ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

IN VITRO MATURATION OF OOCYTES RETRIEVED THROUGH TRANS VAGINAL OOCYTE ASPIRATION FROM BORAN AND BORAN *HOLSTEIN FRIESIAN CROSSBRED COWS

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In vitro maturation of oocytes retrieved through transvaginal oocyte aspiration from Boran and Boran *Holstein Friesian crossbred cows

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LISTS OF ABBREVIATION

AI	Artificial insemination
ANOVA	Analysis of variance
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BCS	Bovine calf serum
BO	Brackett and Oliphant's
CO_2	Carbon dioxide
BSA	Bovine serum albumin
CCE	Cumulus cell expansion
COC	Cumulus oocyte complex
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factors
FAO	Food and agriculture organization
FCS	Fetal calf surem
FF	Follicular fluid
FSH	Follicle-stimulating hormone
FBS	Fetal bovine serum
GH	Growth hormone
GnRH	Gonadotrophin releasing hormone
GSH	Glutathione sulfhydryl
GV	Germinal vesicle
GVBD	Germinal vesicle Break down
HA	Hyaluronic acid
IVC	In vitro culture
IGF	insulin-like growth factor
IVEP	In vitro embryo production

IVF	In vitro fertilization
IVM	In vitro maturation
MTOCs	Microtubule-organizing centers
MEM	Minimum essential medium
mBECM	Modified bovine embryo culture medium
MOET	Multiple ovulation and embryo transfer
mKSOM	Modified potassium Supplemented Optimized Medium.
mRNA	Messenger ribonucleic acid
OPU	Ovum pick up
ROS	Reactive oxygen species
ZP	Zona pellucida

ABSTRACT

The developmental competence of immature oocytes largely depends on the origin and method of retrieval. This is known to determine the success of *in vitro* embryo production. This study aimed to evaluate the *in vitro* maturation difference of oocytes from pure Boran and Holstein Friesian*Boran crossbred cows obtained through ovum pickup. The OPU was carried out on 10 cows, 5 cows from each breed. The overall oocyte recovery rate was 43.4% (288/663); 39.3% (131/333) for Boran and 47.6% (157/330) for Crossbred with no breed influence ($p>0.05$). Oocytes aspirated from the 288 follicles (all ≥ 3mm) were matured in either TCM-199 or BO-IVM maturation mediums. Proportion of viable oocytes were comparable between Boran (81.60%) and B*HF crossbred (78.9%). The mean number of oocytes collected per ovum pickup session for Boran and Crossbred cows were 2.18 ± 1.90 and 2.62 ± 1.86, respectively. The overall maturation rate was in the order of 79.4% and 79% for Boran and crossbreeds, respectively, and comparable between the breeds. Considering all maturation indices, oocyte maturation was significantly higher ($p<0.05$) in BO-IVM (88.5%) compared to TCM-199 (70.4%). Maturation of Boran oocytes in BO-IVM was relatively higher (90.3%) compared to Boran*HF oocytes (86.2%); whereas it was 73.1% for Boran and 68.2% for Boran*HF oocytes in TCM. Maturation rate in terms of cumulus cell expansion was relatively better in BO-IVM (59.3% fully expanded and 30.1% partially expanded) compared to TCM-199 (46.9% fully expanded and 24.4% partially expanded). Overall, there was extrusion of the polar body in 55.75% of oocytes and increased perivitelline space in 69.2% of oocytes. Zona quality was relatively better in BO-IVM (69.2% of oocytes) compared to TCM-199 medium (60.9% of oocytes). In conclusion, oocyte maturation following OPU was influenced by media type but not by breed. It can also be concluded that some of the maturation indices were more affected by breed and media type even though the overall maturation was closely similar.

Keywords. *Boran crossbreed, in vitro, maturation, oocytes,*

INTRODUCTION

Over the past several years, the use of assisted reproductive technologies (ART) in animal breeding has become very important to the genetic improvement of dairy cattle in the world. The reproductive technologies were designed to facilitate a great application of the best germ Plasm. Artificial insemination (AI), gamete and embryo freezing, multiple ovulation and embryo transfer (MOET), and in vitro embryo production (IVEP) are all involved in assisted reproductive technologies (Rodriguez, 2012). Among the ART, there are Collection and *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of likely embryos up to a level that is compatible with transfer to the recipient uterus are all part of in vitro embryo production (Freitas and Melo, 2010). The techniques have evolved into effective commercial applications, allowing for a boost in output through reducing generation intervals, extending reproductive lifetimes, controlling disease, and lowering production costs (Bertolini *et al*., 2009; Verma *et al*., 2012).

Each mammalian ovary contains hundreds of thousands of oocytes at birth, but the majority are lost owing to atresia. Harvesting oocytes from the ovary and employing IVEP procedures could help to mitigate this massive loss of genetic material (Hasler 1998; Mapletoft and Hasler, 2005). Bovine IVEP has become a well-known and cost-effective procedure. Furthermore, using OPU at frequent intervals in conjunction with IVF has been shown to improve or increase the production of embryos from chosen donors. Moreover, IVEP can be utilized to salvage endangered genetic material after slaughter for a variety of reasons (Hasler, 2003). *In vitro* fertilization by intra-cytoplasmic sperm injection, so prominent in assisted human reproduction has become feasible in cattle, even with freeze-dried sperm though not yet widely applied (Mapletoft, and Hasler, 2005).

For several decades, the large-scale production of embryos *in vitro* has been possible in domestic mammals, particularly cattle, for purpose of generating large numbers of embryos for research, or as a route to other technologies, such as nuclear transfer and trans-genesis. The oocytes for bovine *in vitro* embryo production could be obtained from ovaries of slaughtered donors and live donor cows. Transvaginal ultrasound-guided oocyte collection or Ovum pick-up is a common method of recovering oocytes from live animals. This procedure can be used in conjunction with in vitro embryo production (IVEP) to improve the number of offspring from genetically valuable cows. (Galli *et al*., 2001).

The most significant or key stage in the IVEP is oocyte maturation, which influences the subsequent successful fertilization, zygote formation, blastocyst stage, and normal embryo growth and development. The production of competent oocytes during IVM is important for cattle reproduction concerning the ability to increase the production of valuable, healthy offspring, (Rizos *et al*., 2002). During the stage of COC maturation, oocytes undergo, through several molecular and cellular modifications, to reach full developmental competency (Crozet and Dubos, 1994).

IVP is suggested to produce embryos from genetically improved dairy cattle for developing countries, production of hybrid genotypes (i.e. *Bos Taurus* x *Bos indicus*) with the potential for better productive performance for the tropics. The expansion and increasing of genetically improved and potentially productive herd can be achieved in a faster way with IVF than with traditional genetic schemes (Galli *et al*, 2003).

Regardless of the huge and genetically diversified cattle population (65.35million heads) resources in Ethiopia, the dismally low outcome of cross-breeding is evident in the very low proportion (1.91%) of hybrids (CSA, 2020). According to Degefa *et al*. (2016a and b); Gadisa *et al*. (2019) there is a limitation on the availability of information regarding the application of advanced ART techniques on zebu and crossbred dairy cattle and there are very few published data concerning IVEP in Ethiopia. Demand for hybrid replacement heifers remains very high amid the need for dairy expansion that deems the application of advanced technologies mandatory.

Among the pool of naturally available follicles, according to various reports, oocyte retrieval potential could be affected by breed. Previous studies also confirm that the reproductive physiology of *Bos- indicus* (zebu) cattle is not identical to *Bos-Taurus* cows (Degefa *et al.,* 2016). Due to the larger number of antral follicle counts, *Bos indicus* cattle were known to have higher oocyte recovery rate or greater numbers of retrieved oocytes resulting in higher percentages of viable oocytes, and a better number of fertilizable COCs, when compared with *Bos Taurus*.

The type of maturation media and additives used are the other common factor which affects the developmental competence of bovine oocytes (Ayman *et al.,* 2016). IVM medium including; TCM-199, Hem's F 10, SOF, and MEM (Ravindranatha *et al.,* 2001) have been used for oocytes maturation in mammals. There is a great deal of variation in the chemical composition and maturation efficiency among the maturation mediums because of their difference in composition.

TCM-199 is the most widely used culture medium for bovine oocytes (Thompson, 2000). Similarly, BO-IVM complete medium comprises important substances that enhance oocyte maturation in cattle (Pryor *et al*., 2016). However, both these media are experimented with oocytes derived from *Bos Taurus*. In this experiment, it is hypothesized that *in vitro* maturation of oocytes would not be influenced by breed although subtle physiological differences exist in folliculogenesis and oocyte development *in vivo*. Hence, the aims of this study were:-

To evaluate the *in vitro* maturation of OPU derived oocytes retrieved from Boran and their HF cross cows.

