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IN VITRO PRODUCTION OF EMBRYOS AT RESEARCH AND DEVELOPMENT STATION FOR CATTLE BREEDING DANCU, IASI – FIRST BOVINE EMBRYOS PRODUCED *IN VITRO* IN NORTH-EASTERN ROMANIA

S.I. BORŞ[1]*, Şt. CREANGĂ[1,2], D.L. DASCĂLU[1], Elena RUGINOSU[1], Mădălina Alexandra DAVIDESCU[1], Alina BORŞ[2], I. IBĂNESCU[1]

*E-mail: bors.ionut@yahoo.com

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ABSTRACT. The *in vitro* production (IVP) of bovine embryos increases the selection intensity in cattle and reduces the generation interval, which is very important in the genetic gain. In Romania, this reproductive biotechnology has shown a timid evolution in the last years, although the need for genetic improvement in the area is present. The aim of this paper is to describe the work that resulted in first bovine embryos obtained through IVP in North-Eastern Romania. Oocytes were collected by slashing ovaries from slaughtered cows, matured in a TCM199-based medium and fertilized in TL-based medium microdrops with sperm processed by swim-up procedure. The presumptive embryos were cultured one day in TCM199 and 8 days in SOF-based medium and evaluated in days 7, 8 and 9 after fertilization. We retrieved an average number of 83 usable oocytes/IVF session, which represents 73.8% from the total harvested oocytes. The average number of cleaved embryos was 50.8 per IVF, reflecting an average cleavage rate of 61.2%. An average of 8.6 blastocysts/IVF session was obtained, representing 10.4% of the selected oocytes or 16.9% of the number of cleaved embryos. Although suboptimal, the results were comparable with other reports on IVP in cattle. The adapted IVP protocol, based on maturation with TCM199, fertilization in microdrops of TL and culture of presumptive embryos one day in TCM199 and afterwards in SOF seems to offer acceptable results and will be used for further attempts to produce bovine embryos.

Keywords: IVP; TCM199 medium; TL medium; SOF medium; swim-up.

REZUMAT. Producerea *in vitro* de embrioni în cadrul S.C.D.C.B. Dancu, Iaşi - primii embrioni bovini produşi *in vitro* în nord-estul României. Producerea *in vitro* de embrioni bovini determină o

[1] Research and Development Station for Cattle Breeding Dancu, Iaşi, Romania
[2] University of Agricultural Sciences and Veterinary Medicine of Iaşi, Romania

creştere a intensităţii de selecţie a bovinelor şi o reducere a intervalului între generaţii, elemente importante în realizarea câştigului genetic. În România, această biotehnică de reproducere asistată la taurine a prezentat o evoluţie timidă în ultimii ani, deşi există o nevoie crescută în ceea ce priveşte ameliorarea şeptelului de bovine pentru lapte. Scopul acestui studiu este de a descrie rezultatele obţinute ca urmare a cercetărilor efectuate pentru producerea primilor embrioni *in vitro* la specia *Bos taurus* în zona de Nord-Est a României. Ovocitele au fost recoltate prin realizarea unor incizii multiple în corticala ovarelor provenite de la vaci sacrificate în abator, apoi maturate în mediul TCM 199 şi fertilizate în mediul TL, cu material seminal procesat prin metoda swim-up. Embrionii au fost apoi cultivaţi o zi în mediul TCM 199 şi 8 zile în mediul SOF, evaluarea lor făcându-se pe parcurs în zilele 7, 8 şi 9 după fertilizare. S-a obţinut o medie de 83 ovocite de bună calitate recoltate/sesiune fertilizare *in vitro* (FIV), ceea ce reprezintă 73,8% din totalul ovocitelor recoltate. Numărul mediu de embrioni divizaţi a fost de 50,8/sesiune FIV, ceea ce reflectă o rată de clivaj de aproximativ 61,2%. Din totalul acestora s-a obţinut un număr mediu de 8,6 blastocişti/sesiune FIV, reprezentând 10,4% din totalul ovocitelor selectate sau 16,9% din numărul de embrioni clivaţi. Deşi rezultatele nu sunt optime, pot fi comparate cu alte raportări privind producerea *in vitro* de embrioni bovini. Protocolul FIV utilizat, bazat pe maturarea ovocitelor în TCM 199, fertilizarea în picătura TL şi cultivarea embrionilor o zi în TCM 199, iar apoi în mediul SOF reprezintă o metodă care conferă rezultate acceptabile şi va fi utilizată pentru producerea de embrioni bovini în viitor.

