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Research Article

Evaluation of Bovine Embryo Biopsy Techniques according to Their Ability to Preserve Embryo Viability

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The purpose of this research was to evaluate three embryo biopsy techniques used for preimplantation genetic diagnosis (PGD) in cattle and to recommend the least invasive one for current use, especially when PGD is followed by embryo cryopreservation. Three hundred bovine embryos were biopsied by either one of the needle, aspiration or microblade method, and then checked for viability by freezing/thawing and transplantation to recipient cows. The number of pregnancies obtained after the transfer of biopsied frozen/thawed embryos was assessed 30 days later using ultrasounds. The results were significantly different between the three biopsy methods: the pregnancy rate was of 57% in cows that received embryos biopsied by needle, 43% in cows that received embryos biopsied by aspiration, and 31% in cows that received embryos biopsied by microblade. Choosing an adequate biopsy method is therefore of great importance in embryos that will undergo subsequent cryopreservation, as it significantly influences their viability after thawing.

1. Introduction

Preimplantation genetic diagnosis (PGD) has lately become a very useful tool in human as well as veterinary medicine, enabling the very early diagnosis of various genetic disorders and allowing the selection of desired embryos for further transplantation. PGD also includes embryo sexing, a biotechnology that has lately become commercially available for large animals (cattle, horses) as it allows for gender selection according to the owner's desire (females for dairies, males for beef operations, etc.). In order to perform PGD, a small biopsy of the inner embryonic cell mass needs to be collected using a micromanipulator and various sharp instruments, and then further used for DNA extraction. The only problem is that the biopsy implies the damage of the zona pellucida which is literally drilled in order to reach the embryonic cells. Therefore, the protective role of this structure can no longer manifest itself properly, especially if biopsied embryos cannot be transplanted immediately and need to be frozen for later use. Various embryo biopsy methods have been described so far, each of them showing different advantages and disadvantages over the others. Three of them are presently used more often, as previous studies have proven their adequacy: the needle technique [1–3], the aspiration technique [4–6], and the microblade technique [7–9]. Nevertheless, there is currently no work that compares these biopsy methods in the same study and evaluates their capacity to preserve embryo viability after cryopreservation. As the zona pellucida plays a very important role in the process of cryopreservation, we hypothesized that the survival chance of frozen/thawed embryos is biggest in undamaged embryos and decreases according to the degree of its destruction. Therefore, the purpose of this research was to evaluate the three embryo biopsy techniques used for preimplantation genetic diagnosis in cattle from the point of view of their invasiveness and recommend the least aggressive one for ordinary use, especially when PGD is followed by embryo cryopreservation.

2. Materials and Methods

2.1. Embryo Production and Recovery. The animals included in this experiment were 39 Holstein-Frisian cows (2.5 to 3.5 years of age) and 11 Romanian Spotted cows (2.5 to 4 years

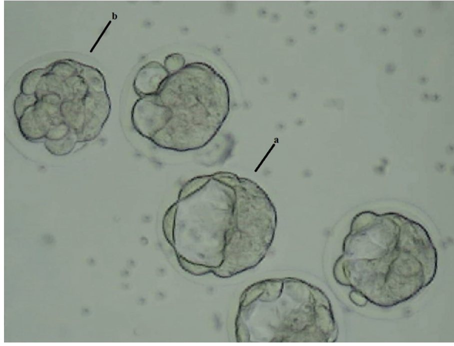

Figure 1: Grade 1 bovine blastocyst (a) and morula (b).

