Advances in Embryo Production Technologies in Buffalo

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ABSTRACT

Reproductive performance in buffalo herds has a significant impact on production and profitability. The use of assisted reproductive technologies such as superovulation (SOV) and *in vitro* embryo production (IVEP) has increased rapidly in recent years and is now applied worldwide for genetic improvement in beef and dairy buffaloes. MOET technique has been shown to be feasible in buffalo, however presents low efficiency and limited commercial application has been documented. These results could be associated with low ovarian follicular pools, high levels of follicular atresia and failures of the oocyte to enter the oviduct after superstimulation of follicular growth. Among current reproductive technologies, IVEP is an important tool for multiplying genetic material of superior merit. Promising results have been achieved using this technology. Furthermore, the control of the emergence of follicular waves and of ovulation at predetermined times, without the need for estrus detection in buffalo recipients, has facilitated the management and improved the efficiency of embryo transfer (ET) programs in buffalo herds. Conclusively, due the scarce results of *in vivo* embryo recovery in superovulated buffaloes, the association of ovum pick-up (OPU) with IVEP represents an alternative method of exploiting genetics and reproductive performance of buffaloes.

Keywords: animal reproduction; buffaloes; *in vitro* embryo production; superovulation.

INTRODUCTION

The combined use of timed-artificial insemination (TAI), SOV, OPU, IVEP and timed-embryo transfer (TET) has a great potential to improve reproductive outcomes and disseminate selected genetics, diminishing the interval of generations and improving buffalo herds genetic gain.

The use of SOV followed by TAI is a technique that generates greater numbers of embryos per donor in cattle (Mapletoft et al., 2002). These techniques, which are associated with ET to recipients, are powerful tools that facilitate the dissemination of genetic material of high quality (Baruselli et al., 2011, Bó et al., 2002). However, buffalo donors generally have lower embryo recovery rates than bovines. While buffaloes have shown follicular responses after superovulation treatment (mean of 15 follicles > 8 mm), only a moderate ovulation rate (approximately 60%) and CL yield at the time of flushing (approximately 9 CL) and low embryo recovery rates (34.8%) have been obtained (Baruselli et al., 2000). The embryo recovery rate in buffaloes (approximately 20 to 40%) is lower than in bovines (63 to 80%; Boland et al., 1991). This divergence in embryo recovery rates was hypothesized to be related to a failure of oocyte capture and/or of oocyte transport along the oviduct (Baruselli et al., 2000).

OPU associated with IVEP is another interesting technology for embryo production in selected donors (Boni et al., 1996, Neglia et al., 2003, Sá Filho et al., 2009). This technology has the potential to enhance genetic progression through the female lineage in buffaloes. The success of OPU–IVEP is directly related to oocyte quantity and quality. However, the use of this technology in buffaloes has been limited by the innately low number of follicles and hence of cumulus–oocytes complexes (COCs) that can be recovered per ovary

(Gasparrini, 2002, Gimenes et al., 2010), as well as by the seasonality of follicle production (Di Francesco et al., 2011).

The knowledge of the particularities of buffalo ovarian follicular growth manipulation, follicular population predictors, and metabolic and environmental aspects that interfere with ovarian environment and, consequently, oocyte quantity and quality is crucial to optimize the ET programs. This review aims to elucidate some factors that affect embryo production technologies and must be well known in order to improve the efficiency of this biotechnology in buffalo.

ADVANCES IN SUPEROVULATION (SOV)

Numerous studies were performed around the world with the aim to evaluate the efficiency of SOV and ET in buffalo species (Baruselli et al., 1994b, Campanile et al., 2010, Carvalho et al., 2002, Oba et al., 1994). Although births of buffaloes achieved using SOV and ET have been reported in several countries (Baruselli, 1994, Baruselli et al., 1994a, Drost et al., 1983, Misra et al., 1990, Zicarelli, 1992), the use of this technique still has limitations, mainly related to the low embryo recovery rate (Ambrose et al., 1991, Baruselli et al., 2000, Carvalho et al., 2002, Madan et al., 1996, Misra et al., 1990, Taneja et al., 1995a, Zicarelli et al., 1994a). Currently, superovulated buffaloes produce on average 1-3 viable embryos for harvest (Carvalho et al., 2002, Misra and Tyagi, 2007, Neglia et al., 2010). This average remains lower than the mean number of recovered embryos in the bovine [10 total and 6 transferable embryos (Boland et al., 1991)].

