African Journal of Biotechnology Vol. 11(19), pp. 4459-4462, 6 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3886
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

The influence of biopsy method on the survival rates of sexed and cryopreserved bovine embryos

Mihai Cenariu, Pall Emoke and Groza Ioan*

Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania.

Accepted 14 February, 2012

The purpose of this research was to find the most suitable embryo biopsy technique that would yield the highest pregnancy rates after the transfer of sexed frozen-thawed bovine embryos. Three methods of embryo biopsy (needle, blade or aspiration) were applied on 120 bovine embryos divided into 3 batches (n = 40). Subsequently, the embryos were frozen/thawed and transferred to synchronized recipients. Pregnancy diagnosis was performed 30 days later using an ultrasound scanner. The results show a significant difference in pregnancy rates according to the biopsy method: 55% for the needle biopsy, 45% for the aspiration method and 30% for the microblade technique.

Key words: Embryo, biopsy, sexing, cryopreservation, pregnancy.

INTRODUCTION

Embryo sexing is one of the biotechnologies that raised a lot of interest among the specialists dealing with *in vitro* fertilization (IVF) and/or embryo transfer (ET). It has lately become almost an industry in bovine and equine reproduction and it is sometimes used in human assisted reproduction, especially when preimplantation genetic diagnosis (PGD) is needed. One of the challenges in embryo sexing is the damage that is done to the embryo through the biopsy performed in order to remove the blastomeres from which DNA is extracted. This results in lower pregnancy rates than with intact embryos and the percentages become even lower if freezing/thawing is also performed. Therefore, the purpose of this paper was to test three embryo biopsy techniques and to find the most suitable one, which would yield the highest pregnancy rates after the transfer of sexed frozen/thawed embryos to recipients.

MATERIALS AND METHODS

The research was carried out on a total number of 120 bovine embryos that were divided into three batches as follows: batch 1 which is made up of 40 embryos that were biopsied using a fine needle; batch 2 which is made up of 40 embryos that were biopsied by means of aspiration; batch 3 which is made up of 40 embryos that were biopsied using a microblade.

Production of bovine embryos

The embryos were obtained after performing the superovulation of donor cows using a total dose of 1000 IU porcine FSH-LH (Pluset, Carlier, Spain), artificial insemination (at 12, 24 and 36 h from the onset of estrus, which usually occurred 36 to 48 h from the last Pluset administration), non-surgical embryo recovery and morphological evaluation, as described before (Robertson and Nelson, 1998). Only grade 1 (excellent and good) and 2 (fair) morulae and blastocysts were used in this study (Figure 1).

Embryo biopsy

The biopsies were carried out using an Olympus Narishige ONO-131 three axis hydraulic micromanipulator. The biopsy medium consisted of PBS supplemented with 0.4% protein (BSA) for the needle and the aspiration technique and Dulbecco's PBS containing no protein for the microblade technique. In the latter, after cutting, the same volume of holding medium with a double concentration of protein (Dulbecco's PBS supplemented with 0.8% BSA) was immediately added to the dish to neutralize the attraction of the cells to the metal blade and to the plastic dish bottom (Lopes et al., 2001).

The needle method of biopsy was used in order to harvest blastomeres from 40 embryos (batch 1) and it consisted of the following steps: the embryo was held in place using a holding pipette and the zona pellucida on the opposite side was punctured

*Corresponding author. E-mail ioangroza55@yahoo.com. Tel: +40-264-596384. Ext: 163. Fax: +40-264-593792.

