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CHALLENGES IN BIOTECHNOLOGY OF REPRODUCTION IN DAIRY CATTLE: THE ANTRAL FOLLICLE COUNTING AS A FERTILITY PARAMETER

Retos en la biotecnología de la reproducción en ganado lechero: recuento de folículos antrales como parámetro de fertilidad

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RESUMEN

La producción lechera juega un papel importante en el escenario socioeconómico, y varias características apuntan a un enorme potencial de crecimiento en la productividad de los hatos. Se trata de una actividad compleja que debe llevarse a cabo de manera eficiente en todos los enlaces que la integran. Además, debido a los aspectos fisiológicos y metabólicos de los animales con aptitud para la producción de leche, se plantean varios desafíos, especialmente en el ámbito reproductivo. En este contexto, las biotecnologías reproductivas aparecen como una buena alternativa al aumento de las tasas de reproducción y mejora genética del ganado lechero. Además, se han estudiado el recuento de los folículos antrales (RFA) como una forma de mejorar la selección de hembras para componer programas reproductivos, ya que se ha demostrado ser un factor de alta repetibilidad sobre la vida reproductiva en el mismo animal. Aunque los estudios han mostrado resultados polémicos en cuanto a la relación entre la RFA y la fecundidad, todavía es necesario investigar las posibles variables que pueden interferir con el desempeño de los donantes en cada biotecnología utilizada. Por lo tanto, esta revisión pretende reunir y discutir la relación de la RFA con los aspectos fisiológicos de las vacas lecheras, su aplicación como parámetro de selección de animales con buena fertilidad y los retos para la aplicación de biotecnologías reproductivas en ganado lechero.

Palabras clave: Bovino, Vaca lechera, conteo de folículos antrales, biotécnicas reproductivas

ABSTRACT

Dairy farming plays an important role in the socioeconomic scenario, and several characteristics point to an enormous potential for growth in the productivity of the herd. This is a complex activity that needs to be carried out efficiently in all the links that integrate it. Moreover, due to the physiological and metabolic aspects of the animals with the aptitude for the milk production, several challenges arise, especially in the reproductive scope. In this context, breeding biotechniques appear as a good alternative to the increase in reproductive rates and genetic improvement of dairy cattle. In addition, ovarian antral follicle counts (AFC) have been studied as a way to improve the selection of females to compose reproductive programs, because it has been shown to be a factor of high repeatability over the reproductive life in the same animal. Although studies have shown controversial results regarding the relationship between AFC and fertility, it is still necessary to investigate the possible variables that may interfere with the performance of donors in each biotechnology used. Thus, this review intends to gather and discuss the relationship of AFC with physiological aspects of dairy cows, its application as a parameter of selection of animals with good fertility and, the challenges for the application of reproductive biotechniques in dairy cattle.

Keywords: Bovine, Dairy cattle, Antral follicle counting, Reproductive biotechnics.

INTRODUCTION

Reproductive biotechnology has been a significant contributor to improved dairy productivity. The animals of greater genetic merit can be multiplied through biotechnology such as artificial insemination and embryo production (Mapletoft and Hasler, 2005; Hansen, 2014). In some countries, like Brazil, the production of embryos in the dairy chain reached 59.3%, with the substitution of in vivo for in vitro production (IETS, 2015).

In this context, antral follicle count (AFC) and its relationship with fertility have been increasingly investigated with the aim of better selecting females for the different biotechniques (Ireland *et al*., 2011; Rico *et al*., 2012). Studies have shown that in females taurus (Burns *et al*., 2005; Ireland *et al*., 2008) and indicus-taurus (Silva-Santos *et al*., 2014a), the AFC is a parameter of great variability in the herd, but with high repeatability in the same individual. Thus, it is possible to classify females in low, intermediate and high AFC categories according to the antral follicle population in the ovary (Guerreiro *et al*., 2014; Silva-Santos *et al*., 2014b).

The repeatability of AFC in a female throughout her life makes it possible to classify it in a strategic and practical way with only a single evaluation of the ovary by ultrasonographic examination. The advantage of performing this evaluation is because in Bos taurus animals the AFC is directly related to the size of the ovarian follicular reserve (Ireland *et al*., 2011). Another way to measure AFC is to evaluate the concentration of Anti-Mullerian Hormone (AMH) in the bloodstream of cows. The AMH is a hormone that has been described to be highly correlated with the population of antral follicles, as well as with oocyte viability (Batista *et al*., 2014). Thus, AMH has been considered an endocrine marker of AFC in the bovine species.

