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COLLECTION and CONSERVATION of MAMMALIAN OOCYTES and EMBYOS for ANIMAL GENETICS RESOURCE PRESERVATION *EX SITU* (a review)

BLAGOJE STANČIĆ, IVAN STANČIĆ, STOJA JOTANOVIĆ, REFIK ŠAHINOVIĆ, SAŠA DRAGIN[1]

SUMMARY: In the last few decades, farm animal genetic diversity has rapidly declined. Therefore, it is in the interest of the international community to conserve the livestock genetics. In situ (live animals herds) model of genome conservation is expensive and limited for practical usage. Becouse, ex situ (ex vivo) conservation model are developed to cryopreserve animal genetic resources in genome (gene banks) to regenerate a particular population in future. Although significant progress has been made in oocyte and embryo cryopreservation of several domestic species, to date a standardized procedure has not been established. Successful long-term cryopreservation of oocytes and embryos would preserve the genetic material from unexpectedly dead animals and facilitate many assisted reproductive technologies. There are the biological, economical and moral imperative and interest of the international community to conserve the livestock genetics.

Key words: *oocyte, embryo, genetic resources, preservation, ex situ.*

INTRODUCTION

Almost all farm animal breeds are experiencing a significant decrease of genetic diversity in the last few decades (Prentice and Anzar, 2011). This is a result of intensive genetic selection for small number of productive and reproductive traits (Buerkle, 2007), application of modern biotechnologies in reproduction, that allowed the production a large number of progeny from a single individual, as well as use the effective methods of transport and long-term storage of sperm cells, oocytes and early embryos (Patterson and Silversides, 2003). Becouse, biodiversity preservation in domestic animal breeds and gene banks formation is in the interest of the international community (Prentice and Anzar, 2011).

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[1]Blagoje Stančić, PhD, full professor, Ivan Stančić, DVM, PhD, assistant professor, Saša Dragin, PhD, assistant professor, Faculty of Agriculture, Novi Sad, R. Serbia. Stoja Jotanović, PhD, associated professor, Faculty of Agriculture, Banja Luka, Republic of Serbska (Federation BiH). Refik Šahinović, PhD, full professor, Faculty of Biotechnology, Bihać, Federaction BiH.
Corresponding author: Blagoje Stančić, e-mail: blagoje.stancic@stocarstvo.edu.rs, phone: +318 21 485-3482.

Gene banks are defined as systematic and organized collection, preservation and exploitation of genetic material, by *in situ (in vivo)* or *ex situ (ex vivo)* methods. The *in situ (in vivo)* method involves preservation and reproduction the small herds of various animal species, breeds, and lines (Bulla, 1996; Wildt, 1999; Stančić, 1999). Method *ex situ (ex vivo)* involves long-term storage of gamets (sperm cells and oocytes) (Johnston, and Lacy, 1995; Stančić, 2000, Stančić et al., 2001; Stančić et al., 2002; Stančić et al., 2005; Stančić and Dragin, 2011; Stanković, 2012) or early embryos by cryopreservation technology (Stančić, 2004; Boettcher, et al, 2005; Pereira and Marques, 2008; Prentice and Anzar, 2011) as well as by cryopreservation of testicular or ovarian tissue somatic cells (Andrabi and Maxwell, 2007; Pereira and Marques, 2008). Longt-term preservation of oocytes and embryos is primarily use for biodiversity preservation and for using genetic material from animals after their biological death, in various selection programes.

The aim of this paper is to review the modren biotechnologies for collection and long-term cryopreservation of domestic animlas oocytes and early embryos *ex situ.*

OOCITES COLLECTION

There are two basic methods for collecting oocytes from domestic animal females: (a) oocytes collection after superovulation induction by treatment with exogenous gonadotrophins (eCG i hCG) and (b) oocyte extractin from the antral ovarian follicles (Stančić et al., 1992; Laurinčik et al., 1992; Stančić et al., 2007).

By the method of superovulation limited number of oocytes can be obtained. Furthermore, there are significant variation in superovulation rate, depending on the animal species and breed, body condition, health status as well as gonadothropin preparations dosage. Additionaly, hormonal treatment can result with various ovarian function disorders. In the cow, it can be obtained average 8.7 ovulation per treatment, with variations from 2 to 50 ovulations. In the sheep, superovulation rate varing from 2 to 15 ovulations, and in the sow superovulation rate varing from 25 to 46 ovulations (Stančić et al., 1992; Šahinović, 1995; Stančić et al., 1998; Stančić and Veselinović, 2002).

