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Research Article

Gender Preselection in the Buffalo Species (*Bubalus Bubalis*): Preliminary Data on Different Strategies and Pregnancy Outcome

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Abstract

In this study, a new comparison was tested in Mediterranean Italian buffaloes (*Bubalus bubalis*), between two different strategies for obtaining sperm cells of the desired sex. In addition, a control group of animals was included in the study, where only ordinary semen straws, containing unselected sper cells were used. Sexed and control straws were then used for Artificial Insemination (AI) in order to confront pregnancy and gender rates. The first of the two strategies to preselect sperm cells relies on the physical determination of DNA content in single spermatozoa though cell sorting machines. The second strategy instead, relies on the coincubation of either fresh or frozen/thawed semen with a specific solution specifically designed for the species of interest. The trial involved a total of 206 heifers and 211 pluriparous buffaloes belonging to a buffalo farm in the south of the Lazio Region in Italy. In this study, no significant differences between treated and control groups were detected in terms of pregnancy rates both on heifers (P=0.6) and pluriparous buffaloes (P=7). With regard to gender rates, pregnancy rates were significantly different among the three groups of heifers (P=0.001) and the three groups of pluriparous buffaloes (P=0.002). Sperm cells preselected for gender determination through Cell Sorting (CST) resulted effective in shifting the gender ratio towards the female, when compared to both the Coinbubation Technique (COiT) and control group of animals.

Introduction

The possibility to obtain progeny of the desired sex in the realm of animal production, has been a goal long expected. A number of different strategies over the last decades [1] have been proposed and tested with a high degree of results, until gender preselection has become a practical reality through the implementation of the sperm cell sorting technology [2]. This last ensures, with a high degree of efficiency, the selection of single spermatozoa of the desired sex once the operating setups have been put in place in the sorting machine. From a physical standpoint in fact, it relies on the differential amount of DNA present in either the X or the Y sperm cell, being the first of the two in higher content and in parallel to a different size of the two chromosomes. This technology has guaranteed then, so far, the possibility to make available straws harbouring sperm cells of the desired sex, with an expected efficiency of 90% in cattle. In buffaloes, this same technology has been proved to be similarly effective since 2004, when the first buffalo calves were born in Italy [3]. Recently though, a novel procedure has been put at service of breeders, that relies on a different strategy, this time possibly of chemical origin. We are in fact referring to a product that is nowadays commercialized by a North American Company, "EMLAB", which is named "Heiferplus™" [4]. This product can be used on both fresh semen prior to freezing, and to frozen/thawed semen just before artificial insemination. In cattle, the same Company claims a shift calf production to 75-90% female when coincubation is made with fresh semen prior to freezing. When coincubation is made with frozen/thawed unsexed semen though, no precise value of a higher shift toward female calves is given, to the understanding of this. In this study, a new comparison was tested in the Mediterranean Buffalo between the use of commercialized semen, sexed through cell sorting technique or by using Heiferplus, together with a control group of animals being inseminated with unsexed semen from the same two bulls.

Materials and Methods

Animals

The study was carried out between the months of November to April of the same year, in a period then characterized initially by a higher number of dark compared to light hours of the day and then levelling out when transitioning into the spring months of the year, in a buffalo farm located in the south of Lazio Region (latitude …. and longitude ….). Animals that were part of this study were selected following clinical examination, transrectal palpation and ultrasound monitoring of the ovaries twice, at ten day interval to confirm cyclicity by presence of follicles ≥10 cm and/or functional corpora lutea, and the entire reproductive tract for presence of gross abnormalities. A total of 206 heifers and 211 pluriparous buffaloes were used in this study.

AI and synchronization of ovulation

An ordinary Ovsynch protocol was adopted to synchronize animals for ovulation [5]. The protocol consisted of administering intramuscularly 12 µg buserelin acetate (Receptal®, Intervet, Milan, Italy) on Day 0, followed by prostaglandin on Day 7 and additional 12 µg buserelin acetate on Day 9. As the time needed for submitting all the animals to AI varied due to farm management constraints, the time of AI following the second GnRH administration differed among animals. The interval between the last GnRH administration and performed AI ranged between 18 to 22 hours. Finally, in this study, with regard to prostaglandin administration, animals received 0.524 mg of synthetic prostaglandin (Cloprostenol, Estrumate®, Schering-Plough Animal Health, Milan, Italy).

