Can Anogenital Distance be Used to Select Oocyte Donors with Greater Embryo Production?

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Summary

In vitro embryo production has increased over the last years as a tool to obtain genetically superior animals. Anti-Müllerian hormone (AMH) concentration and antral follicle count are phenotypes associated with in vitro embryo production but are less accessible at the farm level. Anogenital distance (length from the center of the anus to the base of the clitoris) has been proposed as a new proxy for fertility in dairy cattle that can be collected easily and at a low cost. *Bos indicus* dairy cattle oocyte donors of the Gyr breed (n = 552) from six herds in Brazil were used to test the association of the historical in vitro embryo production record with anogenital distance and AMH. The historical record of embryo production was retrieved from the farms, and anogenital distance and blood samples to evaluate the concentration of AMH were collected in 2021. Donors were classified as High or Low AMH, High or Low recovered oocytes, and Short or Long anogenital distance categories. Donors in the High AMH and oocyte categories produced more viable oocytes and embryos than donors in the Low AMH and oocyte categories. However, no differences were observed between Short vs. Long anogenital distance for AMH concentration and in vitro embryo production. A limitation of our study was the use of oocyte and embryo data collected retrospectively in relation to the current anogenital distance of the animal. Thus, further studies should be performed to validate these results and to better understand the association between anogenital distance and in vitro embryo production.

Introduction

The use of in vitro embryo production has increased over the last years as a tool to obtain genetically superior animals. In that sense, Anti-Müllerian hormone (AMH) concentrations and antral follicle count are phenotypes associated with fertility and in vitro embryo production in cattle. However, evaluation of blood samples or an ultrasound exam is necessary to assess these phenotypes, which poses challenges at the farm level.

Anogenital distance (i.e., the length from the center of the anus to the base of the clitoris, Figure 1) in dairy cattle has been proposed as a new phenotypic trait that is associated with fertility and can be collected easily and at a low cost. A study with

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almost 1,700 heifers showed that heifers classified as short anogenital distance had greater pregnancy per insemination at first service, younger age at first service, fewer services per pregnancy, and younger age at pregnancy when compared to heifers classified as longer anogenital distance. Likewise, a recent study with ~4,700 lactating cows validated that lactating cows classified as having shorter anogenital distance resulted in greater pregnancy per insemination at first service when compared to those classified as having longer anogenital distance. The relationship between anogenital distance and in vitro embryo production in dairy cattle remains to be tested. An association between these two traits could facilitate the selection of donors and potentially explain the association of anogenital distance with fertility from previous studies.

Our aim was to characterize the association among anogenital distance, AMH concentrations, and the number of recovered oocytes to select oocyte donors for in vitro embryo production. The working hypothesis was that donors classified as having short anogenital distance have greater AMH concentrations as well as a greater number of recovered oocytes and embryo production when compared to donors classified as having long anogenital distance.

Experimental Procedures

Animals, location, and records of in vitro embryo production

The current study was conducted using 552 *Bos indicus* dairy cattle, Gyr breed, that had been used commercially as oocyte donors for in vitro embryo production. Some donors had records of up to 47 ovum pick-up (OPU) events, whereas others had been collected just one time. Thus, the animals ranged from 1.5 to 15 years of age. The donors were located in the states of Minas Gerais (five herds) and Rio de Janeiro (one herd), Brazil. Donors were housed in grazing systems with *ad libitum* access to water and mineral salt and were supplemented with corn silage and concentrate.

Records of the commercial in vitro embryo production of each donor from May 2010 until February 2022 were collected from dairy management software used in the six herds participating in the study. The records included the number of recovered oocytes, viable oocytes, and embryos produced per donor on each transvaginal oocyte aspiration. The oocyte viability rate was calculated by dividing the number of viable oocytes by the number of recovered oocytes. The blastocyst rate was calculated by dividing the number of produced embryos by the number of viable oocytes for each donor. The number of OPU sessions varied among donors, and thus, a total of 4,785 OPU records were retrieved. A raw average was calculated for each individual donor's number of (1) recovered oocytes, (2) viable oocytes, (3) embryos produced, (4) viability rate, and (5) blastocyst rate.

Anogenital distance measurement, blood sample collection, and AMH assay

Donor's management was approved by the Animal Care and Use Committee of the Federal University of Viçosa (Viçosa, MG, Brazil). A single morphometrical measurement of anogenital distance was collected from each of the 552 donors enrolled in the study from July to December 2021, regardless of the age of the donor or the number of oocyte aspirations. Briefly, each donor was restrained in a squeeze chute, allowing a technician to conduct the measurements. The anogenital distance was measured from the center of the anus to the base of the clitoris with a 200 mm stainless steel caliper

(Figure 1). Donors with apparently vulvar swelling or laceration and cows 30 days before or after calving were not enrolled in the study.

