Ethiop. J. Agric. Sci. 32(4) 88-104 (2022)

Ovum Pick up and *In Vitro* Embryo Production in Boran and Crossbred Dairy Cattle

Jeilu Jemal[1], Asnaku Funga[2], Sayid Ali[2], Mosisa Dire[2], Tamrat Degefa[2], Tefera Yilma[3] and Alemayehu Lemma[3]

[1] Ethiopian Institute of Agricultural Research (EIAR), National Agricultural Biotechnology Research Center, P. O. Box 249 Holeta, Ethiopia

2 Ethiopian Institute of Agricultural Research (EIAR), Debre Zeit Agricultural Research Center, P. O. Box 32 Bishoftu, Ethiopia

[3]Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Clinical Studies, P. O. Box 34 Bishoftu, Ethiopia

Abstract

*This study was conducted to study the potential of Boran (Bos indicus) and Boran * Holstein Friesian Crossbred cows for non-stimulated transvaginal oocyte collections and in vitro embryo production. Follicular aspirations were done using a vacuum pressure pump and Aloka SSD Prosound-2 ultrasound device. 266 aspirations were made in Boran (n=148) and Crossbred (n=118) cattle. The mean number of punctured follicles for combined collections was 7.68 ± 0.4 for both genotypes. The mean number of recovered oocytes for all collections was 2.98 ± 0.2 for both genotypes. The mean number of punctured follicles was 7.27 ± 0.4 and 8.22 ±. 0.44 for Boran and Crossbred cattle, respectively. The mean number of recovered oocytes was 2.65 ± 0.22 and 3.42 ± 0.26 for Boran and Crossbred cattle, respectively. Differences were observed ($p<0.05$) in the mean number of punctured follicles and recovered oocytes between the genotypes. The oocyte recovery rate for the total puncture sessions were 45.8%. There was no difference between the breeds regarding the number of quality oocytes collected. Higher numbers ($p<0.001$) of GIII oocytes were collected in all aspirations compared to GI and GII quality oocytes. The maturation rate of Boran and Crossbred cattle oocytes was 51.65% (47/91) and 56.8% (50/88), respectively. Cleavage rate of Boran and Crossbred cattle oocytes was 40.7% (37/91) and 31.8% (28/88), respectively. There was no difference ($p>0.05$) in maturation and cleavage rate of oocytes between the genotypes. A total of 21.5% (14/65) of cleaved oocytes developed to morula stage embryos from both genotypes. Relatively similar patterns were observed in maturation process and embryo development between Boran and Crossbred cattle oocytes. OPU/IVF procedures could be used in local and crossbred heifers as an alternative breeders technology options in animal breeding programs to best exploit the genetic potential of local and exotic breeds.*

Keywords: Estrus cycle; ovarian follicle; transvaginal ovum pickup; ultrasound.

Introduction

The advent and advancement of reproductive technologies have wide opened research and experimentation avenues to manipulate reproduction both in in-vitro embryo production (IVEP) and multiple ovulation and embryo transfer (MOET) or *in vivo* derived embryo technologies. This has been used to improve reproductive performance in various species of farm animals. Artificial

insemination (AI) is the most widely used assisted reproductive technique (ART) in dairy cattle breed improvement programs. Its application brings a wide range of benefits to dairy cattle production compared to natural mating practices (Baruselli *et al.,* 2018a). Artificial insemination and estrus synchronization deployment in dairy cattle breed improvement has a fairly long history in Ethiopia. The country has a huge cattle population and enormous diversity of genetic resources that could be exploited through the application of advanced reproductive technologies OPU/IVEP included. The female individual has an average of 200,000 oocytes by the time of puberty. Although between 20 and 40 follicles are recruited during follicular selection, only a single dominant follicle will eventually ovulate while the rest will be atretic. This most inefficient natural cycle repeats year round if it is not interrupted by pregnancy culminating in less than 10 offspring as life-time productivity at best.

Technology options such as the ultrasound guided trans-vaginal follicular aspiration (TVFA) or ovum pick up (OPU) has opened a great opportunity to best exploit the surplus follicular reserve which otherwise would be wasted. OPU, after it was originally established by Pieterse and his colleagues in 1988; it has become a field of great interest due to its potential commercial application (Pieterse *et al.,* 1988). OPU is the most flexible and repeatable technique to produce embryos from nearly any given live donor including cattle of high genetic value. The application of this technique to juvenile calves can further accelerate genetic gain by substantially decreasing the generation interval. It also suits bovines with genital tract illness or low sensitivity to super ovulatory treatments.

The adoption of these techniques can improve the options for selective breeding and hence hastens genetic gain from superior dams and sires by facilitating dissemination of genetic material and shortening the generation interval and increased desired genetic progress from the dam side (Meuwissen, 1991).

Reproductive physiology of zebu cattle breed is not identical to *Bos taurus* cows, and differences in a number of reproductive characteristics are known to exist (Viana *et al.,* 2000; Viana *et al.,* 2010a). Greater number of growing follicles throughout the estrous cycle in zebu cattle and recovery of more cumulus oocyte complexes (COCs) per ovum pick up (OPU) session compared to *Bos taurus* cattle accounted as considerable deference between the breeds (de Roover *et al.,* 2008).

OPU/IVEP techniques help to boost reproductive efficiency of local zebu breeds and make the best use of advanced reproductive biotechnology tools. Thus, the aim of this study was to evaluate alternative reproductive techniques such as OPU/IVEP and assess the potential of Boran breed for non-stimulated follicular aspirations and *in vitro* embryo production, for dairy cattle genetic improvement and multiplications.