Semen and AI

Sexed semen was obtained by a collaborating Company at the time of this study (Centro Tori Chiacchierini, Peugia, Italy). Unsexed and sexed semen from the same ejaculate of each of the two buffalo bulls was used for AI. Sperm sorting was performed according to the Beltsville Sperm Sorting Technology [2]. Briefly, following collection, semen was diluted to $80x10^6$ spermatozoa per mL with modified Tyrode's Albumin Lactate Pyruvate (TALP) extender. Subsequently, 50 µL of 5 mg/mL Bisbenzimide (Hoechst 33342; Sigma St. Louis, MO, USA) and 27 µL food dye FD#40 (Warner Jenkinson Company Inc., St. Louis, MO, USA) were added to samples and incubated at 35.5 °C for 30 min, and filtered through a 30 µm filter (Partec, GmbH, Germany). Samples were then sorted at a sorting rate of 5500 cells/s and sorting pressure of 40 psi into 50 mL conical plastic tubes (BD Biosciences, Italy) prefilled with 3 mL egg yolk extender. Following sorting, samples were centrifuged at 800 X g for 20 min. The supernatant was discarded and the pellet resuspended with a TRIS - tris(hydroxymethyl)aminomethane - freezing extender. A total content of 2 million live sorted sperm cells were frozen and packaged into 0.25 mL straws according to the Cogent Breeding Ltd, UK proprietary technology. Reanalysis of sorting purity was performed from frozen-thawed samples, and performed as described above at an event rate of 100 cells/s and purity was analyzed by curve fitting statistics. Main characteristics of sexed semen from the three bulls, following sorting of the two cell populations, were as follows: (1) purity ranged from 92% to 97% and mean±SEM 94±0.7; (2) progressive linear motility ranged from 30% to 60% and mean ± SEM 48.7±4.4; (3) membrane integrity test (propidium iodide test) ranged from 59% to 69% and mean±SEM 62.7±2.1; and (4) osmotic resistance test ranged from 43% to 56% and mean±SEM 48.1±2.3.

Sexed semen as described above, following thawing was then used as such, or underwent treatment as specified by EMLAB Company and animals were randomly assigned to each of the two preselected semen straws (CST and COiT). Briefly, with regard to COiT treatment, the coincubation and the time needed of frozen/thawed semen with the vial content followed the instructions given by the Company, and animals were inseminated accordingly. Control group was artificially inseminated with unsexed frozen/thawed semen from the same two bulls used for this study (UnS). Both non sexed (UnS) and sexed semen (CST and COiT) were deposited in the horn of the uterus ipsilateral to the ovary presenting a follicle ≥10 mm. Therefore, only animals with a follicle ≥10 mm and a tonic uterus with or without vaginal mucous discharge were considered to be in heat with impeding ovulation and subjected to AI. Non sexed (UnS) semen was used in 55 heifers and 62 pluriparous buffaloes, whereas sexed (CST and COiT) semen was used in 71 and 70 heifers, respectively, and 80 and 79 pluriparous buffaloes, respectively. Pregnancy rates were assessed by ultrasound at Day 28 and confirmed at Day 45. Fetal sex determination was assessed by ultrasound between 60 to 70 days of gestation.

Statistical analysis

Differences among percentages were assessed by the chi-square test or, when appropriate, by Pearson exact test. All statistical analyses were performed using STATA software version 11.2 (STATA Corporation, College Station, TX, USA).

Results

Pregnancy rates following fixed time AI within the Ovsynch synchronization protocol did not differ among heifers randomly divided into the two treatment and control groups (Table 1). Similarly, also among pluriparous buffaloes, rates in pregnancy monitored by ultrasound were non significantly different among groups (Table 2). When highlighting differences among groups with regard to gender determination by ultarsound and following confirmation at birth, then both in heifers (Table 3) and in pluriparous buffaloes (Table 4), the Cell Sorting Technology (CST) gave a significantly higher proportion of expected female calves when compared to both the alternative technology (COiT) and control groups of animal.

Table 1: Pregnancy rates in heifers at 28 days following AI and Ovsynch protocol among Control (CTRL), Cell Sorting Technology (CST) and coincubation protocol (COiT). Pearson chi^2=0.7498 Pr=0.687.

	CTRL	CST	COiT	Total
Non pregnant	20 36.36	31 43.66	31 38.75	82 39.81
Pregnant	35 63.64	40 56.34	49 61.25	124 60.19
Total	55 100.00	71 100.00	80 100.00	206 100.00

Table 2: Pregnancy rates in pluriparous buffaloes at 28 days following AI and Ovsynch protocol among Control (CTRL), Cell Sorting Technology (CST) and coincubation protocol (COiT). Pearson chi^2=0.6622 Pr=0.718.

	CTRL	CST	COiT	Total
Non pregnant	27 43.55	35 50.00	39 49.37	101 47.87
Pregnant	35 56.45	35 50.00	40 50.63	110 52.13
Total	62 100.00	70 100.00	79 100.00	211 100.00

Table 3: Gender rates among groups in heifers by ultrasound fetal sex determination at 60 to 70 days following AI. Pearson chi^2=14.2747 Pr=0.001.