A subset of donors (n = 184) was randomly selected from all farms to have blood samples collected on the same day as the anogenital distance measurement. Samples were collected from the coccygeal blood vessels using EDTA evacuated tubes. Samples were placed on ice upon collection and centrifuged at 3,000 g for 15 min. Harvested plasma was placed into 1.5 mL Eppendorf and frozen at – 20°C (-4°F) until assayed. Plasma concentrations of AMH were determined using a commercial bovine AMH ELISA Kit (Ansh Labs, TX, USA).

Statistical analysis

Data were analyzed using SAS version 9.4 (SAS Institute Inc.). The numbers of recovered oocytes and AMH concentration were classified into two categories (High and Low) using the average value as a threshold point. The recovered oocyte rate was used as a category to represent the antral follicle count since the population of follicles in the ovaries is related to the number of recovered oocytes in the OPU section. The donors were also classified into two anogenital distance categories (Short and Long) using the average value as a threshold point. Then, the AMH concentration, recovered oocytes, viable oocytes, produced embryos, viability rate, blastocyst rate, and anogenital distance were compared between the Short and Long anogenital distance categories (Table 1A), High and Low AMH categories (Table 1B), and between High and Low oocyte categories (Table 1C) using GLIMMIX Procedure in SAS and considering the herd and OPU age in the model. A statistical difference was considered when $P < 0.05$, and a tendency for a statistical difference when $0.05 > P < 0.1$.

Results and Discussion

The relationship of anogenital distance with reproduction was initially studied in humans, mice, and, more recently, dairy cattle. Anogenital distance, defined as the distance from the center of the anus to the base of the clitoris, is determined by the androgen concentrations during the reproductive tract development of the fetus in prenatal life. For example, studies conducted in rats showed that female fetuses exposed to elevated androgen concentrations during prenatal life showed a longer anogenital distance and lower fertility than fetuses exposed to lower androgen concentrations. More recently, studies in cattle have shown that anogenital distance is also related to fertility. That is, heifers and cows with shorter anogenital distances have better fertility than those with longer anogenital distances (See Introduction). Thus, we aimed to characterize the association among anogenital distance, other fertility traits such as AMH concentrations, and the in vitro embryo production in *Bos indicus* dairy cattle of the Gyr breed.

Our current results failed to support the hypothesis that donors classified as short anogenital distance have greater AMH concentrations as well as a greater number of recovered oocytes and embryo production when compared to donors classified as long anogenital distance (Table 1A). For instance, Short anogenital distance donors had almost 1.2 mm shorter anogenital distance when compared to donors in the Long anogenital distance category, but no statistical differences were observed in AMH concentrations, number of recovered oocytes, viable oocytes, produced embryos, viability rate, and blastocyst rate between them. A limitation of our study was the use of oocyte and embryo data collected retrospectively and compared to the current anogen-

ital distance of the animal. Future studies are warranted to test the association between anogenital distance, AMH, and the number of follicles in dairy cattle.

Previous studies demonstrated that cows with long anogenital distances tended to have a greater AMH concentration compared to cows with short anogenital distances. However, we did not replicate such an association in our current study. Nonetheless, AMH concentration continues to seem to be a reliable phenotype for predicting oocyte and embryo quantity for in vitro embryo production (Table 1B). Donors in the High AMH category had almost 1.5 times greater number of recovered oocytes, viable oocytes, and produced embryos when compared to donors in the Low AMH category. Our results agree with the theory that AMH is produced by the growing follicles, which makes AMH highly related to the antral follicle population and a reliable trait to measure the ovarian reserve, the antral follicle count, and the total number of embryos. Donors in the High AMH category also tended to have a greater viability rate, but no statistical differences in blastocyst rate between the High vs. Low AMH category. Viability rate and blastocyst rate are linked to the oocyte quality, and their association with AMH is controversial, whereas the association of follicle and embryo quantity with AMH seems to be very consistent.

Donors in the High oocyte category had 1.5 times greater AMH concentrations than donors in the Low oocyte category (Table 1C). Similarly, donors in the High oocyte category produced two times the average number of recovered oocytes, viable oocytes, and embryos when compared to donors in the Low oocyte category. These results are associated with the ones discussed above and highlight a quantitative association of these traits. That is, donors with a greater number of oocytes and AMH concentrations produce more viable oocytes and embryos when compared to donors with a small number of recovered oocytes and lower AMH concentrations. Donors in the High oocyte category had a 7% greater viability rate, but no statistical differences in the blastocyst rate between the High vs. Low oocyte category. This is also expected since the number of recovered oocytes is not considered to be associated with the oocyte and embryo qualities.

