Research Article

Oocyte Source and Hormonal Stimulation for *In Vitro* Fertilization Using Sexed Spermatozoa in Cattle

Giorgio A. Presicce,[1,2] Jie Xu,[3] Guochun Gong,[3] Juan F. Moreno,[4] Sanjeev Chaubal,[3] Fei Xue,[5] Antonino Bella,[1] Elena M. Senatore,[6] Xiangzhong Yang,[5] X. Cindy Tian,[5] and Fuliang Du[3]

[1] *Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy*
[2] *Consorzio di Ricerca e Sperimentazione per gli Allevatori (CRSA), 00161 Roma, Italy*
[3] *Evergen Biotechnologies, Inc., 428 Hartford Turnpike, Suite no. 107, Vernon, CT 06066, USA*
[4] *Sexing Technologies, Navasota, TX 77868, USA*
[5] *Department of Animal Science/Center for Regenerative Biology, University of Connecticut, Storrs, CT 06269, USA*
[6] *ARSIAL, Centro Regionale per la Zootecnia, 00162 Roma, Italy*

Correspondence should be addressed to Giorgio A. Presicce, giorgio.presicce@iss.it and Fuliang Du, fuliangd@renovalife.com

The aim of this study was to investigate the efficiency of in vitro embryo production in cattle utilizing sexed sperm from two bulls and oocytes recovered by OPU. Twenty donor animals were employed in eight OPU replicates: the first four OPU trials were conducted on animals without hormone treatment, and the last four were run on the same animals, following FSH subcutaneous and intramuscular administration. A higher rate of blastocyst development was recorded in stimulated, as compared to nonstimulated animals, (25.2% versus 12.8%, $P = .001$). Ocytes derived from slaughterhouse (SH) ovaries were also fertilized with sperm from the same bulls. Overall, non-sexed sperm used with oocytes derived from SH ovaries was significantly more efficient for blastocyst development than was sexed sperm with these same SH derived oocytes and sexed sperm with stimulated donor oocytes (39.8% versus 25.0% and 25.2%, $P = .001$). In conclusion, the use of sexed sperm with OPU-derived oocytes resulted in a significantly higher blastocyst development when donors were hormonally stimulated; furthermore, the level of efficiency achieved was comparable to that attained when the same sexed sperm was tested on oocytes derived from SH ovaries.

1. Introduction

Reliable sorting of X- and Y-bearing sperm through a fluorescence-activated flow cytometry will impact breeding schemes and enable the cattle industries to enhance and accelerate the diffusion of production traits [1–3]. In addition, it appears that breeders could exploit this new technology for maximizing herd production and reproductive management. Even if the cost of each straw of sexed sperm is doubled compared to unsexed straw, it would still be profitable due to the increased production of replacement dairy heifers, or steers [4–6]. After invention of sperm sorting, sexed sperm have been used for artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in a number of species

[7–9]. A reduced efficiency in the production of embryos *in vitro* is usually reported though, when using sexed spermatozoa. This is largely attributed to substandard sperm quality following sorting/freezing/thawing, and possibly also inadequate fertilization and culture conditions [5, 10, 11]. Furthermore, technical aspects of the cell sorting technology, such as speed and pressure may affect the sperm and lower its quality, and therefore, the quantity of embryos produced [12, 13]. Currently, the retrieval of oocytes through the use of ultrasound guided follicle puncture, or ovum pick-up (OPU), is inescapably linked to the procedures for *in vitro* embryo production, as it can exploit the most elite males and females to combine for an accelerated genetic gain [14]. Ovum pick-up has been shown to be a valuable technology greatly enhancing the potential of IVF systems

in a variety of breeding conditions and species [8, 15–19]. Since its initial development, a number of interesting aspects have arisen highlighting, for example, the donor cow effect on oocyte retrieval and *in vitro* embryo production [20]. Additional aspects related to the OPU/IVF system have been investigated with the goal of optimizing the efficiency, that is, number of oocytes and IVM/IVF/IVC droplet volume and size [21], number and quality of collected oocytes, as well as frequency of collection and hormonal stimulation [15, 19]. Furthermore, evidence has mounted that the number of retrieved oocytes per animal may not be positively correlated with the number of blastocysts produced, and that there is a general tendency for oocyte donors to maintain their position as either good or poor embryo producers throughout OPU replicates [20]. Within the toolbox of reproductive technologies, the availability of sexed sperm cell populations represents an additional implement yet to be fully applied and optimized. Despite the current limitations, constraints, and inefficiencies of *in vitro* embryo production procedures, it is likely that a synergistic positive effect can be achieved by utilizing sperm sexing technology. In fact, IVF requires a much lower number of spermatozoa than does AI, in order to achieve a reasonable number of embryos produced *in vitro* [7]. Moreover, a higher efficiency will ultimately be reached because producing offspring of the desired sex will be cost effective, nearly half cost of those animals produced with unsexed sperm. Expenses associated with embryo biopsy and waste due to compromised postbiopsy embryos, or unwanted gender pregnancies could be virtually avoided. More importantly, in this study, we tested the possibility whether exogenous hormonal stimulation would improve embryo yield when sexed sperm was used on oocytes recovered by OPU. Our objective was, therefore, to evaluate the efficiency of sexed sperm from two bulls following IVF, using oocytes acquired via OPU from nonstimulated versus FSH stimulated donor animals.

