ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF CLINICAL STUDIES

QUALITY OF *IN VIVO* PRODUCED EMBRYOS FROM BORAN AND HOLSTEIN BREEDS WITH ALTERNATE SIRE AND DAM COMBINATION IN DAIRY ANIMALS UNDER CONTROLLED ENVIRONMENT

By

Hamid Jemal

August, 2021

Bishoftu, Ethiopia

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PHD PROGRAM IN VETERINARY OBSTETRICS AND GYNECOLOGY

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Title: **Quality of *in vivo* produced embryos from Boran and Holstein breeds with alternate sire and dam combination in dairy animals under controlled environment**

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QUALITY OF *IN VIVO* PRODUCED EMBRYOS FROM BORAN AND HOLSTEIN BREEDS WITH ALTERNATE SIRE AND DAM COMBINATION IN DAIRY ANIMALS UNDER CONTROLLED ENVIRONMENT

Hamid Jemal Ahmedel

PhD Thesis

Addis Ababa University (2021)

ABSTRACT

*The difference in dairy breed type can determine the success of bovine embryo transfer by influencing the quantity and quality of embryos during in vivo embryo production. This is particularly true since response to the superovulatory hormone treatments varies because of breed and doses of the hormone. In this study, the quantity and quality of embryos were evaluated for Boran and HF*Boran (50% and 75% cross breed) dairy cows alternatively using semen from HF and Boran sires. Total of 42 HF*Boran and HF cows were used to study superovulatory response, 33 HF*Boran cows for evaluation of estrus behavior and 36 HF*Boran and Boran dairy cows for evaluation of embryo quality as three separate experiments. In experiment one, three different dosages of Pluset® (FSH hormone) (500 IU, 650 IU and 800 IU) were evaluated for their best response. In experiment two, types and strength of estrus behaviors were characterized as predictor of superovulatory responses. In experiment three, the donor dams and sires were alternatively used from different breeds to evaluate breed effect on qualities of embryos flushed on Day-7 post AI. In experiment one, the superovulatory response rate was significantly higher ($P<0.05$) in the HF*Boran than HF cows. The mean ± (SE) highest response based on CL count was 14.6 ± 1.1 and the lowest 11.0 ± 1.3, in HF*Boran, at Pluset® dose of 650 IU and 500 IU, respectively. In experiment two, the overall average time required for commencing estrus signs after CIDR removal was 37.4 ± 1.5 hrs and the duration of estrus was 28.5 ± 1.2 hrs. Behavioral estrus was weak in 48.5% (16/33), moderate in 39.4% (13/33) and strong in 12.1% (4/33) of cows regardless of the breeds. Cows with strong estrus behavior had significantly higher superovulatory response ($p<0.05$) and also* *produced a significantly higher (P=0.000, $\chi 2=31.6$) number of UFO than cows who had moderate or weak estrus. In experiment three, 88.9% of the Boran and 83.3% HF*Boran donors responded to the superovulation with no significant breed differences. Total recovery rate was relatively lower (56.5%) in Boran compared to crosses (67.4%). The mean (±SE) total flush output was 6.5±0.8 for Boran and 6.9±0.7 for HF*Boran with no significant breed difference. Mean (±SE) recovery of transferrable embryo was significantly higher (5.3 ± 0.8; $p<0.05$) in HF*Boran dams inseminated with HF sire semen. Boran cows yielded a significantly higher ($p<0.05$) proportion of UFO (3.6 ± 0.6) irrespective of the sire breeds. Boran cows inseminated with HF sire semen yield significantly higher proportion of G_1 embryos (64.7%, n=22) while HF*Boran produced more G_2 embryos (43.1%, n=25). This study demonstrated that doses of FSH determines the ovarian response and the presence of breed related differences in both the quality and quantity of in vivo produced Bovine embryos. Boran breed consistently produced higher proportion of UFO irrespective of the breed of sires while HF*Boran cows produced a significantly higher number of transferable embryos when inseminated with semen from HF sires. It concludes the significance of breed during selection of donors for a successful embryo transfer.*

Keywords: *Donor dam; CL count; embryo quality; embryo transfer; sire; superovulation*

STATEMENT OF AUTHOR

First, I declare that this thesis/dissertation is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF TABLES

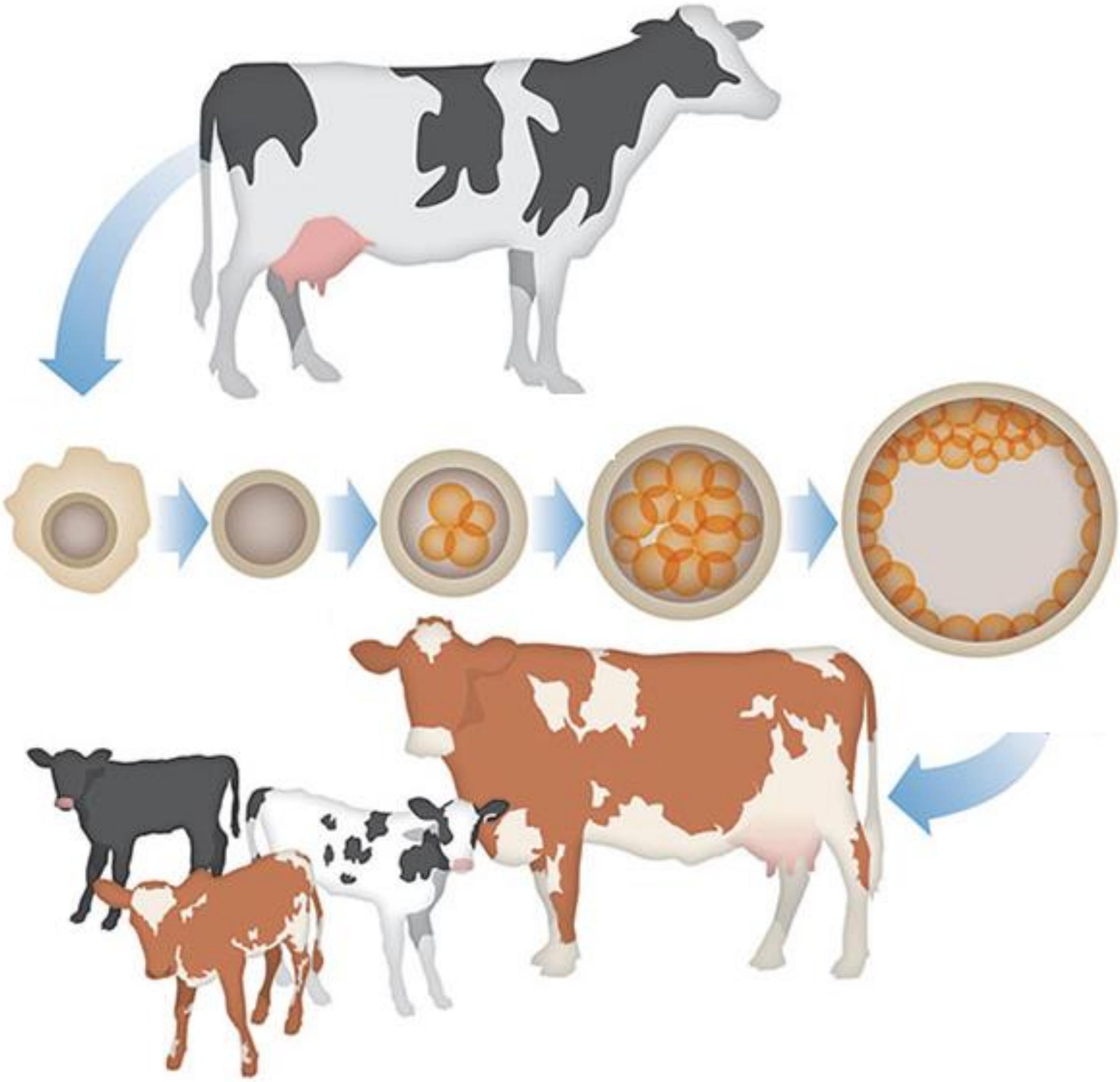
LIST OF FIGURES

ABBREVIATIONS

AnF	Post Anovulatory Follicle
AFI	Agriculture for Impact
AI	Artificial Insemination
BCS	Body condition score
CIDR	Controlled Internal Drug Release
CL	Corpus luteum
CVMA	College of Veterinary Medicine and Agriculture
DZARC	Debrezeit Agricultural Research Center
EIAR	Ethiopian Institute of Agricultural Research
ET	Embryo Transfer
FSH	Follicular Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
HF	Holstein Frisian
IETS	International Embryo Transfer Society
IVF	*In vitro* Fertilization
IVP	*In vivo* Production
IU	International Unit
LH	Luteinizing Hormone
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
MOET	Multiple Ovulatory Embryo Transfer
POF	Preovulatory Follicle
P_4	Progesterone
TCM	Tissue Culture Medium
UFO	Unfertilized Ovum

CONTENTS PAGES

CHAPTER 1



1.INTRODUCTION Pages 2-4

1. INTRODUCTION

In vivo embryo production is a technique by which embryos are collected from donor cows following superovulation and artificial insemination. Multiple Ovulation and Embryo Transfer (MOET) procedures is a reproductive technology generally used to improve the number of offspring from a potentially elite cow with an objective of increasing reproductive efficiency and accelerating the genetic gain (Bó *et al.*, 2019). Still *in vivo* embryo production using MOET is the leading reproductive technology for embryo production and ethically acceptable worldwide (Phillips and Jahnke, 2016). It requires the selection of valuable donors in perfect gynecological conditions and the administration of superovulatory hormones to increase the number of growing follicles (Mikkola *et al.*, 2019). Embryos are generally recovered at 6-7 days after the induction of ovulation and either transferred to synchronized recipients as fresh or frozen, while frozen embryos can be kept for later use (Hasler, 2012; Rocha *et al.*, 2016).

Though embryo transfer (ET) started before 1980s, successful transfers have been reported earlier in cattle and pigs in 1950s by Jim Rowson at Cambridge, England (Youngs, 2007). Bovine embryo transfer technology involves the selection and management of donor and recipient animals, both in physical and pharmacological, and the collection and transfer of embryos within a narrow window of time following estrus (Mapletoft, 2013;Youngs, 2016). Among reproductive biotechnologies from age old to recent; AI, estrus synchronization, MOET, IVF, cloning and transgenesis are of major examples (Yadeta, 2020). When using only AI technique, genetic progress will be only limited to the male side and cows could produce only one calf per year (Curtis, 2015; Jahnke *et al.* 2015). Conversely, the advancement in MOET techniques enables cows to produce many offspring during their reproductive life. This results in more rapid genetic gain, which complements an artificial insemination program (Phillips and Jahnke, 2016). Primarily, MOET is used for genetic improvement rather than for increasing the numbers of a particular breed or phenotype (AFI, 2021).

In vivo and *in vitro* ET techniques in livestock sector are important for several reasons. Livestock is the fastest growing sub-sector in the world, as increasing trends of 114% in demand for meat and 133% for milk attest (Tegegne *et al.*, 2016). To improve the food security, specially it is essential to double livestock production and productivity in the developing world, and hence these methods are clearly required the most efficient ways to accomplish the targets (Getabalew *et al.,* 2019). Although, emergence and availability of different biotechnologies vary from country to country and also their utilization is constrained by different factors with limited applications in African countries like Ethiopia.

Livestock sector in Ethiopia contributes about 12-16% of the national GDP, 30-35% of agricultural GDP, 15% of export earnings and 30% of agricultural employment (Getabalew *et al.,* 2019). However, its economic contribution is still dismally low. The cross breeding program is still challenging implicating the need for alternatives technologies to meet the increasing demand for replacement heifers (Tegegne *et al.,* 2016). According to Central Statistical Authority of Ethiopia (CSA, 2020) only 775,823 (2%) are crossbred and 139,355 exotic breed (0.21%). Lack of access to improved dairy animals is the major problem in Ethiopian dairy sector and hindering dairy expansion in the smallholder system that represent about 85% of the population and responsible for 98% of the milk production. Nevertheless, MOET has not been extensively practiced in Ethiopia except for few recent experimentations with Boran and their Holstein Frisian cross that widely used breed types. Embryo transfer program was initiated in Ethiopia by the international livestock research institute (ILRI) in 1991 but did not continue until very recently as reported by Degefa et al. (2016).

Administration of exogenous hormonal treatment initiates multiple ovulations, with exaggerated and unnatural estrus behavior in cows that are naturally single ovulators (Degefa *et al.,* 2016). The variability of response to superovulatory treatments is also effected by breed, the type and dose of the hormone used. Although estrus behavior is fairy characterized in cattle, variations following superovulatory treatments have not been fully documented (Jiménez *et al.,* 2011; Dolecheck *et al.,* 2015). A number of factors determine the outcome of MOET including breed of animals used including the sire, donor and recipient conditions, nutritional, management, estrus detection, superovulatory response, the AI technique, flushing methods and handling of fresh and/or frozen embryos (Suadsong, 2011; Curtis, 2015; Burnett *et al.,* 2018). Since MOET is an expensive procedure, proper understanding of the reproductive physiology, selection of donors and recipients, sound application of superovulatory protocols and timely insemination with good quality semen followed by efficient flushing protocol must be pursued to eliminate undue failure.