Conclusions

Bos indicus dairy cattle Gyr breed donors with a higher concentration of AMH and a greater number of recovered oocytes per OPU presented, as expected, a greater number of viable oocytes and produced embryos compared to donors with low concentrations of AMH and a small number of recovered oocytes, respectively. However, our study failed to identify differences in the AMH concentration and in vitro embryo production results when comparing Short vs. Long anogenital distance donors. A limitation of our study was the use of oocyte and embryo data collected retrospectively and compared to the current anogenital distance of the animal. Thus, further studies should be performed to validate these results and to better understand the association between anogenital distance and in vitro embryo production.

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Table 1. Association among AMH concentration, recovered oocytes, anogenital distance, and in vitro embryo production results (Mean ± SEM) in Gyr breed oocyte donors classified into two AMH, two oocytes, and two anogenital distance categories

	(A) Anogenital distance category		P-value	(B) AMH category		P-value	(C) Oocyte category		P-value
Endpoint[1]	Short	Long		High	Low		High	Low	
n	276	276	-	80	104	-	223	278	-
AMH (ng/mL)[2]	0.45 ± 0.03	0.44 ± 0.03	0.72	0.69 ± 0.02	0.25 ± 0.01	< 0.01	0.52 ± 0.03	0.36 ± 0.03	< 0.01
Recovered oocytes	23.5 ± 1.7	24.4 ± 1.7	0.37	28.4 ± 1.7	21.1 ± 1.6	< 0.01	34.8 ± 0.6	16.2 ± 0.6	< 0.01
Viable oocytes[3]	16.8 ± 1.6	16.4 ± 1.6	0.59	20.4 ± 1.6	14.9 ± 1.6	< 0.01	25.7 ± 1.3	11.0 ± 1.3	< 0.01
Produced embryos	4.1 ± 0.4	3.9 ± 0.4	0.59	4.7 ± 0.3	3.6 ± 0.3	0.01	5.8 ± 0.3	2.8 ± 0.3	0.01
Viability rate (%)[4]	70 ± 3.6	71 ± 3.6	0.39	73.4 ± 4.0	70.7 ± 4.0	0.10	73.4 ± 3.7	68.6 ± 3.6	< 0.01
Blastocyst rate (%)	27.2 ± 0.0	26.8 ± 0.0	0.65	26.4 ± 2.2	25.0 ± 2.1	0.50	24.4 ± 2.7	26.2 ± 2.7	0.50
Anogenital distance	117.7 ± 0.6	139.8 ± 0.6	< 0.01	131.2 ± 0.0	129.0 ± 1.9	0.29	128.5 ± 1.6	128.7 ± 1.5	0.88

[1] AMH = Anti-Müllerian hormone; Viability rate = number of viable oocytes/number of recovered oocytes; blastocyst rate = number of blastocysts/number of viable oocytes.

[2] A subset of 168 donors randomly selected in the High (81 donors) and Low (87 donors) oocyte category, and a subset of 184 donors randomly selected in the Short (85 donors) and Long (99 donors) anogenital distance category.

[3]Subset of 501 donors evaluated in the Short (275 donors) and Long (244 donors) anogenital distance category.

[4]Subset of 501 donors evaluated in the Short (275 donors) and Long (244 donors) anogenital distance category.

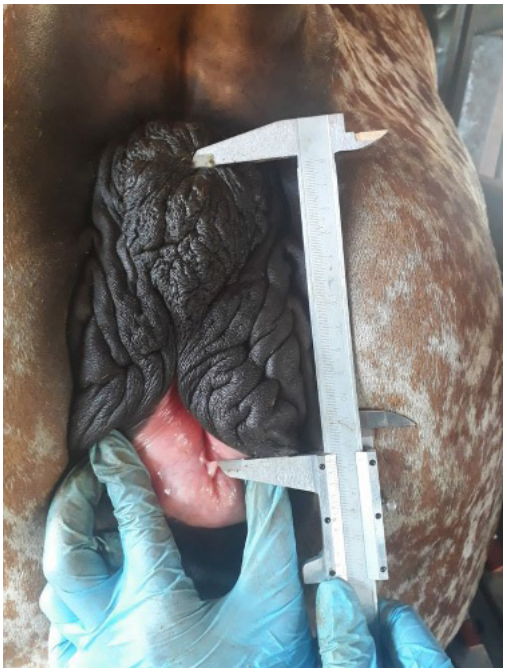

Figure 1. Positioning the caliper to measure the anogenital distance from the center of the anus to the base of the clitoris.