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Research Article

IN VITRO EMBRYO PRODUCTION AND TRANSFER OF BUBALINE EMBRYOS USING OOCYTES DERIVED FROM TRANSVAGINAL ULTRASOUND-GUIDED FOLLICULAR ASPIRATION (TUFA)

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Abstract

Transvaginal ultrasound-guided follicular aspiration (TUFA) has become a popular tool for embryo production *in vitro* due to its high degree of repeatability in terms of recovering oocytes from live animals. In Study 1, the quantity and quality of oocytes from Bulgarian Murrah buffalo cows (n=10) of varying ages (Group 1, 8-12; and Group 2, 13-17 years) were assessed. Group 1 buffalo donor cows yielded significantly higher ($P<0.05$) number of oocytes vs Group 2 buffalo donor cows (71 vs 29 oocytes, respectively), though in terms of oocyte quality, no difference was observed.

In Study 2, oocytes collected (n=100) in Study 1 were matured, fertilized *in vitro* and the resulting zygotes were cultured which developed to blastocyst stage embryos. The maturation, fertilization and blastocyst development rates obtained were 53.0%, 40.0% and 32.5%, respectively. In Study 3, the viability of resulting blastocyst stage embryos was determined by transferring to recipient cows. Of 10 recipients 1 got pregnant and delivered a 35 kg male calf after 310 days gestation period. Overall, the results of the studies conducted demonstrated the potential of TUFA technology in the *in vitro* production of embryos which eventually could be used in the production of live offspring.

Key Words: Transvaginal Ultrasound-guided Follicular Aspiration Bubaline embryos; oocytes; IVEP

Introduction

The *In Vitro* Fertilization and Embryo Transfer (IVF-ET) techniques for improving the genetic improvement of buffaloes have been successfully demonstrated at the institutional farm and at the farmer's level in the Philippines. Success rate using IVF-ET technique is low so to strengthen the implementation of the carabao development program, the Philippine Carabao Center is continuously in search for a practical but efficient technique to enhance the genetic improvement program of buffaloes for milk and meat.

The use of TUFA which was originally applied in human and was first used in cattle in the early 1990s (Boni *et al.*, 1994), has provided the breeding industry the opportunity to increase the number of calves from donors of high genetic merits. It made available oocytes from live cows, giving the opportunity to use germplasm of known source for embryo production. The TUFA technique in combination with *In Vitro* Embryo Production (IVEP) technology has been a routine procedure and widely used particularly in the cattle breeding industry. The use of TUFA technology in buffalo started in 1993 (Boni *et al.*, 1994) and there has been an increasing interest in TUFA-IVP technologies for embryo production in water buffaloes (Boni *et al.*, 1996, Neglia *et al.*, 2003, Techakumphu *et al.*, 2004; Yadav *et al.*, 2006).

The application of TUFA technique was carried out in this study to 1) determine the effects of age of buffaloes on the quantity and quality of the oocytes collected from the live donor cows and 2) monitor the *in vitro* maturation (IVM), IVF, IVC and 3) determine the viability of embryos produced through TUFA using the ET technology.

Materials and Methods

Study1: Quantity and quality of Bubaline oocytes using Transvaginal Ultrasound-Guided Follicular Aspiration (TUFA) technology

The donor buffalo cows

Ten (10) Bulgarian Murrah buffalo cows raised at the PCC-Gene Pool farm were used in the study. The donor cows were assigned into groups, i.e. 8-12 years (Group 1) and 13-17 years, (Group 2), average body weight of 600 kg and a body condition score of 3.5 to 4.0. Each age group consisted of 5 cows. The experimental cows were maintained under complete confinement system of management. The animals were raised in group in a free stall where they were given standard rations composed of Napier soilage (40%), rice straw (40%), spent grain (7%) and supplementary concentrate pellets (13%).