The high AFC in Bos taurus and indicus-taurus cattle (Silva-Santos *et al*., 2014a) is linked with the female reproductive performance and presence of morphologically healthy follicles (Ireland *et al*., 2008, 2011). Also, animals with high AFC have a higher number of oocytes recovered and embryos produced (Guerreiro *et al*., 2014). Several studies have shown that in females classified as high AFC, higher levels of progesterone in the bloodstream and better pregnancy rate were reported when compared to females with low antral follicular population (Jimenez-Krassel *et al*., 2009; Evans *et al*., 2012; Mossa *et al*., 2012).

However, recently the same group of studies found different results when monitoring heifers for a longer period. It was verified that taurus females with high AFC had a shorter productive life in the herd when compared to intermediate and low AFC animals, as well, lower fertility (Jimenez-Krassel *et al*., 2017). It is important to consider that AFC can be influenced by the nutritional state and health of the individual since follicular growth and secretion of reproductive hormones are affected by the general metabolism (Jimenez-Krassel *et al*., 2009; Mossa *et al*. 2010). Also, maternal environment, nutritional status and maternal health throughout pregnancy, will influence the ovarian reserve of offspring (Ireland *et al*., 2011; Evans *et al*., 2012).

In short, considering the AFC as a tool to aid animal breeding biotechnology to maximize gains in genetic improvement and productivity in the dairy herd, this review aims to discuss: i) the relationship of AFC with physiological aspects of dairy cattle; ii) the application of AFC as a fertility selection parameter of cows and, iii) the challenges for the application of reproductive biotechniques in dairy cattle.

Anti-Mullerian Hormone

The anti-Mullerian hormone is a member of the β-growth factor superfamily and is synthesized from granulosa cells of antral and preantral follicles (Morotti *et al*., 2015). AMH can be considered an endocrine marker of AFC in cattle since its concentration in the bloodstream of females is highly correlated with the antral follicular population and oocyte viability (Batista *et al*., 2014).

In Bos taurus animals, females classified as high AFC (> 25 follicles) showed higher blood concentrations of AMH when compared to females with low CFA (<15 follicles). Thus, showing a high correlation between the mean circulating AMH and the ovarian antral follicular population ($R = 0.88$, $P < 0.001$) (Ireland *et al*., 2008). In this context, the measurement of plasma levels of AMH in the bovine species may help in the selection of donor females that have a greater number of antral follicles. Thus, better results can be obtained when these animals are submitted to reproductive biotechniques, such as follicular aspiration guided by ultrasonography (OPU) (Barusselli *et al*., 2016).

A study of 59 Holstein heifers (15 pre-pubescent heifers, 15 cyclic heifers, 14 lactating cows and 15 non-lactating cows) showed a positive correlation of AMH concentration with the total number of both aspirated follicles. Also, the same correlation was observed with the recovered oocytes and the number of embryos produced per OPU session (Guerreiro *et al*., 2014). Furthermore, AMH is recognized as a reliable indicator of ovarian response to superovulation protocols (SOV) (Rico *et al*., 2009). Still, Bos taurus donor females of the Holstein breed followed up for more than one year, who had an AMH concentration lower than 87 ng / mL obtained less than 15 follicles aspirated per estrous cycle and, therefore, low embryo production efficiency (Rico *et al*., 2012). The high plasma concentration of AMH was also associated with a larger number of large follicles and presence of corpus luteum after the SOV protocol (Baruselli *et al*., 2016).

Thus, the measurement of the plasma concentration of AMH in Bos taurus is a tool to be considered in the OPU/IVEP and SOV procedures during the breeding programs of dairy cattle. This parameter may contribute to the identification of donor females with the best potential for production of both embryos in vitro and in vivo (Barusselli *et al*., 2016; Rico *et al*., 2012).

Progesterone Levels

At the end of the follicular wave with ovulation of the dominant follicle, the formation of the corpus luteum (CL) occurs from the granulosa and teal cells, which has the function of producing progesterone (Smith, 1986). This hormone is required to maintain the uterine environment suitable for embryo development, and to maintain pregnancy in mammals (Bazer *et al*., 2010; Pohler *et al*., 2012).