of age), selected according to the following criteria: the females were at least 60 days after their first or second parturition, had a normal reproductive tract on rectal palpation and ultrasound examination, had a cycle length of 20–22 days associated with a history of regular cycles having started at a young age, needed no more than 2 breedings/conception, had no dystocia, no puerperal disorder and were fed an appropriate ration that allowed a body condition score of 3.25 to 3.75 on a 1 to 5 scale (where 1 is emaciated and 5 is obese). They were superovulated using 1000 IU porcine FSH-LH (Pluset, Carlier, Spain), according to the manufacturer's recommendations. Artificial insemination was first performed 48 hours after the last hormone administration and repeated 12 and 24 hours later. Embryo recovery was carried out 6.5–7.5 days after the last insemination using a DISSI CH18 embryo flushing catheter and BoviPRO recovery medium (Minitub, Germany). The embryo morphology was carefully assessed according to the criteria established by The International Embryo Transfer Society (IETS) and described by Robertson and Nelson [10]. A total number of 598 embryos+unfertilized ova were recovered from the donor cows, 407 of which were transferable (grade 1–3 morulae and blastocysts) and 191 were degenerated embryos and unfertilized ova. Out of the 407 transferable embryos, 300 (73.7%) were grade 1, 76 (18.7%) grade 2, and 31 (7.6%) grade 3 embryos. The distribution (mean, variance, minimum-maximum) of total and transferable embryos recovered from the 50 donor cows is shown in Table 1. Subsequently, only excellent and good (grade 1) morulae and blastocysts were selected and included in the present study (Figure 1).

2.2. Embryo Biopsy. Three hundred grade 1 embryos (IETS criteria) were selected and randomly divided into three batches ($n = 100$), according to the biopsy method used. In all cases, a Nikon Eclipse TS100 inverted microscope, equipped with an Olympus Narishige ONO-131 three axis hydraulic micromanipulator, and different cutting instruments were used as follows.

(i) In batch 1 (needle technique), the embryos were placed in PBS supplemented with 0.4% BSA, held in position with a holding pipette and punctured with a fine needle that was inserted carefully through the zona pellucida, not to damage the blastomeres. The embryo was then moved under the holding pipette and moderate pressure was applied to the needle, in an upward direction (towards the holding pipette) while also moving the needle left and right. Finally, a small piece of the zona pellucida was ruptured, enough to insert the biopsy pipette and gently aspirate 2 to 3 cells from morulae or 4 to 5 cells from the trophectoderm of blastocysts that were further transferred into lysate buffer containing proteinase K.

(ii) In batch 2 (aspiration technique), the embryos were placed in PBS supplemented with 0.4% BSA, held in position with a holding pipette, while the zona pellucida was punctured with the biopsy pipette that was also used to gently aspirate 2 to 3 cells from morulae or 4 to 5 cells from the trophectoderm of blastocysts that were further transferred into lysate buffer containing proteinase K. This method also enabled us to remove debris and loose cells (if present) from the perivitelline space, thus increasing the amount of genetic material that was obtained from the embryo.

(iii) In batch 3 (microblade technique), the embryos were placed in PBS only, in a dish whose bottom was previously scratched. The scratches were made in order to help us stabilize the embryos and thus to eliminate the need for a holding pipette, as suggested by Bredbacka et al. [7]. A microblade was gently pressed against an edge of the embryo and slowly moved left and right until a small portion of the embryo was cut off. Subsequently, an equal amount of PBS containing 0.8% BSA was added to the dish, in order to prevent cell attachment to the cutting instruments or dish bottom [9]. The aspiration pipette was then used to remove the biopsy and place it into the lysate buffer containing proteinase K.

2.3. Embryo Sexing. DNA was extracted from biopsied cells using the Isolate Genomic DNA Mini Kit (Bioline, Germany) and further amplified according to the method presented by Peura et al. [11] in order to determine the sex of the embryos. Briefly, the method consists of a duplex PCR, where two sets of primes are used: one that identifies a bovine specific region (1715 bovine satellite DNA, used as marker for the presence of bovine DNA in the sample) and another that identifies a bovine Y-chromosome specific sequence in males (the BRY4a repetitive sequence). The primer sequences are presented below:

1715 bovine satellite DNA: 5′-TGG AAG CAA AGA ACC CCG CT-3′ (forward).

5′-TCG TGA GAA ACC GCA CAC TG-3′ (reverse).

BRY4a: 5′-CTC AGC AAA GCA CAC CAG AC-3′ (forward).

5′-GAA CTT TCA AGC AGC TGA GGC-3′ (reverse).

Following amplification, the PCR products were run on a 2.5% agarose gel and visualised using UV light. The samples that presented only a 216 bp DNA band were considered

Table 1: The distribution of total and transferable embryos recovered from the 50 donor cows.