Transvaginal Ultrasound-Guided Follicular Aspiration (TUFA)

Follicles from each of the ten donor cows were collected using the developed protocol on TUFA technology. The TUFA session was done in the morning while they were restrained in a standing position which involved the aspiration of ovarian antral follicles with diameter size > than 5mm through the vaginal route for the collection of oocytes. Animals were restrained in a chute while in standing position. The ovaries were positioned by rectal palpation in front of a vaginal scanner, TUFA Probe, Model HCV 4710 MV and with the use of an Ultrasonic Scanner Model HS-2000, Honda Electronics Co. Ltd., Japan. The probe is equipped with a 50-cm long needle (FHK #01281350, Japan), 18g, which drew the samples into a sterile tube under 50mm Hg permanent negative pressure with a vacuum pump (FHK, Model #4, Japan). Oocytes were collected using Phosphate Buffered Saline solution (PBS) with 0.3% Polyvinyl Alcohol (PVA), 50 µg/mL gentamycin and 20µg/mL heparin. A clean, labeled, 50 ml collecting tube was prepared and assigned to each of the donor cow every time the TUFA activity was conducted. After each TUFA session, the silicon tubing was thoroughly washed with PBS to ensure that all aspirated oocytes were collected in 50 ml collecting tube. The aspirated follicular samples were maintained at 37°C in a portable incubator and brought to the laboratory for further processing. The contents of the tube were washed and searched using an Encom Filter (Fujihira, Japan) under stereomicroscope (Nikon, Japan).

Searching and evaluation of follicular aspirates

The aspirates derived from follicular aspiration were brought to the laboratory. Extra care was observed in handling and transporting in order to maintain its livability. The collected aspirates were placed in the pre-warmed water bath with temperature set at 37° C. The contents of each of the collecting tube were poured into a searching dish using an Encom Filter (Fujihira, Japan). The tubes were washed thoroughly with PBS solution to ensure that no cumulus oocyte complexes (COCs) were left in the tube. The poured aspirates in the Emcon filter were washed thoroughly with PBS solution to ensure that the aspirates were totally clear and free of blood. Searching of the COCs was done under the stereomicroscope (Nikon, Japan). The number of COCs from each donor cow was counted, recorded and were classified according to the morphology of the ooplasm and the appearance of the cumulus cells as followed: rank A with intact 3 layers cumulus cells, rank B with uneven dark ooplasm but had 3 layers intact cumulus cells, rank C homogenously dark granulation of ooplasm and with intact/compact, 1 to 2 layers cumulus cells and rank D without cumulus cells (PCC Reproductive Biotechnology Laboratory Classification System

Study 2: In Vitro Maturation, Fertilization and Culture of Bubaline Oocytes Derived from Transvaginal Ultrasound-guided Follicular Aspiration.

IVM/IVF/IVC

The searched COCs were washed several times with PBS solution and were then placed in a Falcon Petri dish, 35 X 10 mm with droplets of TCM-199, 50 µl each layered with mineral oil, (M-8410 Sigma) which was pre-incubated in a water jacketed incubator for at least 2 hours. After washing the oocytes in the maturation medium, they were finally placed in the maturation droplet and incubated for 22-24 hours maturation period.

In vitro fertilization (IVF) of matured oocytes was done using frozen buffalo semen thawed at 37ºC for 15 seconds. The thawed semen were washed in a 15 ml centrifuge tube with pre-incubated modified Brackett and Oliphant medium (BO medium, Brackett and Oliphant, 1975) by centrifugation at 800 x g for 5 minutes. After centrifugation the supernatant was discarded, leaving only about 200ul of the sperm pellet.

Semen concentration was determined using a hemocytometer and adjusted to 4 x 10^6 sperm/mL concentration with BODM. Twenty five µL of the semen suspension was added to pre-equilibrated fertilization droplets consisting of 25ul (equal volume of IVF medium and sperm pellet) BODM supplemented with 3mg/mL BSA and 2.5mM theophylline, to have a final concentration of $2x10^6$ sperm/mL. The *in vitro* matured oocytes were transferred to the fertilization droplets. The spermatozoa and oocytes were co-incubated for 16 to 18 hr at 38.5ºC under 5% CO_2.

The preparation of the *in vitro* culture medium was done using the modified Synthetic Oviductal Fluid medium (mSOF). *In vitro* culture working medium was prepared by adding 30 mg of Bovine Serum Albumin (BSA 6003) to 10ml mSOF and was again filtered. In a 35 x 10 mm Falcon Petri dish, several microdroplets with 50ul droplets each of the IVC medium were pipetted and layered with mineral oil.