To evaluate and compare the maturation condition of OPU derived oocytes from Boran and their HF cross cows in selected IVM media (TCM-199 and BO-IVM Complete medium).

2. LITERATURE REVIEW

2.1 Folliculogenesis in cattle

In mammals, the formation of Primordial germ cells begin during the early stage of the fetus, multiplied by mitosis, and migrated from the embryonic sac to the genital ridge to settle in the developing gonadal crest. The primordial germ cells differentiated in Oogonia. In cattle, Oogonia divided mitotically and reach about 2.7 billion at 4 months of fetal life (Aert and Bols, 2010a). which later on decreases to 130,000 at birth (Rosale *et al.,* 2015). The Oogonia build egg nest in the ovarian cortex, enter the first prophase of meiosis, and halted in the diplotene stage called primordial follicles Aerts JMJ, Bols, (2010b).

Folliculogenesis is a protracted process in which numerous small primordial follicles develop into large preovulatory follicles. It occurs throughout a female's reproductive life and concludes with either ovulation or atresia death. Folliculogenesis starts before birth in the cow. Folliculogenesis classifies the growing follicles in primordial, primary, secondary tertiary (antral). There is two follicular development phase, the FSH-LH independent while the follicles transit from primordial to the antral stage. The second one is gonadotropin dependent where the follicle becomes growing fully and reach the preovulatory stage, the FSH required for growth and LH surge required for ovulation. It takes 3 to 4 months for follicles to mature from the primordial to the antral stage (Knight and Glister, 2001; Webb *et al* 2004; Paulini *et al*., 2014; Rosale *et al*., 2015).

2.1.1. Primordial follicle

In the first stage of folliculogenesis, Primordial follicles are the fundamental unit of the mammalian ovary. The primordial follicles are located in the peripheral cortex of the ovary, later on, when follicles and oocytes begin to grow they migrate deeper into the cortex of the ovary through the developmental stage as reach the prenatal stage become visible on the surface of an ovary. Primordial follicles detected around day 90 gestation in cow measures a diameter of approximately 40µ contain one oocyte measuring about 30 µm in diameter (Picton, 2001; Palma *et al* 2012). These follicles consist of oocytes surrounded by a single layer of granulosa cells flatten in shape. The primordial follicles remain in the process of the diplotene (or dictyate) stage of meiosis (the first meiotic division). Follicles will remain in the primordial state until they are recruited into the growing population (Rosale *et al* 2015).

2.1.2. Primary follicles

The primordial follicles shift from a flat to a cuboidal form as the granulosa cells layer proliferates, signaling the start of the primary follicle. The oocyte genome is activated at this point, and genes begin to be transcribed. The development of zona pellucida around the oocytes begins in the late stage of the primary follicle. The gap junction pathways that are vital for communication between the follicle and oocyte are formed, the FSH receptor developed despite gonadotropin-independent. Both the oocyte and the follicle grow increasing to almost in diameter (Picton, 2001; Rajesh and Jaiswal, 2017).

2.1.3. Secondary (pre antral follicle)

During the transition primary to a secondary follicle, the granulosa cells are activated to proliferate the second layer. this stage characterized by the appearance of second granulosa cell, small accumulations of fluid in the intracellular spaces called follicular fluid**,** the complete formation of zona pellucida glycoproteins around the oocyte material, formation of cortical granules within the oocyte cytoplasm the beginning of theca cell layer formation mRNA synthesis in the oocyte and gonadotropin responsiveness (McLaughlin *et al.,* 2010; Araújo *et al*., 2014).

2.1.4. Tertiary (antral follicle)

Tertiary follicles are defined as those in which the cells around the egg continue to proliferate and differentiate. Primary oocytes are also found in cumulus cells, theca interna, and theca externa, which have a fluid-filled antral cavity (antrum) at this stage. When the follicle reaches around 3 cm in diameter at the conclusion of its development, it may begin the process of recruitment, selection, and dominance, which occurs in waves during the estrous cycle (cow 2-3 waves/cycle). (Rajesh and Jaiswal, 2017)

2.1.5. Preovulatory follicle

The surge of LH maintains the dominant follicle's growth, makes it pre-ovulatory, and the oocyte within undergoes final maturation, culminating in follicle rupture and matured oocyte ovulation (Fair, 2003). The hormonal secretion, gonadotropin-releasing hormone (GnRH) from the hypothalamus, and downstream hormones follicle-stimulating hormone (FSH) luteinizing hormone (LH), estrogen, progesterone, and others, become the main actors during the preovulatory

stage). Preovulatory follicles contain secondary oocytes that resume meiosis and progress to metaphase II (Jones and Shikanov, 2019). The granulosa cells changed into cumulus cells and respond to the LH surge by secreting hyaluronic acid and achieve a so-called cumulus expansion (Eppig, 2018).

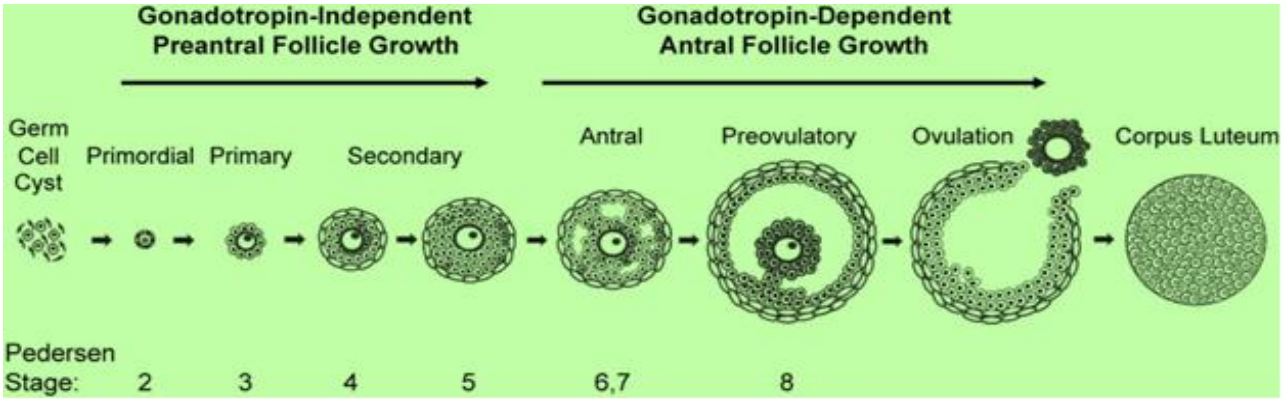


Fig 1.Schematic detailing the stages of mammalian folliculogenesis taken from Jones and Shikanov (2019)

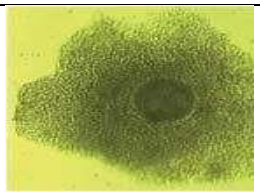
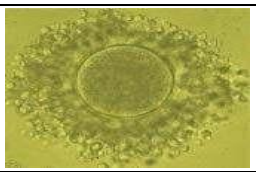
2.2. Oocyte collection and selection for *in vitro* maturation

Bovine oocytes could be harvested from slaughterhouse ovaries of any age of females. These oocytes have the potential of high developmental competence to produce large numbers of embryos which can result in live calves through IVF procedures (Natumanya *et al.*, 2008). Slaughterhouse ovaries especially it is a source of oocytes of great importance large-scale production of genetically improved embryos (Nagai *et al*., 2014). Despite the fact that slaughter ovaries are inexpensive and enable the harvesting of all follicles visible on the surface of the ovaries, the oocyte population is highly heterogeneous that resulted in a variable in their developmental competence during *in vitro* maturation (Karadjole *et al*., 2010). *In vitro* production of an embryo in animals is most of the time dependent on a supply of in addition to the slaughterhouse, currently oocytes are routinely harvested from cattle donors by transvaginal ultrasound-guided collection procedures. The technique is a non-invasive method of extracting oocytes from antral follicles in living animals. Together with the IVEP of oocytes, OPU has been considered as the most flexible and repeatable technique to produce embryos from a genetically valuable donor (Galli *et al*., 2001). OPU does not affect the normal reproduction and production cycles of the donor. Any female including young

cyclic heifers, cows at the third month of pregnancy, and even soon after calving (2-3 weeks) could be suitable donors (Wang *et al*., 2013). The efficiency of OPU recovering oocytes in terms of number and qualified of competent oocytes can be determined by donor breed, the number of follicles present, climate condition, nutritional status of the donor, operator's experience, vacuum pressure, and needle type during ovum pick-up (OPU) (Ward *et al*., 2000).

