COLLECTION OF BLASTOMERES IN ORDER TO ESTABLISH SEX AND ISOLATE GENETIC MATERIAL-REVIEW

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Abstract

Globally, limited access to food needs in relation to meat or milk production has required the establishment of the sex of offspring from the embryonic stage. While the meat industry uses males, the dairy industry relies on females. During the period of exploitation, the number of products obtained from a female bovine is 5-6 individuals, their sex being able to be influenced by means of sexed semen. Embryo sexing programs can result in a large number of conception products, in a shorter period of time taking into account the desired sex. The use of the desired sex embryo facilitates the improvement of the genetic value. Embryonic sexing procedures involve the collection by biopsy of a minimum amount of genetic material that can ensure the determination of sex. Both invasive and non-invasive biopsy and sexing procedures can influence the subsequent viability of embryos prepared for embryo transfer. This paper highlights the methods of embryonic sexing along with the advantages and disadvantages of each technique involved in determining sex.

Key words: Embryo, biopsy, sex determination.

INTRODUCTION

Embryonic sexing has significant economic implications in the dairy and beef industries because it can meet the requirements of the producer by determining the sex of the future conception product. Thus, the use of the embryonic sexing technique can bring a greater economic potential for dairy and beef cattle farmers (Yasuhiro O. *et al*, 2015). Obtaining products by embryonic sexing, which will cover the requirements of cattle breeders is very topical (is of great relevance), by determining the sex during the formation of the (conception) product suggesting the economic importance of this technique in involving the growth of animal production. Embryonic sex is also involved in the diagnosis of genetic disorders in the prenatal stage. Embryo sexing increases the efficiency of embryo transfer, facilitates their transfer at choice, based on the desired sex (Bredbacka P., 2001;Cenariu M. *et al*, 2008). The use of sex-sorted semen is an important technique for obtaining the desired sex by artificial insemination or *in vitro* fertilization (IVF), but it is very expensive (Seidel G.E., 2007) and less effective compared to conventional, unsorted semen (Trigal B. *et al*, 2012). The use of the technique to examine the chromosome in bovine embryo cells has led to the sexing of embryos, a new approach to sex selection. The sexing of the embryos performed before the embryonic transfer, will lead to an improvement of the genetic material by determining the sex of the transferred product, thus it will generate a rapid increase of the genetic value from the respective farm (Sachan V. *et al*, 2020).

Blastomere harvesting technique

Penetration of the zona pellucida (ZP) can be done either:

- mechanical,
- chemical,
- using a laser device.

Mechanical penetration of ZP (also called partial area dissection) was the first method used to form a gap in the ZP membrane and is still applied clinically, although to a lesser extent. The method involves creating a slot in the ZP using a sharp micropipette (Kokkali G. *et al*, 2020).

Chemical perforation of the zona pellucida involves the use of an acid solution (Tyrode's acid) for its local dissolution. This method has been widely used since the first embryonic biopsies; it is used during embryonic segmentation. However, the subsequent implementation of laser technology and the disadvantages caused by the toxicity of acid substances (Tyrode's acid) on the viability of embryos led most laboratories to abandon the chemical method of perforating ZP (Kokkali G. *et al*, 2020).

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Laser is currently the most popular ZP penetration method for polar body (PB), cleavage stage and blastocyst biopsy. The method involves the use of a non-contact guided laser beam, which can be adjusted to create a ZP aperture of the desired size in a precise and fast way. Laser beam power and exposure (pulse length / width) should be carefully addressed according to the manufacturer's specifications to avoid damage to PBs or embryonic cells (Kokkali G. *et al*, 2020).