Materials and Methods

Location

Experiments were conducted at Holeta and Debre Zeit Agricultural Research and National Agricultural Biotechnology Research Centers, Ethiopia. Holeta is located 29 km west of the capital city, Addis Ababa, with Longitudes 38º38' east' Latitude 09º04' north and with Altitude of 2,390 meters above sea level. The minimum and maximum temperatures at Holeta are 6.13°C and 22.8°C, respectively. The rainfall pattern is bimodal with short rains from March to May and long rains from June to August. The mean annual rainfall is about 1,243.7 mm with peak rain in August (HARC, 2019). Debre Zeit Agricultural Research Center is located 45 km east of Addis Ababa (8°46′13.57"N, 38°59′50.45"E) at an altitude of 1920 meters above sea level. The average annual temperature is 18.7°C with an average annual rainfall of 757.05 mm (DZARC, 2020). The National Agricultural Biotechnology research center is located in Holeta town and has similar agro ecology with that of HARC.

Experimental Animals

Experimental animals at Holeta (n=12) and Debre Zeit (n=25) Agricultural Research Centers were employed for oocyte aspiration and IVF work. Indigenous Boran (n=20) and Boran * Holstein Friesian (HF) Crossbred (n=17) cows were employed in this study. Animals used in this experiment (heifers n=12 and cows n=25) were less than ten years of age. Breeding and clinical records of all animals were reviewed, and females with reproductive problems were excluded from the study. Cows underwent a thorough reproductive examination prior to the commencement of the study. Animals were allowed to graze on natural pastures. Hay made from straw and grass constituted the major proportion of the roughage supply. Concentrates, composed of wheat by-products or maize; Noug seed cake (*Guizotia abyssinica*) and salt were used as supplementary feed. Water was provided ad-libitum.

Media preparation

The media for oocyte collection, maturation, fertilization and culture were purchased from Mini Tube readymade stock solution for IVEP. The stock solutions were reconstituted for the final working solution. The BO IVEP ready-to-use media was also used for oocyte maturation, fertilization and culture. Tissue culture grade double distilled water purchased from the local market. Distilled water produced at the laboratory was used for non-biological laboratory routines. Media was prepared under a biosafety cabinet, pH was adjusted accordingly and syringe filtered using 0.22µ filter.

Ovum pick up

OPU could be performed routinely twice a week without pre-stimulation and without any detrimental effects on fertility of the donor cows (Galli *et al.*, 2001). The OPU set-up had a real-time B-mode ultrasound system (Aloka SSD Pro-Sound 2, Japan) with a 6.5-MHz convex sector probe transducer (Hitachi Medical Co., Tokyo, Japan) that was used to visualize the ovaries and a needle guide system (Mini-tube, GmbH, Germany). Visible follicles ≥ 2mm in diameter were aspirated using a disposable 1.2x75mm (for cows) hypodermic needle that was connected to a 50ml falcon tube via a silicon Teflon tube through the OPU probe holder to the warm block of the vacuum pressure. The 0.9x70mm hypodermic disposable needle was used for heifers (Mini-tube, Germany). A vacuum pressure pump (Mini-tube, GmbH, Germany) with adjustable aspiration volume, adjusted between 72-80 mmHg to optimize the pressure, was employed for aspiration. The vacuum pressure had a medium warming block temperature (37^{0}C) at a flow rate of 15-20 ml/min, and was used to recover follicular fluid. Oocytes were collected into a phosphate buffer solution (PBS) preparations supplemented with heparin 20µg/ml, 2% FCS, 50µg/ml gentamicin, and 2.4mg/ml HEPES and maintained at 37^{0}C. Each aspirated animal was given 2-3 ml of 2% lidocaine for epidural anesthesia before follicular aspiration procedures.

In vitro oocyte maturation

Prior to *in vitro* maturation, COC's were washed three times in TCM supplemented with 2.4mg/ml HEPES, 10% FCS and 0.1 mg/ml gentamicin sulfate and 10 µg/ml Pluset® FSH+LH. At each collection procedure COCs of each breed were separately cultured for 24hr in 500 µl drops of maturation media supplemented with 10%FCS, 10 µg/ml Pluset® FSH+LH, 0.1mg gentamicin, 2.2mg/ml NaHCO3 in five well embryo culture dishes under mineral oil at 39°C under an atmosphere of 5% carbon dioxide at maximum humidity. The pH of the maturation media was adjusted between 7.2-7.4. Quality oocytes, grade I, II and III were used in the maturation experiment. Denuded grade IV oocytes were discarded from the experiment. After 24hr of incubation oocytes were examined morphologically for cumulus cells expansion, increase in perivitelline space, extrusion of first polar body and change in cytoplasmic color and zona pellucida shape, denoting maturation and readiness for exposure to sperm. Maturation rate was calculated from the total oocyte ready for insemination over total incubated oocyte for maturation.

Semen preparation

Frozen semen was subjectively checked for motility and viability before semen was processed for in vitro fertilization. Straws of semen less than 40% motility were not used in this experiment, So as to not influence the result of fertility rate of oocytes.

Swim up

For swim up semen preparation procedure; 2ml TALP semen capacitation medium added and equilibrated at 5% CO_2 at 39^{0}C for an hour in 15 ml test tube. Two straws of semen were thawed at 35 ^{0}C for 30 seconds and added to a 15ml conical tube during every fertilization experiment. The thawed semen was expelled to 2ml semen preparation media in a 15ml conical tube. The semen centrifuged at 200g for 5 minutes and 1ml of the supernatant discarded. 250µl sperm suspensions added to four 2ml test tubes containing 1ml sperm preparation media (TALP) and the test tubes placed in 39^{0}C incubator at 5% CO_2 for an hour. The supernatant (800ul) from each test tube taken and the combined samples were centrifuged at 100g for 5 min. 500µl of the supernatant was discarded from the final sample. Sperm concentration was checked using a hemocytometer and a microscope (40 X magnifications).

