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Anti-Mullerian hormone (AMH) concentration in follicular fluid and mRNA expression of AMH receptor type II and LH receptor in granulosa cells as predictive markers of good buffalo (*Bubalus bubalis*) donors

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ABSTRACT

High individual variability in follicular recruitment and hence in the number of embryos produced is a major factor limiting the application of reproductive technologies in buffalo. Therefore, the identification of reliable markers to select embryo donors is critical to enroll buffaloes in embryo production programs. Better understanding of factors involved in follicular growth is also necessary to improve the response to superovulation in this species. The aim of this work was thus to determine the anti-Mullerian hormone (AMH) concentration in follicular fluid (FF) recovered from different size follicles and evaluate the mRNA expression profiles of development-related (*AMHR2*, *CYP19A1*, *FSHR*, and *LHR*) and apoptosis-related genes (*TP53INP1* and *CASP3*) in the corresponding granulosa cells (GCs) in buffalo. Another objective was to evaluate whether the AMH concentration in FF and gene expression of GCs is associated with the antral follicular count. Ovaries were collected at the slaughterhouse, and all follicles were counted and classified as small (3–5 mm), medium (5–8 mm), and large (>8 mm). Follicular fluid was recovered for AMH determination, and the mRNA expression of *AMHR2*, *FSHR*, *LHR*, *CYP19A1*, *TP53INP1*, and *CASP3* was analyzed in GCs. The AMH concentration in FF decreased ($P < 0.01$) at increasing follicular diameter. The mRNA expression of *AMHR2* and *FSHR* was higher ($P < 0.05$) in small follicles, whereas that of *LHR* and *CYP19A1* was higher ($P < 0.05$) in large follicles. The intrafollicular AMH concentration was positively correlated with the antral follicular count ($r = 0.31$; $P < 0.05$). Interestingly, good donors (≥12 follicles) had a higher ($P < 0.05$) concentration of AMH and *AMHR2* levels in small follicles and higher ($P < 0.05$) *LHR* levels in large follicles than bad donors (<12 follicles). These results suggest a potential use of AMH to select buffalo donors to enroll in embryo production programs, laying the basis for further investigations.

1. Introduction

The profitability of buffalo (*Bubalus bubalis*) breeding greatly depends on genetic improvement and hence on the utilization of reproductive biotechnologies. Since the first

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buffalo calf produced by the transfer of *in vivo* produced embryos [1], the attention has been focused for over 3 decades on the improvement of superovulation protocols and embryo recovery. Nevertheless, the application of multiple ovulation and embryo transfer still leads to poor embryo yield compared with cattle [2–4]. As a consequence, ovum pickup and *in vitro* embryo production is the most efficient tool to enhance the maternal contribution to genetic improvement in this species, allowing greater production of embryos on a long-term basis [5,6].

One of the main factors limiting the application of both technologies in buffalo is the high between-animal variability in the number of embryos produced [6,7]. Moreover, the intrinsic species-specific lower number of primordial [8] and antral follicles [9,10] compared to cattle accentuates the problem of the high variability in follicular recruitment in this species. It was recently found that follicular recruitment is predetermined within each individual, allowing desirable and undesirable donors [6,11] to be distinguished, as in cattle [12]. Therefore, in addition to the screening of the antral follicular count (AFC), identification of a reliable marker to select good embryo donors is fundamental before enrolling buffaloes in embryo production programs, to offset laboratory costs [6].

Very recently, attention has focused on the anti-Mullerian hormone (AMH), a molecular marker of the ovarian follicular pool and follicular responsiveness to superovulatory treatments in women, cattle, goats, and other species [13–15]. The AMH is a 140-kDa glycoprotein that belongs to the transforming growth factor-β family and is expressed only in the gonads [16]. In cattle, as in other mammalian species, AMH is specifically expressed by ovarian granulosa cells (GCs) [17,18]; its expression is highest in GCs of preantral and small antral follicles and decreases during terminal follicular growth [19], with low AMH concentrations found in the follicular fluid (FF) of large antral and preovulatory bovine follicles [20]. It is also known that AMH expression in GCs and AMH concentration in FF are both low in atretic follicles [21].