Superovulation in Holstein Frisian cows require higher doses (1000IU) of FSH to get the best response. The presence of differences in ovarian follicular activities and sensitivity to the superovulatory treatments in *Bos indicus* has been previously explained (Degefa *et al.*, 2018). Accordingly, the best response for Boran breed was found at a lower dose (250IU Pluset®) of the superovulatory hormone treatment. Based on this finding, it is plausible to assume that response to such treatment can be influenced by genotype and blood level when Boran's are crossed with Holstein. In the same report by Degefa and colleagues (2018), estrus behavior was weak and recovery of UFO was higher which was described as a challenge to embryo production. Besides to that age and breed has been shown to affect ovarian dynamics, and conception rates in HF and Boran breeds used for AI (Jemal and Lemma, 2015; Degefa *et al.*, 2016). Investigations carried out in Nelore cattle breeds also showed that embryo genotype influences resistance to heat shock thus leading to the question as to whether embryos sired by thermo-tolerant breeds found in the tropics (like Boran breeds) exhibit the same resistance maintaining good quality embryo (Silva *et al.,* 2013). The establishment of pregnancy by these recovered embryos mainly depend on the quality of embryos proper and the novelty of recipient cows maintaining the pregnancy to term (Curtis, 2015).

In this study it was hypothesized that breed of the dam and sire can influence the quality of *in vivo* produced embryo and determine the pregnancy rate during embryo transfer. Hence the general objective was to evaluate how *in vivo* embryo production is influenced by genotype and supeovulatroy treatment in Boran and HF*Boran breeds. The specific objectives were:

- To determine superovulatory hormone doses for HF*Boran cross breed donor cows
- To characterize behavioral estrus in superovulated HF*Boran cross breed donor cows
- To evaluate quality of *in vivo* produced embryos from pure HF semen in Boran cows and pure bred Boran semen in HF*Boran cows
- To evaluate pregnancy rate from transferred embryos

CHAPTER 2

2.LITERATURE REVIEW Pages 6-30

2.1. Background to Bovine Embryo Production and Transfer

Embryo transfer is a process of producing embryos from genetically elite dam and placed an embryo in to the uterus of a recipient female to establish pregnancy (Youngs, 2016). Embryo transfer technique is widely employed in dairy and beef industries and remained as one mode of business in world market (Blondin, 2015; Jaton *et al*., 2019; Souames and Berrama, 2020). Recently, the dairy and beef sector used state of the art reproductive biotechnologies with detailed techniques of genetic engineering with molecular manipulation for the maximum utilization and benefit from farm animals (Cremonesi *et al*., 2020). All the techniques and options of assisted reproductive technologies are for utilizing the genetically superior germplasm from either of the parents (dam or sire). Among the farm animals, bovine are by far the species in which embryo technologies are mostly employed (Ali *et al*., 2012; Viana, 2019). But developing countries are still staggering with the age old AI technique with few attempts of embryo technologies (Degefa *et al.,* 2016). Embryo transfer technique requires knowledge of the basic reproductive biology to the detail techniques of superovulatory procedures for quality embryo production. Hence, this review is objectively to incite the overview of the overall reproductive biology, superovulation and embryo production with emphasis of factors affecting *in vivo* embryo production in bovine species.

2.2. Follicular dynamics, Fertilization and Embryo Development in Cattle

The cattle have series of biological events in reproductive processes which enable to multiply the species. These processes require follicular development, ovulation, fertilization and development of embryos (Sirard, 2019).

2.2.1. Follicular dynamics

Naturally it is known that follicular dynamics in cattle when reach to puberty start cyclic development of follicles in the form of waves (commonly two to three follicular waves) in every estrus cycle. There are major cyclic events in each estrus cycle such as follicular phase (phase of preovulatory follicle) and luteal phases (from development of CL to the next ovulation). These natural ovarian cyclic events are associated with different events of morphological changes in ovary due to follicular development (Kim, 2018). Ovaries have two different pools of follicles, the non-growing and the growing pool. The non-growing pool contains the primordial follicles, whereas the growing pool contains the primary, secondary and tertiary follicles. Follicular waves and growth of follicles from primary to secondary and even tertiary level are gonadotropin-dependent processes (Kanitz, 2003). From the cohort of selected follicles, one follicle is selected for continued growth and becomes dominant based on the expression of mRNA for LH receptors than the subordinates. Once the selected follicle maintains dominancy (mostly 18-28mm in size) during the growth phase then reach to the final maturation and the default program of luteolysis (action of $PGF2\alpha$) launched and ovulation occurs (Kim, 2018), otherwise if luteolysis does not occur the fate of dominant follicle is atresia and the next selection continued (Kanitz, 2003; Ferreira *et al.*, 2014) under the normal cycling condition.

Oogenesis is a prenatal process by which producing the female gamete cells from the primordial germ cells to the point of producing primary oocytes. And hence, female heifer calve are born with all of the primary oocytes that they will ever have as primary oocytes which do not develop until puberty (Britt, 2008; WikiVet, 2012). But ovogenesis is the differentiation of the ovum (egg cell) into a cell competent to further development when fertilized. These primary oocytes are committed to a gamete fate and either become secondary oocytes or degenerate in latter development (WikiVet, 2012) while the development of competent oocyte would be in progressive transformation that occurs in a matter of days in the last part of the follicular wave (Sirard, 2019) as shown in Figure 1.

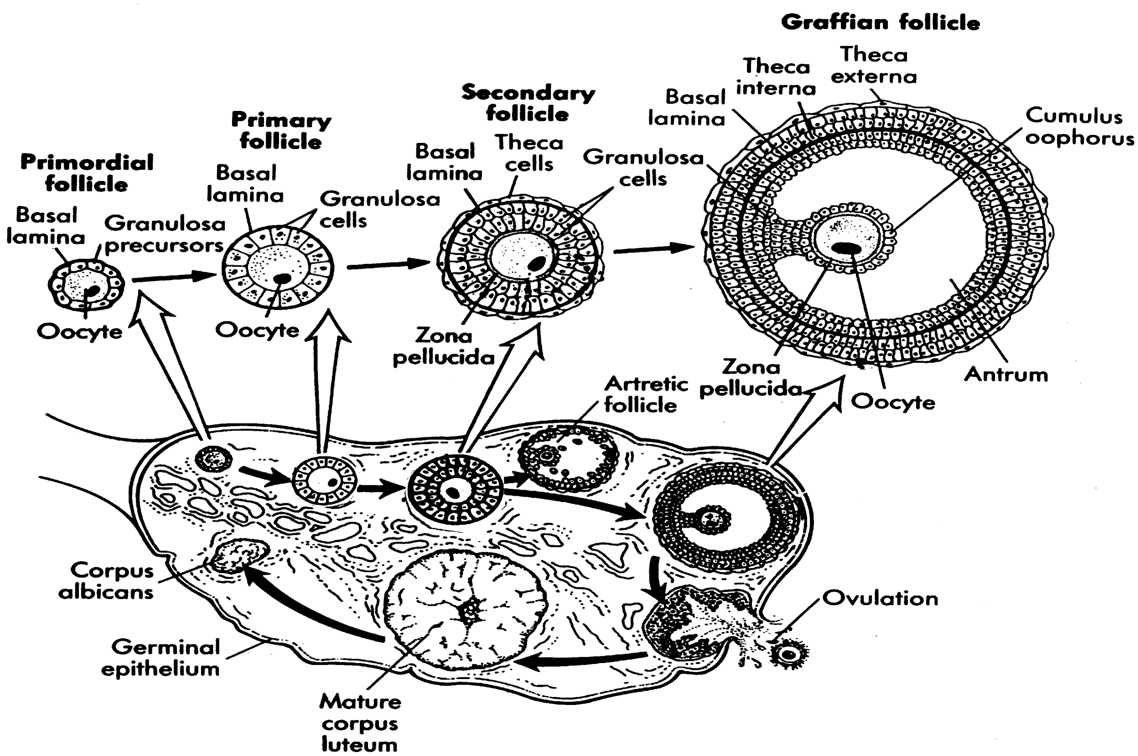


Figure 1. The follicular and oocyte development in cattle

Source: WikiVet (2012)

As practical point of view, effective embryo production and transfer procedures in cattle requires understanding the dynamics of follicular wave using veterinary ultrasound and the invention of real-time ultrasonography made breakthrough for exploring secretes behind the pool, follicular hierarchical emergence and development (Ferraz *et al.,* 2017). This knowledge enables researchers to interrupt and control the natural hormonal interplay and steering the endogenous command by exogenous administration (Bó *et al.,* 2019). When superovulation is done in cattle, deviations in hormonal signaling and endocrine profiles, plus the emerging of heterogeneous group of oocytes recruitment during the process of follicle growth and dominance, some oocytes will lead to asynchrony between development and follicular status (Merton *et al.,* 2003).

2.2.2. Fertilization

Fertilization is the process by which a sperm cell meets with an egg cell in the fallopian tube when successfully fuse to each other. The stages of fertilization are divided in to 6 processes. 1) sperm capacitation, 2) sperm-zona pellucida binding, 3) acrosome reaction 4) penetration of the zona pellucida, 5) sperm-oocyte binding and 6) egg activation with the cortical or zona reaction (Sartori *et al.,* 2010). Once a cow inseminated, the spermatozoa reach to the oviduct, starts its capacitation, which is the change that occur to the spermatozoa that make them capable of fertilizing the oocyte. Providing time for sperm capacitation is very important for the optimal timing of insemination. Following insemination, viable spermatozoa traverse the cervix through the uterus to the oviduct to fuse with the oocyte, undergo the acrosome reaction to penetrate the zona pellucida and fuse with the oocyte plasma membrane. After fusion with the plasma membrane, the fertilizing spermatozoa enters the oocyte cytoplasm and its nucleus decondenses for successful fertilization (Gordon, 2003). In this cascade, different literatures (Kanitz *et al.*, 2002; Gordon, 2003; Sartori *et al.* 2010) reported that concentrations of progesterone (P4) negatively affected sperm migration at the time of insemination of the cow and this alter of gamete transport could affects the facilitation of sperm in to the oviduct which might lower the fertilization success (Ferreira *et al.,* 2014).

2.2.3. Early phase of embryo development

Biologically, an embryo can be formed from when male and female gamete cell are combined for fertilization, and that newly fertilized cell is called zygote. Zygote continues its development in oviduct until it moves down to uterus. Even though multiple ovulations in MOET, the process of male and female gamete fusion (male and female pro nuclei), and zygote formation after a series of mitotic divisions (cleavage divisions), and blastomere formation remains similar with the case of mono-ovulation at normal condition. Once zygote is formed, it starts genome activation and the zygote develops into a morula by day 4 of development (Gordon, 2003 and 2004). Developmentally when embryo reaches to morula stage, then starting compaction, and the morula move down to the uterus by day 5. Then the first embryonic cell differentiation process is commenced and the outer cells of the morula develop in to the trophectoderm, whereas the inner cells form the blastocyst (Gordon, 2004).

2.3. Factors affecting expression of Estrus Behavior in Cattle

2.3.1. Events of Estrous cycle in cattle

Estrous cycle in cattle is a series of events that occur in a definite order over a period of days which repeats itself in a definite period of time (Proestrus, Estrus, Metestrus, and Diestrus). There are terms, which are time bounded biological processes of estrous cycle, i.e. Estrous cycle - an interval or the chain of physiological events that begins at one estrus period and ends at next estrus (21 days in cattle); Estrus - an event with in one cycle where period of sexual desire including the time of starting to end of estrus (12-32 hrs in cattle); Heat is the specific peak time at the mid of estrus when standing to be mate/ fully receptive for mating (4-8 hrs). Sometimes a cow may have silent estrus which is characterized by absence of overt signs of estrus manifested either due to lack of expression of estrus or failure of its detection (Degefa *et al*., 2016; Burnett *et al.* 2018).

2.3.2. Factors affecting expression of estrus behavior

There are different risk factors for expression of estrus in dairy cows. Especially magnitude of estrus behavior manifestation will be affected by various factors related to environment and cow factor, such as:

- ✓ The number of cows/ heifers at a time, status of herd mates (stage of estrous cycle, number of herd mates in proestrus and estrus vs diestrus), type of housing, footing surface, feet and leg problems, variation during the day (hot or cold), nutritional factors, lactation number, days postpartum, and milk production have effects on estrus expression in cows/heifers (Gordon, 2004). Mounting activity (mounts per hour) of estrus females is lowest during very cold weather than warm condition. Other than heat stress in temperate area, in tropical dairying environment seasonal depressions of fertility in cattle is majorly due to nutritional stress (Sankar and Archunan, 2012).
- ✓ Functional disorder of the normal cyclical order of reproductive cycle: There are different types of anestrus conditions manifested by dairy cattle. These include postpartum anestrus (true anestrus) which is characterized by the absence of estrus behavior, which

may be an indication of suboptimal conditions (e.g., inadequate peripartum nutrition) or pathologic conditions (e.g., chronic debilitating diseases or uterine and ovarian diseases). The other type is lactational anestrus which is due to production stress (NADIS, 2021). Also, nutritional anestrus is observed in early postpartum period or lactating dairy cows (Kumar, 2014).