Percoll density gradient centrifugation

Percoll 90% was prepared by adding 1 volume of sperm-TALP medium as a diluent to 9 volumes of Percoll (1:9 v/v) and Percoll 45% was prepared by adding 1 volume of Percoll 90% to 1 volume of sperm-TALP medium (1:1 v/v) (Garcia-Herreros *et al.*, 2010).

Two straws (0.5 ml) of frozen bull semen were thawed at 35^{0}C for 30 second and gently layered over the two layers of Percoll 45% and Percoll 90% into a 15 ml conical centrifuge tube. The sperm sample was centrifuged for 10 min at 800g at room temperature. The supernatant was discarded by aspiration. The re-suspended pellet washed and centrifuged at 200g for 5 minute at room temperature. Recovered spermatozoa were re-suspended with 0.5 ml of sperm-TALP medium. Sperm concentration was calculated with a hemocytometer. 250 µL of the diluted sperm suspension was added to each well. Fertilization plates were returned to the incubator and the sperm and COCs were co incubated for 18 to 22 hrs. To evaluate efficiency of DGC, viable and dead remaining cells in the different percoll layers were checked by subjectively counting under microscope (100X).

In vitro fertilization

Fertilization plates were prepared by placing 500 µl drops of IVF-TALP in a five well embryo culture dish and covered with 500 µl mineral oil. The COCs were washed by HEPES-TALP prior to fertilization. Fertilization plates with IVF-TALP were placed in an incubator at 39^{0}C in an atmosphere of 5% CO_2 and allowed to equilibrate for at least 2 hrs. The PH of the capacitation and fertilization media was adjusted at 7.4 and 7.8, respectively.

10 to 20 COCs removed from maturation drops and washed. Each group of 10 to 20 COCs was placed in a fertilization media of TL fertilization supplemented with 6mg/ml BSA, 0.11mg/ml Na pyruvate and 10µg/ml Heparin.

Statistical Analysis

Data were grouped according to breed and parity of animals, oocyte quality/grade for once and twice weekly oocyte collection scheme. Analysis of variance was used to compare the mean number of viable oocytes collected from the two OPU procedures and each breed. Maturation rates were calculated by the number of oocytes presenting expanded CCs, first polar body formation and increased perivitelline space on the total evaluated oocytes that resulted from different breeds; and cleavage rate were analyzed by Mann-Whitney U-Test, Kruskal-Wallis H-Test and Fisher exact test. Results are presented as mean ± SEM and SD and level of significance was held at $P<0.05$.

Results

Oocyte collection

A total of 1800 follicles ≥ 2mm were punctured and 825 oocytes were retrieved during the entire experiment. The proportion of oocyte recovery for the whole puncture sessions was 45.8%. The mean number of punctured follicles for heifers and cows was 13.53±5.10 and 7.93±.85, respectively. The mean number of aspirated follicles from heifers and cows was 5.72±2.09 and 3.47±1.07, respectively. There was significant difference ($p<0.001$) in mean number of pre OPU follicular count between heifers and cows. There was also a difference ($p<0.001$) in the mean number of aspirated oocytes between the groups.

There was a difference ($p<0.001$) in mean number of recovered oocytes per session between once (5.70 ± 2.10) and twice (2.96 ± 2.0) a week collection scheme. There was no difference between the breeds regarding the number of quality (morphological assessment) oocytes collected by both (once and twice weekly) collections. However, higher numbers ($p<0.001$) of GIII oocytes were collected in both aspirations compared to GI and GII quality oocytes.

In vitro oocyte maturation

The overall maturation rate of oocytes aspirated from Boran and Crossbred cattle was 54.12% (97/179). The maturation rate of Boran and Crossbred cattle oocytes was 51.65% (47/91) and 56.8% (50/88), respectively. Maturation rate in terms of COCs expansion was 74.7% (n=72) and 78.4% (n=45) for Boran and Crossbred cattle oocytes, respectively. The proportion of increased perivitelline space in matured Boran and Crossbred heifers oocytes were 45.1% (n=72) and 52.3% (n=45), respectively. 54.9% (n=72) of Boran and 67.0% (n=45) Crossbred cattle oocytes exhibited the first polar body at the end of maturation. There was no difference ($p>0.05$) in maturation rate of Boran and Crossbred cattle oocytes. The color and shape of aspirated oocytes were not identical. Small, partially denuded and very dark oocytes had shown no palpable change during maturation.

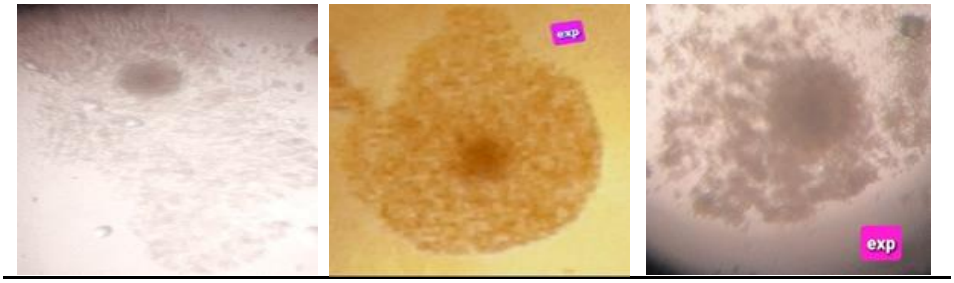

Plate A. Comulus cells expansion after 24hrs maturation

Plate B. First polar body extrusion

Figure 1: Plate A) Cumulus-cells expansion after 24hrs maturation; Plate B) 1PB extrusion.