The advances provided by the SOV technique have revealed that buffaloes have satisfactory responses to superovulatory treatment, although embryo recovery in buffaloes is less efficient than in cows (Baruselli et al., 2000). A low number of embryos recovered in buffaloes has also been described by several authors (Karainov, 1986; Madan, 1990; Drost, 1996; Zicarelli, 1997). According to Baruselli et al. (2000), only 34.8% of buffalo ovulations obtained through superstimulation of follicular growth resulted in recovered embryonic structures, a percentage much lower than that found in bovines by Adams (1994) who recorded rates of 63% to 80%. This disparity between embryo recovery rates may be related to failures in the collection and/or transportation of oocytes in the oviduct.

According to Hunter (1988), the mechanisms involved in oocyte transportation (ciliary beats of the epithelium of the oviduct and waves of contraction of the myosalpinx) are controlled by ovarian steroids. The low number of embryos obtained in buffalo SOV could be attributed to high estrogen (E2) levels during the superovulation treatment, as postulated by Misra et al. (1998). Prolonged exposure to elevated concentrations of 17β-estradiol may change the intrauterine and/or oviductal environment and, consequently, impair normal embryonic development and transport. It is also possible that buffalo are more sensitive to high 17β-estradiol levels during superstimulation treatments than bovines (Beg et al., 1997). To test this hypothesis, our group conducted experiments to reduce estradiol levels during superstimulation treatments (Baruselli et al., 2002, Carvalho et al., 2002) using exogenous sources of progesterone during preovulatory periods or deslorelin bioimplants during superovulation. However, no increase in embryo recovery rate was observed.

To further test this hypothesis, our group performed a sequence of trials to investigate in detail the anatomical and physiological inter-relationships between ovarian steroids and the genital system of buffaloes and bovines (Carvalho et al., 2011, Carvalho et al., 2012). We studied morphometric characteristics of females with single or multiple ovulations and we compared the direction of ciliar movements in oviducts exposed or not to E2 in the culture medium. We observed no effect of E2 on embryo transport or on ciliar movement of oviducts. However, we found that buffaloes have a higher number of anovulatory follicles, a more rigid ovary-mesovarium connection, and a thicker infundibulum muscle layer than bovine females. These factors could be partially responsible for the low embryo recovery rates in buffaloes.

As previously mentioned, low oocyte quality in buffaloes may be associated with a fragile connection between the oocyte and granulosa cells, in contrast to what occurs in

bovine species (Gasparrini, 2002). Some studies infer that rbST can enhance this connection by a direct and/or indirect effect of IGF-1 and increase the population of small antral follicles (Lucy, 2000, Pavlok et al., 1996). Additionally, rbST can stimulate the expansion of cumulus cells (Izadyar et al., 1998a, Izadyar et al., 1998b), contributing to oocyte adhesion to fimbria and ciliated cells of the endosalpinx, which can improve embryo recovery in superstimulated animals. To confirm this hypothesis, studies were performed to investigate the effect of different doses of rbST (0, 250, or 500 mg) on embryo recovery in superovulated buffaloes (Baruselli et al., 2003, Carvalho et al., 2007). The results were mostly inconclusive; in the first study, 500 mg of rbST increased embryo recovery (50.0 vs. 33.3%; P=0.06) and the number of structures recovered (5.1±6.8 vs. 1.6±1.7; P=0.18); however, in the second experiment, none of the doses of rBST used had any impact on the efficiency of SOV in buffalo.

