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CRYOPRESERVATION OF BĂLȚATĂ CU NEGRU ROMÂNEASCĂ CATTLE IMMATURE OOCYTES BY SLOW FREEZING METHOD: PRELIMINARY RESEARCH

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ABSTRACT. Research on bovine oocytes cryopreservation is important for successful preservation of genetically valuable animal. The transvaginal ultrasound-guided follicular puncture coupled with in vitro embryo production has become competitive and alternative method for MOET (Multiple ovulation and embryo transfer) in dairy cattle. The aim of this preliminary research is to presents the result of Bălțată cu Negru Românească (BNR) cows oocytes recovery by two different protocols and its cryopreservation by slow freezing method. By applying the recovery oocytes from slaughterhouse ovary we obtained an average of 16.34 ± 6.71 oocytes per cow, much higher compared with the Ovum Pick Up (OPU) method, which reveals an average of 2.75 ± 0.2 oocytes per cow. After applying the slow freezing procedures using the ethylene glycol cryoprotectant we observed the oocytes with cumulus cells normal with the spherical shape and normal zone pellucida.

Key words: Bălțată cu Negru Românească cows; immature oocytes; slaughterhouse ovary; OPU; cryopreservation.

REZUMAT. Crioconservarea ovocitelor imature la bovinele din rasa Bălțată cu Negru Românească, utilizând metoda congelării lente: cercetări preliminare. Cercetările efectuate asupra crioconservării gameților femeli sunt importante pentru păstrarea cu succes a animalelor de mare valoare genetică. Recoltarea gameților femeli la specia bovină prin puncție

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transvaginală ecoghidată, asociată cu producere de embrioni *in vitro*, reprezintă, la ora actuală, o metodă alternativă şi competitivă pentru ovulaţia multiplă, asociată embrio transferului. Scopul acestor cercetări preliminare constă în prezentarea a două metode standard de recoltare a gameţilor femeli, aplicate la rasa Bălţată cu Negru Românească, şi crioconservarea lor prin utilizarea metodei de congelare lentă. Recoltarea gameţilor din ovarele provenite de la vacile sacrificate în abatoare a relevat o medie de 16.34 ± 6.71 gameţi, superioară metodei de recoltare prin puncţie transvaginală ecoghidată a foliculilor ovarieni (OPU – Ovum Pick Up), care a prezentat o medie de 2.75 ± 0.2 gameţi. După aplicarea metodei de criocongelare lentă, prin utilizarea etilenglicolului ca şi crioprotectant, s-au observat, după decongelare, gameţi de formă sferică, înconjuraţi de celule cumulus normale şi cu zonă pelucidă normală.

Cuvinte cheie: Vaci din rasa Bălţată cu Negru Românească; ovocite imature; ovare din abatoare; OPU; crioconservare.

INTRODUCTION

Cryopreservation of bovine oocytes will permit germplasm of genetically valuable animal to be preserved for extended times. This benefits improving animal programs by increasing the female impact and other biotechnologies that use oocytes, such as cloning by nuclear transfer and transgenic animal production (Massip, 2003). The cryopreservation of bovine oocytes remains a challenge for the dairy cows industry because of a very few calves, which have been born following the transfer of blastocysts derived from frozen or vitrified oocytes (Vajta *et al.,* 1998; Fuku *et al.,* 1992; Hamano *et al.,* 1992; Otoi *et al.,* 1995; Suzuki *et al.,* 1996; Le Gal *et al.,* 2000). The main source of oocytes is from abattoir cattle ovaries and from Ovum Pick Up biotechnologies (OPU).

Oocyte cryopreservation has been performed in several mammalian species. However, in spite of some successful reports, the efficiency is still very low. It was demonstrated (Wu *et al.,* 1999) that immature bovine oocytes are very sensitive to low temperatures of 4 or 0^0C, with a normal meiotic spindle formation that inhibit the normal development of oocyte after *in vitro* fertilization. Mature bovine oocytes are sensitive to cooling, with occurrence of cytoskeleton rupture and meiotic spindle injuries (Aman and Parks, 1994).

In humans, the long-term storage of oocytes rather than embryos would help reduce legal, ethical, and moral issues, associated with storing embryos (Gosden and Nagano, 2002; Grundy *et al.,* 2001). Storage of oocytes would also allow cancer patients without partners to preserve their genetics for later use (Fabbri *et al.,* 2001; Porcu *et al.,* 2004).