The maturation rate of oocytes in BO maturation media in terms of COCs expansion, first polar body extrusion and increased perivitelline space was 88.2%, 68.2% and 47.3%; (n=64), respectively. The maturation rate in TCM based maturation media in terms of COCs expansion, first polar body extrusion and increased perivitelline space was 57.97%,49.3% and 50.7%: (n=53), respectively. The proportion of oocytes matured in BO media was (53.5%, n=40) and (48.7%, n=24) for purebred Boran and crossbred cattle, respectively. The proportion of matured oocytes in TCM-199 based maturation media was (69.2%, n=32) and (43.3%, n=21) for Boran and their crosses, respectively. There was no difference (p>0.05) in maturation rate of Boran and crossbred cattle oocytes cultured in TCM-199 based or BO maturation media.

Table 1: Comparison of Boran and Crossbred cattle oocyte maturation rate (% ± SD)

Breed	N	Cultured oocyte	COC expansion	1PB extrusion	Increase in perivitelline space
Boran	72	68.9±0.42	74.7 ±0.50	54.9 ± 0.41	45.1 ±0.37
Crossbred	45	73.9±0.33	78.4 ±0.43	67.0 ± 0.42	52.3 ± 0.40

Semen viability and in vitro fertilization

Semen viability

The percentage of subjective motility of the HF bull frozen thawed semen that used for percoll density gradient centrifugation treatment was 59.3% ± 3.63 immediately at thawing prior to centrifugation. The percentage of sperm motility

after processing by Percoll separation techniques was 74.4%. There was a significant difference (p<0.05) in motility between initial and processed semen motility. The proportion of motile spermatozoa at the 45% and 90% percoll layers was 27.4% ± 3.48 and 33.9% ± 2.63. The proportion of motile and dead sperm cells at the bottom (pellet) was 74.4 ± 2.78 and 25.6 ± 2.78, respectively. The mean concentration of sperm cells for percoll and self-migration separation techniques was 58.0% and 18.4%, respectively. There was a significant difference ($p < 0.05$) in the sperm cells concentration values between the sperm separation methods. There was a significant difference ($p < 0.05$) between sperm viability before and after processing. However, there were no significant differences for the sperm motility between the sperm preparation methods.

The comparison of dead and motile sperm cells at different layers of percoll gradient centrifugation treatment indicated in **Table 2**.

Table 2: Sperm viability after percoll density gradient centrifugation (%) ± SEM

Motility	Initial	45% percoll	90% percoll	Pellet
Viable	59.30 ± 3.63	27.40 ± 3.48	33.90 ±2.63	74.40 ± 2.78
Dead	40.70 ± 3.63	72.60 ± 3.48	66.10 ± 2.63	25.60 ± 2.78

In vitro fertilization

The cleavage rate for all inseminations combined was 36.31% (65/179). Cleavage rate of Boran and Crossbred zygotes was 40.7% (37/91) and 31.8% (28/88), respectively. Twenty two percent (14/65) of cleaved zygotes were developed to embryos in 72 hrs post insemination. There was no difference (p>0.05) in cleavage rate between the zygotes of the two genotypes at 48 hrs of post insemination. The proportion of cleaved zygotes was slightly higher in Boran oocytes than their crosses. The cleavage rate for BO and TCM-199 based media was (30.0%) 32/110 and (46.4%) 33/69, respectively. There was no difference (p>0.05) in cleavage rate with the two media treatment groups (p>0.05). A total of 21.05% (14/65) of cleaved zygotes were developed to morula stage embryos from both genotypes. 24.3% (9/37) of Boran and 17.86% (5/28) of Crossbred cattle cleaved zygotes were reached morula stage embryos after 72 hrs of post insemination incubation. Comparison of maturation and cleavage rate between the breeds and media employed indicated in **Table 3**.

Table 3: Comparison of Boran and Crossbred oocyte after in vitro maturation and fertilization (%) ± SEM

Breed	Media	n,(oocyte)	n, %(Matured)	n, %(cleaved)
Boran	BO	57	26 (45.6)	20 (35.1)
	TCM-199	34	21 (61.8)	17 (50.0)
Crossbred	BO	53	31 (58.5)	13 (24.5)
	TCM-199	35	19 (54.3)	15 (42.9)
Total		179	97 (54.2)	65 (36.3)

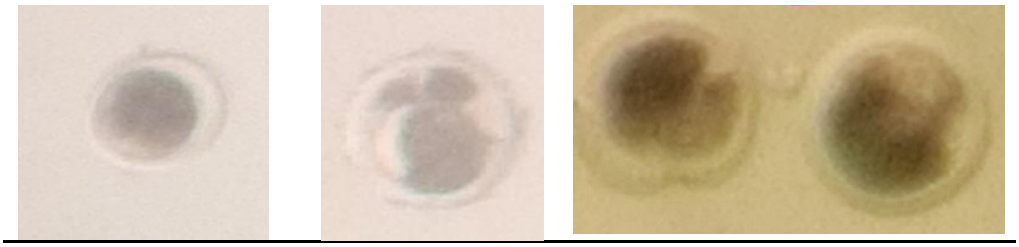

Plate A. Cleaved Boran zygote at 48 hrs. post insemination

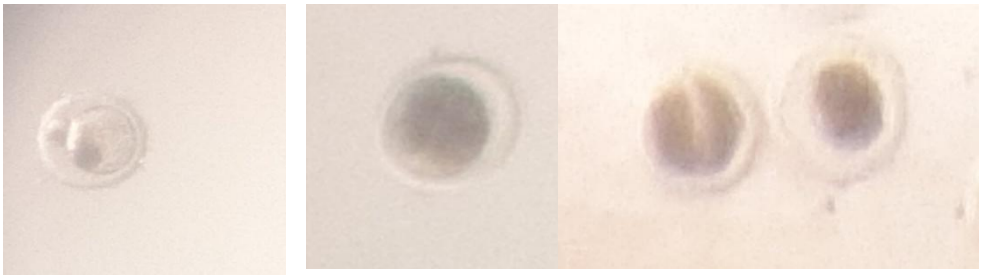

Plate B. Cleaved Crossbred zygote at 48 hrs. post insemination

Figure 2: Plate A Boran and Plate B Crossbred cleaved zygote at 48 hrs. post insemination