Following maturation, fertilization, and culture *in vitro*, only about 30-40 percent of bovine oocytes grow to the blastocyst stage. This is most likely due to the poor quality of the oocytes at the beginning of maturation, which leads to a low success rate. Selection criteria have been used to predict the quality of these oocytes and to improve embryo development. Selection is based on the morphology of the oocyte, the degree of COC compaction, and the presence of homogeneous ooplasm (Sirard *et al.*, 2006). *In vitro*, oocytes with evenly granulated ooplasm and several layers of cumulus cells show higher developmental competence than oocytes with irregularly granulated ooplasm and fewer cumulus layers. The cumulus-oocyte complexes collected from ovarian follicles are classified according to the compactness of the cumulus and characteristics of oocyte cytoplasm (Wang and Sun 2007) the excellent and good qualities are mostly suggested to maturation.

Table 1: Different Quality grade of COCs layer and ooplasm characteristics for maturation (Saha *et al.*, 2014; Paul 2019)

Quality	**Criteria**	Representative Image
Excellent (Grade I)	Oocytes had Compact multi-layered cumulus cells (≥5) and homogeneous ooplasm	
Good (Grade II)	Oocytes that had compact multi-layered cumulus cells (at least 3-5) and less homogeneous ooplasm	
Fair (Grade III)	oocytes that had less compact cumulus cells (at least 3 layer and irregular ooplasm	
Poor (Grade IV)	oocytes had no cumulus (denude)	

2.3. Determination of *in vitro* oocytes maturation

In vitro maturation is a procedure that involves taking immature antral follicles from the GV stage and growing them in a controlled environment until they reach MII and are ready for fertilization and embryonic development. The activation and inhibition of enzymes, hormones, and growth factors occur during oocyte maturation, resulting in nuclear and cytoplasmic maturation (Gilchrist and Thompson, 2007). Nuclear maturation occurs spontaneously, due to the mechanical removal of the oocyte from the follicle is capable of triggering the process, while cytoplasmic maturation occurs more gradually (Brevini *et al.*, 2007). The success of fertilization, progression to blastocyst, and embryo growth are all determined by the maturity of oocytes (Brevini *et al*., 2007).

Maturation of oocytes is a very important stage, which determines the success of fertilization, development to blastocyst, and embryo growth. Germinal vesicle (GV), germinal vesicle break down (GVBD), formation of first mitotic spindle, and extrusion of first polar body are all

characteristics of maturation. Nuclear and cytoplasmic maturation are the two stages of oocyte maturation (Ayman *et al* 2016).

2.3.1 Nuclear maturation

During nuclear maturation, the process of developmental phases includes the acquisition of meiotic competence, meiotic resumption, completion of meiosis I, and the maintenance of metaphase-II arrest. (Saadeldin and Islam 2020). Follicular oocytes in mammals begin meiosis during fetal life and are halted at the diplotene stage of prophase I. (germinal vesicle stage). The oocytes remain at this stage until the onset of puberty. Under the influence of gonadotropins, particularly in response to the LH surge, oocytes mature fully and reach late antral stage, resume meiosis soon before ovulation, and are ready for fertilization (Lonergan and Fair 2016). The resumption of meiosis initiates the release of the nucleus from GV to form GVBD. GVBD characterized by the release of the nucleus from GV to create GVBD begins with the resumption of meiosis. GVBD is characterized by chromosomes rapidly condensing to metaphase and moving towards the animal pole of the oocyte, where they are divided into two haploid sets, one to be given off with the first polar body and the other to remain in the oocyte, nucleus absent, and enter meiosis II (Maziero 2014).

2.3.2 Cytoplasmic maturation

Cytoplasmic maturation refers to the cytoplasmic processes that prepare the oocyte for fertilization and developmental competence, and it takes place throughout oocyte development and at the same time as nuclear maturation. Migration of several organelles (mitochondria, ribosomes, endoplasmic reticulum, and cortical granules), modulation of energy levels, and accumulation of mRNA, protein, and substrates are all structural and molecular changes that occur during cytoplasmic maturation before the embryonic genome is activated (Watson, 2007; Mao *et al.,* 2014). Organelle redistribution, cytoskeleton dynamics, and molecular maturation are the three fundamental stages of cytoplasmic maturation, and they can be used to assess the mature oocyte's ability to undergo normal fertilization, cleavage, and blastocyst development in an indirect and retrospective manner (Ferreira *et al*, 2009; Mayes and Sirard 2002). The distribution of the organelles is controlled by the action of cytoskeleton microfilaments and microtubules (Mao *et al.,* 2014), during metaphase, long and generally stable microtubules are dispersed throughout the cytoplasm. According to Fan and Sun, (2004) cytoplasmic microtubule function is resumption of meiosis. During interphase, organizer centers (MTOCs) are phosphorylated and the activity of microtubule organization

increases. Mitochondria synthesize ATP needed for the production of proteins used during late embryo development as well also regulate the process of cellular apoptosis (Hammed *et al*., 2020). The main source of calcium ion required for continuation and completion of second meiotic division, suppression of polyspermy, and recruitment of maternal mRNAs critical for embryonic genome activation is the endoplasmic reticulum (ER), which reorganizes during cytoplasmic maturation (Ajduk *et al*., 2008). Golgi also apparatus required to transport intracellular protein. Golgi apparatus, ribosome, and lysosomes become less as oocyte maturation progresses and are very rare in fully mature oocytes (Fan and Sun, 2019). Failure to complete cytoplasmic maturation can halt development during fertilization, embryonic genome activation, blastocyst formation, and even after implantation (Zheng 2007).

3. FACTORS AFFECTING IN VITRO MATURATION

3.1 Maturation medium additives or supplementations

IVM determines the developmental competence of bovine oocytes. The media composition is the major factor that determines the maintenance of oocytes and achievement of the maturation and fertilization (Gardner, 1999, Alofi and Alhimaidi 2004). Similarly Shirazi *et al*., (2012) suggested, composition IVM has the capability of influencing the synthesis and storage of certain mRNA and protein that are important for further development.TCM-199, Hem's F 10 SOF, and MEM are some of the basic mediums (Ravindranatha *et al.,* 2001) used for oocytes maturation in mammals. There is a difference between basic mediums in their effectiveness for the maturation of oocytes even when used the same supplementation. When compared to synthetic oviductal fluid (SOF) supplemented with serum, supplementing TCM-199 with FBS during *in vitro* maturation is more successful in enhancing oocyte maturation. (Shirazi *et al.,* 2012). Many significant additions are added to the growing media to contribute to oocyte maturation and fertilization in an environment similar to the living body. These additions help in oocyte development because they contain essential materials for the process of oocyte maturation (Mohammed 2019).

The most extensively used culture media for IVM of bovine oocytes is TCM-199. (Arunakumari *et al,* 2007). The positive effect of this base medium on animal oocyte IVM could be due to some of the ingredients in it, such as essential amino acids and glutamine, which promote DNA and RNA synthesis and cell division, Enhanced the rate of maturation *in vitro* (Gordon 2003; Roushandeh *et al*, 2006). Pyruvate lactate, amino acids, sodium bicarbonate vitamins fetal bovine serum (FBS) and Bovine serum albumin (BSA) are the substances used to modify and improve the base maturation medium in all laboratory

3.1.1 Protein

Protein supplementation fetal calf serum (FCS) or Bovine serum albumin (BSA) are commonly used to enhance mammalian oocyte and embryo development (Lim *et al.,* 2007). The main protein supplements in the IVM medium are FCS and BSA, which contain small peptides, energy substrates, and growth factors that improve maturation, fertilization, blastocyst formation, and hatching rates *in vitro*. (Thompson, 2000, Chaudhry *et al*., 2008). Despite increasing blastocyst yield, the serum also increases the accumulation of cytoplasmic lipids, reduces embryo survival after cryopreservation (Abe *et al*., 2002), increases the male to female embryo ratio, and interferes

with normal gene expression (Rizos *et al*., 2002). Its use in culture media has been implicated in diverse phenotypic alterations observed during gestation and in bovine new borne such as placental defects and large offspring (McEvoy, 2003). For the reason that BSA and FCS are prepared and purified from bovine blood products, they present a risk of contamination by pathogens and viruses (McDowall, 2004).

3.1.2 Glucose

Glucose is an important energy substrate when added acceptable amount to IVM, it is basic and essential for oocyte metabolism. The importance of supplementing IVM with glucose in cattle is to boost the rate of meiosis resumption, embryo cleavage, morulae, and blastocyst formation. (Wrenzycki and Stinshoff, 2013). During IVM glucose via glycolysis produces ATP and other substrates like pyruvate to produce energy used for oocyte metabolism. High glucose concentrations during IVM enhance the production of reactive oxygen species (ROS) and decrease intracellular GSH pools, causing cellular elements to be damaged and cell function to be disrupted, which impairs subsequent embryo development. (Hashimoto *et al*. 2000b).