The prepared dish was placed in a triple gas incubator. Thereafter, the *in vitro* fertilized oocytes were transferred into the IVC dish containing the mSOF microdroplets with BSA. Sperms attached to the oocytes were totally removed before they were placed in the IVC dish. This process was done carefully in order not to harm the oocytes. The pipette that was used to remove the sperms attached to the fertilized oocytes was prepared with fine surface and with a small bore just good enough to remove the sperms surrounding the fertilized oocytes. The fertilized oocytes were washed in the separate IVC droplets and were then placed in the final culture droplets. The culture dish containing the fertilized oocytes was incubated in a humidified incubator with a gas phase of 5% O_2, 5% CO_2 and 90% N_2 level. The cleavage rate was observed 24 hours post-culture. The number of embryos that developed into blastocysts on the Day-7 of *in vitro* culture was observed and recorded.

Study 3. Embryo Transfer of Embryos Produced through Transvaginal Ultrasound guided Follicular Aspiration- *In Vitro* Embryo Production Technology

Preparation of the surrogate cow

To ensure the receptivity of the ten recipient cows, timing of estrus was properly observed. Based on the ovulation of the recipient, the transfer of the blastocyts was done on the 5^{th} or 6^{th} day. However, some embryos were transferred based on the signs of estrus of the recipient cow, where ET was done at the 6^{th} or 7^{th} day.

Preparation of Embryos before Transfer

The embryos that were produced for ET were placed in a 0.25 ml straw with the aid of a stereomicroscope. Each straw contained 1 embryo. The *in vitro* culture medium, mSOF medium was used for the actual conduct of the ET. First, a little amount of mSOF was aspirated to the straw then an air space followed in between the mSOF medium with the embryo then another air space and finally, aspirated mSOF medium again which wet the cotton plug that rendered the embryo safe from getting lost. The embryo was in the mid-part of the straw. The straw with the embryo was then loaded in the ET gun and embryo transfer followed.

Embryo Transfer

Before the transfer of the embryo(s), the presence of corpus luteum in the ovaries was checked by rectal palpation. For easier insertion of ET gun into the cervix, a cervix expander (FHK, Japan) was first inserted to the vagina of the recipient animal. After expanding the cervix, the expander was removed and the ET gun containing the embryo was inserted to the entrance of the cervix with an outer sheath. The tip of the ET gun is placed into the external os of the cervix and is then pushed through the sheath before it is guided as gently as possible through the remainder of the cervical canal and into the uterine horn on the side of the corpus luteum. The embryo is then gently deposited, approximately one-third of the way to the uterine horn and the ET gun is withdrawn slowly. Embryo transfer was done by a technician of the PCC.

Confirmation of Pregnancy

Recipient cows were taken cared of and properly monitored after ET. Rectal palpation of the ten recipient cows was done 60 days after the transfer. The cow that was confirmed pregnant was continuously monitored up to the calving period.

Results and Discussion

Results in study 1 involving the average quantity and quality of collected oocytes in the 22 TUFA sessions in ten donor cows were summarized in Table1. There were more COCs (71) collected in Group I than in Group 2 cows with 29 COCs. The same trend was observed where Su *et al.* (2009) aspirated fewer follicles and obtained lower COC numbers in old cows than in middle-aged and young cows. The collected COCs from Group I were classified into rank A, 5.63% (n=4) with intact 3 layers cumulus cells, rank B, 2.82% (n=2) with uneven dark ooplasm but had 3 layers intact cumulus cells, rank C, 32.39% (n=23) homogenously dark granulation of ooplasm and with intact/compact, 1 to 2 layers cumulus cells and rank D, 59.15% (n=42) without cumulus cells. In the older donor cows (Group 2), the COCs derived were classified into rank A, 6.90% (n=2), rank B, 6.90% (n=2), rank C, 31.03% (n=9) and rank D, 55.17% (n=16).

Table 1: Average quantity and quality of collected COCs by TUFA technique in Bulgarian Murrah buffalo cows.