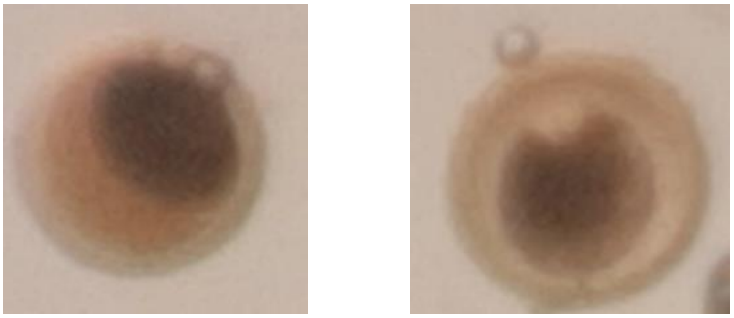

Plate C Plate D

Figure 3: Plate C & D. Cleaved Crossbred zygotes with 2nd polar body at 48 hrs. post insemination.

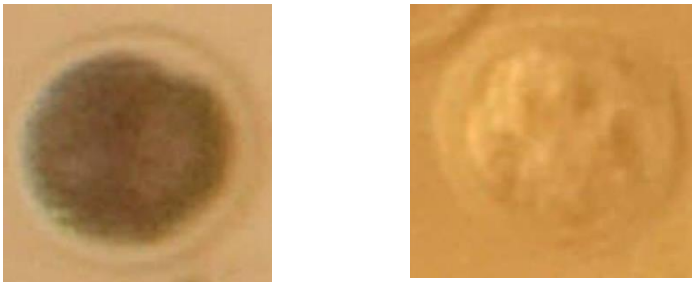

Plate A Plate B

Figure 4: Plate A Boran & Plate B Crossbred cattle morula stage Embryos after 72hrs incubation.

Discussion

Ovum pick up

In this study there was a difference ($p<0.001$) in the mean number of oocytes collected per session between once and twice a week collection scheme. The mean number of oocytes collected in once weekly collection schemes is higher than the number of oocytes collected per session in twice weekly collections. This result agreed with Imai *et al.* (2006) that reported the once weekly OPU scheme produced two times more oocytes than twice a week per donor per OPU session collection. Imai *et al.* (2006) also reported that the number of collected oocytes per each session and the percentage of oocytes developed to the blastocyst stage in once weekly OPU schemes are higher than twice a week OPU collections.

The oocyte recovery rate in both breeds was less than 50% which was lower than the average value reported in literature (Tamassia, et al., 2003). This could be due to technician skill on aspiration and searching, vacuum pressure and also animal handling facility. The Aloka ultrasound used for this experiment has had high screen resolution; however the open crush for animal restraint allows sun rays that disturb vision. Bols *et al*. (2004) reported that ultrasound screen resolution and probe characteristics have a great impact on oocyte recovery efficiency and, in reducing the risk of causing unnecessary damage to the ovarian tissue.

In vitro maturation

Morphological assessment of the proliferation of cumulus oophorus cells is the measure for the success of in vitro oocyte maturation (Lojkić *et al.*, 2014). The success of in vitro oocytes maturation is approximately 90%, even though only 30% of these oocytes develop to the stadium of the blastocyst (Lonergan, *et al.*, 2003). In this experiment the maturation rate of oocytes aspirated from Boran and Crossbred cattle was lower (54.12%) than the normal oocyte maturation reports in the literature by (Lonergan, *et al.*, 2003). The lower maturation result could be attributed to the newly established laboratory set up; mainly to media optimization and maintenance of culture environment (temperature regulation, gas phase and pH). Culture conditions can influence the kinetics of early development (Langendonckt *et al.*, 1997); however the main factors controlling this parameter are intrinsic to the oocyte (Brevini-Gandolfi *et al.*, 2000), the sperm (Ward *et al.*, 2001) or both. Problematic power fluctuations in incubation conditions that occurred regularly believed to be so damaging to the gametes within the laboratory set up, altering gas phase, pH and overall incubation conditions. Small pH deviations in culture conditions are actually large changes in H^+ concentration (Swain, 2010). Additionally, factors that influence oocyte quality, such as age of the donor, the stage of the estrous cycle, nutritional status, genetic potential, presence of a reproductive disorder, and others (Lonergan *et al.*, 2016), might have impact on the quality of aspirated oocytes in both genotypes of cattle. Further studies are elaborate the intrinsic quality of the oocyte as one of the major factors

affecting early embryonic development (Krisher, 2004), and also the crucial role of embryo culture conditions in determining blastocyst quality (Rizos *et al.,* 2002), the precise selection of competent oocytes is vital for IVP technologies in livestock.

Lonergan *et al.* (2001) demonstrated that events further back along the developmental axis determine the proportion of immature oocytes reaching the final developmental stage or its ultimate fate of development. There was heterogeneity in oocyte maturation which was arising from the variability of aspirated oocytes. The total number of aspirated grade I oocytes was less than 20%. The mean number of oocytes per collection session was 11.41 ± 0.18 and few grade I quality oocytes were cultured per each incubation. According to Douville and Sirard, (2014), antral follicles in random ovaries will be in the growing, plateau and atretic phase. This situation is reflected in the morphology of the cumulus-oocyte complexes recovered from these follicles and is partially associated with outcome (Blondin and Sirard, 1995). Oocytes with a partially denuded or atretic-like cumulus have a lower competence compared to other groups (Sirard, 2011).

Selection of uniform oocytes from a group of a limited number of high grade oocytes per OPU session was a limiting factor. Boran cattle contributed for most of the denuded oocytes. Though, there was no difference (p>0.05) in the total number of discarded oocytes between Boran and Crossbred cattle.