- ✓ Nutritional deficiency affects the normal cyclical order of estrous cycle in different ways. It is often stated that malnutrition or specific nutrient deficiencies may interfere with or inhibit estrous behaviors. Nutritional factors can promote or inhibit cyclic ovarian function. Severe mineral and protein deficiency interferes with reproductive processes in dairy cows (Kanitz, 2003).

Energy restriction influences reproductive function through depression in gonadotropin releasing hormone (GnRH) in hypothalamic centers in the brain. Nutritional, metabolic and hormonal signals which may influence hypothalamic function and GnRH secretion include specific amino acids absorbed from the diet (e.g. tyrosine), glucose, ketone bodies, and insulin. Typically, in most dairy herds fewer than 15% of cows should be anestrous beyond 40-50 days post calving before and after insemination. If anestrous cows are a higher proportion, then the nutritional program should be examined (Orihuela, 2000). The negative energy balance and fat mobilization stimulate dry matter intake and cows progress to a positive energy balance by about 8 weeks post calving (Galina *et al*., 2016). On the contrary Sankar and Archunan (2012) reported that as compensatory, high yielding dairy cows require high dry matter intake and that intern affect reproductive cycle through the mechanism as shown in the following Figure 2.

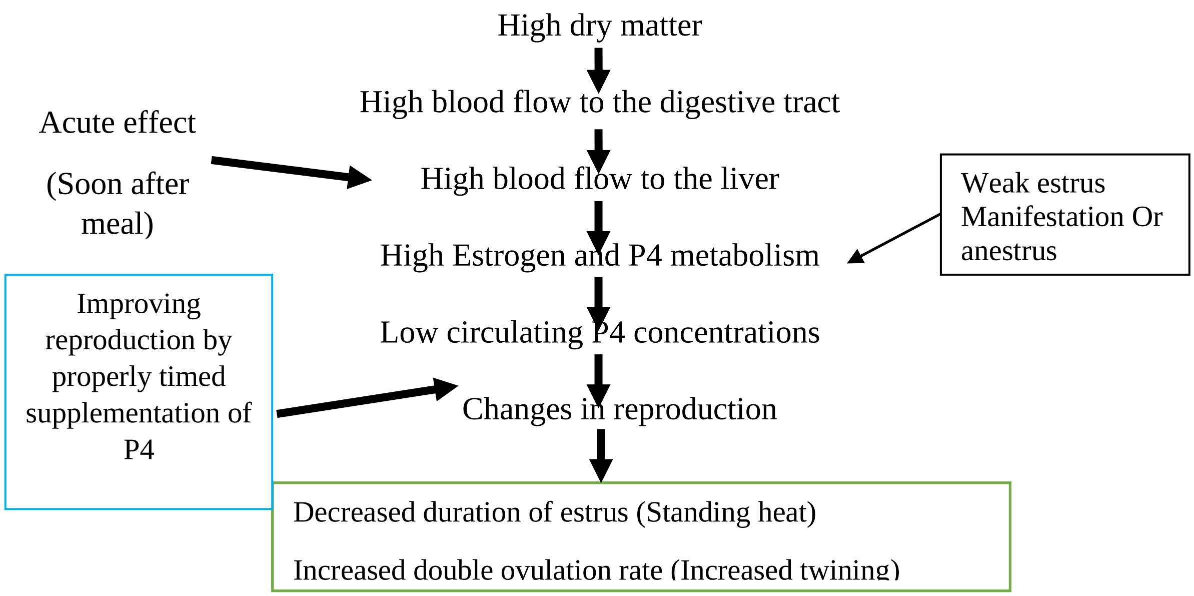


Figure 2. Effect of high dry matter intake on hormonal metabolism and reproduction.

Source: Bindari et al. (2013)

2.4. The World Experience in Embryo Production

Bovine embryo production is mainly as a mode of conventional and sexed embryo types. Now a day, embryo as commercial commodity, continents such as North and Latin American countries, Europe, and little Oceania have big companies that shared about 95% of the world embryo production (Davis-Rairdan, 2020). Whereas, developing countries are the market ends for embryo producing companies from these countries at large with the limited domestic knowledge and facilities (Viana, 2019). In the recent scenario, specially world leading embryo producing countries are pertaining embryo commercialization, such as USA, Canada, Brazil, New Zealand, and other EU countries (Davis-Rairdan, 2020). Export grade embryos prepared and certified according to the International Embryo Transfer Society (IETS) standards and accredited embryos exported to most countries around the world produced in *in vivo* and *in vitro* techniques (Davis-Rairdan, 2020). Brazil is one of the world's known embryo producing country (86% of world share) among the Latin American countries and export to different countries of the world (Viana, 2019). Standard embryos are those embryos with intact outer shells (zona pellucida) and washed with an enzyme called trypsin to remove potential viral and / or bacterial agents or making zero risk from any known diseases, including BSE and FMD (Davis-Rairdan, 2020). The trend of world experience in production and transferring embryo is increasing time to time (Phillips and Jahnke, 2016) as shown in Table 1.

Table 1. Bovine embryo production and ET in the USA between 2002 and 2013

Year	Viable Embryos	Viable embryos/Flush	Fresh transferred	Total frozen transferred
2002	172,118	7.46	59,687	69,978
2007	332,864	5.69	110,223	137,772
2013	301,671	6.80	85,876	215,699

Source: Phillips and Jahnke (2016)

2.5. Pros-and-cons of *In vivo* and *In vitro* Embryo Production

In vivo embryo production (ET) refers to the process of stimulating ovaries and collecting embryos from uterus of the donor using Foley catheter using embryo flushing fluid (Abdoon *et al*., 2020). The main pros in embryo transfer are increase in the number of offspring per female, easier and more rapid exchange of genetic material between countries, less transport of live animals, thereby reducing risks of disease transmission and storage and expansion of rare genetic stock; where as the main cone in embryo transfer is the high cost of the technique (Mapletoft, 2013). There have been an increasing demand in manipulating female animals by using hormonal therapy, and synchrony of female animals using superovulation technique for research and business purposes intensively in both *in vivo* and *in vitro* basis (Sanches *et al*., 2019) as shown in Table 2.

Table 2. *IVD* and *IVP* beef and dairy herds embryo production in Brazil from 2015 to 2017

Years	*In vivo*			*In vitro*		
	Beef	Dairy	Total embryos	Beef	Dairy	Total embryos
2015	73 %	27 %	22,355	43 %	57 %	353,539
2016	48 %	52 %	31,685	46 %	54 %	346,817
2017	24 %	76 %	29,533	48 %	52 %	345,528

IVD- in vivo development; IVP- in vitro production

Source: Baruselli et al. (2019)

According to Viana (2019) report, 1,499,367 transferrable embryos were collected worldwide from different farm animals in 2018 G.C. while 96.7% were from cattle both *in vivo* and *in vitro*. Besides, *in vivo* embryo collecting approach is taken as one way to support for the fastest genetic progress in dairy and beef industry using superovulation technique (Youngs, 2016). Largely *in vivo* technique is enable to have obtaining many ovulations from the targeted genetically superior dam according to the plan of the breeder without sophisticated procedures (Cremonesi *et al.*, 2020). Some reports have shown that still comparatively high number of bovine embryos are produced from the *in vivo* technique (Blondin, 2015; Viana, 2019). Developmentally, even more competent embryos can be collected from *in vivo* technique than those matured from *in vitro* condition (Lonergan *et al.*, 2001) as depicted in Figure 3 below.

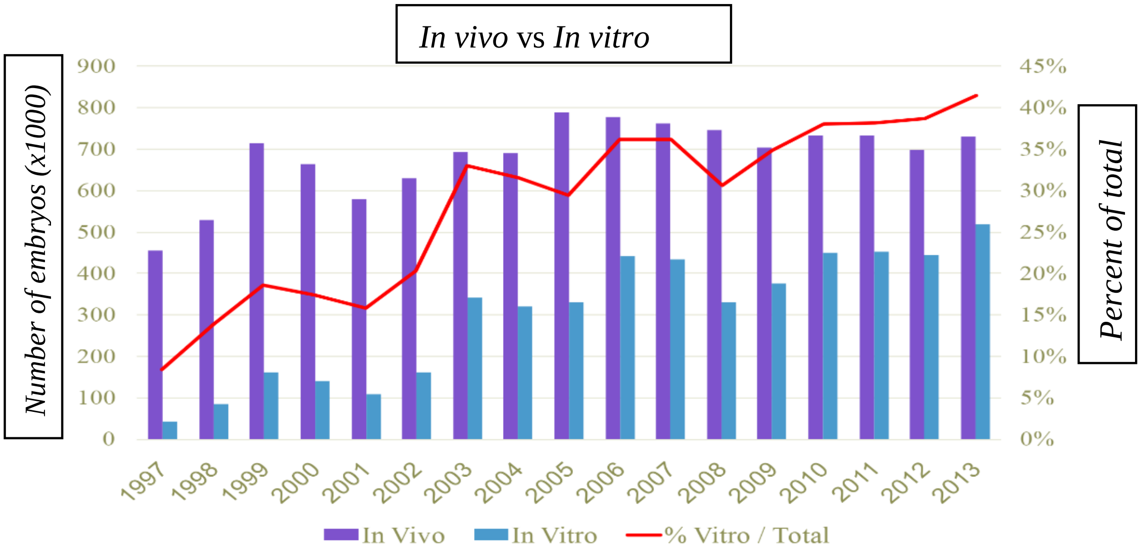


Figure 3. Trend of worldwide bovine embryo production between 1997 and 2013

Source: Blondin (2015)

Embryo transfer makes easier the exchange of genetic material between countries without transport of live animals, thereby reducing risks of disease transmission. Besides ET used as a tool for increasing the number of offspring per female in multiplication of endangered genetic stocks (Youngs, 2007; Jaton *et al*., 2019). Besides, this technique is used as an alternate breeding option to improve pregnancy rate bypassing stressful seasons and environmental conditions (Jahnke *et al.,* 2015). Recent reports has shown greater pregnancy rate from embryos of *in vivo* origin compared with *in vitro* (Pontes *et al.*, 2009; Lopes *et al.*, 2020). The number of embryos recovered after superovulation is slightly higher when the service is performed *in vivo* and lower when the protocol is performed *in vitro*. And hence, considering the higher cost of producing embryos *in vitro*, usually only very valuable cows with reproductive problems or very valuable young heifers will undergo an *in vitro* protocol by traversing their oocytes (Jaton *et al.,* 2019). As part of assisted reproductive biotechnologies, *in vivo* and *in vitro* embryo transfer techniques are applied to enhance transferring the genetic material and used to increasing the female reproduction index, selection intensity and to increase the replacement animals by decreasing the generation interval (Albuquerque *et al.,* 2012). Meanwhile, ET started beyond 40 to 50 years back, it was very challenging until knowledge of extraction of reproductive hormones were established and it was the first entry point for the current existence of the commercial embryo biotechnology (Pontes *et al.,* 2009). In *in vivo* embryo production, the main challenges are the variability of female responses to hormonal treatments, fertilization failures and poor conception rates due premature regression of corpora luteum (Ongubo *et al.,* 2015).

2.6. Quality of *In vivo* produced embryos

An output of embryo flush from donors varies for breed of animals or different intrinsic and environmental factors (Jaton *et al.,* 2019). On the other hand success of embryo production is measured by the number of quality embryos per individual donor collected per each flush (Merton *et al.,* 2003).

Unfertilized ovum: Unfertilized ova collected 6–8 days after the onset of estrus can have many different physical appearances. The three major keys to identifying a UFO are (i) the vitelline membrane (also called the oolemma) that surrounds the cytoplasm of the ovum is smooth and nearly perfectly spherical unless the ovum is fragmented or degenerate; (ii) the cytoplasm is granular and not cellular in appearance; and (iii) there is a moderate amount of perivitelline space (the space between the ovum and the zona pellucida), except in cases of degeneration (Jahnke *et al.*, 2015).

The one reason that incriminated for increasing number of UFO during recovery might due to increased estrogen concentration since more follicles matured concurrently (Youngs, 2007). Intensity of behavioral estrus might also be a potential reason for unfertilized ovum (Sartori *et al.,* 2010). Also breed of the donor, concentration of semen and time of insemination can be taken as reason for increased number of UFO during embryo collection (Sartori *et al.,* 2010).