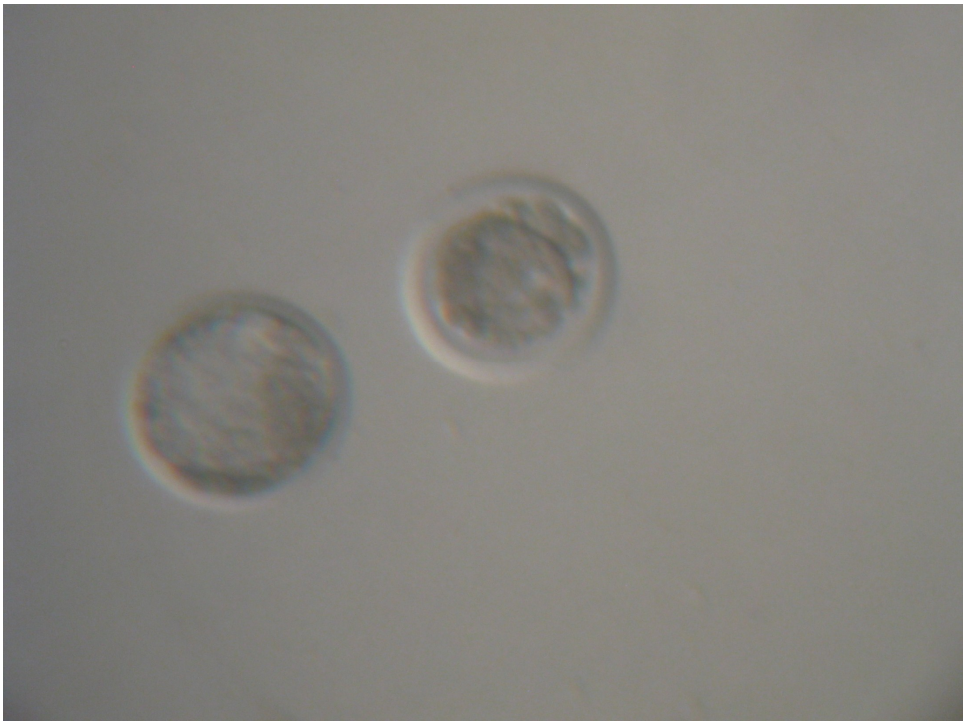

Figure 1. Grade 1 blastocyst (left) and morula (right). The assessment of embryo quality was made according to the guidelines suggested by the International Embryo Transfer Society (IETS).

using a fine needle, it was done carefully not to stab any blastomeres. Once the needle was completely through the zona pellucid, the embryo was repositioned on top of the holding pipette and the needle was moved back and forth in order to remove a small portion of the zona pellucida. When this was accomplished, the biopsy pipette was inserted through the orifice and a small number of cells were removed by gentle aspiration and then transferred into lysate buffer containing proteinase K.

The aspiration biopsy was used in order to harvest blastomeres from 40 embryos (batch 2) and it consisted of the following steps: the embryo was held in place using a holding pipette and through the zona pellucida on the opposite side the embryo was punctured using the biopsy pipette. Once the biopsy pipette was inserted through, gentle aspiration was applied and cells were then transferred into lysate buffer containing proteinase K.

The microblade biopsy was used in order to harvest blastomeres from 40 embryos (batch 3) and consisted of the following steps: there was no need for a holding pipette as the embryo was stabilized using the scratch bottom technique. The microblade was placed on top of the embryo, close to one of the edges, and moved back and forth until a small part of the embryo was literally sliced away. The sliced portion was then removed by aspiration and placed in the same lysate buffer containing proteinase K.

Embryo cryopreservation and transfer

After the biopsy, the fresh embryos were frozen in 1.5 M ethylene glycol as described by Voelkel and Hu in 1992, thawed, and transferred immediately into synchronized recipients. The evolution of pregnancies was carefully monitored until day 30 when pregnancy diagnosis using an ultrasound scanner was performed and the pregnancy rate was assessed. The results obtained were statistically analyzed using the GraphPad InStat (ANOVA) software, and applying the unpaired t test with Welch correction. The results are considered to have statistic significance if $p \leq 0.05$. The pregnant females were further monitored until delivery.

Embryo sexing

The DNA obtained from cells was used for PCR sexing of embryos using the method described by Peura et al. (1991). Duplex PCR was performed, using one set of primers targeted for the 1715 bovine satellite DNA (5' – TGG AAG CAA AGA ACC CCG CT – 3' downstream: 5' – TCG TGA GAA ACC GCA CAC TG – 3') and another for the BRY4a repetitive sequence, highly specific for the Y chromosome (5' – CTC AGC AAA GCA CAC CAG AC – 3' and downstream: 5' – GAA CTT TCA AGC AGC TGA GGC – 3').

RESULTS AND DISCUSSION

The pregnancy diagnosis performed on day 30 after the embryo transfer of biopsied frozen/thawed embryos showed the following results: In batch 1, where embryos were biopsied using a fine needle, 22 of the 40 cows were diagnosed to be pregnant, meaning a pregnancy rate of 55%; in batch 2 where biopsy was made by aspiration, 18 of the 40 cows were pregnant, yielding a pregnancy rate of 45%, while in batch 3 only 12 of the 40 cows were diagnosed to be pregnant, representing a pregnancy rate of 30% (Figure 2). All pregnancies survived to term and resulted in normal developed calves.