Significantly higher number of oocites per one female can be obtained by using methods for oocyte extraction from antral ovarian follicles (so-called „follicular oocytes"). This method is often used in pigs, because hormonal induced superovulation gets only a slightly more oocytes than after spontaneous ovulation (10 to 51 oocytes in gilts and 18 to 24 in sows). On the contrast, in the cow, it can be obtained 8 to 14 follicular oocytes (Wiebke, 1993).

It has been obtained average 7.9 oocytes per ovary by aspiration of antral follicles, and 45 oocytes per ovary by total ovary resection, in the sexualy mature gilts, post mortem (Stančić et al., 1993). The advantage of this method is the possibility of getting a large number of oocytes from sacrificed animals and avoiding the harmful effects of exogenous hormone treatment. Namely, it has been demonstrated that superovulation induction with higher doses of gonadotropins, frequently result with increasing number of degenerated oocytes, as well as increasing number of unovulated and/or cystic follicles (Moor et al., 1985; Stančić et al., 1991). However, the crucial disadvantage of this method is the fact that follicular oocytes are not possible for fertilization immediately afeter extraction from the ovarian follicles. Namely, the nucleus of more than 95% ovarian oocytes is in the germinal vesicle stadium (GV), i.e. diplothen of the first meiotic division (Crozet,

1991). Therefore, it is necessary to perform *in vitro* maturation (IVM) of obtained follicular oocytes, to riche the metaphase of second meiotic division (so-called MfII-oocytes). These oocytes are capable for fertilization, i.e. for activation by sperm penetration (McDonald, 1989). Before *in vitro* cultivation, follicular oocytes must be denuded (i.e. cumulus cells complex must be removed from the zona pelucida surface). The quality of cumulus complex directly influence the *in vitro* maturation rated follicular oocytes. Only oocytes with compact (i.e. GV-oocytes) or expanded cumulus (i.e. GVBD-oocytes, germinal vesicle brake down) are capable for successful *in vitro* maturation (Laurinčik et al., 1992). After 24h to 48h of cultivation, about 80 to 90% of oocytes mature, i.e. riche the MfII stage of nuclear division (Fukui, 1989). The number of matured oocytes can be improved by cultivation medium supplementation with p-FSH (Laurinčik et al., 1993), FSH and LH (Šahinović et al., 1994), follicular fluid (Nagai, 1994), epidermal growth faktor or other bioactive supstance (Singh et al., 1993),

EMBRYOS COLLECTION

Early embryos, 5 to 8 days of age (morula or early blastocyste stage), are use for long-term cryopreservation in the liquid nitrogen, at temperature – 196°C (Paynter et al., 1999). Such embryos can be obtained by: (a) flushing from ovidicts or uterus of superovulated and inseminated donor female or (b) after in vitro fertilization of follicukar oocytes or oocytes obatained by flushing donors oviductes. Embryo flushing can be dun by laparotomic or laparoscopic approach to reproductive organs (i.e. surgical method), as well as by transcervical flushing of uterus (nonsurgical method) (Besenfelder et al., 1998). Nonsurgical method is usualy prformed in large animals (cow, mare), and surgical in small animal (sheep, goat, pig) (Stančić and Veselinović, 2002).

In vitro fertilization (IVF) of in *in vitro* matured (IVM) oocytes, or oocytes obtained after hormonal superovulated donor females, mainly dipend of matured oocyte (MfII-oocytes) quality, proper sperms *in vitro* capacitation, composition of maturation and fertilization medium and conditions of cultivation microclimate (temeperature, composition and percentage proportion of gases, such as O_2, CO_2 and N_2) (Thibault et al., 1988; Courot and Vallard-Nail, 1991). Success of *in vitro* fertilization is calculated by the percentage of monospermic penetrated oocytes, as well as by the number of oocytes reched 2-blastomere or 4-blastomere stadium of embryo division. In the cows, more than 70% monospermic and about 4% polyspermic penetrated oocytes has been obtained after 48h of *in vitru* follicular oocytes cultivation (Šahinović, 1995). However, in the pig, about 85% oocytes were polyspermic penetrated, after *in vitro* fertilization of follicular oocytes. Polyspermic oocytes is not possible to undergoing normal embryo development (Šahinović, 1995; Šahinović et al., 1997). This phenomenon is not yet clearly elucided, buth methods of sperms *in vitro* precultivation and capacitation, as well as sperms and oocytes coincubation procedure, seems to be of the mayor influence (Besenfelder et al., 1998; Cshum et al., 1990). Additionaly, success of IVF is influenced by cultivation medium composition, period of cultivation and microambient cultivation conditions (Edwards, 1989).