In rabbits, the administration of sequential doses of $PGF_{2\alpha}$ during the periovulatory period stimulated the contraction of oviduct smooth muscles, allowing the activation of the oviduct fimbriae to capture the oocytes (Osada et al., 1999). Based on this observation, our research group recently (Soares, 2015) performed an experiment that evaluated the use of $PGF_{2\alpha}$ (injectable or using a mini osmotic pump) during the periovulatory period in superovulated buffaloes. However, no differences were found on the total number of recovered structures (G-CONT=2.1±0.8 vs. GPGF-INJ=2.1±0.6 vs. G-PGF-OP=1.4±0.4; P=0.58).

Another recent study evaluated the effect of follicular growth superstimulation (FGS) anticipation on follicular and superovulatory responses, and on embryonic structures recovery rate of superovulated buffaloes. For this, buffaloes were submitted to initiate the FGS on D3 or D4 of the superstimulation protocol. The authors verified that the FGS anticipation did not increase the follicular and superovulatory responses, and the embryonic structures recovery rate of superovulated buffaloes (Soares et al., 2016 in press).

Generally, the reason for the low embryo recovery rate in superovulated buffaloes remains unknown, compromising the efficiency and the application of SOV technology in this species. Further studies are needed to enable the use of SOV in buffalo, to allow this technique to be widely used by farmers and to accelerate genetic gain and productivity of buffalo herds.

ADVANCES IN OVUM PICK-UP (OPU) AND *IN VITRO* EMBRYO PRODUCTION (IVEP)

Due the variable results of *in vivo* embryo recovery in superovulated buffaloes, the association of OPU with IVEP represents an alternative method of exploiting and multiplying genetic material of superior merit. Historically, OPU-IVEP in buffaloes produced lower outcomes (Gasparrini, 2002, Gimenes et al., 2010, Sá Filho et al., 2009) than in bovines (Lonergan and Fair, 2008, Pontes et al., 2011). However, a series of recent studies have demonstrated the commercial potential of these techniques in the buffalo species.

Two main biological problems seem to be related to the inefficiency of the OPU-IVEP technique in buffaloes: low number of follicles on the ovary, low oocyte recovery per OPU, and poor oocyte quality (only 27.3 to 31.3 % of oocytes are classified as viable (Campanile et al., 2003).

The first problem can be related to the lower number of follicles recruited per follicular wave (Baruselli et al., 1997), as observed in studies comparing buffaloes with *Bos indicus* cattle (Gimenes et al., 2015, Ohashi et al., 1998). Additionally, a higher level of follicular atresia was reported (Danell, 1987, Van Ty et al., 1989) and, consequently, a lower number of total recoverable and viable oocytes. Buffaloes and cattle raised with contemporary nutrition and management were compared *post mortem* by Ohashi et al. (1998), and *in vivo* by Gimenes et al. (2015). In both studies, lower number of follicles and viable oocytes were observed in buffaloes than in *Bos indicus* cattle.

The second problem with the OPU-IVP technique in buffaloes can be attributed to a more fragile *zona pellucida* (Mondadori et al., 2010) and a more fragile bonding between

cumulus cells and the oocyte (Gasparrini, 2002, Ohashi et al., 1998) in buffaloes than in cattle.

To improve oocyte quality and recovery, studies were conducted by our research group to upgrade this biotechnology in buffaloes. Initially, we tested the hypothesis that bST could elevate circulating IGF-1 levels, promoting recruitment of a greater number of follicles and enhancing oocyte quality (Sá Filho et al., 2009). Although bST treatment resulted in greater numbers of aspirated follicles and retrieved oocytes per donor per session, was observed reduced blastocyst formation rate for animals submitted to this treatment (Ferraz et al., 2007, Ferraz et al., 2015, Sá Filho et al., 2009).

An important factor that is known to directly influence the quantity and quality of oocytes obtained by OPU and, consequently, IVP, is the phase of the estrous cycle (Vassena et al., 2003). Oocytes from follicles with a mild degree of atresia have higher rates of embryonic development and require less time for *in vitro* maturation (Vassena et al., 2003). In another study performed by our research group, buffalo, Nelore (*Bos indicus*) and Holstein (*Bos taurus*) heifers were synchronized to be submitted to OPU 1, 3 or 5 days after the follicular growth wave emergence. The results showed that the synchronization to OPU did not affect the IVEP in any of the genetic groups and that the OPU-IVEP procedure is less efficient in buffalo and Holstein than in Nelore heifers (Gimenes et al., 2015).