Therefore, the low circulating concentration of progesterone is associated with lower reproductive efficiency of females, decreasing the number of healthy oocytes and increasing rates of embryonic mortality due to slower endometrial growth (Diskin and Morris, 2008). Studies with Bos taurus females have reported a relationship between the number of ovarian antral follicles and the level of progesterone in the circulation of these animals (Evans *et al*., 2012; Martinez *et al*., 2016). Females classified in high AFC had higher levels of plasma progesterone during dieting and pregnancy when

compared to females with low CFA, as well as higher luteinization (Jimenez-Krassel *et al*., 2009)

Moreover, high AFC was shown to be associated with increased endometrial thickness and consequently higher rates of embryo implantation (Basir *et al*., 2002). Corroborating in this way, the use of AFC as a tool for selection of females with the best potential to be submitted to reproductive biotechniques.

AFC and efficiency in embryo production

The success of bovine embryo production is directly affected by factors such as oocyte recovery rate and donor superovulatory response (Taneja *et al*., 2000; Ireland *et al*., 2007, 2011). Thus, the higher the number of oocytes recovered, the will have a greater the number of blastocysts at the end of the IVP, the larger the number of embryos collected and transferred to the recipients. Therefore, the highest AFC provides quantitative benefits for the final rates of embryo production, either by the in vitro method or in vivo (Taneja *et al*., 2000; Singh *et al*., 2004; Ireland *et al*., 2008).
In a study carried out in Europe, the females classified in low AFC obtained a lower average number of embryos produced in vitro when compared to the high AFC females (1.3 vs. 4.9 embryos, respectively). The same ratio was found for in vivo embryo production in which donors with low AFC (15 follicles) obtained the final mean of 3.8 embryos produced, while high AFC donors (>25 follicles) obtained the average production of 5.4 embryos (Ireland *et al*., 2007).

On the other hand, the use of IVEP in Girolando cattle donors has recently increased due to better efficiency in the use of sexed semen for in vitro fertilization (Pontes *et al*., 2010; Xu *et al*., 2006). The sexed semen ia a strategy that facilitates the production of a vast number of females for the dairy industry. Another important benefit of this breed is that physiologically Bos indicus bovine has more ovarian follicles than Bos Taurus. Thus, allowing the greater quantity of oocytes recovered by OPU from the crossing indicus-taurus. In Brazil, a project was carried out for the large-scale production of bovine embryos of dairy breeds, involving Gir (Bos indicus), Holstein (Bos taurus) and Girolando (indicus-taurus) donors submitted to a total of 5000 OPU with the recovery of approximately 90000 oocytes. For Gir cows, the viable oocytes and the average number of embryos produced by OPU/IVEP procedure were 12.1 ± 3.9 and 3.2 (12243/3778), and, 8.0 ± 2.7 and 2.1 (2426/1138) for the Holstein breed. Already for the crossed races, the values were 16.8 ± 5.0 and 3.9 (1033/267) for the crossbred 1/4 Holstein and 3/4 Gir, and, 24.3 ± 4.7 and 5.5 (1222/224) for ½ Holstein-Gir (P<0.01) (Pontes *et al*., 2010).

These results evidenced the high yield of oocytes aspirated in the bovine subspecies Bos indicus races compared to Bos taurus. Several hypotheses have been investigated to explain such event. Among them, the possibility that Bos indicus females have a greater number of germ cells in the fetal stage, the existence of distinct mechanisms of follicular atresia, or even the hypothesis of follicular renewal by neo folliculogenesis (Johnson *et al*., 2004, 2005).

However, crossbreeding indicus-taurus has been shown to be beneficial for the dairy chain, because besides generating high productivity animals and greater resistance to tropical regions. Moreover, crossbreeding indicus-taurus provided an increase in the production of embryos in vitro through the use of females with high AFC.

CFA and fertility parameters

As previously described, the hypothesis that fertility is influenced by the number of follicles in the ovaries has been reported by several authors over the years (Hunter, 1787; Erickson, 1966). Some parameters such as plasma progesterone level (Martinez *et al*., 2016), CL functionality, endometrial thickness (Jimenez-Krassel *et al*., 2009) and, calving intervals (Mossa *et al*., 2012), were positively correlated with high antral follicular population (Ireland *et al*., 2008). However, the association between AFC and fertility may present controversial results. In New Zealand, dairy cows that were separated into groups according to the antral follicular population and subsequently submitted to artificial insemination and diagnosis of gestation. Pregnancy rates as shown in table 1 (Martinez *et al*., 2016).

Table 1. Pregnancy rate in New Zealand dairy cows submitted to artificial insemination protocol.