3.1.3 Follicular fluid

FF consisting of electrolytes, hormones, amino acids, growth factors, provides an appropriate environment for bovine oocyte development it increases the degree of cumulus cell expansion and improves embryonic development (Helena *et al.,* 2014). Supplementation of the IVM medium by bovine follicular fluid (bFF) at the 10-20% level favorably influence oocyte quality and subsequent embryonic development in cattle whereas supplementation with a higher amount of FF having an inhibitory effect on nuclear maturation, as a result, increase rate of oocyte degradation (Ayman *et al* 2016).

3.1.4. Hormone

Gonadotropins, steroids, and growth factors separately or in combination can be a supplement of *in-vitro* culture media used to stimulate or inhibit cumulus expansion and/or nuclear and cytoplasm maturation mammalian oocytes. The supplementation of growth factors, e.g., epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), stimulate oocyte maturation and had beneficial effects on blastocyst production rates in several species (Byrne *et al*., 2002). The addition of FSH, LH, and GH in IVM media is common. FSH promotes the production of the LH receptor by cumulus cells and potentiates the activity of growth factors like EGF and PDGF. LH has a direct

effect on the oocyte's metabolism, enhancing glycolysis and oxidative phosphorylation. GH enhances the rate of development to the blastocyst stage by speeding up nuclear maturation, stimulating cumulus expansion, and increasing the rate of development to the blastocyst stage in the absence of serum and gonadotrophic hormones (Kandil *et al*., 2000).

3.2 Quality of Cumulus vestment

Using appropriate criteria to choose oocytes for maturation and subsequent embryonic development is an important element. The morphology of the COC in particular the cumulus vestment the compactness of cumulus cell layers and the homogenous appearance of the cytoplasm has been the most common marker of immature oocyte capacity to undergo maturation and develop to embryo (Ayman *et al* 2016). The oocytes surrounded by intact cumulus cells better in developmental competency than oocytes surrounded by compromised vestments (Sirard *et al*., 2006). Cumulus cells provide cell communication through gap junctions, Paracrine and gap junction-mediated bidirectional communication between the oocyte and its cumulus cells is significant for efficient maturation of the oocyte (Vansoom *et al.*, 2002; Sutton *et al.*, 2003). In ovarian follicles, cumulus cells play an important role in oocyte growth and differentiation by providing nutrients and by controlling both nuclear and cytoplasmic maturation when oocytes are selected for ovulation (Boni *et al*., 2002). IVM of bovine oocytes in the presence of bovine LH resulted in increased embryonic development after IVF. Oocytes denuded of cumulus cells did not respond to LH, thereby implicating the cumulus cells as the mediator of the LH effect. During IVM of bovine oocytes, the absence of cumulus cells impedes nuclear maturation (Sutton *et al*., 2003).

3.3 Effect of follicular and size presence of dominant follicle

Because the oocyte's growth is essentially complete in cows when the follicle reaches a diameter of 3 mm, oocytes from small bovine follicles (under 3 mm) have lower developmental competence. There is a link between follicle size and oocyte competence; as the follicle grows larger, so does the oocyte competence (Lojkic *et al.*, 2016). Shabankareh, (2014), classifies the size of follicle < 3mm as small, 3-6 mm as a medium, and > 6 mm as large, and found the higher blastocysts rate where there were medium and large follicles than oocytes from <3 mm, (26.65% and 25.69%) respectively. Oocytes recovered from follicles >5 mm showed a rapid rise in the rate of cleaved embryos by 27 hours post insemination for both oocytes collected from =5 mm and >5 mm follicles, at which time the total percentage of cleaved embryos was significantly affected by

follicle size (39.5 percent vs. 58.3 percent for =5 mm and >5 mm follicles, respectively (Lojkić *et al*., 2016).

It could be linked to the fact that bovine oocytes from 2-mm follicles have not yet completed their growth. According to Farin, (2003), the higher developmental capability of oocytes derived from large follicles (>6 mm) is likely attributable to differentiation that happened later in the follicular development process.

The changes in large follicles, like an expression of LH receptors by granulosa cells, decrease of IGF-binding protein and increase of IGF-I within the follicular fluid and increased expression of growth factors such as TGF-β, activin, and inhibin happen simultaneously with ultrastructure changes within the oocyte and cumulus cells and with further growth of the oocyte (Hendriksen *et al*., 2000).

During the growth of oocytes inside the follicles, some factors influence the quality and development of their competence. These factors include follicular diameter, day of the estrous cycle, atresia levels, and influence of other follicles as DF (Hageman, 1999). A higher number of blastocysts are observed when the oocytes are collected during the follicular growth phase than those collected during the follicular dominance phase. The dominant follicle has an inhibitory effect on the development of subordinate follicles causing their atresia, mainly through inhibin and estradiol 17-β secretion (Hageman, 1999).

3.4 Effect of oocyte recovery method

Oocyte quality is one of the major factors determining the success of embryo production. The cumulus cells that surround the oocyte are known in playing an important role through providing nutrition, energy substrates, and mediating the beneficial effects of hormones on the cumulus-oocyte complexes (COCs) (Krisher, 2004). The effectiveness of the oocyte collection procedure has a big impact on cumulus cell investment. Ovaries from abattoir animals are the cheapest and most plentiful source of primary oocytes for embryo development. COC recovery has been used in numerous approaches to harvesting high-quality oocytes with higher cumulus cell layers from ovaries taken from slaughtered cows, including aspiration of follicles, puncturing of follicles, and slicing of ovaries (Mehmood *et al.*, 2011) to harvest high-quality oocytes with more number of cumulus cell layers from ovaries collected from slaughtered animals. However, the oocyte yield variation in quality with the harvesting technique employed (Mahesh 2014). According to the study Sonowal *et al*., (2018) on the effect of the recovery on

COC quality, the recovery rate of quality COC using aspiration was excellent, good, and fair quality was 62.27 ±1.60, 23.73 ±1.55, and 13.98 ±1.41 respectively. Where the recovery rate of the slicing method was 30.40b ±1.74, 51.36a ±2.01, and 18.23c ±1.31 respectively. This indicates methods we use to remove the COCs from the ovaries influence the recovery rate of excellent quality of COC, this influence longer the maturation because oocyte quality significantly determines the maturation rate Trans-vaginal oocyte pick-up (OPU) is an important technique for oocyte retrieval in living donor cow, the success of OPU is measured in part by the recovery rate of oocytes. The rate of recovery is influenced by a variety of factors, including aspiration vacuum, puncture frequency, hormonal pre-treatment of animals, and the operator's experience. The rate of recovery of grade 1 oocytes reduced dramatically as the vacuum pressure increased, with a corresponding increase in the number of denuded oocytes recovered, which has an impact on oocyte fate (Mahrous *et al*., 2016).

3.5 Effect of duration of maturation

Duration of the maturation culture period is an important factor for successful maturation. Most of the time majority of oocytes cultured *in vitro* can reach MII within 18-24h (Sybrand, 2003). Both Extended and too short maturation time has adverse effects on the developmental potential of the embryo. For example, excessive maturation time causes the aging of oocytes that leads to the condition to favor genetic associated risks, where culture for a too-short time affects the synchronization of nuclear and cytoplasm therefore the subsequent embryonic development will impaired (Aguila, 2020). Incubation temperature also has an important effect on oocytes maturation. For instance, According to the suggestion of (Şen and Kuran, 2018), low incubation temperature decreased the percentage of first polar body extrusion in Grade II oocytes. The percentage of first polar body extrusion of grade II bovine oocytes after 24h incubated on 36.5^{0C} and $38.5^{0\,C}$ was 63.0 and 74.1 respectively.

3.6 Effect of breed

The reproductive physiology such as ovarian follicular populations, production of growth factors, and Anti Müllerian hormone, circulation level of P4 differences between *Bos indicus* and *Bos Taurus* cattle influence their follicle count, quantity, and quality oocyte (Barbosa *et al.,* 2020). According to Alvarez *et al.*, (2000), *zebu* cows of the Brahman breed higher IGF-1 concentrations in the plasma where lower in follicle-stimulating hormone (FSH) concentrations compared to *taurine* cows of the Angus breed. A high concentration of IGF-1 is the factor for a greater quantity

of follicles in the ovary recruited per follicular wave (high follicles population) as consequence better in COC recovery rate during OPU than *boss Taurus*. The greater follicle population favors obtaining a high number of quality oocytes than that of *boss Taurus* (Batista *et al.*, 2014).