Cuvinte cheie: FIV; mediul TCM199; mediul TL; mediul SOF; metoda swim-up.

INTRODUCTION

In vitro production (IVP) of bovine embryos has become widely used around the globe. It increases the selection intensity and reduces the generation interval, which is very important in the genetic gain (Merton *et al.,* 2003). However, not all the countries and regions in the world are equally interested or capable to use IVP in cattle. For example, Blondin (2015) reported that in 2013 South America alone produced 73% of the embryos obtained through *in vitro* fertilization (IVF). In Europe, the interest for such assisted reproductive techniques is lower, presumably due to a general negative attitude towards the biotech products (Galli & Lazzari, 2005), but certainly not absent. The European countries with the highest activity in bovine IVP are Netherlands, Germany and Italy (Perry, 2015). In Romania, some successful efforts to produce bovine embryos through IVP were made in western (Milovanov *et al.,* 2015) and central (Groza *et al.,* 2008) parts of the country, but no IVP in cattle were performed so far in North-Eastern Romania, although there are numerous farms that could benefit from it.

The IVF biotechnologies seem to be an important innovation, that will assure the trade of genetics of some mammalian species across the globe in order to assure the sustainability of global livestock and meat demands (Blondin, 2015). The implementation of these assisted reproductive

biotechnologies in new regions will create the possibilities to improve the genetic gain in areas where such thing is desired, for example in Northeastern Romania.

The aim of this paper is to describe the results of *in vitro* production of bovine embryos by using an adapted protocol at Research and Development Station for Cattle Breeding Dancu, Iaşi, Romania.

MATERIALS AND METHODS

The protocol used for obtaining bovine embryos through IVP was based on the current literature, adapted to the available possibilities and resources of Research and Development Station for Cattle Breeding Dancu (R.D.S.C.B.), Iaşi.

The oocytes were harvested from ovaries of slaughtered cows in a regional abattoir. Ovaries were recovered right before evisceration, in order to keep contamination to a minimum and were preserved in a saline solution (NaCl 0.9%) enriched with 0.5% PenStrep (Dopharma Vet, Romania) for the entire period of transport to the IVF laboratory (approximately 2.5 h). In the laboratory, before recovering the oocytes, the ovaries were washed several times with the pre-warmed saline solution to remove residual blood and then maintained at room temperature in a fresh saline solution until time of oocyte collection.

The oocytes were retrieved on OPU medium (Minitube GmbH, Germany) using the slashing method, as previously described (Hansen, 2014). After slashing the ovaries, the medium containing oocytes was kept in 50 ml centrifuge tubes for 10 min at 34ºC. For aspirating the immature oocytes from the bottom of the centrifuge tubes, the 1 ml Pasteur pipettes (Deltalab, Spain) were used. Identification and selection of the good quality oocytes (based on surrounding cumulus cell layers and homogeneity of ooplasm, was performed with a stereo microscope, model Olympus SZ-51. After classification in different grades according to their quality, the oocytes were cleaned of debris by washing them three times in TCM 199 medium (Minitube GmbH, Germany), enriched with 5% estrus cow serum, transferred in four well NUNC dishes, which contained 400 µl/well maturation medium for oocytes (TCM 199 enriched with 5% estrus cow serum and 0.02 UI/ml FSH - Sigma Aldrich, Germany) under mineral oil and placed in incubator at 38.5ºC, 5% CO_2 and 90% relative humidity for 20 h .

Spermatozoa were selected by swim-up procedure: for each semen straw (0.25 ml) two tubes with 1 ml sperm capacitation medium were prepared. The sperm capacitation medium contained 10 ml TL Sperm (Minitube GmbH, Germany), 60 mg bovine serum albumin (BSA, Sigma-Aldrich, Germany), 1.1 mg Na-Pyruvat (Sigma-Aldrich, Germany), 10 µl Gentamicin solution (50 mg Gentamicin-Sigma-Aldrich in 1 ml PBS Dulbeco-Sigma-Aldrich). The tubes with semen were placed in incubator oblique at an angle of 30º and incubated for one hour at 38.5ºC. At the end of incubation 0.9 ml supernatant of each tube was collected in an extra tube and centrifuged 10 min at 200 g. After centrifugation the surplus capacitation medium was aspirated and the semen pellet was mixed with the TL fertilization medium before it was used for fertilization.