2. Materials and Methods

All chemicals were provided by Sigma Aldrich (St. Louis, MO) unless otherwise noted.

2.1. Animals and Hormonal Stimulation. Twenty Holstein animals, ranging from heifers to 6–8 year old pluriparous cows, were used for OPU as oocyte donors. The study lasted for four weeks in the month of August, and OPU sessions were conducted on a twice-weekly schedule for a total of 8 OPU sessions. The animals were stall fed and maintained in the barn under controlled conditions. Their feeding regimen was standard for sustaining energy input and milk production. Mean values for temperature and humidity relative to the period of study were $21.2 \pm 0.6°C$ and $73.1 \pm 1.2°C$, respectively. As shown in Figure 1(a), the 20 donors were subjected to OPU during the first 4 replicates (first 2 weeks) without hormone stimulation, considered in this study as the "control". The same 20 donors were then stimulated by hormone injection, and subjected to an additional 4 OPU sessions (last 2 weeks), and considered
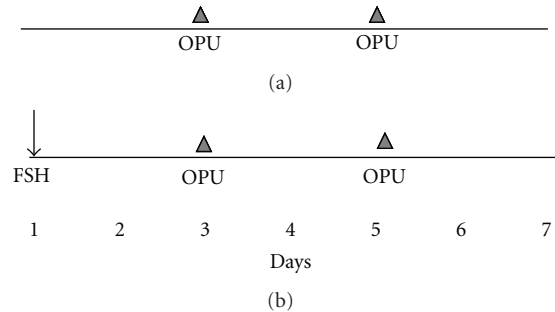

Figure 1: Weekly scheme and hormone treatment for Ovum pick-up (OPU). (a) The animals were not treated with hormone FSH. Transvaginal OPUs (triangle) were performed on Monday and Thursday of each week for consecutive two weeks, resulting in a total of four sessions. (b) FSH treatment: FSH (arrow) at dosage of 80 mg IM and 120 SC was simultaneously administered on Day 1 (Saturday). Ovum pick-ups were performed 48 and 96 hours post FSH injection, meaning on Day 3 and Day 5 of each week for consecutive two weeks, resulting in a total of four sessions.

as "treatment" (Figure 1(b). Hormone administration was implemented as follows: the shot of an intramuscular (80 mg), followed by a subcutaneous (120 mg) injection of FSH (Follitropin, Vetrepharem, Canada) was given to each animal at the beginning of the third and fourth week of oocyte retrieval, followed by 48 hours of a resting, or "coasting" period prior to the 5th and 7th OPU sessions (indicated as in Figure 1(b)). All procedures and protocols applied to animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut.

2.2. Sperm. Semen from two bulls sexed for the production of female calves was provided by Sexing Technologies, Inc. (Navasota, TX, USA). Sperm cell separation was accomplished according to the Beltsville sperm sorting method [22]. Briefly, fresh ejaculates were stained by Hoechst 33342 before being subjected to flow cytometry separation. The X- and Y-chromosome-bearing sperm were sorted into different tubes by their 3.8% inherent difference in DNA content, at the rate of 25,000 to 30,000 sperm cells/sec. Approximately 2.0×10^6 sorted sperm cells were packaged in 0.25-mL straws, frozen on racks in a liquid nitrogen vapour phase, and stored in liquid nitrogen. The purity of X-sperm cells used in this study was $90 \pm 3\%$ based on manufacturer's specifications. Nonsexed, frozen sperm cells from the same sires were used as controls. The nonsexed sperm cells were packaged at approximately $15–20 \times 10^6$ in each 0.25 mL straw. While only sexed sperm was used for IVF with the OPU-derived oocytes, as a control both sexed as well as nonsexed sperm from the same bulls was used in IVF with slaughterhouse-derived oocytes.

2.3. Oocyte Collection by Ovum Pickup. A portable Aloka 500 ultrasound unit equipped with a 5-MHz sector scanner vaginal probe (Aloka Co. Ltd, Tokyo), together with a 17-gauge, 60 cm single lumen needle fitting a proper metallic

needle guide was used for transrectal oocyte retrieval. Animals were restrained in a squeeze chute and prepared for follicular aspiration as described by Pieterse et al. [23]. Aspiration medium consisted of phosphate-buffered saline (PBS) with the addition of 10 IU/mL heparin and 0.1% polyvinyl alcohol. Negative suction pressure was applied to maintain a low constant flow rate ranging from 10 to 15 mL/min in an effort to retrieve oocytes without major disruption of the surrounding cumulus cell layers. The OPU was scheduled twice weekly for 4 weeks utilizing 20 animals for each replicate, thus, a total of 8 replicates and 160 oocyte retrieval sessions were performed. The collected oocytes were graded morphologically based on their cumulus investment, as follows: Grade A, >4 layers of cumulus cells; Grade B, 3 or 4 layers of cumulus cells; Grade C, 1 or 2 layers of cumulus cells; Grade D, denuded oocytes; Grade E, oocytes with expanded cumulus. Only oocytes completely deprived of cumulus cell investment (Grade D) or with gross, evident cytoplasmic abnormalities (Grade D and E) were discarded. All Grade A, B, and C oocytes were included in IVM and IVF experiments, and selected oocytes from each donor were cultured separately from selected oocytes of the other donors.