OOCYTE and EMBRYOS CRYOPRESERVATION

Cryopreservation involves preservation of oocytes, early embryos or whole tissues at very low temperatures, usually in liquid nitrogen (- 196°C) (Woods et al., 2004). At such a low temperature, biological activity is effectively stopped, and the cells functional status may be preserved for centuries. However, to avoid the intracellular ice formation, that influence cell death, freezing technology must include crioprotect substances addition in semen extender, dehydratation, freezing point depression, supercooling, and intracellular vitrification (Wolfe and Bryant, 2001). Other factors, such as osmolarity changes, toxicity of cryoprotectants, increasing intracellular electrolites and other sterssors can also result in cells damage or death (Vajta, 2000). It has been demonstrated that antifreezing-proteins, sugars or antioxidants act to stabilize the cell membrane (Ledda, 2001).

During cryopreservation, a significant number of oocytes or embryos being damaged and, after thawing, is uncapable of further development. The degree of damage depends on the shape and size of the cell, oocytes or embryos quality, permeability of cell membranes, and these factors vary depending on the type of animal (Vajta and Kuwayama, 2006). Previous studies have shown that early embryos are more tolerant to cryopreservation than oocytes. The exact cause is not established, but one reason could be the difference the structure and/or plasmal membrane osmotic potencial, between oocytes and embryos (Chen et al., 2003).

The maintenance potential for normal oocytes development after thawing varies between the animal species, developmental satages and origin (Critser et al., 1999, Pereira and Marques, 2008). It would ppear that the stage of embryo development and the number of cells exposed to the sterss of cryopreservation could play an important limiting role for normal embryo development after thawing (Paynter et al., 1999).

Table 1: Differences of animal oocyte and embryo cryopreservation resistance among species, developmental stages, and origin.

	More resistance	Less resistance
Species	Bovine, ovine	Porcine, equine
Developmental stages	Morula, YBL, and BL	Hatched BL and oocytes
Origin	*In vivo* derived embryos	*In vitro* produced embryos, micromanipulated embryos

YBL: young blastocyst; BL: blastocyst; *Pereira and Marques (2008).*

CONCLUSION

According to FAO reports, in all species of domestic animals is observed an increasing decline in biodiversity. Therefore, there is an increasing demand for efficient biotechnological research methods of long-term conservation of the genomes of existing species, breeds and lines of farm animals.

Preservation of genetic resources is carried out using method *in situ (in vivo)*, forming small herds of certain species of animals, or *ex situ (in vitro)*, using long-term cryopreservation of sperm, oocytes, embryos or reproductive tissue somatic

cells (testis and ovarium). Thus it is possible to perform multiplication of desirable genotypes, when the need arises, although the donor animals were dead for a long time. Although cryopreservation technology has progressed in recent decades, the success of survival of frozen oocytes and embryos, are still not satisfactory. Previous studies have shown that early embryos are more tolerant to cryopreservation than oocytes. In addition, cryopreservation technology is complex and expensive, and not available for widespread use. It is therefore necessary to combine the use of methods of *in situ* and *ex situ*, with the aim of successful conservation of biodiversity of domestic animal breeds.

Conservation of genetic biodiversity of domestic animals is a global imperative in the biological, economic and moral sense. Biologically, because biodiversity is a key condition for survival of life on our planet. Economically, because a human population uses a huge number of animal species for food, medicines, chemicals, technological materials and energy. Moral, because man, as dominant species, is responsible for the maintenance and protection of all other species of living organisms, with which it must live on this planet.

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PRIKUPLJANJE I KONZERVACIJA OOCITA I EMBRIONA SISARA ZA ČUVANJE ŽIVOTINJSKIH GENETSKIH RESURSA *EX SITU* (pregled)

BLAGOJE STANČIĆ, IVAN STANČIĆ, STOJA JOTANOVIĆ, REFIK ŠAHINOVIĆ, SAŠA DRAGIN

IZVOD

U poslednjih nekoliko dekada, genetski diverzitet domaćih životinja rapidno opada. S tim u vezi, postoji interes međunarodne zajednice za očuvanje genetike domaćih životinja. Model in situ čuvanja genoma je skup i značajno limitiran za praktičnu primenu. Zbog toga se razvija model ex situ (ex vivo) krioprezervacije animalnih genetskih rsursa (banke gena) za regeneraciju pojedinih poipulacija u budućnosti. Iako postoji značajan napredak u krioprezervaciji oocita i embriona pojedinih vrsta domaćih životinja, do danas nije ustanovljena standardna procedura ove tehnologije. Uspešna dugotrajna krioprezervacija oocita i embriona će omogućiti očuvanje genetskog diverziteta i primenu brojnih tehnologija asistirane reprodukcije domaćih životinja. Postoji biološki, ekonomski i moralni imperativ i interes međunarodne zajednice za očuvanje genetike domaćih životinja.

Ključne reči: oociti, embrioni, genetski resursi, čuvanje, *ex situ*.

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