FIRST EMBRYOS PRODUCED IN ROMANIA BY OVUM PICK-UP AND *IN VITRO* FERTILIZATION IN HOLSTEIN FRIESIAN CATTLE

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Abstract

Reproductive technologies, such as ovum pick-up (OPU) and in vitro fertilization (IVF) can rapidly enhance genetics of cattle through both the female and male linage. In this study, we describe the results of first bovine embryo production in Romania by IVF after retrieval of oocytes by OPU. Also, we evaluated the embryo production by OPU followed by IVF in cows stimulated with a FSH-based treatment (experimental group, n = 5) compared to non-stimulated cows (control group, n = 4). Our results suggest that the follicular growth stimulation with FSH improves the number of the punctured follicle (9.8 ± 0.8 vs. 2.5 ± 0.8, P<0.05) and the hatched blastocyst rate (41.9% vs. 16.7%). No effect was registered in the oocytes recovery rate in experimental vs. control group (63.3% vs. 60%). In conclusion, the use of follicular growth stimulating program is recommended in order to improve the number of recovered oocytes and the blastocyst rate at Holstein Friesian cattle. This is the first report of embryo production in Romania, using OPU in association with IVF in cattle.

Key words: dairy cows, ovum pick-up, in vitro fertilization, bovine embryos, hatched blastocyst

INTRODUCTION

Both ovum pick-up (OPU) and *in vitro* fertilization (IVF) are seen as mature technologies currently applied in cattle which can be used like an important instrument to drive genetic progress. *In vitro* embryo production has remarkably expanded in the last decade compared to *in vivo* embryo production. This is supported by the total number of transferable OPU and IVF bovine worldwide embryos, 326.623 fresh embryos and 121.490 frozen embryos in 2016 [1]. According to Qi et al. [2], Brazil dominated the *in vitro* embryo production by performing 53.019 OPU sessions averaging 15 oocytes and 6 embryos per session.

Although there is a large variation between donors, some IVF labs have achieved the performance to produce over 50 calves per donor cow per year by combining the two technologies, OPU and IVF.

Most of the studies identify donors with highly potential for oocyte production during oocytes retrieval protocols [3], [4]. However, for stimulating follicular growth in the cows from all hormone used in the research studies, FSH has usually given the best results in terms of number of follicles aspirated and oocytes retrieved. Due to the individual variation to FSH stimulation, the number of recovery oocytes varied from 0 to 26 [5]. Most regimens for FSH involved multiple treatments, either 12 or 24h apart, over 2–4 days [6], [7], [8].

In this study we highlight the results of first embryos production in Romania by combining OPU and IVF at Holstein (*Bos taurus*) breed. Also, we test the involvement of FSH follicular growth stimulating program

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for improving the number of recovered oocytes and the rate of hatched blastocyst.

MATERIAL AND METHOD

This study was performed at the Research and Development Station for Cattle Breeding Dancu-Iasi (SCDCB Dancu-Iasi), Romania, which owns a population of 600 Holstein Friesian cows (recognized as a Bălțată cu Negru Românească breed).

In this experiment we used a standard protocol (IVF Bioscience, UK) for obtaining bovine embryos through IVF which is according to the current literature.

Ovarian stimulation program and oocytes recovery

The cows from this experiment were part from the SCDCB Dancu-Iasi culling group with no milk production during our study. Each animal from experimental group (E-group, n = 5) was treated during the luteal phase to prevent spontaneous ovulation. Cows were stimulated for 3 days with FSH (Pluset, Laboratorios Calier, Spain), 2 administration per day at 12 hours interval following the next doses: day 1 - 3ml/3ml; day 2 - 2.5ml/2.5ml and day 3 - 2ml/2ml. Also in day 3 a dose of PgF2α (PGF Veyx forte, GmbH) was administered for each cow from experimental group. OPU was performed at 24 h after PgF2α administration. The cows from control group (C-group, n = 4) did not receive any treatment.

The oocytes were harvested from the living donors by OPU. A real-time B-mode ultrasound scanner (Aloka Prosound 2) equipped with a 5 MHz convex transducer was used during the transvaginal ultrasound guided follicular aspiration. The transducer was mounted in a metal handle with stainless needle guide. A Cova OPU Needle of 17G x 600 mm was attached by a silicone hose to a 50 ml plastic tube (Falcon). Follicular fluid was aspirated using continuous negative pressure, 50–90 mm Hg, applied with a suction pump (Craft Suction Pump-Rocket Medical) and collected in a 50 ml tube containing OPU medium (IVF Bioscience, UK).

