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Evaluation of factors involved in the failure of ovum capture in superovulated buffaloes

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ABSTRACT

The aim of this work was to evaluate factors affecting ovum capture in superovulated buffaloes, by comparing the morphological features of pre-ovulatory follicles and oocytes, the intrafollicular and plasmatic steroid profile, as well as the expression of genes involved in cumulus expansion and steroid cascade in granulosa cells (GCs) and that of genes involved in contraction-relaxation of the oviduct between superovulated and synchronized buffaloes. Italian Mediterranean Buffalo cows were either synchronized by Ovsynch ($n = 25$) and superovulated ($n = 10$) with conventional FSH protocol and sacrificed 18 h after last GnRH. Antral follicular count, recovery rate and oocyte quality were recorded, and plasma and follicular fluid were collected for steroid profile determination. In addition, in 10 animals (5/group), GCs were collected to analyse the mRNA expression of gonadotropin receptors (LHR and FSHR) and genes involved in steroid synthesis, as the cytochrome P450 family 19 (CYP19A1) and the steroidogenic acute regulatory protein (STAR). Moreover, oviducts were collected to evaluate the mRNA expression of estrogen receptor 1 (ER1) and the progesterone receptor (PGR), the vascular endothelial growth factor (VEGF) and the VEGF receptors, i.e. the kinase insert domain receptor (FLK1) and the fms related tyrosine kinase 1 (FLT1). No differences were recorded in steroids plasma concentration between synchronized and superovulated animals whereas intrafollicular E_2 and P_4 concentrations decreased in superovulated group (63.2 ± 10.6 vs 30.3 ± 5.9 ng/mL of E_2 and 130.1 ± 19.8 vs 71.6 ± 8.5 ng/mL of P_4, respectively in synchronized and superovulated animals; $P < 0.05$). Interestingly, both the recovery rate (85.7% vs 56.6%, respectively in synchronized and in superovulated animals; $P < 0.05$) and the percentage of oocytes exhibiting proper cumulus expansion (75% vs 28.1%, respectively in synchronized and in superovulated animals; $P < 0.01$) decreased in superovulated animals. In addition, the expression of *FSHR* and *CYP19A1* increased while the expression of *STAR* in GCs decreased ($P < 0.05$). Finally, in superovulated buffaloes a decreased expression of *PGR*, *ER1*, *VEGF* and its receptor *FLK1* in the oviduct was observed. The results suggest that the exogenous FSH treatment impairs steroidogenesis, affecting both the oviduct and the ovarian function, accounting for the failure of ovum capture in superovulated buffaloes.

1. Introduction

Buffalo cows represent one of the pivotal species ensuring energy and protein in developing countries [1,2]. Reproductive biotechnologies play a critical role in accelerating genetic merit and allowing herd replacement. Several attempts have been carried out in the last years to improve the efficiency of reproductive biotechnologies in buffalo species [3,4]. Despite an efficient response to the synchronization programs and artificial insemination [5,6], further studies are needed to improve embryo production, either in vivo by multiple ovulation and embryo transfer (MOET) and in vitro. In particular, the poor number of embryos recovered per donor is undoubtedly the major constraint of MOET in buffalo. Different studies [7–9] demonstrated that, despite a very low embryo/CL recovery rate (13–35%), superovulation results in the growth of a high number of ovulatory follicles, together with a

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relatively high ovulation rate (50–70%). Therefore, a limited capacity for follicular growth and ovulation, arising from the reduced number of primordial follicles typical of the species [10], cannot account for the poor embryo outcome recorded in superstimulated buffaloes. In superovulated buffaloes sacrificed at different days after AI the ratio of ova recovered in relation to the CLs after flushing of both oviducts and uteri was still low [11]. As a consequence, the most likely hypothesis is that in superovulated buffaloes a failure in the ovum capture by the oviduct fimbria occurs.

A critical point could be the binding of the newly ovulated cumulus-oocyte-complexes (COC) to the ciliary tips of the epithelial cells lining the distal region of the oviduct. The degree of adhesion controls the pick-up and subsequent transport and compaction of the complex that allows its passage into the oviduct lumen, where it will contact the sperm that are swimming up the oviduct. Interestingly, a lower adhesion of cumulus cells to the oocyte, as well as a reduced expansion of cumulus after maturation are observed in buffalo [12]. A reduced extracellular matrix resulting from inappropriate cumulus expansion may lead to a lower cohesion between the oviduct cilia and COCs and to a higher difficulty for COCs to move into the infundibulum, as previously demonstrated in in vitro studies [13]. It was speculated that the imbalance between the levels of steroid hormones may impair the interaction between the ovum and the ciliates cells of endosalpinge during the ovulations in superovulated buffaloes [14]. Another factor involved into the regulation of oviductal contraction-relaxation and gamete/embryo transport is the active oviductal VEGF system during the peri-ovulatory period [15].