The ratio of cumulus cell expansion in crossbred cattle oocytes was slightly higher than Boran cattle oocytes. Cumulus cell expansion was more extensive and broad following 22hr of maturation in the oocytes of both breeds. Sixty percent of the total cultured oocytes exhibited first polar body extrusion. The degree of nucleus maturation is measured as a percentage of oocytes in the stage of metaphases II with the first polar body expelled (Lojkić *et al.,* 2014). The first polar body extrusion was more prominent in Crossbred oocytes (67.0%) than the Boran cattle oocytes (54.6%). Around 90% of cultured immature oocytes will reach MII at the end of maturation under proper conditions (Lonergan, *et al.,* 2003). Though, oocyte cytoplasmic maturation and full acquisition of developmental competence are in many cases not automatically accompanying the nuclear maturation and may account for fertilization and/or development deficiencies (Watson, 2007). The heterogeneous source of the aspirated immature oocytes retrieved from ovarian follicles at different phases of the follicular growth can result in compromised developmental competence due to improper completion of the cytoplasmic and nuclear maturation (Mermillod *et al.,* 1999).

The ultimate test of the quality of an oocyte is its ability to be fertilized and develop to the blastocyst stage, to establish a pregnancy and ultimately to produce

a live calf (Lonergan *et al.*, 2001). To this end, minimizing the factors responsible for poor oocyte and semen quality should get focus above and beyond incubation conditions and working environment optimization. These results indicate the importance of further studies to investigate the local cattle oocyte acquisition of developmental competence and optimization of in vitro culture conditions for the improvement of the bovine IVP system in our laboratory conditions.

Semen viability and in vitro fertilization

Frozen thawed semen samples had shown a range of variability in motility (45-75%) during semen preparation for IVF. The sperm quality parameters (motility, morphology, concentration, viability) were evaluated immediately after thawing and after sperm preparation for IVF. It was reported that mammalian spermatozoa have high expressive heterogeneous traits in morphology, motility and nuclear stability. Spermatozoa metabolic activity in the female genital tract is varied from in vitro conditions and is not adapted to survive for long time in in vitro conditions (Van S. A. and de Kruif A. 1996). The frozen thawed straws of semen for fertilization had a highly inconsistent percentage of motility. There were also observable physical defects, unresolvable clots and debris in the population of thawed semen during sperm separation procedures. Parrish *et al.* (1995) reported that frozen bull spermatozoa after thawing have lower percentage of progressive motility (30 to 70%), though the percentage of morphologically normal spermatozoa in thawed ejaculate is equal to fresh semen. Straws of semen evaluated for less than 40% motility were not included in this experiment.

Percoll density gradient centrifugation and self-migration swim up techniques were used for semen characterization and optimization experiments, for selecting hyper-motile sperm from the pool of semen population. There were significant differences ($p < 0.05$) in sperm motility between initial sperm and sperm after Percoll separation techniques. There were also significant differences ($p < 0.05$) in the sperm cells concentration values between the sperm separation methods. Comparing the results of sperm viability before processing with the results after sperm processing it was found that there were significant differences ($p < 0.05$) between them. However, there were no significant differences for the sperm motility between the sperm preparation methods.

Even though a great deal of sperm cells were found and left at each stratum of the percoll layer, Percoll density gradient centrifugation method was helpful in enhancement of the sperm quality by separating high rate of progressive motility and morphologically normal spermatozoa. In this study the swim up method yields lower concentration of sperm cells than the percoll gradient centrifugation technique. Comparing swim up method and Percoll gradient Parrish *et al.* (1995) obtained similar sperm motility results for both methods, although a lower concentration resulted for swim up method. There was inconclusive report on

semen purification methods however numerous studies investigating which method, among many, are better for sperm enrichment.

The result of this study showed again that the sperm recovery rate following percoll gradient largely depends on initial semen quality; which indicates the importance of initial semen quality evaluations for frozen thawed semen prior to swim up and percoll procedures. These semen preparation methods would not spare the need for proper semen handling. Poor handling of stored semen (improper top-upping and shortage of LN_2 supply) was the culprit for deterioration of the quality of semen on top of individual bull variability. The sperm recovery rate from poor semen samples such as liquefaction defects, unresolvable clots or rare viable sperm was far from satisfactory. The repeated experimentation made on semen prepared for IVF indicated a small proportion of motile sperm was obtained from a relatively poor quality semen sample.

The results of this experiment in which a lower proportion of matured oocytes were developed to fertilization suggests that the events around the time of maturation might be important in determining the developmental competence of the oocyte. However, whether or not maturation was solely responsible for the observed decrease in fertilization was questionable. It should be noted that despite the extrinsic factors surrounding the oocyte the intrinsic factor (quality of each oocyte) might have had effect on both maturation and fertilization of the oocytes. This would suggest that a proportion of the heterogeneous oocytes aspirated from small follicles had not been competent for maturation and contributed to a lower maturation and fertilization rate. For years, there was doubt in defined media that it was the major cause of limited developmental competence of oocytes, although this media was allowing the culture of oocytes up to blastocyst stage (Sirard, 2018). The average success rate plateaued at 30-40% of oocytes developing to the blastocyst stage despite the addition of hundreds of different products, cytokines, growth factors, anti-oxidants, in addition to the introduction of new types of incubators, and reduced oxygen tension (Hansen 2006). Nowadays, attention turned towards the source of oocytes as a potential explanation for the limited success rates of IVF. The morphology of oocyte-cumulus complexes obtained when all follicles are aspirated from ovaries varies greatly (Sirard, 2018).