The fertilization of matured oocytes was conducted in 60 µl microdrops of TL fertilization medium under mineral oil. TL fertilization medium was prepared from 10 ml TL Fertilization (Minitube

GmbH, Germany), 60 mg BSA, 100 µl Na-Pyruvat (solution of 11 mg Na-Pyruvat diluted in 5 ml Dulbeco PBS) and 10 µg/ml Heparin (Sigma-Aldrich, Germany). Before fertilization the matured oocytes were washed once in 200 µl drops of TL fertilization medium and then transferred to the fertilization microdrops. After this procedure, approximately 1 x 10^6 sperm/ml were also submitted to the fertilization microdrops and then the gametes were co-incubated for 20 h at 38.5ºC in an atmosphere with 5% CO_2 and 90% relative humidity. After this period of co-incubation, the oocytes were transferred back to the dishes where they underwent *in vitro* maturation (IVM) and incubated again for 24 hours, until transfer to the culture dishes.

For cultivation of presumptive zygotes, cumulus cells were removed by vortexing the cumulus-oocytes complexes for 2 min in SOF medium (Synthetic Ovidut Fluid) prepared from 9.5 ml SOF medium (Minitube GmbH, Germany) with 0.5 ml ECS (estrus cow serum), 200 µl aminoacids essential (Gibco), 100 µl aminoacids non-essential (Gibco) and 3.63 mg Na-Pyruvat (Sigma-Aldrich, Germany). Afterwards, presumptive zygotes were washed three times to remove remaining granullosa cells and cultured in 400 µl SOF medium under mineral oil at 38.5ºC, 5% CO_2 and 90% relative humidity. The results of the IVP procedure were evaluated in days 7, 8 and 9 (the day of fertilization was considered day 0).

RESULTS AND DISCUSSION

Five sessions of *in vitro* embryo production were performed, in which a total number of 562 oocytes were collected, but only 415 were considered suitable for further procedures (oocytes of grade A, B and C), according to previous reports (Boni *et al.,* 2002). The selection of viable oocytes for IVM is the initial and one of most important steps in IVF (Kouamo *et al.,* 2014), as it eliminates the improper oocytes and retains only those that have developmental potential. In our study, we retrieved an average number of 83 usable oocytes/IVF session (*Table 1*).

In domestic animals, three methods for collection of oocytes have been described: aspiration of the oocyte from the follicles of living cows (a procedure also called Ovum Pick-up - OPU) (Datta *et al.,* 1993; Boediono *et al.,* 1995), slicing the ovaries (Carolan *et al.,* 1992; Mogas *et al.*, 1992; Pawshe *et al.,* 1994) and puncture of visible surface follicles from slaughtered cows (Wani *et al.,* 1999; Shirazi *et al.,* 2005). The slicing technique yields more oocytes per ovary than follicle aspiration technique (Das *et al.,* 1996). Ovum pick-up seems to be preferred nowadays for commercial purposes (Perry, 2016), as it allows a prolonged use of a certain donor cow for IVP, but slaughterhouse ovaries represent a cheaper and abundant source of oocytes (Nandi *et al.,* 2006). Although we performed OPU in this work as well, unfortunately the number of retrieved oocytes was too low and no embryo could be obtained. Therefore, the presented results will refer only to the oocytes retrieved by slashing the ovaries of slaughtered cows.