The statistical analysis showed the following results:

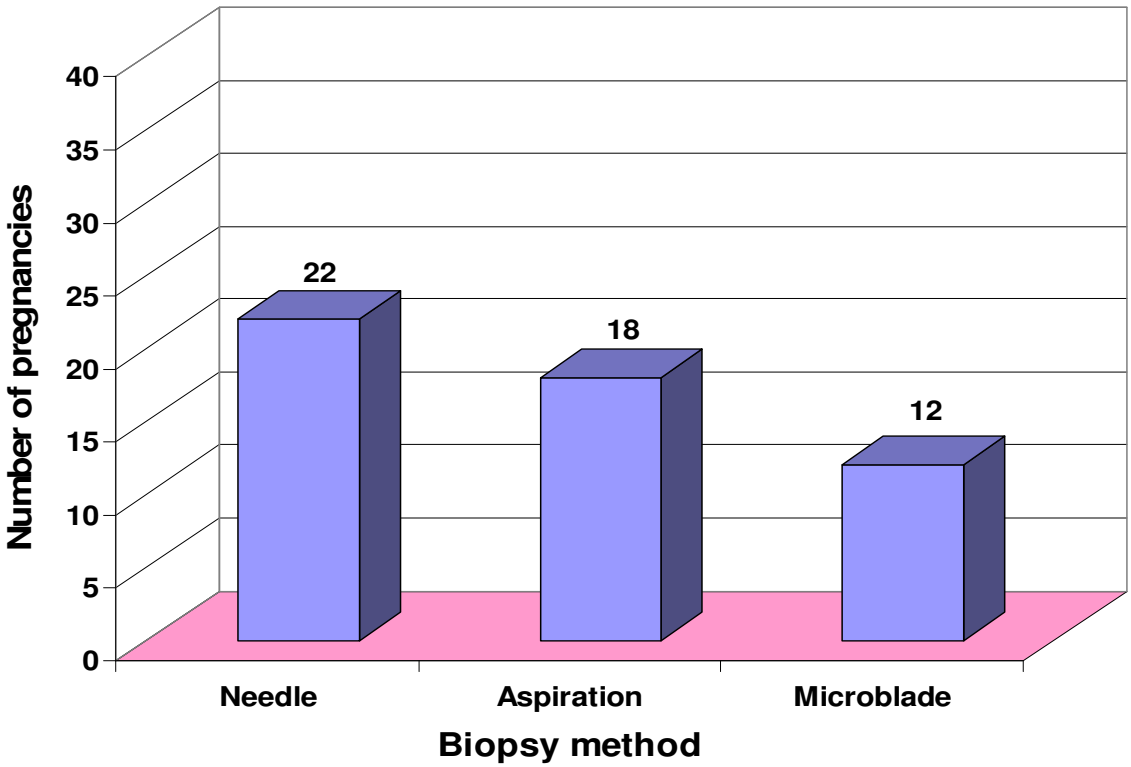

Figure 2. Number of pregnancies obtained in the three batches according to the biopsy technique used.

1. When comparing the results obtained for batch 1 with those obtained for batch 2, the p value was 0.0497, which was considered statistically significant.
2. When comparing the results obtained for batch 1 with those obtained for batch 3, the p value was 0.0004, which was considered extremely significant.
3. When comparing the results obtained for batch 2 with those obtained for batch 3, the p value was 0.0085, which was considered very significant.

The accuracy of the sexing method was assessed at the time of calving, when the actual sex of the calf was compared with the prediction made by PCR. Since all the calves belonging to the three batches presented the sex which was expected according to the PCR, we concluded that the accuracy of the sexing method was 100%.

Our study shows that the biopsy method does influence the rate of survival in frozen/thawed embryos after their transfer to recipients, and therefore the pregnancy rate. The needle and the aspiration techniques imply a minimum damage of the zona pellucida and therefore it can still act as a protective coat for the blastomeres. On the other hand, the microblade biopsy method produces a large opening in the zona pellucida and the subsequent survival rate of the embryo is much lower. Nevertheless, the microblade biopsy method is easier and quicker than the other two, and should be preferred if the embryos are to be transferred directly, without cryopreservation. In the case of frozen/thawed embryos, the best method which least damages the embryos proved to be the needle technique, followed by the aspiration and microblade methods, with statistically significant differences between each other. The accuracy of PCR sexing was not influenced by the biopsy method, as it was 100% in all batches. Although, the size of the biopsy tends to be somewhat smaller when needle or aspiration biopsy is used as compared to microblade biopsy, the former two still contain enough genetic material to make amplification possible. Other studies (Chrenek et al., 2001; Park et al., 2000) have shown that the biopsy of a single blastomere is enough in order to perform sexing and other genotype analysis on embryos.

Various authors, performing embryo biopsy for different reasons (PGD, sexing, etc.), obtained comparable results. The pregnancy rate after the transfer of fresh, biopsied embryos varied from 53 to 62%, the microblade biopsy method being usually preferred (Bredbacka et al., 1996; Herr and Reed, 1991; Roschlau et al., 1997; Thibier and Nibart, 1995). This pregnancy rate is comparable to the one obtained for the transfer of fresh, intact embryos. Sex determination can also be performed in bisected embryos if transferred freshly, without cryopreservation, with good conception rates, up to 56.5% (Lopatarova et al., 2008).