Degenerated or dead embryos: If embryos at stage code 2 are collected on day 7, the stage of embryonic development is not consistent with the expected stage of development. Therefore, these embryos are classified as degenerate (quality grade 4) because they are severely retarded in their development (Curtis, 2015; Youngs, 2016). Degenerate embryos are ones that have been fertilized but which died some time during the developmental process. An embryo could also be considered dead or degenerate if its blastomeres are not fused to one another (i.e., there is lack of tight cell junction formation between the "outer" cells of the embryo). Failure of blastomeres to develop in a tight and cohesive group to compact and form tight cell junctions is a sign that the embryo is in the process of degenerating or dying (Curtis, 2015; Jahnke *et al*., 2015).

Transferable or viable embryos: A number of morphological characteristics of an embryo can be considered as a deviation from normal (Rocha *et al.,* 2016). These abnormalities can include irregular sizes of embryonic blastomeres, large and/or multiple vacuoles within the cytoplasm of embryonic cells, degeneration of one or more cells in the embryo, some cells not adhering to the other cells comprising the embryo (i.e., extruded blastomeres/ cells), and a damaged or misshapen zona pellucida (Sartori *et al.,* 2010). Many viable embryos have some detectable morphological abnormality such as a small number of extruded cells. Embryo quality grading must consider not only the presence or absence of these abnormalities but also the extent/degree to which these deviations are expected to influence post-transfer embryo survival (Jahnke *et al*., 2015; Phillips and Jahnke, 2016).

In general, embryo technology is one of the competent commercial sectors in the dairy world, while it requires much effort in Africa especially Ethiopia. Besides to that embryo production evolved to the level of embryo sexing, embryo splitting and also genome editing technologies adapted for applications in cattle for several purposes including bio-reaction and disease control models. Hence, MOET technologies are bench marks for those counties adopting such kind of advancements in the dairy and beef sector including for Ethiopian production system to exploit the untapped livestock resource.

2.7. Evaluation and Determination of Embryo Qualities

An embryo is defined as *in vivo* fertilized ovum recovered at its early period of development before the commencement of distinct commitment and differentiation of blastomeres. Recently, there are latest approaches for evaluation and screening of quality of embryos after flush including morphological grading system, cellular metabolism, development kinetics and cleavage symmetry, embryo cell pre-implantation genetic diagnosis, zona pellucida birefringence, embryo cells ion release. Except the first one, the rest procedures are quest of sophisticated laboratory setup. Successful in embryo transfer techniques require qualified embryo which selected and graded morphologically under laboratory condition for transfer. In a laboratory, embryos are classified morphologically in two four basic types such as excellent, good, poor and degenerated forms (Rocha *et al.*, 2016).

Embryo evaluation procedures require the use of a stereomicroscope different from semen evaluation where a compound microscope is used. Stereomicroscopes, also called dissecting microscopes, are designed for use with three-dimensional specimens (Rocha *et al.*, 2016). During the recovery procedure, embryos are typically collected using an embryo filtration device that minimizes the volume of flushing medium through which the technician must search to locate the embryos. Some embryo filtration devices also serve as the embryo search dish, whereas other devices require that embryos be transferred from the filtration device to a Petri dish for searching (Curtis, 2015). After embryos are recovered, they must be located in the recovery medium using a stereomicroscope typically under 10–15× magnification (Curtis, 2015; Jahnke *et al*., 2015). Embryos are subsequently transferred to a Petri dish containing embryo holding medium where they will be evaluated at a minimum magnification of 50x for stage of development and embryo quality (Jahnke *et al*., 2015) as shown in Table 3.

Table 3. Level of developmental stage and embryo quality grades

Stages of development	Quality of embryos
1. Unfertilized	1. Excellent or good
2. 2-cell to 12-cell	2. Fair
3. Early morula	3. Poor
4. Morula	4. Dead or degenerating
5. Early blastocyst	
6. Blastocyst	
7. Expanded blastocyst	
8. Hatched blastocyst	
9. Expanded hatched blastocyst	

Source: *Manual of the International Embryo Transfer Society* (IETS, 2010)

On the other hand, there is no method capable of giving reliable and trustworthy results or golden standard for the differences between each grade levels (Rocha *et al.,* 2016). Quality of embryos are generally categorized as Stages 1-9 and Grades 1-4 based on the standards set by as depicted in Annex I.

2.8. Use of Hormones in Reproductive Management

Reproductive hormones are organic substance secreted by animals that functions in the regulation of physiological activities and in maintaining homeostasis (Lunenfeld *et al.*, 2019). The classical view of hormones is that they are transmitted to their targets in the bloodstream after discharge from the glands that secrete them (Lunenfeld *et al.,* 2019). This mode of discharge (directly into the bloodstream) is called endocrine secretion (Anderson and Wallace, 2013). Regulation of the reproductive system is a process that requires the action of hormones from the pituitary gland, the adrenal cortex, and the gonads (Jahnke *et al.,* 2015). During puberty, in both males and females, the hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the production, and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Lunenfeld *et al.*, 2019). These hormones regulate the gonads (testes in males and ovaries in females); they are called gonadotropins (Weltzien *et al.*, 2004).

Reproductive hormones are highly active substances extracted or modified from proteins and carbohydrates (Lunenfeld *et al.,* 2019). They have profound effects on the physiology of the animals, impressively in small doses (Lunenfeld *et al.,* 2019). Commonly hormones and hormone analogues used therapeutically or for other reasons in farm animals (Lovern, 2016). Reproductive hormones are widely marketable and used for reproductive and therapeutical purposes at large in dairy and beef production (Chauvigne *et al.,* 2017).

Reproductive hormones serve as a management tools in farm animals such as synchronization of cattle which reliably induces estrus in presence of palpable corpus luteum using prostaglandins ($PGF_2\alpha$) (Islam, 2011). Hormonal therapy is now become common and one way of increasing reproductive and productive tool in animal reproduction (Lunenfeld *et al.*, 2019). Application of GnRH combined with $PGF_2\alpha$ in the puerperal phase appeared to have positive effects on fertility of cows with endometritis (Ribeiro *et al.,* 2016). In Ethiopia different types of synthetic hormones are imported and, used by practitioners and researchers for estrus synchronization, cystic ovarian disease, dystocia, abortion and other reproductive procedures (Degefa *et al.*, 2016). Besides to therapeutical effects, reproductive hormones are widely used in the manipulation and control of ovarian cycle for the sake of optimizing breeding of animals using superovulation technique.

Superovulation technique is enabling to reverse the trend of single dominant follicle by several secondary follicles to dominant state and more ovulations at a time (Moore and Hasler, 2017; Afriani *et al*., 2018; Cremonesi *et al.*, 2020). The development of this technique has largely contribute to the artificial manipulation of animal reproduction eliminating the inhibitory mechanism of the dominant follicle (immunization against inhibin) and by promoting development of a subordinate follicle with reduced follicular atresia using follicle-stimulating gonadotropins (Cremonesi *et al.,* 2020). Superstimulation using FSH is widely used treatment to induce follicular growth and to improve the efficiency of *in vivo* embryo production and of *in vitro* embryo production (IVP) using ovum-pick up (OPU) (Sakaguchi *et al.,* 2018). The technique is enabling to utilize the large pool of life time ovarian reserve from 14,000 to 250, 0000 follicles and the number of follicles comes to preovulatory stage ranges between 8 and 20 per ovary in a session of superovulation (Ireland *et al*., 2008; Bó and Mapletoft, 2014). The range of variability of response to the superovulatiom is especially due to the breed of the animal, dosage and type of the hormone applied (Afriani *et al*., 2018).

Superovulatory treatment is application of gonadotropin hormone and employed to increase the ovulation rate, thus the number of available oocytes in the donor animal without disrupting the physiological and endocrinological processes associated with oocyte maturation, ovulation, and fertilization (Jahnke *et al.,* 2015). Factors associated with the superovulatory treatment have a marked influence on the outcome, including the type and purity of the gonadotropin and the frequency and route of administration (Mikkola *et al*., 2019). In multiple ovulatory embryo transfer technique (MOET), it is tricky work in replacing and superimposing of natural reproductive processes such as reproductive hormone secretion and time of their effect on reproductive organs (Moore and Hasler, 2017). Also understanding types and origin of these reproductive hormones are very important in manipulation of reproductive biological processes in any area of research interest (Youngs, 2007; Youngs, 2016). Different pharmacological preparations have been used for the process of MOET (Kanitz *et al.,* 2002). CIDR for synchronizing follicular growth, FSH for stimulating the cohort growth of follicles, GnRH and $PGF_2\alpha$ for crushing of the CL and these are the main domains of superovulatory treatments used throughout the world (Merton *et al.,* 2003). Efficacy of these hormones varies according to the choice of the protocols and procedures used by the technician (Phillips and Jahnke, 2016). The most effective commercially available treatment is based on the use of purified porcine and equine pituitary extracts (Mapletoft and Bó, 2014).

There are different and inconsistent results of superovulation protocols used in superovulation for different breeds in different production systems besides to the variability of hormone brands (Sumretprasong *et al*., 2008; Mapletoft and Bó, 2014). This is due to the differences in response of the animals because of the nature of the hormone and their biological response (Sumretprasong *et al.,* 2008). There are different factors affecting effectiveness of superovulatory protocols as indicated in various studies such as breed, age, BCS, parity, nutrition status and ovarian status at the time of superstimulation (Ireland *et al*., 2008).

Testing and optimizing the best responsive doses to the superovulatory procedure will help to reducing the excessive cost associated with hormone treatments (Youngs, 2007; Sumretprasong *et al*., 2008; Degefa *et al*., 2018). Even within the same breed, potential of reproductive variations could be found according to different studies in relation to different superovulatory responses (Youngs, 2016). However there are several limitations for *in vivo* embryo production, especially from variations in superovulatory responses reported as a challenge to enhance pregnancy outcome in the tropics (Ferreira *et al.*, 2014). As a general guideline, FSH dose to be used for a donor cow varies according to the age, body weight, breed, and stage of production of the donor female (Dorice *et al.,* 2019).

Zebu breeds responded better to the superovulatory protocol when compared animals with a greater *taurine* genotype. Also, *Bos taurus X indicus* animals are more sensitive to exogenous gonadotropins and respond better to superovulation (Degefa *et al.*, 2018). As studies have shown that *Bos indicus* breeds have lower threshold of response to the superovulatory treatments compared with *Bos taurus* breed though it is not well characterized (Youngs, 2007). The problem of higher dose is that, FSH as a product manufactured with LH (FSH:LH) and when we administered much increases LH concentration and this leads to the luteinization of follicles and/ or premature ovulation of the dominant follicle (Trigal *et al.*, 2012) and it is a reason for poor response (Youngs, 2007).

2.9. Factors Affecting Embryo Production

2.9.1. Dose of the superovulatory hormone

The misunderstanding of dose application has a drawback effect in embryo production by affecting the biological mechanism of fertilization (Youngs, 2007; Silva *et al*., 2009). When administering booster dose of superovulatory hormone it will be reason for poor response by inhibits release of FSH from anterior pituitary, and increasing LH concentration which luteinize follicles and reason for premature ovulation of the dominant follicle (Youngs, 2007). The breed dose response in pure HF shown that there need more dose of FSH to attain the maximum response. The recommended dose for *taurus* breed is higher (up to 1000IU FSH) to get the maximum response while detrimental to *indicus* breed (Youngs, 2007; Degefa *et al*., 2016).

The variations of ovarian responses in superovulatory treatments reported as a challenge to hinder the enhancement of outcome of *in vivo* embryo production (Kanitz *et al.*, 2002; Ferreira *et al*., 2014). Different dairy breeds respond differently for different Pluset® (FSH) dose treatments stated as factors affecting effectiveness of the protocols (Silva *et al*., 2009; Barros *et al*., 2012; Mapletoft and Bó, 2014). Even to the extent, the dose of Pluset® to be used for a donor cow also varies according to the breed of the donors, age and/ or parity as well as body condition (Silva *et al.*, 2009). For instance, heifers generally receive a lower dose of Pluset® for superovulation than do mature cows (Youngs, 2007). Due to the variations of response of donors, testing and optimizing the best responsive doses of Pluset® to the targeted breed for the superovulatory procedures majorly used to maximize the number of matured graafian follicles which will be the next candidate of preovulatory follicles (Merton *et al.*, 2003). This will help upgrade the outcome of the superovulatory procedures and some reports (Youngs, 2007; Sumretprasong *et al*., 2008; Velazquez, 2008) indicated that approximately 20% of donors do not respond to the superovulatory treatment and do not produce any embryos. Level of superovulatory response is measured by ultrasonographic count of CL is commonly employed for evaluating the responses for superovulaton (Palhão *et al*., 2019; Esposito *et al*., 2020).

2.9.2. Status of follicular dynamics

Though there are controversial reports in this regard, time of superovulation commencement matters the effectiveness of the outcome (Bó *et al.,* 2010). When superovulation is commenced in the presence of functionally dominant follicle, the subordinate follicles regress and the responsiveness to gonadotropin stimulation is poor (Mikkola et al., 2019). Previously different studies (Jahnke *et al*., 2015; Adams and Singh, 2017) have been conducted to found the optimal phase of follicular waves for commencing superovulation. Some studies (Camargo *et al*., 2005; Baruselli *et al*., 2006; Mikkola *et al*., 2019) reported that the superovulatory response was compromised in the presence of a dominant follicle at the time of treatment initiation.