Variables	High ≥ 30 follicles n = 104	Intermediate 21-29 follicles n = 137	Low ≤ 20 follicles n = 200
Conception by AI (day)	82.4 ± 1.6[a]	85.2 ±1.6[ab]	87.3 ±1.2[b]
No. of AI per pregnancy	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Pregnant to first AI (%)	54.6	49.4	48.5
Pregnant after 6 weeks of AI (%)	70.0	70.4	61.2
Pregnant overall (%)	87.1	79.4	81.0

a-b Within the same row differ (P < 0.05). Abbreviations: AI, artificial insemination; CL, luteum corpus. Adapted from Martinez et al. (2016).

A similar result was obtained by our team, considering Holstein donors that were classified as low (≤35 follicles), intermediate (40 to 55 follicles) and high (≥ 60 follicles) AFC and later submitted to artificial insemination. The conception rates obtained were similar among the low AFC group, 26.7% (8/30), intermediate, 36.7% (11/30) and high, 50% (n = 15/30 P ≤ 0.05) (Droher *et al*., Unpublished data).

The relationship between AFC and fertility seems to be even more complex. Jimezes-Krassel and collaborators (2017), recently demonstrated that dairy heifers in the high AFC group (≥25 follicles), had a productive life 180 days shorter in the herd. Also, females with high AFC have more services per conception, and lower pregnancy rates compared to both low (≤ 15 follicles) and mean AFC heifers (between 16 and 24 follicles).

In summary, the divergence among results indicates that other variables seem to interfere in the relationship between AFC and fertility, such as the biotechnology in question. For IVEP, there are clearly quantitative advantages when cows with high ovarian follicular population are submitted to OPU. However, many factors need to be better understood, such as the AFC relationship with follicular growth, female nutritional and health status. Further, research is needed to overcome the challenges related to the use of AFC as a reproductive tool in dairy cows.

The challenges for the application of reproductive biotechniques in dairy cattle

For dairy cattle, the AFC seems not to be related to production parameters. It has been demonstrated, that the

ovarian antral follicular population is a reproductive trait with moderate heredity that may be affected by age and lactation status, but there is no correlation with the genetic merit of characteristics such as milk production (Walsh *et al.*, 2014). Therefore, at the time of selection of females for breeding, the AFC can be used as a secondary tool to the selection for productivity without the occurrence of genetic demerit.

However, the attainment of reproductive efficiency in dairy herds is more laborious when compared to beef cattle, since high production cows with a lactation curve of more than 305 days are unable to obtain an adequate calving interval. The ideal is that this period is 365 days and for this to occur the period of service should last a maximum of 81 days, in which case the cows will present one birth per year (Ferreira, 1991; Ruas *et al.*, 2008). Such challenges are because high production cows in the postpartum period usually present a greater difficulty in recycling due to the energy reserve that is directed to milk production, rather than reproduction. Oocytes are directly influenced by the metabolic and hormonal variations that occur during the prolongation of this period. The fertility is restored only after the recovery of the body condition because at this stage the cows enter into a negative energy balance (Ferreira *et al.*, 1999; Leroy, 2005).

On the other hand, increased dry matter intake affects serum levels of progesterone, which in turn leads to reduced reproductive efficacy. In this way, dairy farming requires an integrated management between genetics, nutrition, sanitation, and reproduction to achieve good results. Lack of attention to any of these items prevents efficiency being achieved.

CONCLUSION

The dairy chain plays an important role in the socioeconomic scenario of the country since to ensure the production of healthy and quality food for human consumption. Moreover, this branch is a generator of income and jobs for the population from the countryside, through the industrial sector and reaching the urban centers. However, the milk chain is an activity that involves high production costs and significant risks, making it a complex activity that needs to be carried out efficiently in all the links that integrate it.

In this way, milk cows routinely face several challenges with reproduction in their herds, due to the physiological and metabolic aspects of the animals that are capable of producing milk.

However, development has reached the rural sector, and reproductive biotechnology emerges as a good alternative for increasing reproductive rates and genetic improvement. In vitro embryo production and fixed-time artificial insemination are examples of techniques that have been applied on a larger scale in the last decades, presenting good results for the producer.

In short, AFC appears as an alternative for the evaluation of the fertility of females in the reproductive programs, despite more studies are necessary due to the several controversial results found. In general, prospects are considered as positive for overcoming the challenges encountered around the improved reproductive efficiency and productivity of the dairy chain in the country.

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