Donor Cow Age	N	No. of TUFA Sessions	No. of Oocytes	Quality and % of collected oocytes							
				A	%	B	%	C	%	D	%
8-12	5	22	71	4	5.63	2	2.82	23	32.39	42	59.15
13-17	5	22	29	2	6.90	2	6.90	9	31.03	16	55.17

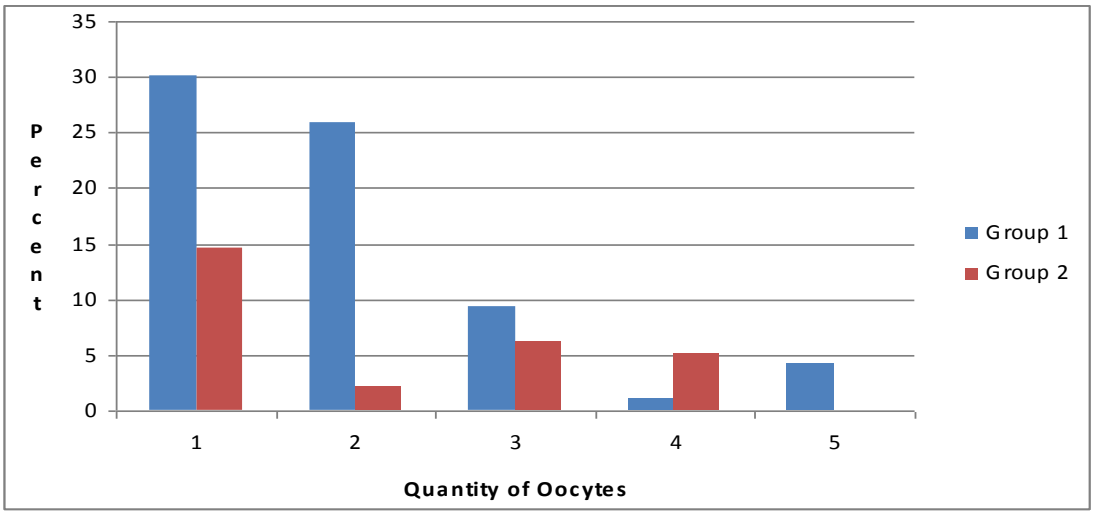

Fig. 1: Quantity of collected bubaline oocytes**, %**

Table 2. Maturation, fertilization and embryo development rates (%) of TUFA- derived oocytes

No of oocytes	Matured	2-cell	Morula	Blastocyst	Unfertilized	Degenerated
100	53 (53.00%)	40 (40.00%)	13 (32.50%)	13 (32.50%)	26 (26%)	16 (16%)

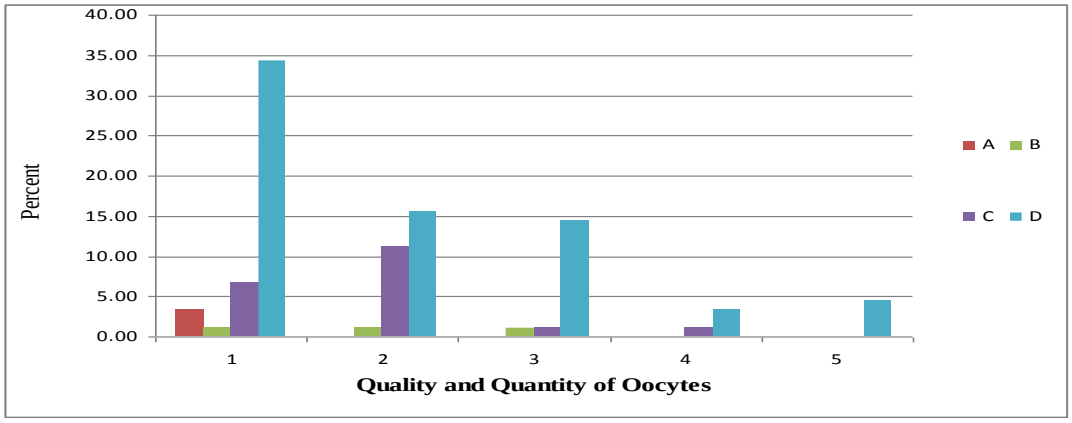

Fig. 2: Percentile of quality ranks of oocytes

For the study 2 age groups there is no significant difference on the quality of oocytes collected but has significant difference only on the quantity of oocytes collected with the younger age group yielded 71 oocytes compared to the older group of cows with only 29 oocytes collected.