4. MORPHOLOGICAL AND VISUAL MARKERS FOR MATURE OOCYTES

4.1 Expansion of cumulus cell

Indirect morphological parameters that can be taken into account to evaluate the maturation cumulus cell expansion, extrusion of the first polar body, zona pellucida, and an increased perivitelline space are clearly detectable under a stereomicroscope (El-raey and Nagai, 2014). Cumulus cell expansion is one significant indicator for oocyte maturation. The degree of expansion also an indicator of developmental potential in bovine. CCs and their expansion play an important role in fertilization by inducing the acrosome reaction and, therefore, promoting higher fertilization rates (Aguila *et al.,* 2020). During meiotic maturation of the oocyte, cumulus cells change their morphology and metabolic activity. Thus, oocyte with meiotic maturation within the follicle, cumulus cells synthesize structural components of the extracellular matrix. The synthesis of glycosaminoglycan rich in hyaluronic acid (HA) into the extracellular space plays a role as the structural component of expanded cumuli and signal molecule regulating oocyte maturation. *In vivo*, cumulus expansion occurs immediately before ovulation, while *in vitro*, it occurs during meiotic maturation. The enlargement of COCs occurs significantly influences oocyte maturation and developmental competence acquisition so that COC expansion is one of the morphological indicators of meiotic maturation or cytoplasm maturation (Han *et al.,* 2006; Aguila *et al.,* 2020).

4.2 Extruded first polar body

First polar body formed after the germinal vesicle breakdown stage completed. Immediately after GVBD chromatin condenses to form chromosomes and a bipolar microtubule formed. In the meiosis, I phase the spindle moves to the oocyte surface and half of the homologous chromosomes segregate into a small unit. This is the mark of completion of first meiosis and reaches MII, This small unit is known as extruded first polar body is the cellular landmark of meiotic maturation indicator of nuclear maturation assurance of a functional component of cytoplasmic competence,(Rose and Laky 2013; Viveiros and Fuente, 2019 ; Hammed *et al.,* 2020).

4.3 Formation of perivitelline space

The perivitelline space is a subcellular structure that exists between the ZP and the oocyte plasma membrane that is rich in extracellular matrix components, which are essential for fertilization, implantation, and embryo development. PVS is commonly formed as a result of meiotic completion

(Inoue *et al.*, 2007). Increased perivitelline space is the indirect morphological parameter to evaluate cytoplasmic maturation (El-raey and Nagai, 2014).

4.4 Condition of zona pellucida

The zona pellucida (ZP) is the extracellular coat that surrounds growing oocytes, ovulated oocytes, and early embryos which plays an important role in oogenesis, fertilization, and pre implantation development participates in blocks polyspermy after fertilization and protects early embryos as they travel through the female reproductive canal. Follicular fluid secretion from granulosa cell and cumulus cells increase as follicles grow resulted in ZP formation (Wassermann 2008). The ability to activate an oocyte for embryonic development can be determined by ZP thickness, which can be used as an indicator of oocyte maturation. (Zhou *et al.*, 2014).

5. MATERIALS AND METHOD

5.1 Study area and animals

The study was conducted from December 2020 to June 2021 at Debre Zeit Agricultural Research Center (DZARC) Animal Biotechnology Laboratory. The area located 47 km southeast of the capital city, Addis Ababa, at 9^0N latitude and 4^0E longitudes, and an altitude of 1850 m.a.s.l. The mean minimum and maximum annual temperature ranges from 10.55^0C to 27.45^0C which makes the mean annual temperature of $19.^0$C; mean annual rainfall of 779.3 mm; and mean monthly relative humidity of 57.12% (DZARC Agro-meteorology 2018). Totally 10 healthy cows (5 Borans and 5 Holstein Friesian X Boran cross) with an average body condition ranging 3 to 4 (on the scale of 1-5) and 1 to 2 parity were used for the OPU procedures. All cows were vaccinated for common contagious diseases and managed under a uniform housing system. The study animals were fed on teff straw and grass hey basal diet and supplemented with concentrate (mixture of 50% wheat bran, 25% wheat short, 24% nuge seed cake, and 1% salt). Additionally, the animals were allowed to graze and had ad libitum access to water.

5.2 Study design

A total of 10 experimental animals were grouped into two breeds (5 indigenous pure Boran breed cows and 5 Holstein Friesian X Boran cross with 75% exotic blood level.

The collected oocytes from both breeds were grouped into two according to media used for the study, BO-IVM, and other TCM-199 base maturation medium. Main effect of Breed and Maturation media type were evaluated based on recovery rate in the OPU sessions, oocyte population, oocyte quality in accordance with Grades, and maturation indices (cumulus expansion, polar body extrusion, conditions of the zona and formation of perivitelline space).

5.3 Ovum pick-up (OPU)

Experimental cows were selected from a pool of dairy herds with no history of reproductive diseases and found to be in good health at the time of the experiment. The cows were subjected to gynecological evaluation using ultrasonography to confirm cyclicity as well as the soundness of the reproductive tract. Cows with disorders such as COD or any other identifiable problem were rejected.

Cows were guided into a chute and administered epidural anesthesia (2 to 5 ml of 2% lidocaine, JEIL.PHARMA.CO, LTD, Daegu, Korea) to prevent straining during aspiration. After emptying the rectum, the vulva and perineal area were thoroughly cleaned and disinfected. Follicular aspiration was performed transvaginal on each visible follicle that was ≥ 3 mm in diameter. An ultrasound (Aloka SSD Pro-Sound 2, Japan) s with a 6.5 MHz convex array transducer that was fitted into an intravaginal needle guide (Hitachi Medical Co., Tokyo, Japan) was used for visualization of follicles during aspiration. The follicular puncture was performed using a disposable 18-gauge x 12 mm aspiration needle that was connected to a 50-ml conical tube via a 2 m long silicon tube fitted to an aspiration pump that has a warming block (mini-tube, GmbH, Germany) adjusted to 38.7^0C. A vacuum aspiration pressure of 72 to 80 mmHg equivalent to a flow rate of 15-25 ml/min was used for follicular aspiration. Follicles were aspirated into a 50ml tube containing about 10ml of the recovery media supplemented with heparin, FCS, gentamicin, and HEPES.

5.4 Media preparation

5.4.1. Recovery media

About 100ml of distilled water was made ready in a sterilized beaker. Using digital balance 0.9g DPBS, 0.05gm gentamicin, and 0.024gm HEPES were weighed and added to the distilled water. A stirrer was used to mix the ingredients thoroughly, then the mixture was filtered using a 2µl filtering membrane, sealed, and then kept at +4 in a refrigerator until day of recovery or oocyte collection. On the day of recovery 200 µl, heparin, and 2ml FCS were added to per mixed ingredients and made ready for use.

5.4.2 Maturation media.

One day before the recovery date, a 10ml TCM-199 stock solution was taken and placed in a 50ml falcon tube. Gentamycin (0.05g), 0.22g $NaHCO_30$, and 022g $NaHCO_3$ were weighted and added to TCM-solution to which 0.2ml FSH and 2ml FCS were also added and mixed. The mixtures were filtered using a 2µl filtering membrane, sealed, and then kept at +4 in a refrigerator.

The first oocyte maturation medium TCM- 199, base maturation medium, was a premade ready to use compound available as a 10ml TCM-199 stock solution (Gibco Grand Island, NY) into which

0.2ml FSH (Pluset® Barcelona, Spain), 2% FCS, 0.05g Gentamycin, 0.22g $NaHCO_3$0 and 022g $NaHCO_3$ were added.

The second maturation medium BO-IVM (IVF Bioscience, United Kingdom) was a complete medium ready to use with optimized formulae that eliminate the need for the addition of further components. The basic differences between the two maturation media are the first medium is free from serum that is substituted by glucose.

The serum provides many beneficial factors to the COCs and embryo such as amino acids, vitamins, growth factors, and energetic substrates; promote COCs maturation additional facilitate fertilization by preventing hardening of Zona, however, as the serum is extracted from a live animal, it may also contaminate the culture media with toxic factors, increases the male to female embryo ratio and disturbs gene expression (Rizos *et al*., 2003). Its use in culture media has been implicated in diverse phenotypic alterations observed during gestation and in bovine newborns such as placental defects and large offspring syndrome.

Glucose is an essential energy substrate when added in an acceptable amount to IVM, provides energy which is the fundamental requirement for oocyte metabolism. Furthermore, glucose improves the resumption of meiosis in cattle COCs which is essential for oocytes to achieve full developmental competence for fertilization, enhance embryo cleavage, development to morulae and increase blastocyst rates.