2.9.3. Ovulation and fertilization failure

The optimal dose for superovulation hormone dose enables to have the optimum ovulation rate from superovulated cows. Deviations in the optimal dose is taken as a reason for failed in ovulation due to overstimulation the ovary by disrupting the normal ovulatory mechanism, altering gamete transport and incapable of the infundibulum to capture the ovum from the larger ovary (Degefa *et al*., 2016). Intensity of estrus behavior has contribution for ovulation positively or negatively. Cows with a corpus luteum present at the beginning of the protocol has shown greater ovulation rates than those that did not have a corpus luteum. The sizes of preovulatory follicle also matters ovulation rate where increased with larger pre-ovulatory follicles compared with cows that had smaller pre-ovulatory follicles reported in timed AI. The lower fertilization rate in superovulated cattle may be due to disturbances in spermatozoa and/or ova transport and suboptimal oocyte quality (Sartori *et al.*, 2010).

2.9.4. Donor factors

Breed*:* *Bos indicus* breeds have lower threshold of response to the superovulatory treatments compared with *Bos taurus* breed though it is not well characterized (Youngs, 2007). This implies that cross breed of this family have also different responses that believed to be different from *taurus* (Mapletoft and Bó, 2014). Several differences in reproductive physiology have been reported between *Bos taurus* and *Taurus indicus* breeds, which can in part explain the variations in superovulatory response between breeds belonging to these genetic groups (Youngs, 2007). Some of these differences include the diameter of the dominant follicle, the duration of estrus expression and greater sensitivity to the exogenous gonadotropins in zebu cattle (Ferreira *et al.*, 2014). Especially efficiency of estrus detection in zebu cows is low because of the reproductive behavior characteristics of these females, which have a shorter estrus that manifests at night, and difficult to use biotechnologies in these cattle herds (Ferraz *et al*., 2017). Boran and HF cross Boran breeds are widely used in Ethiopian dairy and beef production. Boran breed widely reared in Borena area of Oromia region and adapted with stressful conditions. This breed is crossing with *Taurus* breed under the dairy clusters of Oromia and SNNP regions of Ethiopia.

Age: Some variation in the superovulation success among donors can be explained by age (Dorice *et al.,* 2019). However, this applies mostly to old donors, greater than ten years of age, because the numbers of gonadotropin responsive follicles decrease with age (Stockler, 2014). For successful *in vivo* embryo production, the prerequisite is cyclicity of the donor, which sets a limit on the age of the female, exclusively the blastocyst development declines when oocytes collected from young donors (Mikkola *et al*., 2017). The increased donor age reduces the number of recovered structures and viable embryos (Stockler, 2014). As age increases number of CL count and collection will decreased. There would be decreasing rate of fertilization and decreasing numbers and percentages of transferable embryos (Dorice *et al.*, 2019. Also the decreased response to superovulation in older, as compared with younger donors, likely due to a reduction in the numbers of follicles capable of responding to exogenous gonadotropins given (Madureira *et al.* 2020).

Management factors: Management factors can influence the outcome of superovulation, including nutritional management, artificial insemination (AI) timing, competence and semen quality (Afriani *et al.,* 2018). Maintaining optimum body condition is mainly of tissue reserves than body weight (Swecker, 2014). Poor hygiene and production status of donor cow has a potential effect on quality of in vivo producing embryos (Sales *et al.*, 2015). Environmental factors can also affecting the outcome of superovulation such as high ambient temperatures; can interfere with the oocyte and follicular quality and endocrine pathways of superovulation (Albuquerque *et al.,* 2012). Adverse effects of heat stress on reproductive performance represent a well-established problem, not only in subtropical and tropical climates, but also in temperate climates during hot summer periods (Sartori *et al.*, 2010).

2.9.5. Other factors related to embryo recovery

Embryos can be recovered from donor cows by lavaging the uterine horn using embryo flushing fluid. Embryo flushing is the technique by which draining of embryos from uterine horn of the female animal using flushing medium/ fluid (Jahnke *et al.*, 2015). Embryo flushing medium can be used from commercially prepared (Curtis, 2015). There are ready made medium in the market from different manufacturer which contains a surfactant (polyvinyl alcohol) and antibiotics (gentamicin and kanamycin) and other specialized (Curtis, 2015) phosphate buffered saline (DPBS) 1 to 2 liters and flow down 50 to 200ml to wash uterine horns by gravity. Lactated ringers solution will be also used for this purpose where it is available easily in laboratories by adding 0.1% bovine serum albumin as a surfactant for short-term holding (Jahnke *et al.*, 2015). This appears to work well and is much more economic. But in some countries where embryos are collected for export purposes, it is not allowed to use animal byproducts such as bovine serum albumin to be added (due to the scare over bovine spongiform encephalopathy, or BSE). In these countries, use of a complete medium that uses polyvinyl alcohol as the surfactant is acceptable (Jahnke *et al*., 2015; Philips and Jahnke, 2016).

As a limiting factor, time of insemination and fertilization failure may affect the output of superovulation. Naturally and spontaneously ovulation may occur in 24 to 48 hours after the onset of estrus in the cow (Velazquez, 2008; Souames and Berrama, 2020). But in the case of superovulation, more preovulatory follicles emerged while at different maturation status and hence ovulate accordingly at different time interval (Phillips and Jahnke, 2016). By considering this variation insemination of double to triple more dose of spermatozoa is required than the conventional dose and inseminating at 12-13 hrs interval to keep the optimum concentration (Ireland *et al*., 2008). Failures of maintain optimum concentration of semen from the first ovulation of the preovulatory follicle to the last ovulation can cause increased unfertilized ovum (Youngs, 2007). Skill of the technician, embryo flushing environment and docility of the donor are also factor that might affect the performance of withdrawing all embryos and non-embryonic substances from the animal.

2.10. Factors Affecting Embryo Quality

2.10.1. Age and parity

When age of a cow is too early age (4 to 7 months) or late age of the cow embryo quality is under scored (Camargo *et al*., 2005; Rizos *et al*., 2010). Studies conducted on *in vitro* experiment indicate that the oocytes derived from heifers has shown the maximum blastocyst developmental stage than cows (Rizos *et al.*, 2010). Furthermore, the same authors reported that the number of embryos developing to the blastocyst stage significantly higher in heifers at puberty stage than prepubertal heifers and cows. Early reproductive age in cattle is preferred for embryo production because of lower oocyte competence poor fertilization rate at later (Blanco *et al.,* 2011).

2.10.2. Developmental competence of oocyte

Oocyte developmental competence is usually defined as the ability of a female gamete to mature into an egg with its potential to be fertilized to attain embryo development (Rybska *et al.*, 2018).

The oocyte competence is not a single instantaneously event but a progressive transformation that occurs in a matter of days in the last part of the follicular wave (Blanco *et al.,* 2011). Among the various factors, nutrition is main factors especially low tension of trace elements affect oocyte competence. Hence embryos originated from compromised oocyte might be reason for increases UFO and degenerated embryos (Dorice *et al.*, 2019).

2.10.3. Nutrition and body condition

Over-feeding protein or under-feeding minerals can affect pregnancy outcome from recipient side (Swecker, 2014). Carvalho et al. (2014) reported that the percentage of fertilized oocytes, percentage of degenerated and transferable embryos have greater variations among cows having low and optimum body condition associated. High negative esterified fatty acid (NEFA) and low-glucose level at the time of oocyte maturation *in vitro* studies, has demonstrated that NEFA has detrimental effect for the developmental competence and impair early embryo development (Leroy *et al.,* 2008). Negative energy balance in donor might be reason for alteration of the endocrine and biochemical environment of the follicular fluid and that will changing the micro-environment of the growing and maturing female gamete (Leroy *et al*., 2012; Mikkola *et al*., 2019). In relation to ammonia and urea concentrations in the blood may cause alterations in intrafollicular, oviductal and uterine environments in high yielding cows which can be reason for disturbed maturation, fertilization or early cleavage (Sartori *et al*., 2010; Bisinotto *et al*., 2012) while no interference with oocyte numbers and quality due increasing energy diet (Sales *et al.* 2015). Low circulating IGF-I may also be associated with reduced embryo development. High-energy diets decrease oocyte and/or embryo quality in both single and superovulated cattle (Sartori *et al.*, 2010).

2.11. Factors Affecting Successful Embryo Transfer

2.11.1. Failures in recipient selection

Recipients are females that hosts to carry on an embryo for growing in their uterus (Lamb, 2011). It is clear that recipients have no genetic effect on the embryo transferred but gynecological welfare of recipients should be insured for the transferring embryo (Phillips and Jahnke, 2016).

Different literature reported that BCS, age, parity and lactation status can affect success of embryo transfer (Stockler, 2014, Degefa *et al*., 2016; Ribeiro *et al.*, 2016). Hence, as a prerequisite thorough and careful ultrasonographic diagnosis of the uterine environment and associated disorders and uterine diseases should be checked to manage or treat recipients or devoid of the recipient from choice before starting synchronization of the cows for embryo transfer (Islam, 2011; Lamb and Mercadante, 2014; Ribeiro *et al*., 2016).

2.11.2. Carry over effects of postpartum disorders

According to previous findings (Colloton, 2014; Estrada-Cortés *et al*., 2019), inflammations of the uterus in cows, classified as puerperal metritis, clinical endometritis, subclinical endometritis, and pyometra represents one of the most important causes of failure to conception in dairy herds (Phillips and Jahnke, 2016). Ascending and descending bacterial infection of the endometrium of cattle to cause endometritis, uterine disease, and infertility (Ribeiro *et al.*, 2016). Diagnosis of metritis within the first 10 days postpartum is associated with the presence of pyrexia, fetid pus within the uterine lumen, vagina or discharging from the vulva, and delayed uterine involution. Solely monitoring the rectal temperature of dairy cows to diagnose metritis is less reliable than including an examination for abnormal uterine discharge because pyrexia is not consistently associated with pathogenic bacteria in the uterine lumen (Ribeiro *et al*., 2016; Estrada-Cortés *et al*., 2019). Knowledge in types and effect of postpartum risk factors is very important to take a mitigation measures and to control in order to reduce detrimental effects associated to them. Inflammatory disease before transferring the embryo has greater opportunity to impaired early conceptus development to elongation stages and secretion of IFN-τ in the uterine lumen which might be cause for failure to sustain conceptus (Ribeiro *et al.*, 2016). Hence, examination of uterine health should also be performed and evaluated prior to synchronization to sustain the conceptus (Colloton, 2014; Ribeiro *et al*., 2016).

2.11.3. Embryo quality

To achieve successful conception, embryo quality shares half of the opportunity window, on the other hand, the embryo recovered will faces for frequent unnatural touches and thermal changes during embryo flushing, screening and freezing. Removing an embryo from its natural uterine environment increases the level of stress to that embryo (Jahnke *et al.,* 2014). When embryos experience additional external stress, such as freezing and thawing, the result is a decreased survival rate for those embryos. Some reports have shown that decreases in pregnancy rate from 83% with fresh embryos to 69% with frozen-thawed embryos are similar to the 10 to 15% decrease in pregnancy rates (Lamb, 2005). Recently, Erdem et al. (2020) reported that pregnancy rates with Grade I quality embryos found to be higher compared with Grade 2 embryos.

2.11.4. Functional Status of CL at the time of transfer

Existence of functional CL at the time of embryo transfer is mandatory in recipient cows. As a functional structure, CL is formed from a mass of luteinized cells on ovarian surface. It is responsible for the production of progesterone hormone specially during early phase of the pregnancy commonly found ipsilateral to pregnant horn. It is a palpable structure and used to maintain pregnancy. Its functional status collapsed if the default program of cyclic event in recipient female is initiated otherwise this event blocked when intact CL is existed (Curtis, 2015). Sometimes in the existence of CL, early pregnancy could be fail due to suboptimal production of progesterone by the CL. Ultrasonographic diagnosis is very critical to ensure ovarian activity by identifying corpora lutea before embryo transfer (Arthington *et al.,* 2017). Due to its accuracy ultrasound is much better than manual palpation for identifying small or fluid-filled corpora lutea. Since maintaining open animals is costly to the owner so it is imperative to identify all appropriate recipients.

2.12. Synchrony and Preparation of Donor and Recipients

2.12.1. Donors' synchrony

Before starting the FSH treatment to donor cows, CIDR insertion is applied to inhibit further estrus and ovulation by maintaining high concentration of progesterone hormone. This mode of approach maintains all eligible donor cows at their diestrus phase and enables to start injection of FSH using number of cows at once (Velazquez, 2008). CIDR insert for cattle is made by molding a thin layer of silicon and progesterone mixture (10% w/w) around a nylon spine. The CIDR contains 1.38 g progesterone and is designed to maintain elevated blood concentrations of progesterone at least 2 ng/ml for up to 10 days. Being relatively thin, the CIDR is easily inserted into the vagina and has good retention capacity (2.5% loss rate is normal). A flexible nylon tail is attached to the device to allow for easy removal (Islam, 2011).