Methods for harvesting blastomeres

The method by cutting the blastocyst with the help of the microblade is applied at the stage of 7 days from artificial insemination or cultivation. After placing the blastocyst in a biopsy medium with the addition of mineral oil, the Petri dish is inserted under a microscope equipped with a micromanipulator and microblade. About a third of the blastocyst (trophoblastic cells) is removed by cutting.Embryos that have been biopsied are transferred to another environment to be washed. After washing, the embryos were cultured in a special culture medium for 2-3 hours at 38.5 ° C, in a humid atmosphere of 5% CO_2 in the air (Vieira de Sousa R., *et al* 2017**).**

In the method of pipetting or aspirating blastomeres, embryos in the morula stage are exposed for one minute in a solution (pronase) to dissolve the zona pellucida. Embryos without zona pellucida will be transferred to an environment to stop the action of the enzyme. Aspiration of blastomeres is done by light aspiration using a pipette approximately 110 μm in diameter. When the amount of blastomeres required for evaluation has been reached, the embryos will be placed in a culture medium for 48 hours at 38.5 ° C in a humid atmosphere of 5% CO2 in the air.During embryonic biopsies, the amount of DNA extracted must ensure that the sexing procedure is performed.Regarding the biopsy procedure, the main target is the care with which the embryo must be handled in order to not damage the structure and also its viability (Vieira de Sousa R., *et al* 2017**).**

Several procedures for sexing embryos in farm animals by invasive or non-invasive methods have been evaluated. These methods may or may not require embryonic biopsy (Garcia J.F., 2001). Non-invasive methods are considered less harmful to the embryo because the integrity of the embryo is not damaged and the embryos remain intact and viable (Utsumi K.,1993).

A. Non-invasive Methods

Sexation based on cleavage and development

During embryonic sexing by the PCR method and by the karyotyping method, Yindee Kitiyanant *et al*. (2000) observed that male embryos showed a faster cleavage rate than female embryos. The faster growth in male embryos may well be a consequence of the faster gene expression caused by Y-chromosomal genes. A possible effect of Y-linked genes promoting the rapid growth of male embryos has been suggested to be caused by H-Y antigen or Y-chromosome growth factors. Total glucose metabolism was found significantly greater in male than in female bovine blastocysts which might be related to more rapid development of male embryos (Yindee K. *et al*, 2000). This method of embryo sexing has still many limitations,In-vivo produced embryos cleavage time cannot be known, besides the difference in developmental rate is very small and needs high skills in separation of fast and slow embryos (Sharma M. *et al*, 2017).

Detection of X – linked enzymes

Embryos can be differentiated as male or female by measuring the dose of the X-linked enzymes gene. Glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT) are involved in determining embryonic sex based on their concentrations (Kouamo J. *et al*, 2014). In normal mammals, homogametic sex (female) carries two X chromosomes (XX) whereas heterogametic sex (male) possesses only one X chromosome (XY). In female, one of the X chromosomes is in activated in each cell in embryonic life to maintain an equivalent number of genes between sexes **(**Lakshmy K. V. *et al*, 2018). Gutierrez –Adan et al. demonstrated that G6PD and HPRT are almost twice as high in female embryos as in male embryos in the early blastocyst stage (Gutierrez-Adam A. *et al*, 2000). Peippo *et al*. (2002) observed that the level of G6PD was significantly higher in female bovine embryos produced in vitro than in male ones in both the morula and blastocyst stages. The HPRT level was much higher in female embryos than in males, evaluated in the morula and blastocyst (Peippo J. *et al*, 2002). To maintain an equivalent number of genes between the sexes, one of the X-chromosomes in the female is inactivated in each cell early in the embryonic life. Although the exact timing of X-inactivation is not known, studies have suggested that there is a brief period between activation of the embryonic genome and X-inactivation in which genes from both X chromosomes in the female are transcribed **(**Sharma M. *et al*, 2017).

Detection of H-Y antigens

On the cell surface in males embryos is the surface antigen called Histocompatibility Y or HY antigen, this antigen is not present in female

embryos. The HY antigen can be used in embryonic sexing (Sachan V. *et al*, 2020). There are two tests that can detect HY antigen on the surface of embryos, namely: an immunoflorescence test and a cytotoxicity test (Sharma M. *et al*, 2017). In the fluorescent control (test) , embryos to primary H-Y antibody are incubated in the absence of complement for 30 minutes and then for another 30 minutes with secondary antibodies that are labeled with fluorescein isothiocyanate (FITC) (Sharma M. *et al*, 2017). By microscopic evaluation, male embryos show fluorescence (HY positive) and female embryos do not show fluorescence (HY negative) (Lakshmy K. V. *et al* 2018). Through the cytotoxicity test, the embryos are incubated in the presence of complement with polyclonal antiserum that will have an immunological action of cell lysis on embryos that present the H-Y antigen. Through this cytotoxicity test the male embryos will be neutralized and the female embryos will survive (Lakshmy K. V. *et al* 2018).