The results obtained for frozen/thawed biopsied embryos varied quite a lot among various researchers. Pregnancy rate ranged from 33 to 66% when needle or

aspiration biopsy methods were used, but was only 23 to 28% when a microblade was used (Nibart et al., 1997; Shea 1999).

When biopsy was performed before vitrification, 98% of the embryos survived manipulation, and 86% of these re-expanded after vitrification and in-straw dilution. Biopsy after vitrification was less efficient, since only 69% of the embryos survived both processes (Vajta et al., 1997). Moreover, Ito et al. (1999) reported that the short-term culture of bovine blastocysts after biopsy improved their ability to withstand cryopreservation.

The accuracy of the sexing method was reported to be higher when the microblade biopsy method was used. The reason was that the amount of cells harvested was higher in this case as compared to the needle or aspiration method, when insufficient DNA could be found in the biopsied sample (Tominaga and Hamada, 2004). A solution to this problem for frozen/thawed embryos could be to perform the biopsies very carefully and to make sure there is enough cellular material in the pipette before releasing it into the lysing buffer.

Conclusions

Our study shows that the biopsy technique has a great influence on the pregnancy rate of biopsied frozen/thawed embryos, the most suitable biopsy method being the one using a fine needle. The amount of DNA extracted from the blastomeres obtained by one of the three biopsy methods did not influence the accuracy of PCR. These conclusions obtained after performing the biopsies on bovine embryos can easily be extended to embryos belonging to other species (including human) as they are expected to behave the same under similar conditions.

ACKNOWLEDGEMENT

This work was supported by CNCS-UEFISCDI, project PN II RU-PD code 298, contract no. 180/2010.

REFERENCES

Bredbacka P, Peippo J, Jaakma U (1996). A simplified protocol for PCR-sexing of bovine embryos: a field trial. Theriogenology, 45: 218.

Chrenek P, Boulanger L, Heyman Y, Uhrin P, Laurincik J, Bulla J, Renard JP (2001). Sexing and multiple genotype analysis from a single cell of bovine embryo, Thenogenology, 55(5): 1071-1081.

Herr CM, Reed KC (1991). Micromanipulation of bovine embryos for sex determination. Theriogenology, 35(1): 45-54.

Lopes RF, Forer F, Oliveira AT, Rodrigues JL (2001). Splitting and biopsy for bovine embryo sexing under field conditions.Theriogenology, 56(9): 1383-1392.

Ito K, Sekimoto A, Hirabayashi M, Hochi S, Kimura K, Ueda M, Nagao Y (1999). Effect of time interval between biopsy and vitrification on survival of in vitro-produced bovine blastocysts. J. Reprod. Dev. 45: 351-355.

Lopatarova M, Cech S, Krontorad P, Holy L, Hlavicova J, Dolezel R (2008). Sex determination in bisected bovine embryos and conception rate after the transfer of female demi-embryos. Veterinarni Medicina, 53(11): 595-603.

Nibart M, Marquant Le Guienne B, Humblot P, Guerin B (1997). The application of new reproductive technologies in France. Arq. Fac. Vet. UFRGS. 25 (1): 21-35.

Park JH, Lee JH, Choi KM, Joung SY, Kim JY, Chung GM, Jin DI, Im KS (2001). Rapid sexing of preimplantation bovine embryo using consecutive and multiplex PCR with biopsied single blastomere. Theriogenology, 55(9): 1843-1853.

Peura T, Hyttinen JM, Turunen M, Janne J (1991). A reliable sex determination assay for bovine prelmplantatlon embryos using the polymerase chain reaction. Theriogenology, 35(3): 547-555.

Robertson I, Nelson RE (1998). Certification and identification of the embryo. In: Manual of the International Embryo Transfer Society, 3rd ed. IETS, Savoy, Illinois, USA.

Roschlau K, Roschlau D, Roselius R, Dexne U, Michaelis U, Strehl R, Unicki P, Rink N (1997). Over 5 years experience in sexing of bovine morulae and blastocysts during routine embryo transfer. Theriogenology, 47(1): 273.

Shea BF (1999). Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study. Theriogenology, 51(4): 841-854.

Thibier M, Nibart M (1995). The sexing of bovine embryos in the field. Theriogenology, 43(1): 71-80.

Tominaga K, Hamada Y (2004). Efficient production of sex-identified and cryosurvived bovine in-vitro produced blastocysts. Theriogenology, 61(6): 1181-1191.

Vajta G, Booth PG, Holm P, Greve T, Callesen H (1997). Comparison of two manipulation methods to produce in vitro fertilized, biopsied and vitrified bovine embryos. Theriogenology, 47(2): 501-509.

Voelkel SA, Hu YX (1992). Use of ethylene glycol as a cryoprotectant for bovine embryos allowing direct transfer of frozen–thawed embryos to recipient females. Theriogenology, 37(3): 687-697.