The aim of our study was to evaluate at different levels the various factors involved in the failure of ovum capture in superovulated buffaloes. In particular, we compared the morphological parameters of the follicles and the steroid profile (E_2 and P_4) both in plasma and follicular fluid (FF) in the peri-ovulatory period in superovulated vs synchronized buffaloes. Furthermore, to investigate whether the failure is linked to lack of capture, the oocyte morphology and expansion, as well as the mRNA expression of factors involved in cumulus expansion (*FSHR* and *LHR*) and into the steroid cascade (*CYP19A1* and *STAR*) in granulosa cells (GCs) were evaluated. In addition, the mRNA expression of factors playing a role on contraction-relaxation of the oviduct like *ER1*, *PGR*, *VEGF* and its receptors *FLK1* and *FLT1* was analysed.

2. Materials and methods

2.1. Animals and treatments

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments. The study was carried out in Southern Italy (latitude 40.5°–41.5° N and longitude 13.5–15.5) during autumn, i.e. the breeding season, on 35 multiparous Italian Mediterranean Buffalo cows with a mean weight and age of 556.7 ± 13.8 kg and 5.6 ± 0.3 years, over four replicates. Animals were randomly divided into two groups: a group (n = 25) was synchronized by Ovsynch [5] in the presence of a progesterone releasing intravaginal device (PRID®, Ceva Vetem SpA, Milan, Italy) from day 0 to day 7 and another group (n = 10) was superovulated as described below. Only synchronized animals that ovulated after the first GnRH in the Ovsynch protocol (n = 14) were chosen as a control group [16] and slaughtered 18 h after the last GnRH, just before ovulation. With regard to superovulation group, animals received a double administration of $PGF_{2\alpha}$ analogue (Cloprostenol®, 0.250 mg/mL Schering-Plough Animal Health, Milan, Italy) spaced 11 days apart, ovulation was induced by GnRH analogue (buserelin acetate, 12 μg; Receptal®; Intervet, Milan, Italy) 48 h after the last dose of $PGF_{2\alpha}$. The day after, ovulation was assessed and PRID® was inserted. Superovulation treatment, consisted in 400 mg FSH (Folltropin®, Bio98, Milan, Italy) given in decreasing dosages for 4 consecutive days started on day 9 of the cycle. Briefly, the scheme was: 80 mg on day 9, 60 mg on day 10, 40 mg on day 11 and 20 mg on day 12, given twice daily. On day 11 (72 and 84 h after initiating gonadotrophin treatment) buffaloes were administered 0.524 mg PGF_{2a} twice (12 h apart) for inducing luteolysis and PRID® was removed. On day 13 animals received a GnRH analogue to induce ovulation and on day 14, precisely at 18 h after GnRH, were sacrificed at a local abattoir. At slaughter, blood samples were taken for hormone analyses in both groups. All blood samples were separated using centrifugation at 4 °C, 800 × g for 10 min, and stored at −20 °C until the analysis. Moreover, ovaries and oviducts of animals of both groups were recovered individually and transported to the laboratory in physiological saline supplemented with 150 mg/L kanamycin at 30–35 °C.

2.2. Collection of follicular fluid and granulosa cells

At the laboratory, the presumptive dominant follicles (diameter > 0.7 cm) in both synchronized and superovulated animals were measured: follicle size was assessed by measurement of the two perpendicular diameters with a millimeter scale. The FF of the dominant follicle was aspirated by a syringe with a 21-gauge needle and poured into a petri dish for a quick search of the COC. Then, the COC-free FF was recovered in a vial and centrifuged at 600 × g for 10 min to separate the FF and the GCs. After centrifugation, the FF recovered was snap frozen and stored at - 80 °C until hormonal assay determination, while the GCs-containing pellet was previously washed with PBS +0.1% polyvinyl alcohol (PVA), centrifuged again at 600 × g for 10 min, then snap frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

2.3. Evaluation of oocyte quality and maturation status

The FF recovered from the dominant follicles of synchronized and superovulated buffaloes was inspected under a microscope for the presence or absence of the COC. For each run, the recovery rate, i.e. the percentage of COCs in relation to the aspirated follicles, and the percentage of oocytes showing an appropriate cumulus expansion were recorded. The COCs recovered were scored for quality on the basis of morphological features according to our classification [17]. Oocytes were stripped of the cumulus cells by gentle pipetting, allocated on a clean slide and fixed in absolute ethanol overnight. Then oocytes were stained with 4',6-diamidino-2-phenylindole (DAPI, Vectashield® by Vector Laboratories, Inc., Burlingame, CA, USA) to evaluate the nuclear status using a Nikon Diaphot 300 inverted microscope equipped with fluorescence filters.

2.4. Collection of oviduct samples

At the laboratory, both oviducts of each synchronized and superovulated animal were trimmed free of tissue. According to anatomy, the oviduct was divided into four parts: fimbriae, infundibulum, ampulla and isthmus. Tissues from fimbriae were snap frozen into liquid nitrogen and stored at −80 °C until RNA isolation. The infundibulum, ampulla and isthmus of both oviducts were placed into a Petri dish, washed three times with physiological saline and then opened longitudinally. Once opened they were gently scraped with a blade and flushed with 500 μL PBS + PVA (0.1%), to recover epithelial cells. Cells were centrifuged twice at 600 × g for 10 min, PBS was removed and the pellet was snap frozen and stored at −80 °C until RNA isolation.