2.4. Oocyte Collection from Slaughterhouse Ovaries.

Bovine oocytes used for IVF control were retrieved from slaughterhouse Holstein ovaries, and processed as previously described [6]. Only oocytes surrounded with at least 4 intact layers of cumulus cells and displaying homogeneous cytoplasm (Grade A) were selected for further processing. Selected SH oocytes (10 to 15/droplet) were used as controls for OPU oocytes in sexed IVF experiments.

2.5. Maturation, Fertilization, and Embryo Culture In Vitro.

Embryos were produced as previously described by Xu et al. [6]. Briefly, selected oocytes were matured for 22 hours in 75 μL droplets of Medium 199 (Invitrogen) containing Earle's salts, 0.7 mM L-glutamine, 2.2 g/L sodium bicarbonate and 25 mM Hepes, supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone, Logan, UT), 0.5 μg/mL ovine FSH (National Institute of Diabetes and Digestive and Kidney Disease, NIDDK, Los Angeles), 5.0 μg/mL ovine LH (NIDDK), and 1.0 μg/mL estradiol 17-β. Droplets were maintained under sterile mineral oil. Brackett and Oliphant (BO) medium [24] was used for IVF. Briefly, straws containing sexed sperm were thawed for 10 s in a 37°C water bath after 10 s of gentle shaking in air at room temperature. Sperm were washed in 8 mL of BO medium supplemented with 3 mg/mL of BSA and 10 mM caffeine. Sperm pellet was resuspended in additional 8 mL of BO washing medium. Matured oocytes were rinsed in BO medium containing 6 mg/mL BSA and 10 μg/mL heparin. Fertilization droplets (50 μL) containing matured oocytes were prepared in small Petri dishes. Processed sperm was added (50 μL) for a final droplet volume of 100 μL and a final sperm concentration of 0.3×10^6/mL, and overlaid with mineral oil. Following 6 hours of sperm/oocyte coincubation, presumptive zygotes were transferred into 75 μL culture droplets in CR1aa medium containing 6 mg/mL of BSA. Presumptive zygotes

were cultured at 39°C in humidified air and a mixed gas atmosphere consisting of 5% CO_2, 5% O_2, and 90% N_2. Embryo cleavage and rate of development to blastocyst were assessed at day 2 and 7, respectively.

2.6. PCR Confirmation of Sexed OPU Embryos.

The procedure of PCR for confirmation of sexed embryos derived from OPU oocytes was performed as descibed by Xu et al. [6]. Breifly, the proteinase K digested IVF embryos samples were added into one round multiplex amplification of polymerase chain reaction (PCR) with bovine-specific autosome and Y-chromosome fragments. A bovine specific primer pair was designed to amplify a fragment of 219 bp from a bovine 1.715 satellite DNA as follows; forward: 5′-TGA GGC ATG GAA CTC CGC TT-3′; reverse: 5′-GGT GGT TCC ACA TTC CGT AGG AC-3′. The Y-chromosome fragment (131 bp) was amplified using male specific primers (forward: 5′- GAT TGT TGA TCC CAC AGA AGG CAA TC-3′; reverse: 5′-GAA CTT TCA AGC AGC TGA GGC ATT TA-3′). One μL embryo lysate was used for PCR amplification in a total volume of 25 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl (S-5761), 2 mM $MgCl_2$, 0.2 mM dNTPs (DNTP100A), 0.4 μM oligonucleotide primers and 0.5 unit REDTaq DNA polymerase. The PCR was initiated with an initial cycle of 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, primer annealing at 64°C 30 s, primer extension at 72°C 30 seconds, and a final hold at 72°C for 10 minutes. Ten μL of PCR products were analyzed on a 2% agarose gel. One band (219 bp) indicated a female embryo, and 2 bands indicated (219 bp and 131 bp) a male embryo.

2.7. Statistical Analyses.

Values are expressed as Mean ± SEM. Categorical data were compared by χ^2 test and continuous variables by t-test. Differences between replicates were evaluated by nonparametric Wilcoxon signed rank test for paired data. All statistical analyses were performed by STATA statistical software version 8.2 (Stata Corporation, College Station, TX).

3. Results

3.1. Sexed Sperm IVF of OPU-Derived Oocytes.

Hormone injections did not increase the number of available antral follicles (8.62 ± 0.67 versus 7.56 ± 0.32, $P = .47$) and resulted in a significant reduction in the number of oocytes retrieved (4.93 ± 0.46 versus 7.15 ± 0.51, $P = .0001$). In 8 out of the 20 donors used, with or without hormone stimulation, no late stage embryo development was ever observed. Among the 12 donors producing embryos, a range of 3 to 20 collected oocytes were processed for IVF in single droplets of maturation, fertilization, and culture medium. When considering parameters related to embryo development, although the rate of embryo cleavage was similar in nonstimulated and FSH-treated donors (41.2% versus 48.4%), the blastocyst output was, by contrast, significantly higher in FSH stimulated donors (25.2% versus 12.8%, $P = .001$) (Table 1).