	Mean/donor	Minimum number/donor	Maximum number/donor	Variance
Embryos + unfertilized ova	11.96	6	14	3.79
Transferable embryos	8.14	3	10	3.14

Table 2: The male-female distribution of pregnancies/calves according to the developmental stage of the embryos at the moment of biopsy and transfer.

	Biopsied and transferred	Yielded a pregnancy	Male calves born	Female calves born
Morulae	186	82	44	38
Blastocysts	114	49	26	23

to come from a female embryo, while the samples that presented both a 216 bp and a 301 bp band were considered to come from a male embryo.

Fetal sex was determined using ultrasounds on days 55–65 of pregnancy, according to the method described by Curran [12], and thus the accuracy of the PCR sexing method was evaluated.

2.4. Embryo Cryopreservation, Transplantation, and Monitoring of Pregnancies. The biopsied embryos were cultured for 3 hours in TCM199 (Minitub, Germany) supplemented with 20% fetal calf serum (FCS) at 38.5°C under 5% CO_2 in humidified air. Subsequently, they were submitted to the cryopreservation protocol presented by Voelkel and Hu [13] (using 1.5 M ethylene glycol), which allows direct transfer of frozen-thawed embryos to recipient females, and kept in liquid nitrogen overnight. On the next day, the embryos were thawed and transplanted to recipient cows that were previously synchronized using the Ovsynch protocol described by Pursley et al. [14] (1 embryo/recipient). The day of recipient's estrous cycle was matched with the stage of embryo development such that morulae were transferred into recipients on day 6 or 7 and blastocysts on day 7 or 8 of the cycle, as suggested by P. W Farin and C. E Farin [15]. The ultrasound pregnancy diagnosis was made 30–35 days later of occasion on which the pregnancy rate was calculated for each batch. Subsequently, all pregnancies were carefully monitored to term.

The GraphPad InStat (ANOVA) software was used in order to statistically analyze and compare the results obtained in the three batches, applying the unpaired t-test with Welch correction (statistic significance if $P \leq 0.05$).

3. Results and Discussions

Of the three biopsy methods tested, the needle technique was considered to be the most laborious, as it required the longest execution time, followed by aspiration and microblade technique which proved to be the most operative. Nevertheless, the damage of the zona pellucida seemed to be very little when the needle technique was applied, moderate for aspiration and quite severe for microblade (in the latter, a significant part of the zona pellucida was sliced away).

The results of the ultrasound pregnancy diagnosis (the ultimate *in vivo* test for embryo viability) confirmed our hypothesis concerning the influence of the biopsy method on the survival rate of biopsied and cryopreserved embryos. The pregnancy rate was of 57% for batch 1, 43% for batch 2, and 31% for batch 3. All pregnancies progressed to term without incidents and resulted into healthy calves. The statistical analysis of the results revealed the following aspects.

(i) Batch 1 (needle biopsy) versus Batch 2 (aspiration biopsy): $P = 0.0010$, considered extremely significant.

(ii) Batch 1 (needle biopsy) versus Batch 3 (microblade biopsy): $P < 0.0001$, considered extremely significant.

(iii) Batch 2 (aspiration biopsy) versus Batch 3 (microblade biopsy): $P = 0.0030$, considered very significant.

The ultrasound examination, performed on days 55 to 65 of pregnancy in order to assess the accuracy of the PCR sexing method, showed that all fetuses had the same sex as expected. Therefore, the accuracy of PCR sexing was of 100%, which was also confirmed at calving when the morphological sex of each calf was observed. The assumption that male embryos develop at a different rate than female embryos [16] was not confirmed by the results of this work. The male-female distribution of pregnancies/calves according to the developmental stage of the embryos at the moment of biopsy and transfer is shown in Table 2.