In vitro embryo production

After recovery, all the collected cumulus oocytes complexes were cleaned of debrids by washing them three time in Bo-Wash medium (IVF Bioscience, UK) and transferred for maturation step in 4 well NUNC dishes, which contained 500 µl/well BO-IVM medium (IVF Bioscience) and placed in incubator at 38.8ºC, 5% CO_2 and 90% relative humidity for 24 h.

Spermatozoa were selected by using BO-SemenPrep medium (IVF Bioscience, UK) as following: for each 250µl frozen semen straw, two tubes with 4 ml and respectively 2 ml Bo-SemenPrep, were prepared; the semen for one straw was centrifuged at 328g for 5 minutes in 4 ml Bo-SemenPrep tubes; after centrifugation the supernatant was removed until 350–700µl sperm suspension remained in 4 ml tubes; after this procedure we added the additional preheated BO-SemenPrep (from 2 ml tubes), resuspended the pellet and centrifuged; following the second centrifugation, we removed again the supernatant until the same volume of 350–700µl sperm suspension remained; we resuspended the pellet in this volume and used for IVF.

The fertilization of matured oocytes was conducted in 90 µl drops of BO-IVF medium (IVF Bioscience, UK) under mineral oil. Before fertilization, the matured oocytes were washed three times in 100 µl BO-IVF medium and then transferred to the fertilization microdrops. After this procedure, a concentration of 2×10^6 sperm/ml was used for the *in vitro* fertilization and then the gametes were co-incubated for 20 h at 38.8ºC, 5% CO_2 and 90% relative humidity.

For culture of presumptive zygotes in BO-IVC medium (IVF Bioscience, UK), cumulus cells were removed by vortexing the cumulus oocytes complexes for 2 minutes in the same solution. The culture of presumptive zygotes was carried out in NUNC dishes, which contained 500 µl/well BO-IVC medium under mineral oil and placed into the incubator at 38.8ºC, 5% CO_2 and 90% relative humidity for 7–9 days. The results of the IVF procedure were evaluated in days 7, 8 and 9 (the day of fertilization was considered day 0).

The statistical significance of the differences in means of two groups was evaluated by one-way analysis of variance

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(ANOVA) and by Tukey-Kramer Multiple Comparisons Test.

RESULTS AND DISCUSSIONS

Nine sessions of OPU associated with IVF procedures were performed, in which a total number of 59 follicles were punctured (49 in E-group and 10 in C-group). In experimental group the follicular growth stimulation protocol with FSH generated an average of 9.8 ± 0.8 punctured follicles (Fig. 1) per session ($P<0.05$) compared with C-group in which only 2.5 ± 0.8 follicles per session were punctured (Table 1). In this experiment we observed no differences in the recovery rate in E-group (63.3%) vs. C-group (60%), but in the number of recovered oocytes, which was superior ($P<0.05$) for E-group (6.2 ± 0.8) compared with C-group (1.5 ± 0.2). In our opinion this is a promising result considering that it is a premiere in Romania. In domestic animals, four methods for collection of oocytes have been described: aspiration of the oocyte from the follicles of living cows by transvaginal ultrasound guided follicular aspiration (a procedure also called ovum pick-up, OPU) [9], [10], slicing the ovaries [11], [12], [13], puncture of visible surface follicles [14], [15] and laparoscopic ovum pick-up [16]. However, the OPU seems to be preferred for commercial purpose [1] as it allows a prolonged use of a certain donor cows for IVF compared to other methods. Several studies reported a high variation in oocyte retrieval per OPU across breeds [17], [18], [19]. For example, Watanabe et al. [20] presented a high number of recovery oocytes (n = 19.3 ± 0.6) per OPU session at Holstein Friesian cattle, which is superior compared to us. Also, this research highlights the fact that the result was influenced by the decision to use for OPU session only the donor cows with greater potential for oocyte recovery per OPU. This may determine IVF success in some cattle breeds yielding fewer oocytes per OPU [20]. Thus, further research is needed to explore the relationships between the number of oocytes recovered per OPU session and with IVF efficiency, as well as with field fertility (pregnancy results following embryo transfer). It will be an important step in decisions making regarding the donors selection for improving the results of *in vitro* production of bovine embryos.