The influence of season (winter or summer) on oocyte viability (number of viable oocytes and mitochondrial DNA amount) was investigated in nulliparous (n = 8) and multiparous (n = 8) buffaloes (Macabelli et al., 2012). All animals had synchronized follicular wave emergences by hormonal treatment (P4 + E2) before OPU, performed five days after the beginning of the protocol. As expected, cutaneous and rectal temperatures and respiratory frequency were higher in summer than in winter (P> 0.05). It was observed that the number of viable oocytes was greater in nulliparous than in multiparous animals (13.4±2.2 vs. 6.3±0.8) and the percentage of viable oocytes was lower in summer than in winter (55.5±3.6 vs. 64.4±2.6). The amount of mtDNA was decreased in oocytes from nulliparous during summer than winter and increased in oocytes from multiparous during summer than winter. During summer, the amount of mtDNA was lower in oocytes from nulliparous than those from multiparous, but during winter mtDNA amount was greater in oocytes from nulliparous than those from multiparous. The mtDNA analyses do not suggest a negative effect of summer on oocyte viability in buffalo.

Therefore, in tropical climates, the season would not appear to adversely affect oocyte quality and fertility. However, more studies need to be carried out on the oocyte competence during different seasons to confirm these findings.

Commercial applications of OPU-IVEP and TET

Pioneering studies, developed in partnership between Brazilian private IVEP-laboratory (Cenatte Embriões LTDA) and universities (UFMG, USP and UNINA) proved that the production of *in vitro* buffalo embryos for commercial purposes was possible (Saliba et al., 2013). Viable oocytes (grade 1, 2 and 3) were subjected to IVEP procedures, resulting in a blastocyst production rate of 44.9% (262/584). A portion of the embryos produced was transferred fresh or vitrified, resulting in pregnancy rates 30 days later of 43.5% (50/115) and 37.1% (26/70), respectively. Embryonic mortality 60 days after embryo transfer was 4% (2/50) and 5.7% (4/70), respectively. The results obtained in these studies were higher than those previously described in the literature and demonstrate the potential of OPU-IVEP in buffaloes.

Sequentially, we recently evaluated the efficiency of OPU-IVEP-TET technology in buffalo donors (Experiment 1) and recipients (Experiment 2). These studies were conducted in partnership between the University of São Paulo – USP, the research center Unidade de Pesquisa e Desenvolvimento de Registro - UPD/APTA, the Brazilian private IVEP-laboratory - InVitro Brasil S/A, and the private farm - Paineiras da Ingaí.

The aim of the Experiment 1 was to evaluate the number of aspirated oocytes, viable oocytes, and in vitro embryo production in different categories of buffalo donors submitted to OPU. Buffalo donors were randomly assigned to one of three groups: nulliparous (n = 49); primiparous (n = 61) and multiparous (n = 207). All buffaloes underwent a regular transvaginal ultrasound-guided ovum pick-up (OPU) for oocyte recovery at random stage of the estrous cycle. There were no significant differences between nulliparous, primiparous and multiparous buffaloes for aspirated oocytes (11.7±0.4, 12.8±0.5 and 9.7±0.3; P = 0.10), viable oocytes (4.9±0.1, 4.8±0.1 and 4.8 ±0.1; P = 0.95) and blastocysts produced per OPU (1.5±0.1, 1.5±0.1 and 1.6±0.1; P = 0.98). These variables were also analyzed in the pregnant (n = 52) and non-pregnant (n = 139) multiparous buffaloes donors at moment of OPU. Although the aspirated oocytes number (8.8±0.2 and 11.3±0.3; P = 0.003) and the viable oocytes (4.6±0.1 and 5.7±0.1; P = 0.02) were lower in pregnant than in non-pregnant animals, blastocysts produced were similar (1.8±0.1 and 1.7±0.1; P = 0.06).

