika su metodom aspiracije iz jajnika izolovane jajne celije i analizirana njihova citologija kao prvi korak u metodi in vitro sazrevanja (IVM), nakon koje slede in vitro oplodnja (IVF) i embriotransfer. Ove metode su siroko rasprostranjene u modernom stocarstvu (goveda, konji) jer imaju niz prednosti od kojih je najvaznije dobijanje veceg broja potomaka od kvalitetnih zenskih grla. Metoda ima i nedostatak: moguce smanjenje biodiverziteta, i stoga treba voditi racuna da se biodiverzitet ne ugrozi, a da se dobije veci broj potomstva sa visokim proizvodnim osobinama. IVM, IVF i embriotransfer su nov pristup selekciji, brzi, a sa slicnim efektom kao kod klasicne vestacke selekcije, i zbog toga bi bilo dobro da nadju primenu i na nasim prostorima.

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