Fig. 1 presents the percent quantity of collected oocytes in the 2 age groups of donor cows. Group 1 donor cows that gave 1 oocyte are as much as 30.2% compared to Group 2 with only 14.6%. Twenty six percent of Group 1 donor cows gave 2 oocytes while for Group 2, only 2.1 %. Group 1 donor cows collected with 3 oocytes had as much as 9.4% while for the other group, only 6.2%. One percent of the Group 1 donor cow gave 4 oocytes while for Group 2, only 5.2%. Only Group 1 donor cows, 4.2%, gave 5 oocytes.

In study 2, of the 100 COCs, 53% maturation rate was recorded after 22-24 hours maturation period, Table 2. Twenty six (26%) of the COCs were not fertilized and 16% were degenerated. The matured COCs (53%) that were subjected to fertilization for 24 hrs period gave a fertilization rate of 40%. Of the fertilized embryos, 13 (32.50%) developed to morula stage embryo and 13 embryos developed further to blastocyst (32.50%) 7 days after in vitro fertilization.

Fig. 2 presents the percentile ranking profile of the qualities of the oocytes collected from the 2 age groups of donor

buffalo cows. Most of donor cows gave 1 oocyte ranked with different qualities, i.e. 3.3% for rank A oocytes, 1.1% rank B oocytes, 6.7% rank C oocytes and 34.4% rank D oocytes. Some of the donor cows were collected with 2 oocytes belonging to quality ranks B, C and D at 1.1%, 11.1% and 15.6%, respectively. In the figure most of the collected COCs from the donor cows that gave 3 oocytes were dominated by rank D oocytes, 14.4% as against 1.1% for rank B and C oocytes. Very few of the donor cows were collected with 4 oocytes with ranks C and D and with percentage values of 1.1 and 3.3, respectively. Those donor cows that were collected with 5 oocytes gave only rank D oocytes equivalent to 4.4 %.

In study 3, ten of the 13 blastocysts produced were transferred to ten surrogate cows, where an embryo developed to full term resulting to a live birth of a male calf. These TUFA-derived oocytes after IVM, IVF and ET gave a success rate of 10%.

Conclusion

The application of TUFA technology gave significant results in terms of the number of oocytes collected from younger donor buffalo cows. The fertilization rate achieved, the number of *in vitro* produced embryos and the resulting live birth of a calf clearly demonstrated that TUFA-IVEP protocol can be practically used and applied in enhancing the production of genetically superior calves for dairy production purposes.

Recommendations

TUFA-IVEP is a promising technology that could be applied in assisted reproduction of ruminant species. Improving IVEP conditions in the future will require strong research effort to improve the understanding of oocyte and early embryo physiology and gene expression. This technology can be used to develop *in vitro* treatments more adapted to precise requirements of oocytes and early stage embryos.

Donor cows for TUFA as sources of oocytes for IVEP should be those that just have calved once or twice for better in vitro embryo production results.

References

Boni RS, Roviello S and Zicarelli L (1996) Repeated ovum pick-up in Italian Mediterranean buffalo cow. *Theriogenology* **46**:899-909. DOI: 10.1016/S0093-691X(96)00248-8

Boni RS, Di Palo V and Barbiere L (1994) Ovum pick-up in deep anestrus buffaloes. *Proc IV World Buffalo Congress* **3**:480-482.

Neglia G, Gasparini B, Brienza VC, Palo D, Campanile G, Presicce G and Zicarelli L (2003) Bovine and buffalo *in vitro* embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicular aspiration. *Theriogenology* **59**:1123-1130. DOI: 10.1016/S0093-691X(02)01170-6

Su L, Yang S, He X, Li X, Ma J, Wang Y, Presicce G and Ji W (2009) Effect of Donor Age on the Developmental Competence of Bovine Oocytes Retrieved by Ovum Pick Up. *Reprod. Domest. Anim*. **47**(2):184-189. DOI: 10.1111/j.1439-0531.2009.01349.x

Techakumphu M, Promdireg A, Na-Chiengmai A and Phutikanit (2004) Repeated oocyte pick up in prepubertal swamp buffalo (*Bubalus bubalis*) calves after FSH stimulation. *Theriogenology* **61**:705-1711.

Yadav SK, Misra AK, Sharma R, Prasadand S and Gupta HP (2006) Repeated transvaginal ultrasound-guided aspiration of oocytes (OPU) from Murrah buffaloes. Proceedings, 5th Asian Buffalo Congress, Naning China, April 18-22.