In Experiment 2, we aimed to evaluate the effect of animal category (nulliparous, primiparous or multiparous) on timed embryo transfer (TET) protocol efficiency in recipients. 125 buffalo were distributed according to the category into one of three groups: nulliparous (n=42), primiparous (n=27) and multiparous (n=56). At random stage of the estrous cycle (D0), all animals received an intravaginal P4 device (1g) plus EB (2.0 mg im estradiol benzoate). On D9, the P4 device was removed and buffaloes were given $PGF_{2\alpha}$ (530 µg im sodium cloprostenol) plus eCG (400 IU im). On D11, all buffaloes received GnRH (20 µg im). On D17, the females were evaluated for the presence of CL and only ones with CL were submitted to TET. No differences were verified between categories (nulliparous vs primiparous vs multiparous) regarding the diameter of the largest follicle on D11 (10.5±0.3 *vs.*11.1±0.5 *vs.*11.5±0.4 mm; P=0.25) and on D17 (10.5±0.4 *vs.*12.0±1.1 *vs.*11.7±0.6 mm; P=0.30), the CL diameter (16.1±0.3 *vs.* 15.4±0.9 *vs.*16.6±0.4 mm; P=0.24), the conception (38.5 *vs.* 40.0 *vs.* 27.5 %; P=0.56) and the pregnancy rates (23.8 *vs.*14.8 *vs.*19.6 %; P=0.65). However, the ovulation rate was lower in primiparous ($42.3^{\%}$) than in multiparous (71.4^{a} %; P=0.001). The results obtained in the present study allow concluding that is possible to use the TET in the different categories (nulliparous, primiparous and multiparous) of recipients.

Additionally, a field trial with 295 buffalo recipients was conducted to evaluate the pregnancy per TET in six farms. The pregnancy rate was similar between the farms (20.3, 20.8, 25.0, 28.0 33.3 and 50.0%; P=0.20). In average, the pregnancy rate after TET was 27.8%. Regarding the type of embryo transferred, the conception rate was similar between fresh and frozen embryos (26.1 and 31.9%; P = 0.78).

Influence of Anti-Müllerian Hormone on SOV and OPU/IVEP

The number of antral follicles in the early follicular phase directly correlates with ovarian reserve (Frattarelli et al., 2000). Indeed, the antral follicular population (AFP) directly represents the follicle cohort in the ovaries, which is associated with the number of oocytes retrieved per OPU for IVEP.

A large variability of AFP is reported among different cows, however AFP count is highly repeatable within animal (Burns et al., 2005; Ireland et al., 2007), and anti-Müllerian hormone (AMH) can be considered a reliable endocrine marker of ovarian reserve (Ireland et al., 2007, 2008; Monniaux et al., 2012). AMH is a dimeric glycoprotein member of the TGFβ superfamily of growth factors synthesized from granulosa cells of preantral and small antral follicles (growing follicles up to the antral stage or to a diameter of approximately 6 mm) and represents the indirect activity of the follicular pool (Cate et al., 1986; Durlinger et al., 1999; Grootegoed et al., 1994; Weenen et al., 2004). In cattle, circulating AMH concentration can help veterinarians to predict AFP in ovaries (Batista et al., 2014; Ireland et al., 2008; Rico et al., 2009), response to SOV treatments (Monniaux et al., 2010a, 2010b; Rico et al., 2009; Souza et al., 2015), and more recently as a marker to predict IVEP performance of *Bos taurus* (Gamarra et al., 2015; Guerreiro et al., 2014; Vernunft et al., 2015) and *Bos indicus* breeds (Guerreiro et al., 2014).

Aiming to determine the relation between AMH and AFP in different genetic groups, our group recently conducted a study (Baldrighi et al., 2014). Despite the high variability in AFP between individuals within each genetic group, the AFP count was greater in Gir (*Bos indicus*) than in Holstein (*Bos taurus*) and Murrah (*Bubalus bubalis*) heifers (P=0.01). Similarly, AMH concentration was lower (P<0.01) for Holstein and Murrah heifers than for Gir heifers. Despite the differences between genetic groups, a positive relationship between AFP and AMH concentration was detected within buffalo. The studies performed with Holstein and Nelore, suggest a marker to predict AFP and IVEP performance.

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