This study reveals some results obtained by slow freezing Bălţată cu Negru Românească cattle oocytes obtained from slaughterhouse ovary and from Ovum Pick Up biotechnologies. These oocytes were not submitted to any treatment before cryopreservation process.

MATERIAL AND METHODS

Ovaries obtained from slaughterhouse were transported to the laboratory in saline NaCl 0,9% + 10% PENSTREP ject (Dopharma, Olanda) at 30^0C within 2-3 h. For this procedure we used sterile containers filled with saline solution and imprinted with the cows identification number.

Upon return to the lab, the ovaries were homogenized several times with the pre-warmed saline solution until the majority of the blood was removed. Following the washes, the ovaries were place in the beaker containing fresh transport saline and store at room temperature until time of oocyte collection. Cumulus oocytes complexes were recovery by holding ovary above beaker and make 2-3 mm deep incisions across all visible follicles. After that the ovaries with the incision were washed with the OPU recovery medium (Minitube, Germany) (*Fig. 1*). For improving the oocytes collection the slashed ovaries after washing were pressed against the side of the breaker to allow drainage of oocytes.

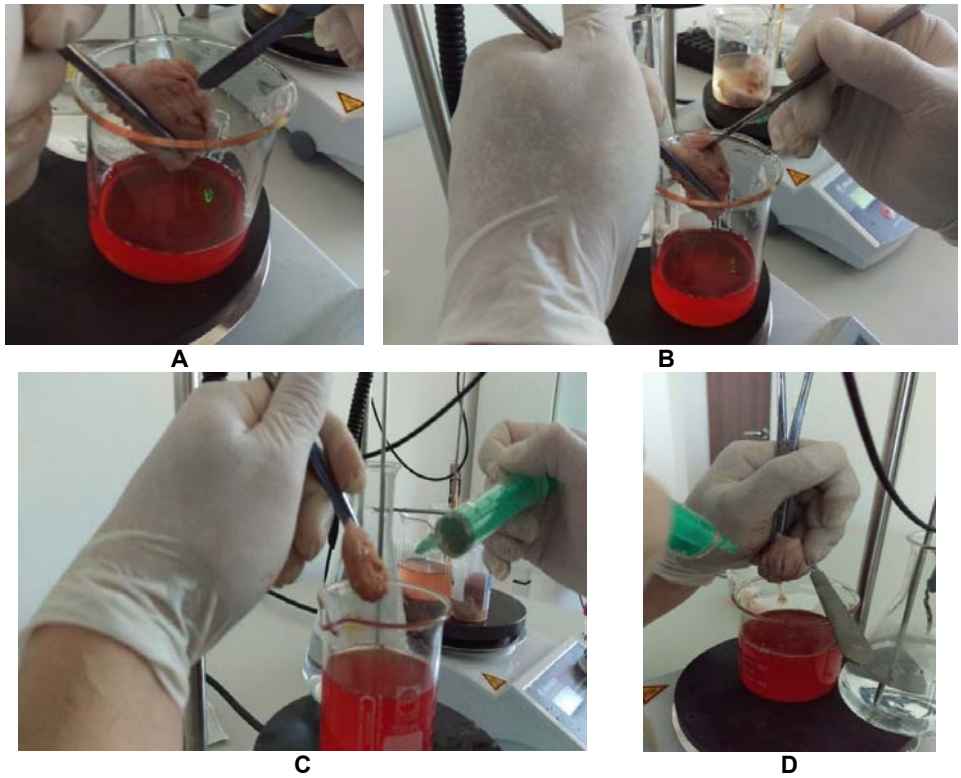


Figure 1 - Recovery the cows oocytes from the slaughterhouse ovary by incisions of follicles (A, B) and washing with the OPU recovery medium (C, D)

Another method used for recovery the oocytes were represented by the OPU recovery biotechnologies. In this case, four dairy cows were used by applied the OPU recovery technique two times per week. Transvaginal ultrasound guided oocyte collection was performed using an ultrasound scanner (Ultrasonic device SSDProSound2 convex real time scanner) with a convex-sector probe 5 MHz (Bold Medical, Romania), attached to an aspiration pump (Rocket medical) and ovum pick-up needle (COVA NEEDLE "type A", 17Gx500 mm, Misawa Medical Industry-Japan) guidance system. The follicles from 2 to 10 mm were punctured. For oocytes aspiration a vacuum of 100 mmHg was used. The following parameters were evaluated: the number of punctured follicles (*Fig. 2*), the oocytes recovery rate and the oocytes quality.