TABLE 1: Embryo production (%) using nonsexed and sexed sperm on slaughterhouse and OPU derived COCs.

Oocyte source	No. of Replicates	No. oocytes	Cleavage (%) (mean ± SEM)	Blastocyst (%) (mean ± SEM)
Nonsexed (SH)	4	106	75.8 ± 2.3a	39.8 ± 2.4a
Sexed (SH)	4	133	57.1 ± 7.8b	25.0 ± 6.1b
Sexed without FSH (OPU)	4	121	41.2 ± 6.1b	12.8 ± 2.3c
Sexed with FSH (OPU)	4	101	48.4 ± 8.9b	25.2 ± 5.0b
*P value			.001	.001

*P values were compared within the same column. SH, slaughterhouse; OPU, ovum pickup.
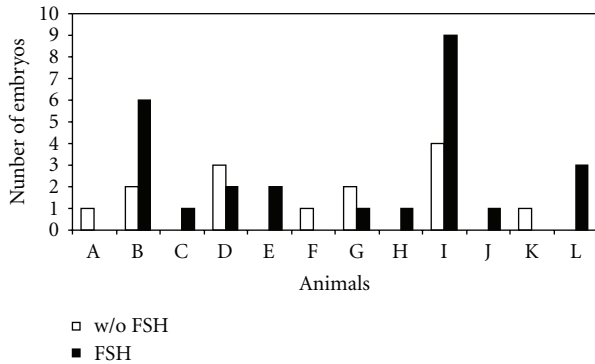

FIGURE 2: Blastocyst yields from the animals subjected to OPU without FSH (open bars) and OPU with FSH treatment (solid bars).

With regards to the remaining 12 donors from which blastocysts were produced, 4 produced blastocysts either when nonstimulated or stimulated, 3 produced blastocysts when nonstimulated, and 5 produced blastocysts when they were stimulated (Figure 2). Blastocyst development rates from individual animals ranged from 5% to 66%.

3.2. Sexed and Nonsexed Sperm IVF Using OPU and Slaughterhouse (SH) Oocytes. Semen from the same two bulls, both sexed and nonsexed, was used with oocytes obtained from SH ovaries, and a significantly higher cleavage rate (75.8% versus 57.1%, P = .001) and blastocyst production (39.8% versus 25.0%, P = .001) was observed with the use of nonsexed sperm compared to sexed sperm. By considering collectively the data when using sexed sperm on oocytes acquired via OPU, the blastocyst rates remained significantly lower than those achieved from the use of nonsexed sperm with SH oocytes (12.8–25.2% versus 39.8%, P = .001). Blastocyst production from sexed sperm with oocytes derived from SH ovaries was significantly higher than that achieved with the same sexed sperm and oocytes derived from OPU on nonstimulated donors (25.0% versus 12.8%, P = .001), but was similar to the blastocyst rate achieved with OPU oocytes from stimulated donors (25.0% versus 25.2%, P = .65) (Table 1).

3.3. Bull Effect. Sexed sperm from bull A attained similar blastocyst rates with oocytes derived from either nonstimulated or stimulated OPU donors (13.0% versus 12.6%, P = .95). Bull B's sperm was more effective in blastocyst

production when using oocytes from stimulated animals (27.0% versus 8.8%, P = .02). Collectively, these two bulls demonstrated a significantly higher rate of blastocysts when fertilizing oocytes derived from stimulated animals compared to nonstimulated animals (24.0% versus 11.5%, P = .01). When examining the effect of sexing the sperm from these two bulls, a similar satisfactory rate of blastocysts was observed when using either oocytes derived from slaughterhouse ovaries (24.0%), or collected from stimulated animals (24.0%).

3.4. PCR Conformation of Sexed OPU Embryos. Twenty five blastocysts derived from OPU oocytes fertilized by sexed sperm were subjected PCR sexing. Blastocysts derived from SH oocytes fertilized with either unsorted or sorted sperm were served as controls for PCR sexing. From unsorted sperm IVF control, 51.8% (n = 27) of embryos were female; whereas 92.3% (n = 26) of embryos from SH oocytes fertilized with X-sorted sperm were female. Twenty three OPU/sexed IVF derived embryos (92%, n = 25) were confirmed as female. This result confirmed the accuracy of the sex-sorting of sperm.