All media including the recovery media were prepared one day before the recovery date. Media preparation was carried out in a sterile environment under a laminar flow cabinet to avoid any contamination. They were thoroughly mixed, filtered using a 2µl filtering membrane, sealed, and then kept at +4 in a refrigerator until the next day of collection. The maturation medium was later withdrawn and kept in a CO2 incubator for at least 2 hours before use.

5.5 Oocytes searching and recovery

The aspirated fluid was diluted in HEPES media and transferred into a 60 mm petri dish and left for 5 minutes to settle. Oocytes were searched under a stereo-microscope (Motis SMZ, Roanoke USA). The COCs were examined and selected based on their morphology, the compactness of cumulus, and the homogeneity of the cytoplasm. All oocytes handling was performed under a

cleaned and disinfected laminar floor. All procedures were carried out within an hour to avoid aging and deterioration of oocytes.

Oocytes were selected and assigned a grade for maturation according to the criteria previously described in Paul, (2019).

Grade I - Oocytes with Compact multi-layered cumulus cells (≥5) and homogeneous transparent cytoplasm.

Grade II - Oocytes with compact multi-layered cumulus cells (at least 3-5) and slightly granulated dark cytoplasm.

Grade II - oocytes with less compact cumulus cells (1 to 3 layer and granulated dark cytoplasm)

Grade IV - oocytes had no cumulus (denuded). Oocytes with Grades I, II, and III were selected for maturation. All necessary data were recorded on a previously prepared excel spreadsheet.

5.6 In vitro maturation

The selected oocytes were then washed three times in their respective media with TCM-199 maturation media for the TCM group or BO-IVM medium for the BO-IVM group. IVM drops of 500µl were prepared in the meantime in a 4-well embryo culture dish and covered with paraffin oil and left in a CO2 incubator for 2 hours for equilibration. About 15-20 oocytes (COC) were then transferred into each droplet under the paraffin oil. The oocytes were then placed in a CO2 incubator set at 39^{o}c under a 90% humidified atmosphere of 5% CO2 in air for 24 h.

5.7 Assessment of maturation parameters of oocytes

All maturation parameters, COCs expansion, extrusion of first polar body, increment of perivitelline space as well as the form of zona pellucida (thickness, and regularity of its shape) were evaluated under the stereomicroscope after 24 hours of incubation. Cumulus cell expansions were rated as indicated below:

- Fully expanded COC - the Cumulus cells spread non- homogenously with no clustered cells
- Partially expanded: the cumulus cells spread homogenously and with the presence of clustered cell
- Unexpanded: the cumulus cells remain attached to the zona

COCs with fully and partially expanded cumulus cell layers were considered as maturated oocytes.

COCs were washed three times in buffered saline solution and made completely devoid of cumulus cells by vortexing. The following maturation characteristics were evaluated.

- COCs exhibiting small unit between zona pellucida and perivitelline space considered as a COC with first polar body extruded
- COCs exhibiting a subcellular space between the ZP and the oocyte plasma membrane are considered as COCs with increased perivitelline space
- COCs rounded with bright and regular outer coat recorded as qualified ZP formation

The oocyte maturation parameters were later on used to study the influence of the maturation media.

5.8 Data management and statistical analysis

Data were grouped according to the breed of the animal, oocytes quality, and maturation media. Descriptive statistics were used to determine the frequency and proportional distribution of oocyte count, quality and maturation rate. ANOVA and t-test, were used to determine the difference in groups of independent variables such as the breed of experimental animals, puncture session, and maturation media with the dependent variables like a number of counted and aspirated oocytes, oocyte quality, and maturation rate. LSD analysis was used to test the mean difference among the groups. The relationship between specific maturation media and oocyte maturation rate was determined using a person correlation test. The result was reported as mean ± SEM/SD. Statistical significance was determined at ($p< 0.05$).

6. RESULT

6.1 Ovum pick up and oocyte recovery rate

A total of 663 follicles ≥ 3mm were counted and 288 oocytes were retrieved from both Boran and Crossbred cows with a recovery rate of 43.4%. A summary of the OPU session and oocyte recovery is presented in Table 2. Oocyte recovery rates for Boran and Crossbred cows were 39.3% (131/333) and 47.6% (157/330), respectively. There was no difference ($p>0.05$) between the Breeds in oocyte recovery rate.

Table 2: Oocyte recovery rate of Boran and Crossbred cows

Breed	Animal no.	Puncture session (n)	Follicle punctured (n)	Oocyte retrieved (n)	Recovery rate (%)
Boran	1	12	86	43	50.0
	2	12	69	29	42.0
	3	12	54	25	46.3
	4	12	60	16	26.7
	5	12	64	18	28.1
Boran*HF	1	12	75	30	40.0
Crossbred	2	12	72	40	55.6
	3	12	60	14	23.3
	4	12	56	27	48.2
	5	12	67	46	68.7
Total	10	120	663	288	43.4

Eighty percent of all collected COCs were incubated for maturation. 20% of collected COCs were discarded due to poor quality while 81.6% (107/131) and 78.9% (124/157) of the recovered COCs from Boran and Crossbred, respectively, were incubated. The quality of COCs for Boran and Crossbred cows is indicated in table 3.

Table 3: COCs collected from Boran and Crossbred cows classified based on quality grades.

Breed	Collected COCs (n)	Grade I %,(n)	Grade II % ,(n)	Grade III %, (n)	Grade IV % ,(n)
Boran	131	22.14(29)	32.5(43)	27.08(35)	18.30(24)
Crossbred	157	25.48(40)	27.4(43)	27.06(41)	21.02(33)
Total	288	23.96(69)	29.9(86)	27.14(76)	19.79(57)

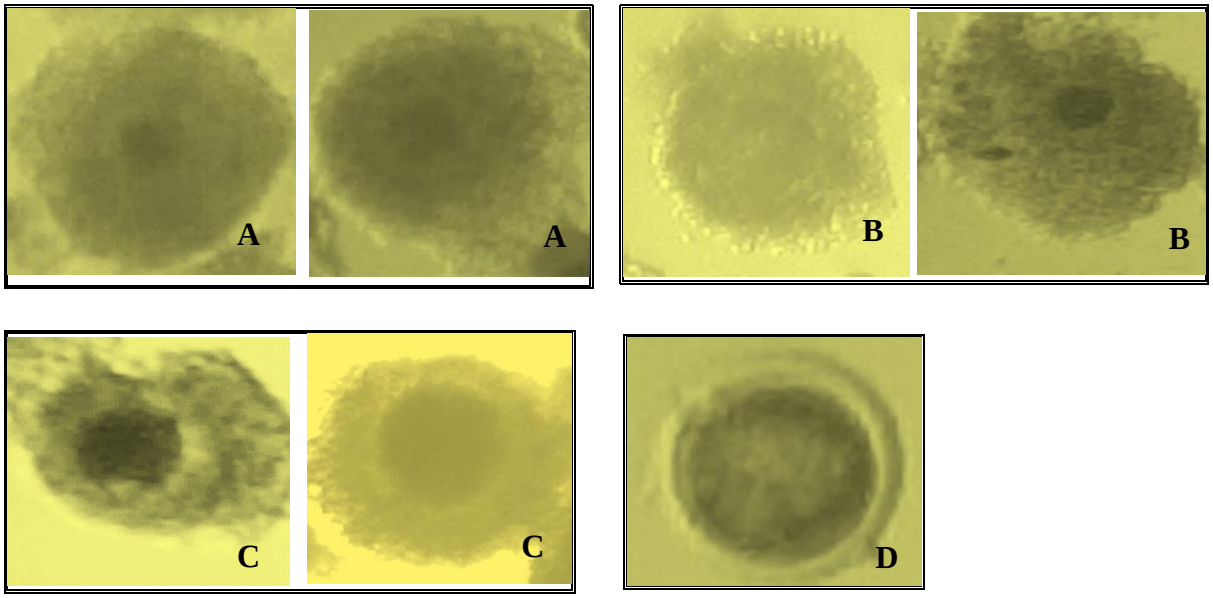


Figure 2. Images of different qualities (**A-** Grade I**, B-** Grade II**, C-** Grade III**, D-** Grade IV) of immature oocytes recovered from experimental cows**.**

The oocyte recovery was undertaken for twelve rounds (12 OPU session) in each breed. There was no difference (p>0.08) between Boran and Crossbred cows in the mean number of oocytes collected per OPU session (Table 4). The mean number of oocytes collected per animal was 2.18 ± 1.90 and 2.62 ± 1.86 for Boran and Crossbred cows, respectively. There was no difference (p>0.05) between Boran and Crossbred cows in the mean number of oocytes collected.