The oocytes with homogenous ooplasm surrounded with multilayer compacted cumulus cells (*Fig. 3*) were selected for experiments by transfer them in the TCM 199 medium, which represented the first washing. The second wash was represented by the TCM 199 medium (Minitube, Germany) + 10% estrus cow serum. The third wash was represented by the 50% TCM 199 medium with 10 % estrus cow serum and 50 % freezing medium (BoviFreeze, Minitube, Germany), which contained ethylen glycol, buffers, sugar, pyruvate, BSA (bovine serum albumin), antibiotics and sucrose.

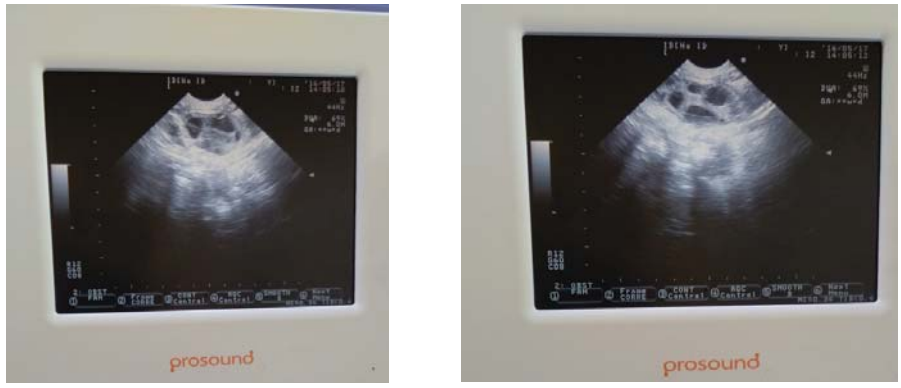


Figure 2 - Ultrasonography images with the cows follicles, which were punctured *in vivo* by using OPU biotechnique

After finalizing the three washes procedures (*Fig. 3*), the oocytes were transferred to the freezing medium (BoviFreeze, Minitube, Germany) and aspired in the 0.25 ml storage straw. Before start the cryopreservation process the storage straw were equilibrated 10 min at the room temperature.

The cryopreservation processes were conducted with the CryoPreservation System CL 8800i. The freezing process involves three steps: 1) holding the straws for 10 min at the - 6^0C temperatures; 2) lowering the temperature with 0.3^0C/min until it reaches -32^0C; 3) equilibration the straw at this temperature for 10 min; 4) immersion the straws in the nitrogen chamber with liquid nitrogen (LN_2) at the -196^0C.

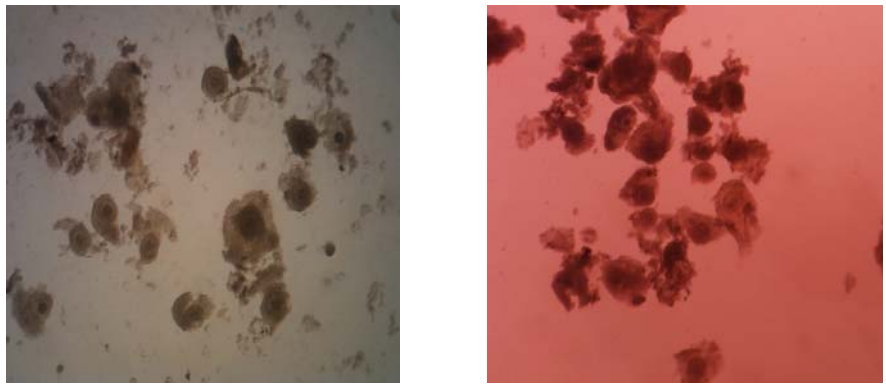


Figure 3 - Microscopic images of the immature bovine oocytes in the washing procedures

RESULTS AND DISCUSSION

The *Tables 1 and 2* presents the results of the oocytes recovery process from the slaughterhouse ovary and by OPU biotechnique.