B.Invasive Methods

The Barr body

Based on the Lyon (1961) hypothesis, all but one X chromosome is randomly inactivated early in embryogenesis, after implantation. The end result is that, when evaluated, female cells have one Barr body, while the male cell has none. In 1948 Barr et al. proved, first in cats and then in humans, that female and not male cells consist of deeply stained body in the nucleus. The origin of the Barr body was established by Lyon (1961), who claimed that the Barr body is a heteropyknotic material (pyknosis-irreversible condensation of chromatin in the nucleus) originating from the X chromosome that is randomly inactivated and can be from either maternal or paternal origin (*O*rnfooy A. *et al*, 2020). The first blastocysts to be evaluated for the presence or absence of Barr's body were rabbits (Bondioli, K. R., 1992). Due to the granular nature of the cytoplasm, observation of the Barr body can be difficult, so some female embryos can be confirmed as male embryos (King W. A., 1984;Shetty, N. K. *et al*, 2018). The presence and detection of the Barr body depends not only on the stage of the cell, but also on the fixation procedure, so the improper stage of the cell or the improper fixation and staining procedure can give a false diagnosis for embryonic sexing. Another limitation of this technique is that due to the need for a large number of cells, embryo damage may be visible (Wakchaure R. *et al*, 2015).

Cytological methods or karyotyping

Cytogenetic sexing or karyotyping of bovine embryos from day 6 or 7 is done by analyzing chromosomes blocked in metaphase (King W. A., 1984). The technique consists in the biopsy collection of embryonic trophoblastic cells and their cultivation with mitosis-arresting agent such as (e.g. colchicines) that stop the division of cells in the metaphase stage of mitosis (Seidel G.E. *et al* 1991). These substances cause the depolymerization of microtubes and prevent the formation of the spindle through their antimitotic effect (Sharma M. et al, 2017). The cells are subjected to a hypotonic solution to osmotically lysis them so that the chromosomes can be dispersed. Giemsa solution can be used for DNA staining so that the metaphase chromosomes can be analyzed microscopically (Sharma M. *et al* 2017). As an interpretation of the results, the presence of two X chromosomes indicates the female embryo and the presence of the Y chromosome the male embryo (Lakshmy K. V. *et al*, 2018). The main advantage of the method is the accuracy of this technique. Another advantage would be the detection of chromosomal abnormalities. This method is affordable requiring a microscope and reagents that are cheap and easy to access (Yindee K. *et al*, 2000). As disadvantages, the embryo must undergo the biopsy stage and the preparation sometimes requires even 12 hours or more, so most laboratories have turned to other embryonic sex techniques (Seidel G.E. *et al* 1991).

Polymerase chain reaction (PCR) method

The polymerase chain reaction (PCR) was developed by Kary Mullis in the 1980s (Schluger N. *et al*, 1995). PCR also took shape in the embryonic sexing technique, giving the possibility to identify the embryonic sex (Mara L. *et al*, 2004). The method of sexing the bovine embryo by amplifying specific DNA sequences of the Y chromosome using PCR proves to be an effective tool for sex identification. Embryonic sexing using PCR includes embryo biopsy (1-4 blastomeres), amplification of DNA fragments and interpretation after analysis of products amplified by electrophoresis technique (Sachan V. *et al*, 2020). If only one band of the bovine-specific product is visible, on the gel, the blastomere is considered to derive from a female embryo, whereas the presence of two bands referred to a male embryo (Sharma M. *et al*, 2017). Regardless of whether the use of PCR requires technical skills for embryonic sexing, this method is almost 100% effective (Kouamo J. *et al* 2014), sensitive and fast (Gokulakrishnan P. *et al***,** 2013; Wakchaure R. *et al*, 2015) and can be achieved in several hours (Bredbacka P., 2001). With this technique, a good percentage of embryos can be sexed obtained without disturbing their capacity for development (Peura T. *et al*,1991). PCR can give false results