4. Discussion

This study has looked into the feasibility of producing *in vitro* embryos using sorted sperm cells with oocytes collected via OPU from either nonstimulated, or hormonally stimulated cattle. In recent years, contradictory results have been reported on the use of sexed sperm for *in vitro* embryo production. The causative factors for reduced embryo production efficiency following the use of sexed sperm cells has been associated with a number of variables, including: (i) bulls and technologies employed: bull effect, speed, and pressure of sorting [5, 13]; (ii) general aspects related to IVF system: that is, IVF procedures, media employed, culture conditions [25, 26], (iii) environmental conditions: that is, season, breed, distance to the embryology laboratory, ovaries' transport conditions [20, 27]. Age of the animals, under current ordinary *in vitro* embryo production procedures is another element of paramount importance, especially when older cows are used for *in vitro* embryo production together wit0068 the use of nonsexed semen [28]. Another variable responsible for altering embryo production efficiency is the retrieval of oocytes through OPU. Mechanical and biological variables affecting OPU efficiency, and consequently the overall IVF procedure, include: vacuum pressure, needle

quality and bevel angle, hormonal pretreatment of animals, puncture frequency, stage of estrous cycle, and skill of OPU technician [17, 27, 29]. In addition, the ability to recover oocytes from individual animals has highlighted the importance of the oocyte donor effect. This effect cannot be examined when oocytes recovered from slaughterhouse ovaries are used for IVF, because the ovaries of many animals are pooled, and usually only Grade A oocytes used for IVF. Maternal influence is apparently expressed during later stages of embryo development, and there is strong evidence that cattle oocyte donors always show a correlation between oocyte and embryo production, and also that often the best and the worst embryo producers tend not to change for the duration of an OPU program [20].

A number of studies have been performed in an attempt to establish the most cost-effective procedure for retrieving the highest number of good quality oocytes via OPU that produce the most blastocysts [30, 31]. Follicle stimulating hormone (FSH) has routinely been used to rescue follicles back into development that would otherwise be doomed to atresia. Scheduled administration of FSH has been typically given as a decreasing dosage, or, in order to reduce time and labour cost, a single dose of equine chorionic gonadotropin (eCG) can be administered [32]. In the latter case, prolonged FSH and LH activity within the eCG can generate an abnormal endocrine profile and reduced embryo quality following either AI or IVF [33]. Alternative schemes include a reduced FSH administration consisting of a single subcutaneous and/or intramuscular injection [34, 35]. In this study, a previously optimized hormonal stimulation protocol consisting of both a subcutaneous as well as an intramuscular injection of FSH [36] has been employed, and although a tendency toward a higher recruitment of antral follicles has been observed, it was not substantiated by a corresponding increase in the number of oocytes retrieved. Conflicting results have been reported regarding follicle availability, oocyte recovery, and blastocyst production following hormonal administration: from increased antral follicle and unaffected oocyte recovery and blastocyst production [30], to increased antral follicles, lower oocyte recovery, but increased blastocyst production in our study.

It has been shown that the administration of FSH in combination with twice weekly OPU does not produce the expected synergistic effect [30, 36–38]. The findings of the present study concur: once all of the visible follicles are aspirated in the first OPU session following FSH treatment, a new cohort of follicles begins to develop, although they cannot benefit from hormone administration because it has already been largely metabolized.

Stimulating an animal with hormone prior to OPU would be recommended only if the costs, in time and expense, could be offset by rendering available a greater number of antral follicles leading to the collection of a larger number of good quality oocytes. Previous studies have established that following hormonal stimulation the number of follicles yielding oocytes resulting in embryo production is a highly variable response. To explain such contradictory and inconsistent results, one has to take into consideration the hormone employed, the dosage and schedule of its administration, as well as the age and condition of the animals [31]. With regards to the production of late stage embryos, although cleavage rates remained similar weather or not an animal was FSH stimulated, blastocyst development was significantly higher when donors received FSH injections. However, there were inconsistencies among the animals in this study, some donor's oocytes (3 donors) developed into blastocysts when nonstimulated, while oocytes from more animals (5 donors) progressed into embryo development only after being subjected to FSH stimulation.

Hormonal stimulation with FSH without a coasting period is believed to force follicles into an accelerated growth phase, thus leaving the oocytes within the follicles insufficient time to acquire developmental competence [39]. A coasting period of 48 h initiates FSH starvation and halts follicular growth, thereby allowing oocyte cytoplasm to "catch up" with follicular maturation and creating an early atresia-like condition [40]. In cattle, early atresia seems to mimic part of the naturally occurring preovulatory maturation, thus providing the oocytes an opportunity to enhance their developmental competence [19, 41]. Consequently, an FSH treatment schedule that allows for a coasting period may result in the production of oocytes with enhanced developmental competence, and lead to significantly higher embryo development when compared to oocytes from nonstimulated OPU donors [17].

In the present study, oocytes retrieved from each individual animal were processed for IVF separately as a group. Groups of oocytes were always treated or maintained in medium droplets of the same volume, although numbers ranged from very few (2–5) to a maximum of 20 per droplet. The high variability in the number of oocytes retrieved from an individual donor animal poses the problem of determining the appropriate volume of medium droplets to be used throughout the various stages of *in vitro* embryo production. A wide variability in the rate of blastocyst development was observed among animals, and only one or two blastocysts were produced when the group of fertilized oocytes in culture was less than 15. There is evidence that a sufficient number of fertilized oocytes are necessary in the culture droplet in order to maintain a good rate of late stage embryo development [42]. This effect on embryo production, as evidenced by increased development, is likely caused by the combined effects of the assembly of developing embryos [43, 44]. Furthermore, when the small number of cattle oocytes is cultured in cell-free medium, a reduced developmental competence has been reported when compared to that attained with a larger number. Even the kinetics of blastocyst formation has been demonstrated to be influenced by the number of embryos cultured per droplet [45]. In other studies, while emphasizing the importance of the number of oocytes cultured in a group versus cultured singly, no effect on oocyte development has been attributed to the culture volume, although a lower medium to oocyte ratio was seen to increase cell number in produced blastocysts [46]. Possible reasons for the reduced developmental potential of oocytes cultured singly may be related to substrate depletion, or toxic buildup that may occur in microdroplets when