These results are very conclusive and clearly point out the importance and great influence of the biopsy method on the integrity and viability of cryopreserved embryos. The integrity of the zona pellucida represents a key element in the survival of embryos submitted to extremely low temperatures as it interferes with the diffusion of cryoprotectants during the dehydration and rehydration processes [17]. Therefore the biopsy method that was able to best preserve its structural integrity was also able to better preserve embryo viability and yielded highest pregnancy rates (57% needle biopsy). The aspiration and especially the microblade biopsy techniques produced more damage to the zona pellucida and therefore the embryo survival rate was lower, as shown by the lower pregnancy rates obtained when the biopsied embryos were transferred to recipients (43% and 31% resp.). The rapidity and ease of the microblade biopsy method yet recommend it as the most suitable technique for the situations when embryos

are transplanted without cryopreservation. In such cases, the integrity of zona pellucida is less important and therefore the viability of embryos is not affected by its damage.

When morulae are biopsied, the place where the zona pellucida is breached is of no importance. Therefore, the opening can be made anywhere on the circumference of the embryo, no matter what biopsy technique is used [18]. In case of blastocysts, all biopsy methods imply the disruption of zona pellucida in an area where only trophectoderm cells are present, which are also harvested during biopsy [18].

All three biopsy techniques were able to provide enough cells, and thus enough DNA, to successfully carry out the PCR reaction and sex determination in all embryos. Moreover, both ultrasound fetal sexing and evaluation of anatomical sex at calving confirmed the results obtained by PCR, which reaffirms the very high accuracy of this sexing tool. Although the amount of genetic material obtained by microblade biopsy was slightly higher than that obtained by needle or aspiration, the latter techniques were still able to provide enough cells and DNA to enable amplification.

Other authors [3] reported a higher accuracy of the sexing method for the microblade biopsy technique, related to the higher amount of cellular material that was harvested. Very small amounts of DNA are actually needed for PGD (including embryo sexing), as previously shown by various studies [5, 19–21] and the biopsy of a single blastomere should be enough, even for the establishment of embryonic stem cell lines [22]. Therefore, the only concern remains the integrity of the zona pellucida, which only matters when the embryo is frozen/thawed after the biopsy.

The immediate postbiopsy transfer of embryos, without cryopreservation, significantly increases the pregnancy rate in recipient females as compared to the transfer of frozen/thawed embryos. Roschlau et al. [23] showed that the pregnancy rate in fresh transferred sexed bovine embryos was of 45,6% and did not significantly differ from that of the embryos transferred without sexing (53%) while deep freezing tended to decrease the pregnancy rate of biopsied embryos (44,4%). Shea [24] obtained 49–60% pregnancy rates when fresh transfers were made and 23–41% for frozen/thawed transfers. Lopes et al. [9] found that pregnancy rates achieved with fresh bisected or biopsied embryos (50 to 60%) were similar to those of fresh intact embryos (55 to 61%). Lopatarova et al. [25] performed bovine embryo bisection and sexing and transferred the freshly split demiembryos without cryopreservation. The pregnancy rates were comparable with other studies (56.5%).

The transfer of frozen/thawed biopsied embryos reported by other authors yielded comparable results. Agca et al. [26] obtained a 23% pregnancy rate when the biopsied embryos were frozen and 50% when they were freshly transferred, without cryopreservation. Ito et al. [27] suggested that a brief cultivation of biopsied embryos prior to freezing is able to significantly increase embryo viability after thawing.

New methods of embryo biopsy have lately been successfully used, especially in human assisted reproductive techniques, in order to harvest cells for PGD. Martinhago et al. [28] developed a real-time method for rapid sexing of human preimplantation embryos where they used a diode laser in order to open the zona pellucida. Other studies reported promising clinical pregnancy rate (31.4%) following the transfer of cryopreserved blastocysts that underwent laser-assisted hatching on the zona on day 3 [29].

4. Conclusions

Biopsied frozen/thawed bovine embryos have a better chance to survive the cryopreservation process if damage of the zona pellucida is minimized as much as possible. This can be achieved by carefully choosing the embryo biopsy method, the needle technique showing obvious advantages, as presented above. As biopsied embryos are rarely transferred directly into recipients, and are usually cryopreserved for later use, the choice of an adequate biopsy method has a direct influence on embryo viability after thawing. The accuracy of PCR sexing was not influenced by the biopsy method, as it was of 100% in all batches. Our results can be extrapolated to other species and should be useful for PGD decisions in these as well.

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