due to the collection of a limited amount of DNA from embryo biopsies, cross-species DNA contamination, DNA contamination during handling of DNA products in PCR procedures and electrophoresis (Geshi M., 2012). It has been reported that the accuracy of the PCR method for sexing bovine embryos gives better results (96.4%) compared to FISH (86.66%) (Cenariu M. *et al*, 2011).

Embryo sexing by fluorescence *in situ* hybridization (FISH)

The in situ fluorescence hybridization (FISH) technique can detect specific DNA sequences of individual chromosomes in a cell (Kobayashi J. *et al*, 2004). The efficiency of the technique is high and the sex of an embryo can be unequivocally determined when two X-chromosome signals in the absence of a Y-chromosome signal or one X-chromosome and one Y-chromosome signal are detected (Staessen C., 1999). By using Y chromosome-specific DNA fragments in in situ fluorescent hybridization (FISH), male and female embryos can be differentiated (Cotinot C., 1991). The evaluation of the embryonic sex by the FISH method can be performed in the morula or blastocyst stage, the collection of a small number of embryonic cells will reduce the damage to the subsequent viability of the embryo. The pregnancy rate following the transfer of biopsied embryos was 51.3% and the accuracy of the FISH sexing technique is 92% (Cenariu M. *et al*,2008). FISH is, however, expensive and has a more complex degree of operation (Xie Y., 2020).

Loop-mediated isothermal amplification (LAMP)

Sexing of embryos based on specific sequences on the Y chromosome was achieved by PCR amplification from a small number of blastomeres (Alves B.C., 2006; Garcia J.F., 2001). Hirayama *et al.* (2004) reported loop-mediated isothermal amplification (LAMP), a simpler method of sexing bovine embryos compared to PCR. LAMP is a new method of amplifying DNA that can amplify a specific DNA sequence in a temperature range of 60 to 65 ° C (Lakshmy K. V. *et al*, 2018). DNA amplification is performed under isothermal conditions using a DNA polymerase and four sets of specific DNA primers (inner and outer) that recognize a total of six distinct sequences on the target DNA (Hirayama H. *et al*, 2013). Moreover, an additional set of primer (called a loop primer) is used to accelerate the LAMP reaction. The inner primer initiates the primary DNA synthesis, and the next DNA synthesis by an outer primer releasing a single-stranded DNA derived from the inner primer (Hirayama H. *et al*, 2013). Amplification of the target DNA is estimated by measuring the turbidity due to a white precipitate of magnesium pyrophosphate, a by-product of DNA synthesis (Geshi M., 2012). This method of DNA amplification is highly specific, efficient, and rapid. Besides, gene amplification and detection can be completed in one step, and amplification could be up to 10^9-10^{10} times in 15–60 min. Since the detection of all target gene sequences can only be determined by either the presence or absence of amplification products, LAMP is considered to be highly specific (Sharma M. *et al* 2017).

CONCLUSIONS

Embryonic biopsy and embryonic sexing have improved embryos production based on the wishes of cattle breeders. The embryonic biopsy method will be chosen depending on the laboratory equipment and equipment costs, but satisfactory results can be obtained using the cutting method.Sexual methods classified as invasive and non-invasive are used in embryonic sexing. The use of invasive methods can affect the subsequent viability of embryos, which is essential in embryonic transfer, gestation and procreation (parturition). Non-invasive methods have a reduced effect on embryonic viability, they are less used. Of the invasive methods of embryonic sexing, the most used would be the PCR method with a sexing rate close to 100%, being simple and fast. The use of embryonic biopsy and embryonic sexing ensures the growth of meat production in the case of male embryos and milk production in the case of female embryos. In the future, some techniques could be improved to increase the efficiency and subsequent viability of embryos.

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