2.12.2. Recipients' synchrony

Synchrony of recipients cows is the technique by which keeping the uterine environment conducive (Islam, 2011). There are different factors affecting pregnancy success after embryo transfer. Failure to the synchrony between embryo and the uterine environment can be reason for failure to conception. It is common to use recipient's synchrony by injecting $PGF_2\alpha$ 2ml i.m. This is done to maintain maternal recognition of pregnancy or to establish the receptive uterine condition to the new coming embryo (Lamb, 2005; Adams and Singh, 2017). One of the main secrete is synchrony of age of embryo and maintaining uterine environment for that particular age of embryo which is called synchrony. Pregnancy rates were not compromised when the recipient was in estrus within 24 hours before or after the donor (Lamb, 2005). Scheduled injection of $PGF_2\alpha$ will conducted according to the interest of technician single, or double injection of $PGF_2\alpha$ considering the dates of donor cow's embryo flush (Bó and Mapletoft, 2014) as shown in Figure 4 below.

2.13. Maternal Recognition of Pregnancy

Maternal recognition of pregnancy is the physiological process by which the early embryo signals its presence to the recipient. This process used to down regulate receptor sites of oxytocin which is precursor for the initiation of the default program ($PGF_2\alpha$) synthesis and release after days 16 post estrus (Forde and Lonergan, 2016). Oxytocin that initiates regression of the functional CL blocked by the chemical called interferon-tau (IFNT). Quality of embryo proper determines success of an embryo implantation process by producing the required amount of IFNT. IFNT is a protein secreted by trophoblast of the early embryo used for the latter communication with the recipient's womb (Lamb, 2005; Adams and Singh, 2017). ET performance in general, depend on the quality of embryo, the novelty of the recipient selected, the existence of functional CL and the management of the recipient during pregnancy. Failure of early embryonic implantation causes for reproductive failure and resulting lower pregnancy rate (Forde and Lonergan, 2016) .

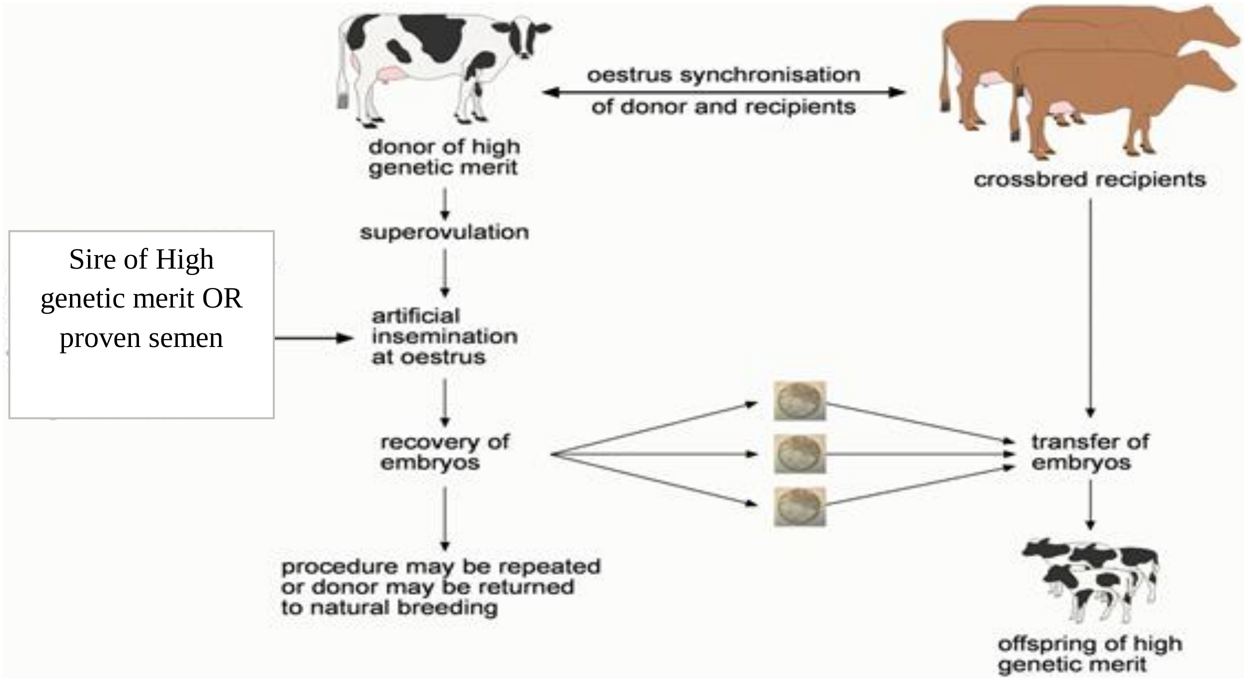
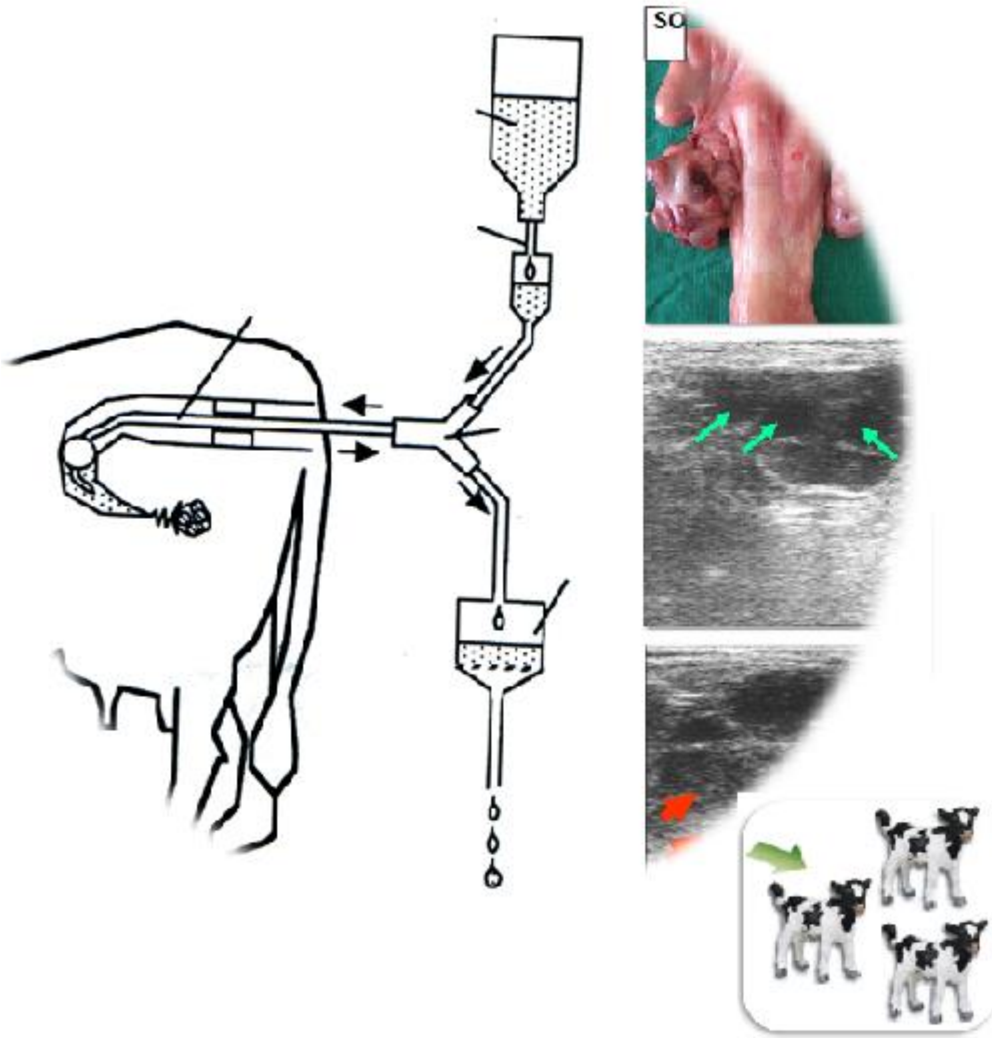


Figure 4. Donor recipient orientation in multiple ovulation and embryo transfer

Source: VeterianKey (2017)

CHAPTER 3



3.MATERIALS AND METHODS Pages 32-42

General description of study area

The study was conducted from February 2019 to March 2020 at Addis Ababa University College of Veterinary Medicine and Agriculture (AAU, CVMA), Debre Zeit Agricultural Research Centre (DZARC) and Nordeli Private dairy farm. CVMA provided all the required research inputs such as Pluset®, CIDR, Foley catheter with embryo filter, imported semen and lactated ringer solution while DZARC and Nordeli farms were sources of all the experimental animals and facilitated to use the standard embryo flushing shuts and all the laboratory equipment and space. The study site is 47km South East of Addis Ababa, located at 08° 44’ N and 38° 58’ E (Latitude/ Longitude) at 1900 meters a.s.l. with the average annual temperature ranging from 9.8°C to 28.3°C. It receives an average annual rainfall of 851 mm in a bimodal season (EIAR, 2020).

Selection of donors and recipients

Breed and blood level of study animals were HF (pure Holstein Frisian), HF*Boran (50% and 75% Holstein Frisian x Boran) and Boran dairy cows. Donor cows were identified based on the criterion indicated by Philips and Jahnke (2016). Cows with high milk yield, age greater than 18 months, and regularly cycling, no history of general and reproductive health problems were selected. Eligible donor and recipient dairy cows were subjected to ultrasound assisted gynecological evaluation to determine their reproductive soundness. During selection of recipient cows, the description in Lamb (2016) was referenced based on parameters such as calving ease, lactation yield, mothering ability, and absence of history of reproductive disorders. All cows were properly housed in ventilated barns with well fenced compound having sufficient free moving area, fed on hay and green fodder daily supplemented with formulated concentrate based on the feeding protocol prepared by the research center to maintain an optimal body condition score (BCS) in both farms. Water was provided ad libitum.

Steps followed in selection of study animals

The experimental animals involved in this study were dairy cows selected from DZARC and Nordeli private dairy farms. Three stage screening for embryo donors was undertaken. All donor animals (n=77) from DZARC and Nordeli dairy farms were subjected to rigorous selection scheme from the total herd (n=135).

Stage 1: Overall (n=77), 65 dairy cows from DZARC and 12 from Nordeli farm were screened based on breed and blood level, history of good reproductive performance and optimal BCS at the time of selection.

Stage 2: The study population of HF, HF*Boran, Boran herd from farms was assessed for the fitness of the experiment and to make decisions for eligibility and suitability of physiological status of the donors. Based on the selection criterions (Table 4), donor cows were those having anatomically normal cervix and bear with ovarian corpus luteum (CL) besides to their healthy uterine information from ultrasound diagnosis. All cows with straight cervix were admitted for the experimental protocols.

Table 4. Selection parameters for donor cows from DZARC and Nordeli dairy herds

Breed	Total animals examined	**Cervix condition**		**Ovarian status**			**Exceptions**
		Straight cervix	Dented cervix	Right CL	Left CL	without CL	Bilateral CL
		Selected					
HF	25	**24**	1	23	1	1*	0
HF*Boran	29	**27**	2	27	0	1	1
Boran	23	**18**	5	21	1	1	0
Total	77	**69**	8	71	2	3	1

**- A cow with fluid like accumulation in endometrium when scanned;*

Note: Anatomically cows with straight cervix were better suited for catheterization when flushing

The superovulatory treatment protocol

The superovulatory protocol was a 4 days Pluset® (FSH, Barcelona, Spain) injection applied as shown in Figure 5.

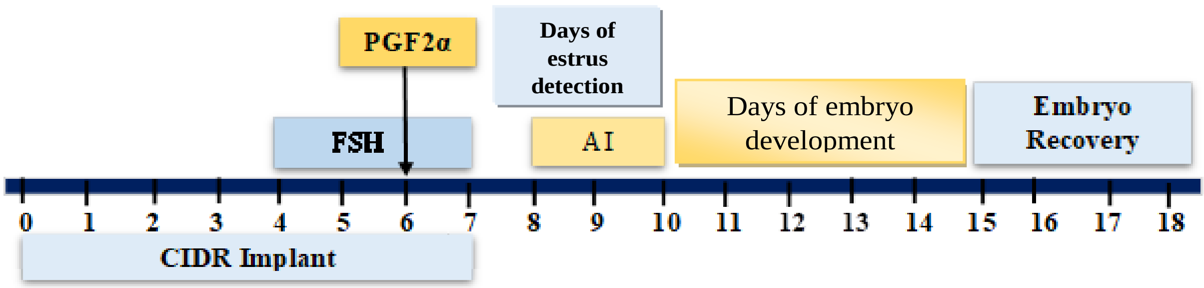


Figure 5. Schematic designation of the superovulation protocol and schedule of embryo recovery

CIDR insertion: According to the manufacturer's recommendation (Zoetis Inc, 2015), Day 0 marked as the insertion of Controlled Internal Drug Releasing (CIDR) which is T-shaped intravaginal nylon spine molded with a silicone rubber skin and coated with progesterone. The CIDR insert was removed on Day 7 following the end of the treatment period by pulling the plastic tail that protrudes from the vulva.