Irrespective of the breed and the media used for maturation and fertilization, only a very low number of oocytes were cleaved (19), few of them (4) grew to morula stage and none of them developed to blastocyst stage. As reported by Lequarre *et al.* (2003), the frequency of embryos reaching the blastocyst stages is far from what is normally observed in embryos produced in vivo. In this experiment 66% of matured oocytes could not reach cleavage and embryonic development. Research report indicated 60 to 70% laboratory losses of in vitro matured oocytes

due to the inability of the embryo to properly undergo cleavage and development to blastocyst stage (Lequarre *et al.*, 2003).

There are many factors that can interfere in embryo development in cattle causing embryo cleavage to cease, and most of the embryonic block occurs during the fourth or between the fourth and fifth cell cycle transition (Memili and First, 2000). This developmental block is observed in many species, with the peculiarity that it shows up at different stages (i.e., fourth cell cycle in cattle; fifth in rabbits, third to fourth in humans; second in mice; (Memili and First, 2000), and in the fourth cell cycle in cats (Hoffert *et al.*, 1997). This species-specific block moment is concurrent with the maternal-embryo transition, the developmental stage when embryos conclude the major genome activation (De Sousa *et al.*, 1998b) and must rely on the mRNAs transcribed from its own genome to continue development.

It would seem that a number of factors such as donors' age, nutritional status, oocyte aspiration pressure, media composition and optimization, pH, temperature regulation and incubator and working environment might have affected embryo production efficiency. Even though IVF technology progressed from where it was 40 years ago and its techniques employed in multiple animal species, the technology is not yet completely matured or satisfactory; a number of problems remain to be solved and several procedures still need to be optimized (Sirard, 2018). IVEP procedures are not easily repeatable and adapted in a new laboratory set up. Every step of the procedure should be optimized and the incubation environment should upgrade to optimal condition. In a new laboratory set up most of the approach was fraught with technical difficulties which cloud the overall effort exerted to adapt and optimize the procedures.

The challenge for the future is optimization of different stock solution preparations, incubator conditions and working environment, and to secure the source of quality oocyte and semen for the attempt to amplify the maturation, fertilization and embryo culture conditions. The development of a complex technology such as in vitro embryo production requires years of experimentation, to create the right incubation environment for oocytes, spermatozoa and early embryos (Sirard, 2018).

The quest for defining optimal culture conditions allowing for the development of bovine zygotes to blastocysts took place including focus on co-culture systems (Edwards *et al*., 1997), media composition (Holm *et al*., 1999) as well as the physical design of the culture platforms (Smith *et al*., 2012). These efforts all became extremely relevant to prevent adverse effects of improper in vitro culture conditions, particularly in new IVF laboratory setups.

Conclusion

The lower maturation rate of both Boran and Crossbred cattle oocytes could be attributed to the newly established laboratory set up; mainly to media optimization and maintenance of culture environment. The decrease in the number of matured oocytes that developed to fertilization suggests that the events around the time of maturation might be important in determining the developmental competence of the oocyte. Uncontrolled factors such as electric flex laboratory equipment optimization and less exposed lab technicians might also have contributed to the low outcome. The intrinsic factor (quality of each oocyte) might have an effect on maturation, fertilization and developmental competence of the embryo. Relatively similar patterns of maturation process and embryo development were observed between Boran and Crossbred cattle oocytes. OPU/IVF procedures could be used in local and crossbred heifers without adverse effect on the normal ovarian activity. Alternative ART techniques (OPU, IVF) for dairy cattle genetic improvement and multiplication work were tested and adopted in the country.

Recommendations

Further research and experimentations are needed on the extrinsic and intrinsic factors affecting oocyte yield & quality, to optimize and create the right *in vitro* environment for oocyte maturation, fertilization, embryo culture and development, sperm purification and quality analysis methods, embryo freezing and cryopreservation procedures. Capacity building in human and physical infrastructure is crucial to be benefited from the knowledge based economy such as IVEP.

Acknowledgements

The authors gratefully acknowledged the financial and research inputs support of this study by the Ethiopian Institute of Agricultural Research (EIAR), National Agricultural Biotechnology Research Center (NABRC).The financial support of Agricultural Growth Project (AGP II) of the (EIAR).The provision of experimental animals by Holeta and Debre Zeit Agricultural Research Centers.

Reference

Baruselli P.S., Ferreira R.M., Sá Filho M.F., Bó G.A. (2018a): Review: Using artificial insemination v. natural service in beef herds. *Animal*, **12:** 45-52.

Blondin P., Sirard M.A. (1995): Oocyte and follicular morphology as determining characteristics for developmentalcompetence in bovine oocytes.Mol. Reprod. Dev., 41: 54-62.

Bols P.E., Leroy J.L.M.R., Vanholder T. and Van Soom A. (2004): A comparison of a mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. *Theriogenology*, **62:** 906-914.

Brevini-Gandolfi T., Favetta L.A., Lonergan P. and Gandolfi F. (2000): The mechanism regulating maternal mRNA stability and translation is affected in bovine embryos with low developmental competence, *Theriogenology*, 53: 268 abst.

De Roover R., Feugang J.M., Bols P.E., Genicot G. and Hanzen C.H. (2008): Effects of ovum pick-up frequency and FSH stimulation: a retrospective study on seven years of beef cattle in vitro embryo production. *Reprod. Dom. Anim.*, **43:** 239-245

De Sousa P.A., Watson A.J. and Schultz R.M. (1998b): Transient expression of a translation initiation factor is conservatively associated with embryonic gene activation in murine and bovine embryos. *Biol. Reprod.*, **59:** 969-977.

Douville G. and Sirard M.A. (2014): Changes in granulosa cells gene expression associated with growth, plateau and atretic phases in medium bovine follicles. *J. Ovar. Res.* **7:** 50. (https://doi.org/10.1186/1757- 2215-7-50)

Edwards L.J., Batt P.A., Gandolfi F. and Gardner D.K. (1997): Modifications made to culture medium by bovine oviduct epithelial cells: Changes to carbohydrates stimulate bovine embryo development.*Mol. Reprod. Dev.*, **46:**146-154.