Table 4 the mean number of oocyte collected per OPU session

Breed	N	Mean (±SD)	Range
Boran	12	10.92±3.37	6-19
Crossbred	12	13.08±2.23	9-16
Total	24	12.00±3.01	6-19

6.2 In vitro oocyte maturation

The overall maturation rate of oocytes aspirated from Boran and Crossbred cows was 79.2%. The maturation rate of COCs recovered from Boran and Crossbred were 79.4% (85/107) and 79% (98/124), respectively. The maturation rate in terms of fully expanded COCs was 52.4% and 54.4% for Boran and Crossbred cows, respectively while 27.2% of Boran and 25.6% of Crossbred COCs were only partially expanded during maturation. The proportion of unexpanded COCs from Boran and Crossbred cows was 17.5% and 15.2%, respectively. The proportion of increased perivitelline space in matured Boran and Crossbred COCs were 64.1% and 60.0 %, respectively. First polar body extrusion was recorded in 47.6% of Boran and 49.6% Crossbred cows COCs at the end of maturation. There was no difference ($p>0.05$) in the maturation rate of Boran and Crossbred cattle oocytes.

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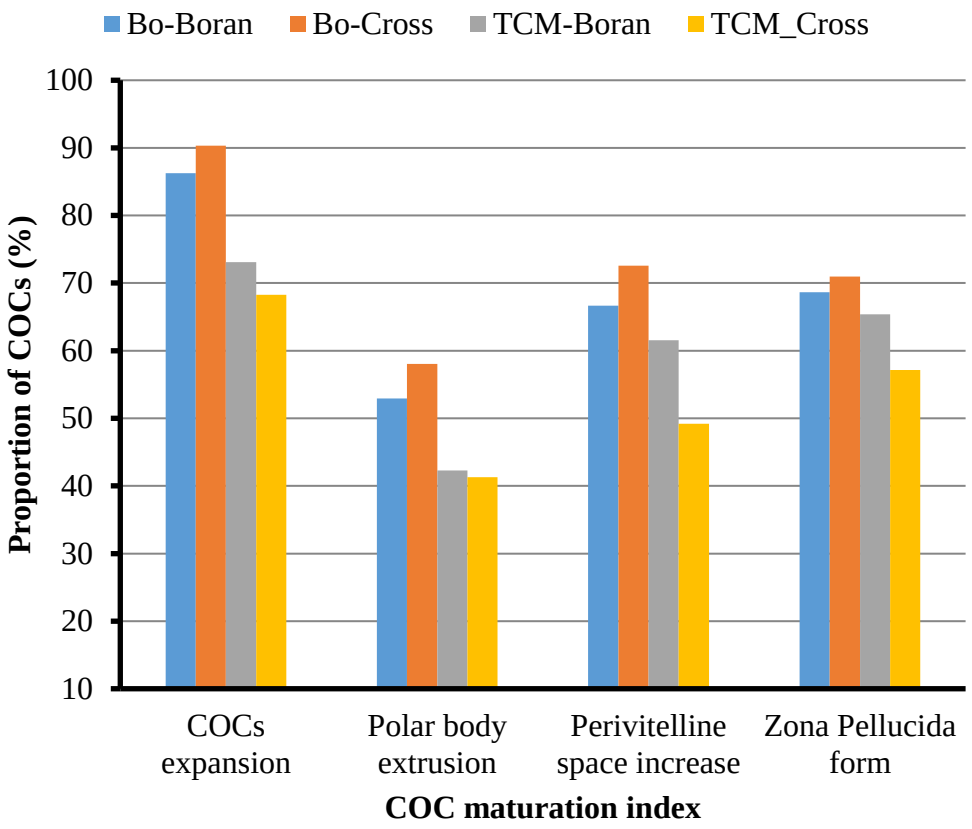

Figure 3: Percentage of COCs expansion, Polar body extrusion, PSI and ZPF for Boran and Crossbred cows in BO and TCM-199 maturation media

88.5% and 70.43% COCs matured in BO and TCM-199 maturation media, respectively. Fully expanded COCs were 59.3% and 46.9% in BO and TCM-199 maturation media, respectively. 30.1% and 24.4% of COCs were partially expanded in BO and TCM-199 maturation media, respectively. The proportion of unexpanded COCs in BO and TCM-199 maturation media was 7.1% and 25.2%, respectively. There was a significant difference (p<0.001) in the proportion of fully expanded and unexpanded COCs in BO and TCM-199 maturation media. The maturation rate of oocytes in BO maturation media in terms of first polar body extrusion increased perivitelline space and zona pellucida form was 55.75%, 69.62%, and 69.8%, respectively. The maturation rate in TCM-199 maturation media in terms of first polar body extrusion increased perivitelline space and zona pellucida form was 41.7%, 44.8%, and 60.9%, respectively. The proportion of oocyte matured in BO media was 86.3% and 90.3% for Boran and crossbred cows, respectively. The proportion of matured oocyte in TCM-199 maturation media was 73.1% and 68.3% for Boran and

Crossbred cows, respectively. There was no difference ($p>0.05$) in maturation rate between Boran and crossbred cows oocytes, However there was significantly difference ($p<0.05$) between BO-IVM compared to TCM-199.

Table 5: Proportion of matured COCs in BO and TCM-199 media

Media	Breed	Incubated COCs	Matured %,(n)	Fully expanded %,(n)	Partially expanded %,(n)	Unexpanded %,(n)
BO-IVM	Boran	53	86.27 (46)	56.86 (30)	29.41 (16)	7.84 (7)
	Cross	62	90.32 (56)	61.29 (37)	30.65 (19)	6.45 (6)
TCM-199	Boran	54	73.08 (39)	46.15 (25)	25.00 (14)	26.92 (15)
	Cross	62	68.25 (42)	47.62 (29)	23.81 (13)	23.81 (20)
Total		231	79.22 (183)	53.07 (121)	27.19 (62)	16.23 (48)

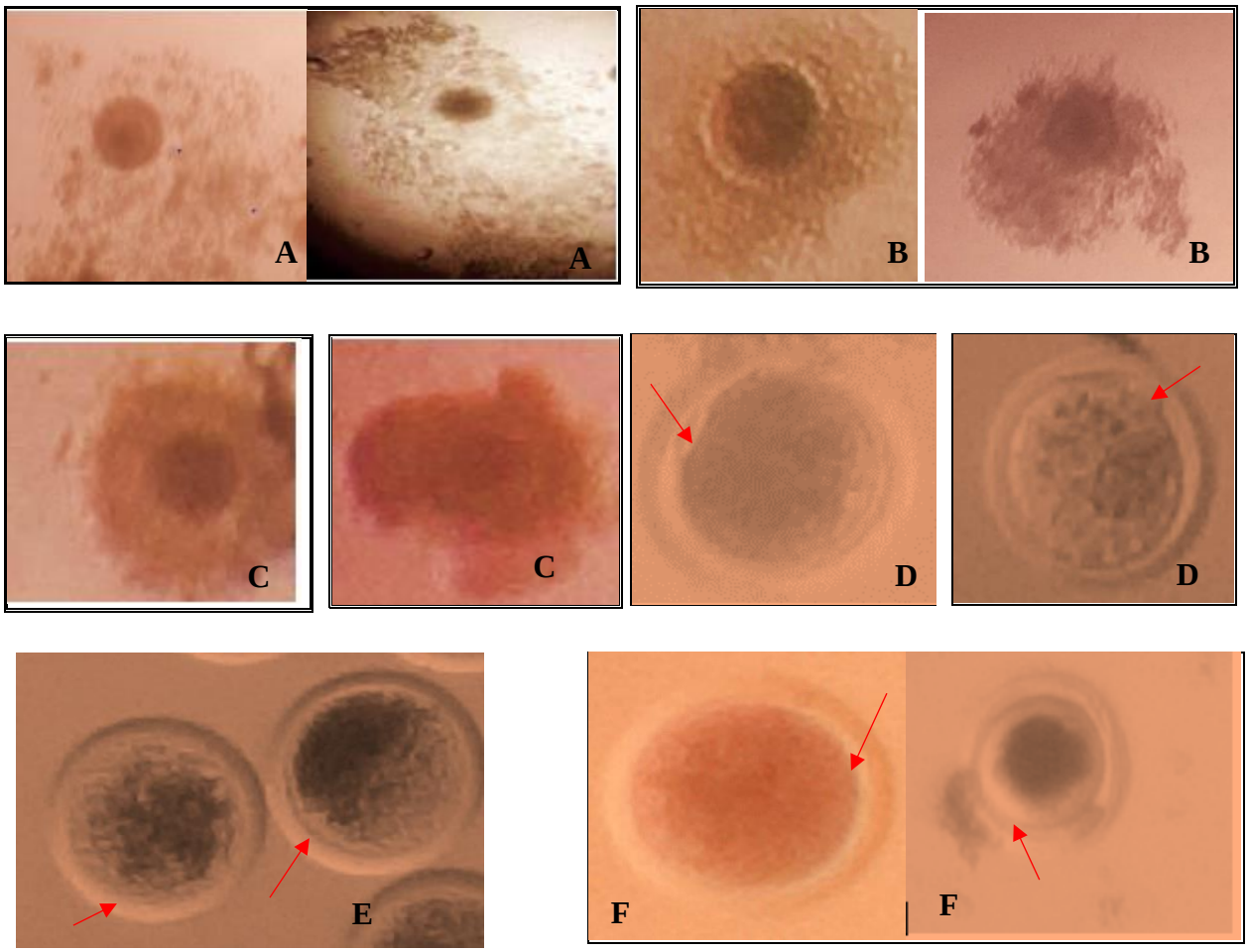

Figure 4. Images of mature oocytes **A**, fully expanded, **B**-partially expanded, **C**- unexpanded, **D** extruded **1PB, E**-smooth, and regular shape ZP, **F**- increased PS