medium is not changed or concomitantly due to somatic cell coculture [47]. On the other hand, in our study, an increase in blastocyst production was detected when the number of fertilized oocytes in culture was equal to, or higher than 15. Unfortunately, due to the small number of embryos generated in this study, it was not possible to ascertain a statistical correlation between the number of oocytes fertilized and the number of resulting embryos. In a different study, in order to tackle this problem and to standardize culture conditions for cleaved embryos derived from OPU, we are devising a protocol in which each droplet will contain the same threshold number of oocytes. We propose accomplishing this by supplementing droplets with cleaved embryos derived from oocytes from slaughterhouse ovaries having little or no commercial value, while identifying them as separate by embedding them in agar chips that allow the promoting factors from embryos to diffuse into medium. Our previous study showed that such a system can be highly effective, and that a threshold number of 10 cleaved embryos per droplet with a volume of 50 μL is sufficient to sustain an optimal rate of late stage embryo development [48].

The sperm sorting parameters employed in this study have been thoroughly tested and shown to give satisfactory embryo development rates [6]. Nonsexed and sexed sperm from both bulls was demonstrated to be effective in producing blastocysts from either source of oocytes, although a significantly higher production rate was achieved by the use of unsexed sperm with oocytes derived from slaughterhouse ovaries. When considering the use of sexed sperm only, FSH stimulation was advantageous in increasing the rate of blastocyst production from OPU collected oocytes to the level attained when using sexed sperm with slaughterhouse derived oocytes.

5. Conclusion

In conclusion, we have demonstrated the effective use of sexed sperm for fertilizing OPU-derived oocytes. We have emphasized a considerable oocyte donor variability, as well as the interaction of hormone treatments on embryo production. Sexing PCR confirmed that the sex status of OPU/sexed IVF derived embryo contained the same accuracy as the sex-sorting of sperm. Our study suggests that stimulation with FSH prior to OPU can increase the yield of transferable embryos, and thereby maximize the use of donors of high genetic merit.
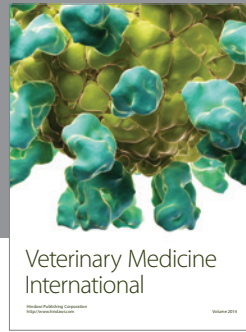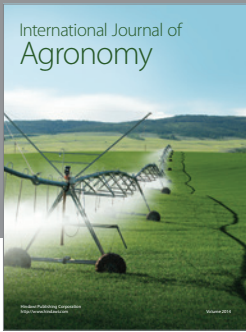
Acknowledgments

References

[1] D. G. Cran, "XY sperm separation and use in artificial insemination and other ARTs," *Society of Reproduction and Fertility Supplement*, vol. 65, pp. 475–491, 2007.

[2] A. De Vries, M. Overton, J. Fetrow, K. Leslie, S. Eicker, and G. Rogers, "Exploring the impact of sexed semen on the structure of the dairy industry," *Journal of Dairy Science*, vol. 91, no. 2, pp. 847–856, 2008.

[3] D. L. Garner and G. E. Seidel Jr., "History of commercializing sexed semen for cattle," *Theriogenology*, vol. 69, no. 7, pp. 886–895, 2008.

[4] R. D. Wilson, K. A. Weigel, P. M. Fricke et al., "In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows," *Journal of Dairy Science*, vol. 88, no. 2, pp. 776–782, 2005.

[5] R. D. Wilson, P. M. Fricke, M. L. Leibfried-Rutledge, J. J. Rutledge, C. M. S. Penfield, and K. A. Weigel, "In vitro production of bovine embryos using sex-sorted sperm," *Theriogenology*, vol. 65, no. 6, pp. 1007–1015, 2006.

[6] J. Xu, Z. Guo, L. Su et al., "Developmental potential of vitrified Holstein cattle embryos fertilized in vitro with sex-sorted sperm," *Journal of Dairy Science*, vol. 89, no. 7, pp. 2510–2518, 2006.

[7] W. M. C. Maxwell, G. Evans, F. K. Hollinshead et al., "Integration of sperm sexing technology into the ART toolbox," *Animal Reproduction Science*, vol. 82-83, pp. 79–95, 2004.

[8] G. A. Presicce, "Reproduction in the water buffalo," *Reproduction in Domestic Animals*, vol. 42, supplement 2, pp. 24–32, 2007.

[9] X. W. Liang, Y. Q. Lu, M. T. Chen et al., "In vitro embryo production in buffalo (Bubalus bubalis) using sexed sperm and oocytes from ovum pick up," *Theriogenology*, vol. 69, no. 7, pp. 822–826, 2008.