Galli C., Crotti G., Notari C., Turini P., Duchi R. and Lazzari G. (2001): Embryo production by ovum pick up from live donors. *Theriogenology*, **55:** 1341-1357.

Garcia-Herreros M., Bermejo-Alvarez P., Rizos D., Gutierrez-Adan A., Fahey A.G. and Lonergan P. (2010): Intra follicular testosterone concentration and sex ratio in individually cultured bovine embryos. *Reprod. Fertil. Dev.*, **22:**533-8

Hansen P.J. (2006): Realizing the promise of IVF in cattle:-an overview. *Theriogenology*, **65:** 119-125. (https://doi.org/10.1016/j. theriogenology.2005.09.019)

Hoffert K.A., Anderson G.B., Wildt D.E. and Roth T.L. (1997): Transition from maternal to embryonic control of development in IVM/IVF domestic cat embryos. *Mol.Reprod. Dev.*, **48:** 208-215.

Holm P., Booth P.J., Schmidt M.H., Greve T. and Callesen H. (1999): High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology*, **52:** 683-700.

Imai K., Tacawa K., Yoshioka H., Matoba S., Narita M., Inaba Y., Aikawa Y., Ohtake M. and Kobayash S. (2006): Efficiency embryo production by ovum pick up and in vitro fertilization in cattle. *J. Reprod. Dev.*, **52:**19-29.

Krisher R.L. (2004): The effect of oocyte quality on development. *J. Anim. Sci.*, **82** (E-Suppl): E14-E23.

Langendonckt A.V., Donnay I., Schuurbiers N., Auquier P., Carolan C., Massip A. and Dessy F. (1997): Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium, J. Reprod. Fertil. 109: 87-93.

Lequarre A.S., Marchandise J., Moreau B., Massip A. and Donnay I. (2003): Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. *Biol. Reprod.*, **69:** 1707-1713.

Lojkić M., Čavlek M., Bačić G., Getz I., Samardžija M., Maćešić N., et al. (2014): Morphology evaluation of bovine embryos produced in vitro. *Vet. stn.*, **45(3):**187-193.

Lonergan P. and Fair T. (2016): Maturation of Oocytes in Vitro. *Annu. Rev. Anim. Biosci.*, **4:** 255-268.

Lonergan P., Rizos D., Fair T. and Boland M.P. (2003): In vitro production of bovine embryos: factors affecting blastocyst yield and quality. Proceedings of the 4th Middle-European Buiatric Congress, 23-27 April. Lovran, Croatia; Pp. 33-38.

Lonergan, P., Rizos, D., Ward, F. and Boland, M.P. 2001: Factors influencing oocyte and embryo quality in cattle, *Reprod. Nutr. Dev.*, **41:** 427-437.

Memili E. and First N.L. (2000): Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote,* **8:** 87-96.

Mermillod P., Oussaid B. and Cognie Y. (1999): Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J. Reprod. Fertil.,* **54:** 449-460.

Meuwissen T.H.E. (1991): The use of increased female reproductive rates in dairy cattle breeding schemes. *Animal Production,* 52: 21-31.

Parrish J.J., Krogenaes A. and Susko-Parrish J.L. (1995): Effect of bovine sperm separation by swim-up or percoll on success of in vitro fertilization and embryo development. *Theriogenology,* **44:**859-69.

Pieterse M.C., Kappen K.A., Kruip T.A.M. and Taveme M.A.M. (1988): Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology, 30: 751-761.

Rizos D., Ward F., Duffy P., Boland M.P. and Lonergan P. (2002): Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol.Reprod. Dev.,* **61:** 234-248.

Sirard M.A. (2018): A review: 40 years of bovine IVF in the new genomic selection context. *Reproduction,* **156:** R1-R7.

Sirard M.A. (2011): Follicle environment and quality of in vitro matured oocytes. *Journal of Assisted Reproduction and Genetics*, **28**: 483-488. (https://doi.org/10.1007/s10815-011-9554-4)

Smith G.D., Takayama S. and Swain J.E. (2012): Rethinking in vitro embryo culture: new developments in culture platforms and potential to improve assisted reproductive technologies. *Biol. Reprod.,* **86:**62.

Swain J.E. (2010): A review; Optimizing the culture environment in the IVF laboratory: impact of pH and buffer capacity on gamete and embryo quality. *Reprod. Bio-Medicine,* Online **21:** 6-16.

Tamassia M. Heyman Y. Lavergne Y. Richard C. Gelin V.Renard J. Chastant-Maillard S., (2003): Evidence of oocyte donor cow effect over oocyte production and embryo development in vitro. *Reproduction,* 126:629-637.

Van Soom A., de Kruif A. (1996): Oocyte maturation, sperm capacitation and pre-implantation development in the bovine: implications for in vitro production of embryos. *Reprod. Dom. Anim.***, 31:**687-701.

Viana J.H.M., Ferreira A.M., Sá W.F. and Camargo L.S.A. (2000): Follicular dynamics in zebu cattle. *Pesquisa Agropecuária Brasileira,* **35:** 2501-9.

Viana J.H.M., Palhao M.P., Siqueira L.G., Fonseca J.F. and Camargo L.S. (2010): Ovarian follicular dynamics, follicle deviation, and oocyte yield in Gyr breed (Bos indicus) cows undergoing repeated ovum pick-up. *Theriogenology,* **73:** 966-972.

Ward F., Rizos D., Corridan D., Quinn K.M., Boland M.P. and Lonergan P. (2001): Paternal influence on the time of the first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo, Mol. Reprod. Dev. 60: 47-55.

Watson A.J. (2007): Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence. *J. Anim. Sci.,* **85:** E1-E3.