Table 1 - The results of IVP procedures in cattle at R.D.S.C.B. Dancu, Iaşi

No. of IVF session	No. of ovaries	No. of recovered oocytes	No. of grade A, B, C oocytes	No. of cleaved embryos	Cleavage rate (%)	No. of blastocysts	% blastocysts developed from total selected oocytes	% blastocysts developed from cleaved embryos
1	10	110	90	60	66.7%	8	8.9%	13.3%
2	13	150	80	46	57.5%	10	12.5%	21.7%
3	6	60	54	20	37.0%	0	0.0%	0.0%
4	10	150	120	88	73.3%	10	8.3%	11.4%
5	10	92	71	40	56.3%	15	21.1%	37.5%
Average	**9.8**	**112.4**	**83**	**50.8**	**61.2%**	**8.6**	**10.4%**	**16.9%**

The average number of excellent and good oocytes (grade A, B and C) in our study was 83, which represents 73.8% from the total harvested oocytes. As stated above, only those oocytes were used for subsequent maturation and fertilization leading to an average number of 50.8 cleaved embryos (*Fig. 1*) per IVF session (61.2% average cleavage rate). This is comparable with other reports from literature and reflects and acceptable rate of *in vitro* maturation and fertilization of the oocytes using the media and protocol that we described in the Materials and Methods section. Based on literature reports, there is no standard culture medium for bovine preantral follicles. The lack of standardized protocols may affect in vitro follicle culture and can also explain the different results from several research groups. Among the commercial culture media, TCM-199 and α-MEM have been the most commonly used to maintain follicular survival and viability and to improve the development of bovine follicles (Araújo *et al.,* 2014). *In vitro* culture media have nevertheless improved significantly in the last 15 years. Recent defined IVF media result in embryos that survive slow freezing protocols making it possible to apply direct transfer techniques just like *in vivo* produced embryos (Blondin, 2015). Multiple variations of media have been tested over time (Araújo *et al.,* 2014) and nowadays the range of products and combinations that provide acceptable results is relatively wide.

Although a good cleavage rate was achieved, unfortunately, the further development was below the expected rate with bovine oocytes fertilized *in vitro*. Only 8.6 blastocysts/IVF session were obtained, representing 10.4% of the selected oocytes (*Table 1*), which is less than other results reported in literature (Luo *et al.,* 2006; Watanabe *et al.,* 2008). When the number of obtained blastocysts (*Figs. 2,3 and 4*)

is reported to the number of cleaved embryos (*Fig. 1*), the percentage is 16.9%. Our results were, however, not profoundly lower than the European average as reported by Perry (2016) and comparable with other results of IVP in Romania (Groza *et al.,* 2008). Further efforts will be focused on improving the development rate and transfer of the obtained embryos.