Although we did not select the oocytes according to A, B, C quality grade [21], [22] we obtained an acceptable embryo development rate (Table 1) objectified by 41.9% hatched blastocyst (n = 13, $P<0.05$) in E-group compared with only 16.7% hatched blastocyst in C-group (n = 1). In our opinion, this result is influenced by the higher number of fertilized ova in E-group compared to C-group. Similar results were obtained by Watanabe et al. [20], which concluded that the number of blastocysts per OPU is greater for dairy donors with higher number of oocytes recovered per OPU. Our best performance was to obtain 6 hatched blastocysts (Fig. 3) from 9 recovered cumulus oocytes complexes (Fig. 2), generating a hatched blastocyst rate of 66.6%.

Table 1 The results of first OPU and IVF procedures at SCDCB Dancu Iasi in Holstein Friesian cattle

OPU group	Crt. no. of OPU sesion	Punctured follicles	Recovered oocytes	Oocytes recovery rate	Hached blastocysts	Hatched blastocyst rate
E-group	1	9	5	55.5%	1	20%
	2	13	9	69.2%	6	66,6%
	3	9	4	44.4%	1	25%
	4	10	6	60%	3	50%
	5	8	7	87,5%	2	28.5%
	Average± SEM	9.8 ± 0.8*	6.2 ± 0.8*	63.3%	2.6 ± 0.9*	41.9%
C-group	6	2	2	100%	0	0
	7	2	1	50%	0	0
	8	1	1	100%	0	0
	9	5	2	40%	1	50%
	Average± SEM	2.5 ± 0.8	1.5 ± 0.2	60%	0.25 ± 0.2	16.7%

* $P<0.05$ statistically significant; E-group is experimental group; C-group is control group

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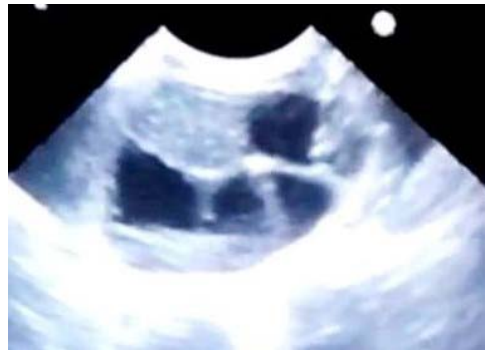

Fig. 1 Representative image from OPU session
on a FSH-stimulated ovary (E-Group)

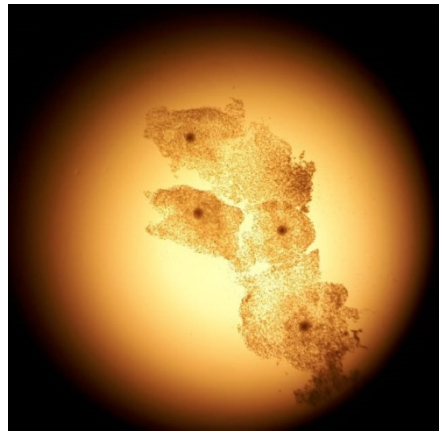

Fig. 2 Representative image of matured cumulus
oocyte complexes, recovered by OPU, during *in vitro* embryo production procedures

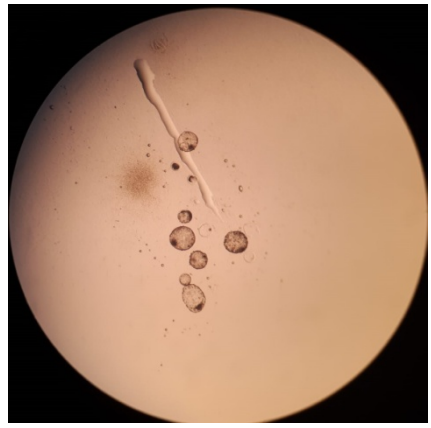

Fig. 3 Representative image of hatched blastocysts
during *in vitro* embryo production procedures

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Although the number of samples was suboptimal to obtain high impact results, our study generate a hatched blastocyst rate at E-group similar with those from the study of Watanabe et al. [20], which have been achieved in a specialized laboratory. Further efforts will be focused at SCDCB Dancu-Iasi for improving the results of OPU in association with IVF and to transfer the obtained embryos to the recipient cows.

CONCLUSION

This is the first report of bovine embryos production (hatched blastocysts) in Romania by the association of OPU and IVF. This could be a corner stone for implementation of embryo production by OPU and IVF in Holstein Friesian cattle in north-eastern Romania to extend elite genetics provided by both proven donors and rare or expensive sires. Although a small sample size was used, this paper is a proof of the fact that at SCDCB Dancu-Iasi the *in vitro* embryo production in *Bos taurus* species is in a continuous development for further attempts to produce genetically highly valuable animals.

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