[10] M. B. Wheeler, J. J. Rutledge, A. Fischer-Brown, T. VanEtten, S. Malusky, and D. J. Beebe, "Application of sexed semen technology to in vitro embryo production in cattle," *Theriogenology*, vol. 65, no. 1, pp. 219–227, 2006.

[11] K. H. Lu and G. E. Seidel Jr., "Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically-sorted sperm," *Theriogenology*, vol. 62, no. 5, pp. 819–830, 2004.

[12] G. A. Palma, N. S. Olivier, CH. Neumüller, and F. Sinowatz, "Effects of sex-sorted spermatozoa on the efficiency of in vitro fertilization and ultrastructure of in vitro produced bovine blastocysts," *Anatomia, Histologia, Embryologia*, vol. 37, no. 1, pp. 67–73, 2008.

[13] M. Zhang, K. H. Lu, and G. E. Seidel Jr., "Development of bovine embryos after in vitro fertilization of oocytes with flow cytometrically sorted, stained and unsorted sperm from different bulls," *Theriogenology*, vol. 60, no. 9, pp. 1657–1663, 2003.

[14] T. A. Kruip, R. Boni, Y. A. Wurth, M. W. M. Roelofsen, and M. C. Pieterse, "Potential use of ovum pick-up for embryo production and breeding in cattle," *Theriogenology*, vol. 42, no. 4, pp. 675–684, 1994.

[15] R. De Roover, G. Genicot, S. Leonard, P. Bols, and F. Dessy, "Ovum pick up and in vitro embryo production in cows superstimulated with an individually adapted superstimulation protocol," *Animal Reproduction Science*, vol. 86, no. 1-2, pp. 13–25, 2005.

[16] P. Humblot, P. Holm, P. Lonergan et al., "Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes," *Theriogenology*, vol. 63, no. 4, pp. 1149–1166, 2005.

[17] S. A. Chaubal, L. B. Ferre, J. A. Molina et al., "Hormonal treatments for increasing the oocyte and embryo production in an OPU-IVP system," *Theriogenology*, vol. 67, no. 4, pp. 719–728, 2007.

[18] G. A. Presicce, E. M. Senatore, G. D. Santis et al., "Hormonal stimulation and oocyte maturational competence in prepuberal Mediterranean Italian buffaloes (Bubalus bubalis)," *Theriogenology*, vol. 57, no. 7, pp. 1877–1884, 2002.

[19] R. De Roover, J. M. N. Feugang, P. E. J. Bols, G. Genicot, and CH. Hanzen, "Effects of ovum pick-up frequency and fsh stimulation: a retrospective study on seven years of beef cattle in vitro embryo production," *Reproduction in Domestic Animals*, vol. 43, no. 2, pp. 239–245, 2008.

[20] M. Tamassia, Y. Heyman, Y. Lavergne et al., "Evidence of oocyte donor cow effect over oocyte production and embryo development in vitro," *Reproduction*, vol. 126, no. 5, pp. 629–637, 2003.

[21] T. Fujita, H. Umeki, H. Shimura, K. Kugumiya, and K. Shiga, "Effect of group culture and embryo-culture conditioned medium on development of bovine embryos," *Journal of Reproduction and Development*, vol. 52, no. 1, pp. 137–142, 2006.

[22] L. A. Johnson, J. P. Flook, and H. W. Hawk, "Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting," *Biology of Reproduction*, vol. 41, no. 2, pp. 199–203, 1989.

[23] M. C. Pieterse, P. L. A. M. Vos, TH. A. M. Kruip et al., "Transvaginal ultrasound guided follicular aspiration of bovine oocytes," *Theriogenology*, vol. 35, no. 4, pp. 857–862, 1991.

[24] B. G. Brackett, D. Bousquet, M. L. Boice, W. J. Donawick, J. F. Evans, and M. A. Dressel, "Normal development following in vitro fertilization in the cow," *Biology of Reproduction*, vol. 27, no. 1, pp. 147–158, 1982.

[25] P. Lonergan, T. Fair, D. Corcoran, and A. C. O. Evans, "Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos," *Theriogenology*, vol. 65, no. 1, pp. 137–152, 2006.

[26] Y. Nagao, R. Iijima, and K. Saeki, "Interaction between embryos and culture conditions during in vitro development of bovine early embryos," *Zygote*, vol. 16, no. 2, pp. 127–133, 2008.

[27] A. S. Lopes, T. Martinussen, T. Greve, and H. Callesen, "Effect of days post-partum, breed and ovum pick-up scheme on bovine oocyte recovery and embryo development," *Reproduction in Domestic Animals*, vol. 41, no. 3, pp. 196–203, 2006.

[28] L. Su, S. Yang, X. He, et al., "Effect of donor age on the developmental competence of bovine oocytes retrieved by Ovum Pick Up," *Reproduction in Domestic Animals*. In press.

[29] P. E. Bols, A. Van Soom, M. T. Ysebaert, J. M.M. Vandenheede, and A. De Kruif, "Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes," *Theriogenology*, vol. 45, no. 5, pp. 1001–1014, 1996.