	CTRL	CST	COiT	Total
Male	17 54.84	4 11.43	16 39.02	37 34.58
Female	14 45.16	31 88.57	25 60.98	70 65.42
Total	31 100.00	35 100.00	41 100.00	107 100.00

Table 4: Gender rates among groups in pluriparous buffaloes by ultrasound fetal sex determination at 60 to 70 days following AI. Pearson chi^2=12.2303 Pr=0.002.

	CTRL	CST	COiT	Total
Male	16 55.17	4 12.90	15 41.67	35 36.46
Female	13 44.83	27 87.10	21 58.33	61 63.54
Total	29 100.00	31 100.00	36 100.00	96 100.00

Discussion

This study confirms once more the feasibility of using sexed semen in the buffalo species on both heifers and adult animals, at least when using the fine-tuned technology of cell sorting [3,6,7]. This reproductive technology improves the efficiency of buffalo farm management under intensive care, and again, it is clearly evident that in buffaloes both the sorting process and the low dose of sexed spermatozoa employed to inseminate animals, do not affect significantly the final outcome in terms of pregnancies obtained. In fact, similar rates can be reported when confronted to ordinary unsexed semen used for AI. Furthermore, as previously discussed [7], and similarly to what occurs for cattle semen, the process of semen sorting may help in selecting the most viable spermatozoa within the ejaculate, eliminating those sperm cells which are unable to bypass the hurdles posed by the sorting process itself followed by the freezing/thawing procedure.

This study was undertaken and completed in half a year, encompassing an initial period of predominant dark hours, corresponding to the favourable season for reproductive efficiency in buffaloes, and terminating in the spring months of the year, when the photoperiod shifts and bring buffaloes into their unfavourable period. The focus of interest of this study though, was a comparison between a well-established technology, the Beltsville Sperm Sorting Technology [2], currently and worldwide adopted for use of sexed semen in Artificial Insemination and for *in vivo* and *in vitro* embryo production in cattle [8-12] and buffalo as well [13], and a more recently developed technology which claims to bypass the complex technology of cell sorting, and to give the possibility to make use of ordinary unsexed semen within straws ready for AI by coincubating the straw content with a solution containing substances able to evidently boost sperm cells harbouring the X chromosome, with a final outcome represented by a significant shift towards female offspring. This can be only surmised, considering that for obvious commercial reason, the Company cannot unveil the complete mechanism that gives reason of skewing the sex ratio following AI. It has been suggested that this alternative technology contains substance/s that would enable a differential pre-capacitation of spermatozoa containing the X chromosome which, as a consequence, would reach the site of fertilization earlier, giving thus more chance to X-bearing spermatozoa to fertilize the ovulated oocyte. For this to occur, and in order to be successful, the procedure of artificial insemination should be performed as closely as possible to the timing of ovulation, giving the X-bearing spermatozoa a time advantage over the Y-bearing spermatozoa. For this to happen, and when using AI following visual aid of standing heat in the herd, the Company advise to inseminate animals around 18 hours from the initial standing heat in heifers and 24 hours in adult animals. Alternatively, when adopting fixed time protocol of insemination following synchronization of ovulation, indications are given to inseminate 18 to 24 hours following the second GnRH administration of the Ovsynch protocol, heifers and adult cattle, respectively. In our study, the time encompassing the last GnRH administration and insemination in both heifers and adult buffaloes ranged between 18 to 21 hours, falling adequately within the range of hours suggested by the Company.

In buffaloes, the coincubation protocol suggested by the Company, in one reported trial on the effect on some functional parameters of buffalo sperm cells following *in vitro* exposure to the vial content, did show a reduced viability of spermatozoa [14]. In line with these findings, the same authors reported a significant reduction in conception rates when compared to control animals, when buffalo semen was treated according to the company guidelines, and following artificial insemination at 16 and 18 hours following the last GnRH administration of the Ovsynch synchronization protocol [15], whereas in this study we did find some significative differences not so much in the pregnancy rates obtained following AI among control and the two semen sexing technologies, but definitely among the gender rate following fetal sexing and calf birth. In fact, the Beltsville Sperm Sexing Technology gave satisfactory rates of female calves born following AI, whereas the alternative semen sexing technology did not perform, in our hands, as expected. In such a process, where a complex number of variables are involved, from the physical processes of the cell sorting technology, to the chemical mechanisms only surmised of the alternative technology in this study tested, together with the bull factor, all this may account for differences detected and possibly confirmed by the collection of a higher amount of data in other trials to follow.

Conclusion

From the data generated by this study, it can be concluded that the technology of cell sorting for obtaining spermatozoa carrying the desired cell population for gender preselection in the buffalo species, is currently the method of choice. An alternative technology, tested and confronted in this study and based on a strategy that uses chemical substance/s with the intent to boost a pre-capacitation process in X-bearing sperm cells, was not similarly effective. More trials, on semen samples from different buffalo bulls, used for AI in other buffalo farms are possibly needed to confirm these preliminary results.

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