7. DISCUSSION

The recovery rate for both Boran and crossbred cows oocytes was identical. In spite of the higher number of follicles aspirated from crossbred cows there was no difference in oocyte recovery rate between the genotypes. This finding was in agreement with previous finding reported by Jemal *et al.* (2020), 42.3% and Neglia *et al.* (2011), 49.11% , but higher from the recovery rate in Thai indigenous cows 28% and 18.1% reported by Pellerano *et al*. (2017), and Sakhong *et al,*. (2012) respectively. There was no difference between breeds in quality of COCs. More than eighty percent of recovered COCs were with recommended quality standard for maturation. There was a consistent trend with previously report by Ali *et al*. (2021), however the result in this study was slightly greater than the finding of, Kang *et al.* (2019), in Howoo cows immature COCs quality and Nelore cows COCs quality Santos *et al*., (2005).

There was no difference in the mean number of oocytes collected per OPU session for purebred Boran and HF*Boran crosses. The mean number of recovered oocytes in this study was lower from the previous report in Ethiopian Boran and their crosses by Ali *et al*. (2021), however, it was comparable to the study by Sakhong *et al.,* (2012) in Thai indigenous beef cattle. *Boss indicus* breeds are known to grow and produce more follicle population and better recovery rate as well as better COCs quality than *Boss Taurus* breeds (Batista *et al.,* 2014). This is due to differences in their reproductive physiology in the production of hormones responsible for growth factors, and circulating P4. Differences were not observed in antral follicle count, oocytes recovery rate, and COCs quality between the breeds. Climate temperature and nutritional status also contribute effect on several growing follicles. The experience of the operator is one of the factors that affect the obtaining of good COCs numbers during OPU (Dode and Adona, 2001; Silva *et al.,* 2011).

Cumulus cell expansion is one significant morphological indicator of meiotic competence of oocyte maturation and the degree of expansion also an indicator of developmental potential in bovine (Deb *et al.,*2011; Jahan *et al*., 2012; Aguila *et al*., 2020). In the present study, the overall oocytes maturation rate was comparable with the result of Islam *et al.* (2007), in Zebu and Vijayalakshmi *et al.,* (2020) in Buffalo oocytes. The maturation in terms of COC expansion was greater compared to the maturation rate of abattoirs oocytes reported by Deb *et al.,* (2011). The proportion of oocytes extruded their first polar PB were lower than the result reported by (Deb *et al.* 2011), and greater than the reported by Vijayalakshmi *et al.,* (2020).

Cumulus cells surround the egg and play an important role in the acquisition of full developmental competence during maturation by facilitating cell-to-cell contact via gap junctions, as well as mediating the favorable effects of hormones and delivering nutrients and energy substrates (Krisher, 2004). According to Nevoral *et al.,* (2015) the synthesis of glycosaminoglycan contains hyaluronic acid (HA) into extracellular space is the foundation of COC expansion, and HA important for COCs expansion. For COCs effectively expanded the adequate amount of HA is a prerequisite, this means the amount of HA synthesized has a direct relationship with the compactness of the COCs layer (Salustri *et al* 1999; Kimura *et al*., 2002). Hence in addition to other factors for partial and unexpansion of cumulus cell may be a result of poor quality oocytes or oocytes with less cumulus cell layers at the beginning of the maturation. However, the partially expanded COCs are accepted as intermediate maturity and suggestive for fertilization (Lasiene *et al.,* 2014; Shrikant *et al.,* 2020).

The overall maturation rate of oocytes matured in BO and TCM was greater than reported by Rahman *et al.* (2018), TCM, mSOF, and TALP was 74.5%, 67.7%, and 58.2%, respectively, Bari and Farida (2015). 75.5 % and 62.2 in TCM and SOF respectively. Mohammed, (2019) TCM -199, SOF, MEM, SOF + BCS, SOF + BSA, (57.31 ± 3.68), (37.02%), (51.57%), (55.65%), respectively, and comparable with the result of Sonjaya and Hasbi (2019), 91.53%.

The media composition is one of the major factors that determine the maintenance achievement of the maturation of oocytes (Alofi and Alhimaidi 2004). The main variation among the maturation medium is significant additions that are added to the growing media to contribute to oocyte maturation and fertilization in an environment similar to the living body, for instance, the addition of gonadotropins hormones improve quality and development ability by enhancing metabolic processes the oocyte (Sonjaya, 2017). The addition of essential amino acids and glutamine in the base medium increased the rate of in vitro maturation by accelerating DNA and RNA synthesis and increasing cell division (Gordon, 2003; Roushandeh *et al,* 2006).

The maturation rate of oocytes matured in BO-IVM was higher than the oocytes matured in TCM-199 on both breeds. The higher maturation rate of oocytes matured in BO-IVM media seemed may be due to the favorability of glucose in oocyte maturation. Wrenzycki and Stinshoff (2013) described that Glucose is an essential energy substrate when added acceptable amount to IVM, energy is an important requirement for oocyte metabolism. Glucose enhances the resumption of

meiosis in cattle COCs which is fundamental for oocytes to achieve full developmental competence for fertilization. COCs consume glucose in a different way over the different stage of maturation For instance glucose support induction of meiosis by increasing the production of substrates involved in nuclear maturation in pentose phosphate pathway. Glucose also support COCs maturation by converted to extra-cellular matrix component which involved in COCs expansion (Downs *et al.,* 1998; Mcdowall, 2004).

The serum is one of the popular protein source supplement to maturation media contains essential components such as growth factors, lipids, albumin, hormones, steroids, cholesterol, peptides vitamins that an important to stimulate cell growth and proliferation, promotes differentiated functions, support the rupture of germinal vesicle and induces oocytes maturation. Moreover, serum favors fertilization by preventing the hardening of the zona pellucida (Gstraunthaler, 2003; Puri *et al.,* 2015,). In contrast, the highly undefined nature of serum makes it undesirable in many research aspects due to the risk of batch variation and contaminating characteristics of undefined compounds (Mcdowall, 2004). Hence the effect of undefined compounds may be the cause for the lower maturation rate of COCs matured in TCM-199 contain 2% serum used in current study. In general, the different media additives (composition) support the growth and development of COCs in different mechanisms as well as produce some side effects. Therefore, such kinds of nature of additives may be one of the reasons for variation in maturation rate among the COCs cultured in different maturation mediums.

8. CONCLUSION AND RECOMMENDATIONS

Media is one of the vital and determining factors in *vitro* embryo production particularly the *in vitro* maturation of COCs. The media employed for this study was showed their influence. Thus, it can be concluded that oocyte maturation following transvaginal oocyte aspirations was influenced by employed media type but not by cattle genotype. It can also be concluded that some of the maturation indices were more affected by breed and media type even though the overall maturation was closely similar.

In general, the result indicated the impact of media compositions on *in vitro* embryo production, procedures, which is a crucial step in the IVEP procedures.

Although IVEP has a great deal of contribution to breed improvement, laboratory inputs are scarce for laboratories such as those in Ethiopia. Therefore, it is highly recommended that a small scale media optimization work is carried out for indigenous breeds before scaling up.

A successful oocyte maturation is a prelude to IVEP, in areas where the more expensive OPU set up is not available, alternative use of slaughterhouse ovaries must be sought as a source of oocyte for more detailed maturation and in vitro fertilization studies such as embryo sexing, embryo freezing, oocyte freezing, oocyte and embryo vitrification.

The fact that breed influences are limited in *in vitro* oocyte maturation hints a promising fact for propagation of cross bred animals that are highly needed to meet the demand for replacement heifers in the newly growing commercial dairying.

9. REFERENCE

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ANNEX I. Media preparation and COCs searching during experiment