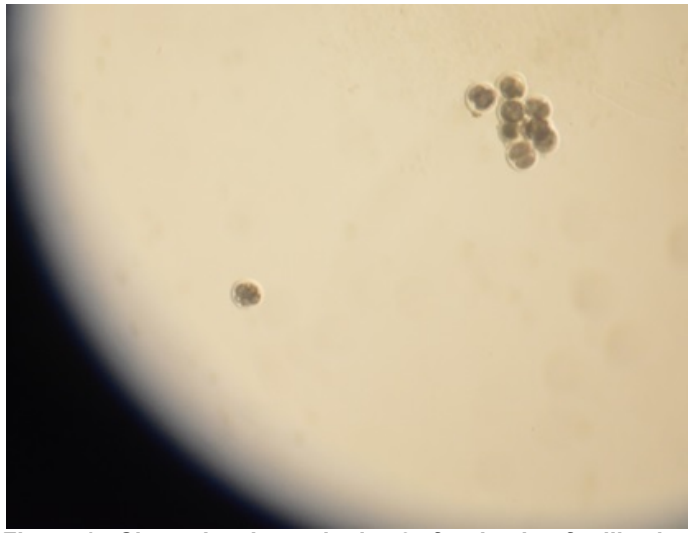

Figure 1 - Cleaved embryos in day 2 after *in vitro* fertilization

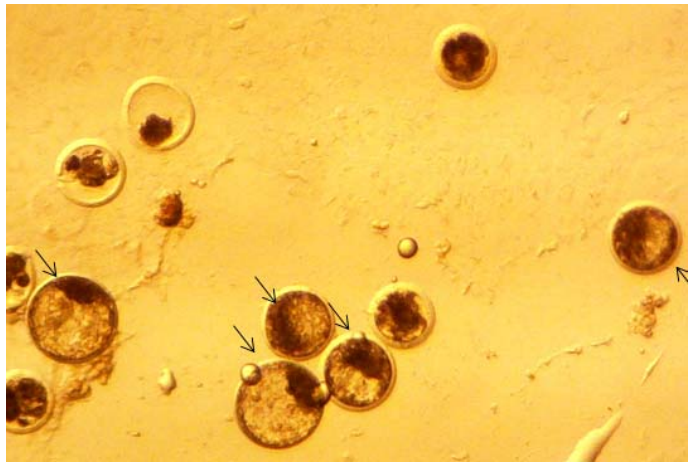

Figure 2 - Bovine blastocysts in day 7 after *in vitro* fertilization

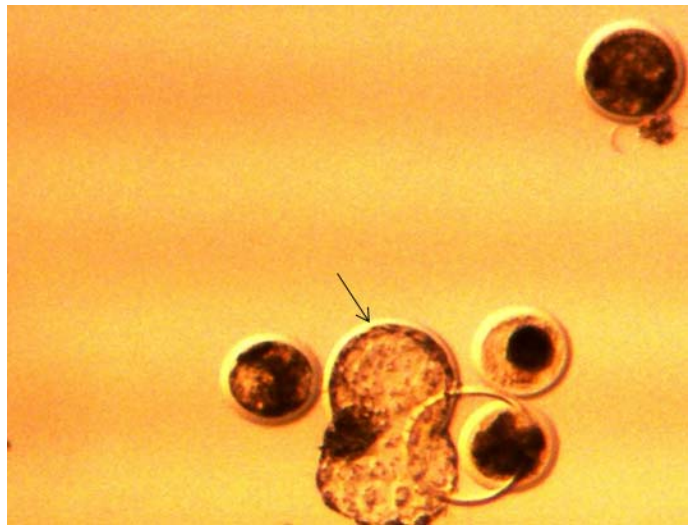

Figure 3 - Hatching blastocyst in day 8 after *in vitro* fertilization

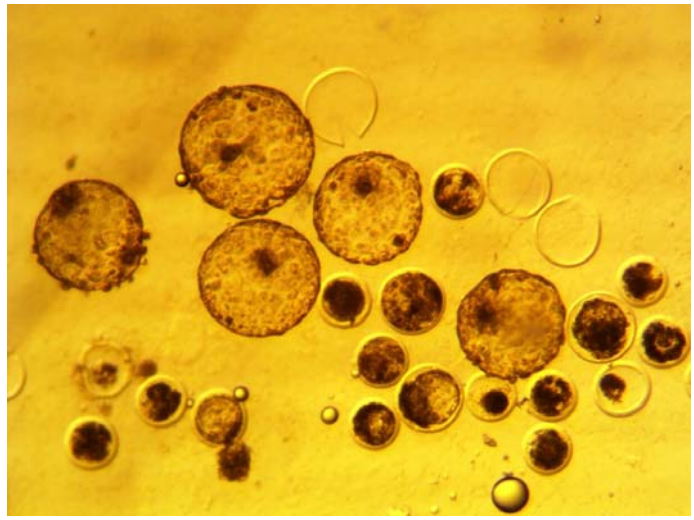

Figure 4 - Hatched blastocysts in day 9 after *in vitro* fertilization

Continuous efforts are made in Europe and other parts of the world to improve the success rate of IVP procedures and to use this biotechnology for improving the genetic pool in dairy cows. IVF embryo production has increased significantly year after year. In 2015, bovine IVP recorded a total of 671,111 embryos available for transfers worldwide, exceeding the 660,221 bovine *in vivo derived* (IVD) embryos collected, the first time this has happened (Perry, 2016). Our work will hopefully help in implementing such assisted reproductive

technologies in North-Eastern Romania, where they haven't been used yet.

In conclusion, the Laboratory for Assisted Reproduction at the R.D.S.C.B. Dancu, Iaşi, is able to produce bovine embryos through *in vitro* fertilization and this could be the corner stone for the implementation of IVP in North-Eastern Romania. Although the success rate is yet suboptimal, the results are encouraging considering the fact that it is a newly constructed laboratory and these were the first attempts of the research team to perform IVP. The adapted IVP protocol, based on maturation with TCM199, fertilization in microdrops of TL and culture of presumptive embryos one day in TCM199 and afterwards in SOF seems to offer acceptable results and will be used for further attempts to produce bovine embryos.

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