Injection Prostaglandin hormone: On Day 6, each cow received an IM injection of 2 ml prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (Estrumate®, Cloprostenol Sodium, Germany) twice a day (b.i.d) and inseminated twice upon the detection of standing estrus starting Day 8 following the end of superovulatory treatment and removal of the CIDR implant. Besides, all flushed cows injected 2ml $PGF_2\alpha$ to avoid further conception.

Dispensation of Pluset® (FSH)

Superovulatory hormone was dispensed over four days in equally divided doses given twice per day at decreasing fashion as previously described by Degefa et al. (2016) and presented in Table 5. The vial containing 500IU lyophilized or freeze dried Pluset® (Barcelona, Spain) was reconstituted with sterile water of 10.5ml. After injection of the first portion, the remaining portion was kept at a refrigeration temperature (+2^0 C - +8^0 C).

Table 5. Pluset® treatment schedule for superovulation of donor cows

Pluset® (IU)	Reconstituted amount (ml)*	Time of day	Treatment fraction of Pluset®			
			Day1	Day2	Day3	Day4
500	10	Morning	2ml	1.5ml	1ml	0.5ml
		Evening	2ml	1.5ml	1ml	0.5ml
650	13	Morning	2.5ml	2ml	1.25ml	0.75ml
		Evening	2.5ml	2ml	1.25ml	0.75ml
800	16	Morning	3ml	2.5ml	1.5ml	1ml
		Evening	3ml	2.5ml	1.5ml	1ml

**-Volume of reconstituted Pluset®, 1ml=50IU*

Superovulatory response was evaluated based on an ultrasonic count of the CL and persistent or unovulated follicles (AnF). Arbitrary parameters were used for the superovulatory responses and categorized as: Poor when <5 CL and/or AnF are detected; Moderate for 5-10 CL and/or AnF and Good for 11-14 CL and/or AnF and Excellent for ≥15 CL and/or AnF are recorded during ultrasonography. Rate of response to superovulation was computed as the number of cows with CL and/or AnF / Total number of cows treated x100. AnF were those unovulated preovulatory follicles having ≥15mm diameter.

Embryo flushing

Embryos were flushed after seven days post AI or on day 15 of CIDR insertion. Donor cows were received 3-5ml of epidural anesthesia (2% Lidocaine) 2-3 minutes prior to embryo flushing. The perineum was cleaned and the external genitalia was also disinfected before every procedure. Embryos were flushed using a two-way Foley's catheter with 1 liter of pre-warmed (to body temperature) lactated ringer solution into which 1-5% calf serum was added (Purohit *et al*., 2013).

Total embryo recovery or flush output was used interchangeably to represent the total UFO and embryos recovered. Recovery of flush output was computed as: Recovery rate (%) = Total flush output (UFO + embryos)/Total number of (CL+AnF) *100; Rate of UFO recovery = Total number of UFO/ Total recovery or flush output * 100; Rate of transferable embryos = Total number of transferable embryos/ Total recovery or flush output* 100.

Evaluation and classification of recovered embryos

Embryos were classified, based on their stages of development, into four different qualities as follows;

1. Excellent- An ideal embryo, spherical, symmetrical and uniform sized blastomers, with uniform color
2. Fair- Few extruded blastomers, slightly irregular shape, few vesicles
3. Poor- Definite but no severe problem, extruded blastomers, vesiculation of few degenerated cells
4. Degenerated- Severe problems, numerous extruded cells, degenerated cells with different size, large vesicles with dark color

Based on the above classification, embryos were evaluated for their developmental stage (from stage 1 = 1-cell to stage 9 = expanded hatched blastocyst) and for their quality (from Grade 1 = excellent to Grade 4 = degenerate/ dead) according to the International Embryo Transfer Society guidelines described in Jahnke et al. (2015).

Further classification of embryos

According to Baruselli et al. (2006), embryos were further classified in to 5 categories:

1. Transferable embryos (Grades 1, 2 and 3)
2. Freezable embryos (Grades 1&2)
3. Poor quality embryos (Grades 3 and 4)
4. Degenerate embryos (Grade 4)
5. Fertilized embryos (Grades 1, 2, 3 and 4)
6. Unfertilized ovum (UFO)

Ethical procedures

All clinical study procedures were carried out according to the guidelines of the Research Policy on Animal Ethics and Welfare of the Addis Ababa University and approved with the reference number: VM/ERC/01/11/11/2019. Animals that were considered for more than one experiment were rested for 2-3 months between the experiments until the usual reproductive cycle resumed.

Endpoint definitions:

- *Superovulation*- The process of stimulating an ovary to nurture more than one preovulatory follicle to release more than one egg at a time in one and/ or both ovaries
- *Non-responders*- When an ovary bears only one CL and/or AnF (from each ovary) when superovulated
- *Poor-responders*- It is a relative term for those cows responded below the level of the parameters fixed other than the non-responders (such as weak, moderate, good and excellent responders) under the superovulatory protocol used in this study
- *Superovulatory response rate*- The extent of ovulation of preovulatory follicles in terms of CL and AnF
- *Persistent unovulatory follicles (*AnF*)*- One or more preovulatory follicles failed to ovulate and remining persistent until days of embryo flush
- *Total recovery*- The recovery of the total substances (UFO and embryos) compared with the total ovulation in terms of the total CL counted
- *Total flush output*- The total average of recovered substances (UFO and embryos) per individual donor cow
- *Proportion of UFO*- The ratio of recovery of unfertilized ovum versus the total flush output
- *Dented cervix*- The situation that refers to the deviation of the normal cylindrical straight cervical anatomy of cows found to be curved or dented in shape when rectally palpated
- *Controlled environment*- Is the specific place where for experimental purpose and used to block the involvement of external factors that affect the experimental procedures and outcomes

Experiment 1: Determination of the Optimal Dosage of Superoulatory Hormone

The HF*Boran cows (n=42) were grouped randomly into three treatment groups (500IU, 650IU and 800IU) to determine the optimal dose of Pluset® and also these cows were further subdivided in to three groups based on their blood level. A 3x3 factorial analysis of the random effect was designed as presented in Table 6 as described in Kaps & Lamberson (2004). BCS was determined on a 1-5 scale (1- Poor, 2 – Moderate, 3 – Good, 4 – Very Good, and 5 – Fat) according to Klopčič et al. (2011). Animals with 1 and 5 BCS were not included in the experiment. Selected cows were from parity one to parity four with age range of 18 months to 5 years to fix age effect.

Superovulatory response of treated animals was evaluated by counting number of CL, POF (CL+ AnF) were used to compare efficiency of the three-dosage regimens (Mehmood *et al*., 2012). Response to superovulation of all donor cow was confirmed ultrasonically by counting number of CL and/or AnF on both ovaries on Day 7 post AI.

Table 6. Treatment groups of superovulated dairy cows and the breed blood level (n=42)

Breed blood level*	Dose level tested, Pluset®		
	500 IU (n=14)	650 IU (n=15)	800 IU (n=13)
50% HF*Boran (n=14)	TR_1	TR_2	TR_3
75% HF*Boran (n=13)	TR_1	TR_2	TR_3
HF (n=15)	TR_1	TR_2	TR_3

**Blood level of donors fixed based on the DZARC dairy herd recording book; TR-Treatment*

Experiment 2: Characterization of Estrus Signs in Superovulated HF*Boran Dairy Cows

A total of 33 dairy cows, 50% HF*Boran (n=17) and 75% HF*Boran (n=16) crossbred dairy cows were used in this experiment. Age of the cows were from 2 to 8 years with BCS of poor, fair and good were considered. Animals were meticulously observed for one hour on daily basis three times per day (at 6am, 1pm and 5pm) starting the day of CIDR removal until the complete cessation of signs. Estrus signs manifested during this schedule were recorded and evaluation was made for their onset, duration and frequency of occurrence. The behavioral estrus signs monitored were mounting on others, standing to be mounted, sniffing, bellowing, restlessness, chin resting, supporting to others, urination and trailing. The cutoff point for ending of estrus follow up was refusal for standing to be mounted by others and refusal. Computation was made as follows:

$$\mathbf{MoB} = \frac{\left(\frac{\text{sum of frequency of each behavior}}{\text{no.of rounds for daily follow up}}\right)}{\textbf{No. of days of follow up for each of estrus behavior manifested}}$$

Where; *Mo- Mean of frequency of behavioral signs count; B- Each behavioral sign*

Characterization of estrus behaviors in superovulation:

Evaluation of the intensity and category of estrus signs were carried out based on the description given in Dobson et al. (2018) and Röttgen et al. (2018). Accordingly, vaginal color and discharge was observed to determine the grade category of the primary estrus signs as:

Grade-1: Noticeable swelling and hyperemia of the vulva with a clear mucus discharge
Garde-2: Absence of noticeable swelling with fairly sign of hyperemia and presence of a clear mucus discharge
Grade-3: The vulva is fairly hyperemic but there is no discharge
The intensity of the estrus signs was categorized as: Good, Moderate and Weak viz:
Strong estrus: cows exhibited with ≥ 4 behavioral signs and Grade 1 primary estrus sign
Moderate estrus: cows exhibited 2 to 4 behavioral signs and Grade 2 primary estrus signs; and
Weak estrus: cows exhibited with ≤ 2 behavioral signs and Grade 3 primary estrus signs
Ovulation rate (OVR) was computed as:

$\mathrm{O_V}R = \frac{CL}{CL + AnF}$; Where; cows with ≤ 2 CL were considered as non-responders

Experiment 3: Evaluation of Embryo Quality Produced from Different Breeds of Dam and Sire

A total of 36 dairy cows, Boran (n=18) and HF*Boran (n=18) were superovulated. HF*Boran were given an IM injection of 650 IU (13 ml) Pluset® while Boran were given 250 IU (5ml) of same Pluset® based on the superovulation protocols in experiment 1 and 2.

The two-level (a 2x2 design) of Dam and Sire combination method was applied during embryo production in which the sire breeds were assigned to the two breeds of donor cows alternatively (Figure 6). Imported high-quality purebred Boran semen and progeny tested HF-Friesian (HF) Sire semen were used.

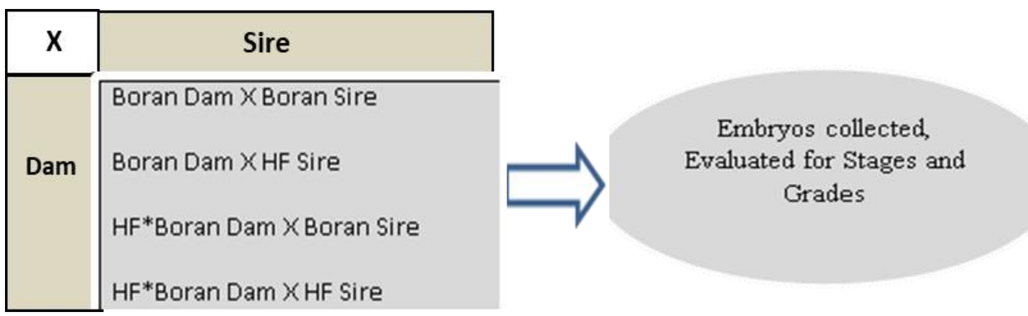


Figure 6. Dam-Sire breeding combination model

Embryo freezing:

Those embryos that were not used for fresh transfer (Grade 1 and 2) were cryopreserved as described in Youngs (2011) for later use as frozen embryos. Embryos were placed for 10 minutes into a commercially prepared embryo freezing medium containing 1.5M ethylene glycol and 0.3M sucrose (BoviPRO™, MOFA®, Canada) before being loaded individually into a 0.25 ml straw. All straws were placed into the heat transfer chamber of a portable embryo freezing machine (EFT-3002, BELTRON Instruments®, Colorado, USA) filled with 90% ethanol at a temperature of -6.5 °C. Embryo loaded straws were seeded for 2 minutes, held for an additional 8 minutes, and then slowly cooled at a rate of 0.6 ^{0}C per minute to -32 ^{O}C after which they were quickly plunged into the liquid nitrogen for longer time storage. Cryopreserved embryos were withdrawn from the container and thawed at +37°C for 25-30 seconds to when recipients were ready for transfer.

Embryo transfer and pregnancy diagnosis:

After embryos were collected, they were evaluated and assigned into their respective grade and stage of development. Those assigned transferrable embryos (Grade 1, 2 and 3) were transferred fresh to synchronized recipient cows that were on Day 7 &/or 8 of their post ovulatory reproductive cycle. On the other hand, Frozen thawed embryos were also transferred to the recipients by holding straws at room temperature air for 3-5 seconds, and placed into a water bath at +37°C for 25-30 seconds. After blot drying the straw, the heat-sealed end of the straw was cut off and inserted the straw in to the transferring catheter by pointing the cut end in to upward position of the catheter and then capping the catheter with plastic sheath, then transferred to the recipients aseptically. Embryos were transferred to recipients based on the following combination as described in Table 7. Early pregnancy diagnosis was done using ultrasonography with 5.0 MHz linear-array transducer between 30-35 days post transfer (Gunn and Hall, 2018).