Table 1 - Results of oocytes recover from slaughterhouse ovary

Cows identification number	No. of recovery oocytes
RO240001042890	16
RO505000112376	18
RO241001042864	20
RO502000112335	14
RO242001058679	25
RO 246000218528	5

Table 2 - Results of oocytes recover by OPU biotechnique

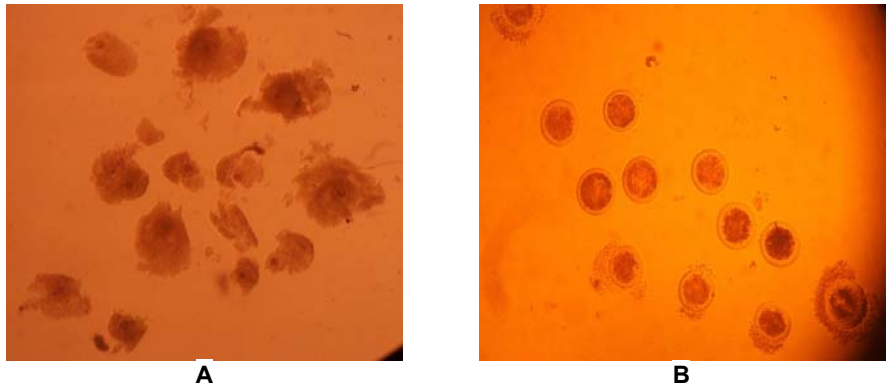
Cows identification number	No. of punctured follicles	No. of oocytes recovery	No. of punctured follicles according with size		
			3-4 mm	5-6 mm	≥ 7 mm
RO503002730703	6 ± 0.2	2 ± 0.1	3 ± 0.1	1 ± 0.2	2 ± 0.3
RO509001166316	8 ± 0.4	3 ± 0.3	3 ± 0.1	3 ± 0.2	2 ± 0.1
RO508000954090	10 ± 0.9	4 ± 0.1	6 ± 0.3	2 ± 0.3	2 ± 0.1
RO2470001744322	7 ± 0.3	2 ± 0.1	4 ± 0.2	1 ± 0.2	2 ± 0.1

*The results are presented like average ± standard deviation

By applying the recovery oocytes from slaughterhouse ovary we obtained a total of 98 oocytes and an average of 16.34 ± 6.71 oocytes per cow. From this total number of oocytes, 78 were selected for freezing

process. For the OPU recovery procedures the mean numbers of recovery oocytes were 2.75 ± 0.2. The recovery rate was in this case 35.49 %. Our results are not so satisfying, compared with the result reported by Bungartz *et al.* (1995), which reveals a number of 7.0 ± 0.6 oocytes in pFSH stimulated Holstein Friesian cows and 5.8 ± 0.5 for non-stimulated Holstein Friesian cows. This difference can be influenced by many factors, such as: different breeding conditions, the presence of the dominant follicle, the heat stress etc. (Boni, 2012).

For checking the freezing procedures, one straw were thawed in a water bath at 37^0C for 1 min. After that, the oocytes were transferred in TCM 199 solution and vortexed for denuded the cumulus oocytes complex. The first evaluation reveals the oocytes with normal cumulus cells, with the spherical shape and normal zone pellucida (*Fig. 4*).



A **B**

Figure 4 - Microscopically images with the thawed oocytes before (A) and after (B) vortexing

CONCLUSION

In conclusion, it is possible to cryopreserve immature bovine oocytes by using BoviFreeze medium and CryoPreservation System CL 8800i. However, the efficiency of this method is not totally evaluated in this preliminary study and further studies are necessary to evaluate the oocyte dysmorphisms (OD) [perivitelline space (normal/large), perivitelline debris (no/yes), oocyte shape (spherical / non-spherical), zone pellucida (normal/abnormal), first polar body morphology (normal /fragmented or irregular), cytoplasmic granularity (normal / excessive), cytoplasmic vacuoles (no/yes) and colour of cytoplasm (normal/dark)], the protection of ethylene glycol in the slow freezing process considering permeability and its toxicity. The development rate of the freezing oocytes must be evaluated in futures studies.

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