[30] L. Bungartz, A. Lucas-Hahn, D. Rath, and H. Niemann, "Collection of oocytes from cattle via follicular aspiration aided by ultrasound with or without gonadotropin pretreatment and in different reproductive stages," *Theriogenology*, vol. 43, no. 3, pp. 667–675, 1995.

[31] J. S. Merton, A. P. W. De Roos, E. Mullaart et al., "Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry," *Theriogenology*, vol. 59, no. 2, pp. 651–674, 2003.

[32] J. F. Hasler, A. D. McCauley, E. C. Schermerhorn, and R. H. Foote, "Superovulatory responses of Holstein cows," *Theriogenology*, vol. 19, no. 1, pp. 83–99, 1983.

[33] A. M. Jensen, T. Greve, A. Madej, and L.-E. Edqvist, "Endocrine profiles and embryo quality in the PMSG-PGF2α treated cow," *Theriogenology*, vol. 18, no. 1, pp. 33–44, 1982.

[34] G. A. Bo, D. K. Hockley, L. F. Nasser, and R. J. Mapletoft, "Superovulatory response to a single subcutaneous injection of Folltropin-V in beef cattle," *Theriogenology*, vol. 42, no. 6, pp. 963–975, 1994.

[35] M. Yamamoto, M. Ooe, M. Kawaguchi, and T. Suzuki, "Superovulation in the cow with a single intramuscular injection of FSH dissolved in polyvinylpyrrolidone," *Theriogenology*, vol. 41, no. 3, pp. 747–755, 1994.

[36] S. A. Chaubal, J. A. Molina, C. L. Ohlrichs et al., "Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows," *Theriogenology*, vol. 65, no. 8, pp. 1631–1648, 2006.

[37] J. R. Gibbons, W. E. Beal, R. L. Krisher, E. G. Faber, R. E. Pearson, and F. C. Gwazdauskas, "Effects of once- versus twice-weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development," *Theriogenology*, vol. 42, no. 3, pp. 405–419, 1994.

[38] G. Rick, D. G. Hadeler, E. Lemme, et al., "Long term OPU in heifers from 6 to 15 months of age," *Theriogenology*, vol. 45, no. 1, p. 356, 1996.

[39] P. Blondin, K. Coenen, L. A. Guilbault, and M.-A. Sirard, "Superovulation can reduce the developmental competence of bovine embryos," *Theriogenology*, vol. 46, no. 7, pp. 1191–1203, 1996.

[40] J. R. Gibbons, M. C. Wiltbank, and O. J. Ginther, "Functional interrelationships between follicles greater than 4 mm and the follicle stimulating hormone surge in heifers," *Biology of Reproduction*, vol. 42, pp. 405–419, 1997.

[41] P. Blondin and M.-A. Sirard, "Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes," *Molecular Reproduction and Development*, vol. 41, no. 1, pp. 54–62, 1995.

[42] C. Carolan, P. Lonergan, H. Khatir, and P. Mermillod, "In vitro production of bovine embryos using individual oocytes," *Molecular Reproduction and Development*, vol. 45, no. 2, pp. 145–150, 1996.

[43] C. L. Keefer, S. L. Stice, A. M. Paprocki, and P. Golueke, "In vitro culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors," *Theriogenology*, vol. 41, no. 6, pp. 1323–1331, 1994.

[44] I. Donnay, A. Van Langendonckt, P. Auquier et al., "Effects of co-culture and embryo number on the in vitro development of bovine embryos," *Theriogenology*, vol. 47, no. 8, pp. 1549–1561, 1997.

[45] G. A. Palma, A. Clement-Sengewald, U. Berg, and G. Brem, "Role of embryo number in the development of in vitro produced bovine embryos," *Theriogenology*, vol. 37, p. 271, 1992.

[46] F. A. Ward, P. Lonergan, B. P. Enright, and M. P. Boland, "Factors affecting recovery and quality of oocytes for bovine embryo production in vitro using ovum pick-up technology," *Theriogenology*, vol. 54, no. 3, pp. 433–446, 2000.

[47] L. Ferry, P. Mermillod, A. Massip, and F. Dessy, "Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage," *Theriogenology*, vol. 42, no. 3, pp. 445–453, 1994.

[48] E. M. Senatore, M. E. Mannino, M. V. Suarez Novoa, et al., "Synergistic effect on embryo development by inclusion of supplemental embryos embedded in agar chips," *Reproduction, Fertility and Development*, vol. 21, no. 1, p. 208, 2009.

International Journal of
Agronomy

Scientifica

Veterinary Medicine
International

The Scientific World Journal

International Journal of
Ecology

International Journal of
Microbiology

Journal of
Viruses

Biotechnology Research International

Psyche

Journal of
Insects

Journal of
Veterinary Medicine

Case Reports in Veterinary Medicine

International Journal of
Zoology

International Journal of
Evolutionary Biology

International Journal of
Genomics

International Journal of
Cell Biology

Journal of
Parasitology Research

Journal of
Animals

Applied & Environmental Soil Science

Hindawi

Submit your manuscripts at
http://www.hindawi.com