Anti-Mullerian hormone is known to play a critical role in folliculogenesis by modulating the follicle-stimulating hormone (FSH) function and thereby limiting follicular recruitment [22,23]. In addition, AMH is a key mediator in regulating steroidogenesis, as it inhibits estradiol secretion by reducing the expression of the aromatase enzyme CYP19 [24] and progesterone production in GCs *in vitro* [25]. Furthermore, AMH is negatively associated with the expression of luteinizing hormone (LH) receptors in small human antral follicles [25]. The pattern of expression in the GCs of growing follicles and the role in regulating folliculogenesis and steroidogenesis make AMH an ideal marker for the size of the ovarian follicle pool. Moreover, AMH has been shown to be a reliable endocrine marker of oocyte and embryo quality in humans [26,27].

The only work carried out so far in buffalo reported a correlation between plasma AMH concentration and AFC in Murrah heifers, suggesting that AMH is a potential marker of follicular reservoir in this species [28]. However, many aspects still need to be investigated to clarify the role of AMH in influencing follicular growth and oocyte quality in buffalo. Therefore, the aim of our study was to characterize the population of AMH-producing antral follicles in buffalo ovaries by determining AMH concentration in FF recovered from different size follicles and evaluating the mRNA expression profiles of four follicular development-related genes (*AMHR2*, *CYP19A1*, *FSHR*, and *LHR*) in the corresponding GCs. Another objective was to evaluate whether AMH concentration in FF and gene expression of GCs are associated with the AFC. Furthermore, we compared reproductive parameters, including AMH concentration and gene expression, in good versus bad donors ($\geq$12 and $<$12 follicles, respectively). Finally, apoptosis-related genes, such as tumor protein p53-inducible nuclear protein 1 (*TP53INP1*), and caspase 3 (*CASP3*) were assessed in GCs from good and bad donors.

2. Materials and methods

All chemicals and reagents, if not otherwise stated, were purchased from Sigma (Sigma–Aldrich, Milan, Italy).

2.1. Experimental design

The study was carried out in Southern Italy (latitude 40.5°–41.5° N and longitude 13.5–15.5) during autumn, that is, the breeding season, on 54 multiparous Italian Mediterranean Buffalo cows, with a mean weight and age of 565.3 ± 14.5 kg and 5.3 ± 0.3 years, respectively, over four replicates. To induce luteolysis and synchronize the occurrence of estrus, buffaloes received a double administration of a PGF2α analogue (cloprostenol, 0.250 mg/mL Schering-Plough Animal Health, Milan, Italy), spaced 11 days apart and were slaughtered at a local authorized abattoir 6 days after the second administration, that is, after the emergence of the first follicular wave but before development of a dominant follicle [20,29]. The Ethical Animal Care and Use Committee of the University of Naples Federico II (Naples, Italy) approved the experimental design and animal treatments.

On all animals AFC, recovery rate and oocyte quality were recorded, and FF was collected for AMH determination. In a representative number (n = 22), the mRNA expression of six genes (*AMHR2*, *CYP19A1*, *FSHR*, *LHR*, *TP53INP1*, and *CASP3*) was analyzed in GCs. Finally, buffaloes were classified according to the AFC as good ($\geq$12 follicles) and bad ($<$12 follicles) donors, to evaluate the differences in reproductive parameters. The criterion used to classify the donors was chosen on the basis of our previous experience on abattoir-derived ovaries during the favorable season.

2.2. Collection of FF and GCs

At slaughter, ovaries were recovered individually and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30 °C to 35 °C. All the ovaries were weighed and measured: ovarian size was assessed by measurement of two perpendicular diameters of each ovary with a millimeter scale. For each antral follicle, antrum size was estimated by measurement of two perpendicular diameters with a millimeter scale. Thus, all follicles with an antrum larger than 3 mm were counted (AFC) and allocated to three size classes: small (3–5 mm),