Table 7. Status and breed of embryos transferred to the recipients

Embryo status	Breed of embryos	Recipient
Frozen	HF*Boran	HF
	Boran	HF
Fresh	HF*Boran	Boran
	Boran	Boran

Statistical models used:

Poisson regression:

$$P(x; \mu) = \frac{(e - \mu)(\mu x)}{x}$$

; Where, x is the actual number of successes of flush output that result from the experiment; e is approximately equal to 2.71828; the mean of the distribution is equal to μ and the variance is also equal to μ.

ANOVA equation:

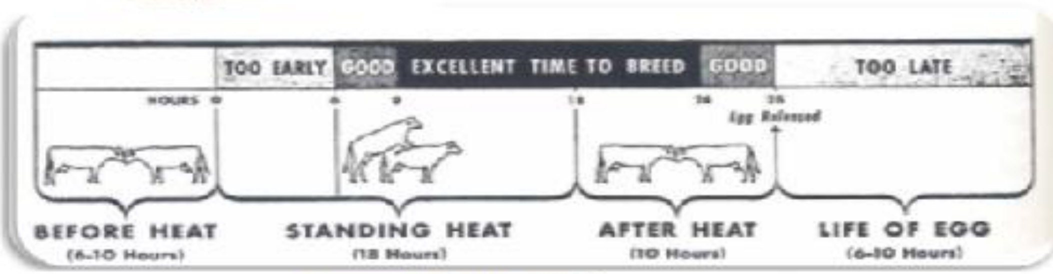
$$F = \frac{MST}{MSE} \;;\; MSE = \frac{SSE}{N - p} \;;\; SSE = \sum (n-1)\, s^2$$

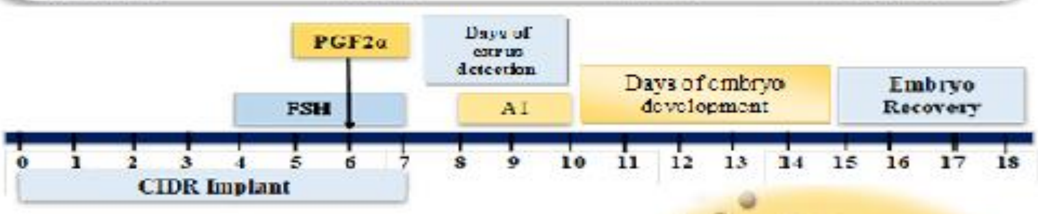
; Where, F = Anova Coefficient; MSE = Mean sum of squares due to error; SST = total Sum of squares; SSE = Sum of squares due to error; p = Total number of populations; n = The total number of samples in a population; s = Standard deviation of the samples; N = Total number of observations.

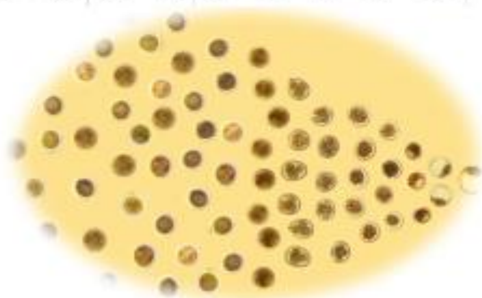
Statistical analysis

All data collected from the three experiments were summarized and managed before analysis using Microsoft excel spread sheet for filtering the dataset. Datasets were tested for their normality (for their skewness and kurtosis) by running normality histogram plot to test the level of skewness and skewed variables were transformed by using "Zero-skewness log transform technique" command under STATA® version 14.0 software. Mean of estrus sign parameters recorded three times per day, OVR, UFO/embryos were assigned for plotting. Post estimation and pairwise comparison of the means of estrus behavior and embryo collection, and their mean comparison adjustment was done using Bonferroni's method. Poisson's regression was used for establishing test of significance for all count datasets while GLM was run to declare test of significance for those transformed variables appear with negative values and Chi-square test was used to compare estrus behavior parameters. Tested variables were onset and duration of estrus behaviors, POF, AnF, CL, OVR, overall recovery of UFO/embryos. Clustered column charts (SE-bar) plots were used to depict variations in superovulatory treatments and different grades of embryos. All transformed variables (parametric forms) analyzed for the within and between computation with One-way ANOVA to test significance of parameters and Two-way ANOVA was used to measure interaction terms accordingly. The quality of embryos collected from the dam-sire combination method was evaluated for the breed of the dam or sire and computed by two-way factorial ANOVA. All average values were reported as mean ± (SE) and level of significance was held at $P<0.05$.

CHAPTER 4







4. RESULTS Pages 44-57

4.1. Determination of Optimal Dosage of the Superovulatory Hormone

The overall superovulatory response rate of dairy cows (n=42) for the treatments of 500 IU, 650 IU and 800 IU showed at least good superovulatory response (number of CL $\geq$ 11) in the three TR groups were 35.7%; 86.6% and 46.1%, respectively. The mean (±SE) number CL in 500 IU, 650 IU and 800 IU regardless of blood level were also 10±0.7, 13.1±0.7 and 9.7±1, respectively. Explicitly, in treatment doses of 500 IU and 800 IU there was no excellent responder cows while 92.8% and 69.2% cows were responded at moderate and good level, respectively. As typical case, large ovarian size was noticed in 50% HF*Boran for a dose of 800 IU and ovary was bear with fewer CL count in this cow. Moreover, the largest sized AnF were also 30mm in diameter in this cow and in average 20.1mm were recorded in other cows (n=5). Differently HF has shown lowest response for the lowest doses applied (500 IU) and increasing as dose increased (Table 8). Among the three levels of Pluset® dose administered, 650 IU had the highest count for POF and CL than 500 IU and 800 IU doses treated in crossbred cows. The overall mean (±SE) of the superovulatory response of the treated cows were 11.6±0.6, 14.6±0.5, 11.5±0.5 (POF) and 10.1± 0.7, 13.8±0.7, 9.7±0.9 (CL) for 500 IU, 650 IU and 800 IU, respectively (Table 9).

Table 8. The superovulatory hormone treatment response in dairy cows for the three treatment doses

Pluset® dose level	Exotic blood level	N (42)	Superovulatory response category			
			Poor (%)	Moderate (%)	Good (%)	Excellent (%)
500 IU	50% HF*Boran	4	0	25	75	0
	75% HF*Boran	4	0	75	25	0
	HF	6	16.6	66.8	16.6	0
650 IU	50% HF*Boran	5	0	0	40	**60**
	75% HF*Boran	5	0	20	20	**60**
	HF	5	0	20	80	0
800 IU	50% HF*Boran	5	**20**	40	40	0
	75% HF*Boran	4	0	75	25	0
	HF	4	0	25	50	**25**

Table 9. Effect of dose variation in response to CL and POF (Mean ± SE) in different TR groups of donor cows

Pluset® (IU)	TR category	POF	CL	AnF	P value
500	50%HF*Boran	13.0 ± 1.0	11.7 ± 1.3	1.2 ± 0.5	P>0.05
	75% HF*Boran	12.5 ± 1.0	11.0 ± 1.3	1.5 ± 0.5	
	HF	10.2 ± 0.8	8.5 ± 1.0	1.7 ± 0.4	
650	50% HF*Boran	15.2 ± 0.9*	13.6 ± 1.1*	1.6 ± 0.4	P=000
	75% HF*Boran	16.0 ± 0.9*	14.6 ± 1.1*	1.4 ± 0.4	
	HF	14.5 ± 1.0 *	13.2 ± 1.3*	1.2 ± 0.5	
800	50% HF*Boran	11.4 ± 0.9	8.4 ± 1.1	1.8 ± 0.4	p>0.05
	75% HF*Boran	10.2 ± 1.0	7.7 ± 1.3	1.5 ± 0.5	
	HF	12.6 ± 0.9	11.2 ± 1.1	1.4 ± 0.4	

** significant at 0.05*

The column bar chart plots shown that increasing superovulatory response in 50% and 75% HF*Boran from the dose 500 IU and picked for the dose 650 IU. But the general effect of dose increment has shown decreasing the superovulatory response in crossbred cows while increasing in HF and the variation would vary for the level of the dose administered as shown in the equations below in Figure 7.

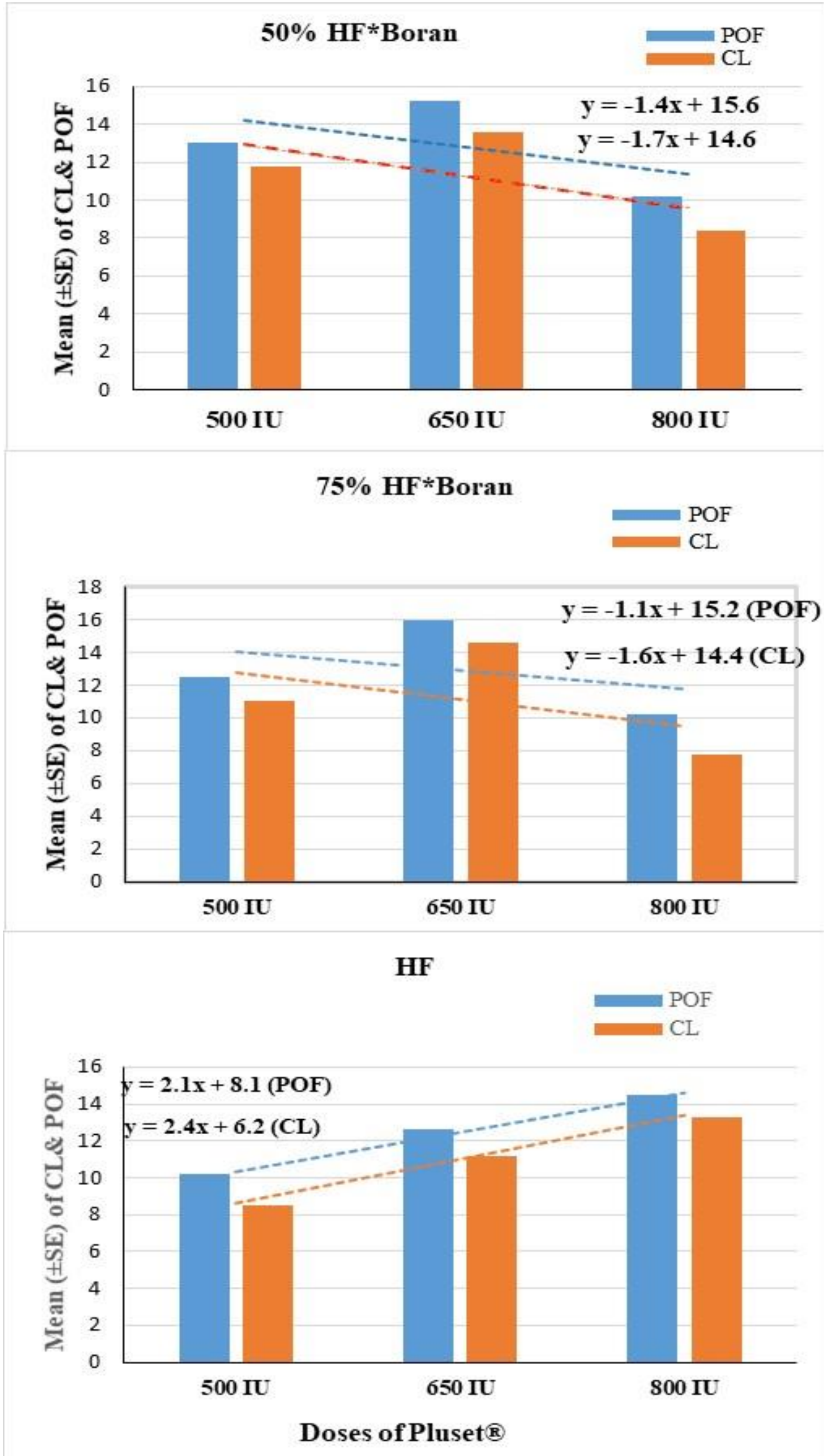


Figure 7. Trend of supeovulatory response in treatment groups for different doses of Pluset®

4.2. Estrus Characteristics in Superovulated HF*Boran Dairy Cows

All cows (100%) manifested behavioral estrus by considering at least one of the primary and/ or secondary estrus signs exhibited. Out of this, estrus was weak in 48.5% (n = 16), Moderate in 39.4% (n=13) and Strong in 12.1% (n=4) with different interplayed signs regardless of the breed blood level. Both mounting other cows and standing to be mounted were expressed by 73.3% (n=12/17) and 49% (n=8/16) cows, for 50% and 75% HF*Boran, respectively. But 93% (n= 31/33) of the study animals did not exhibit bellowing as a sign (Figure 8).

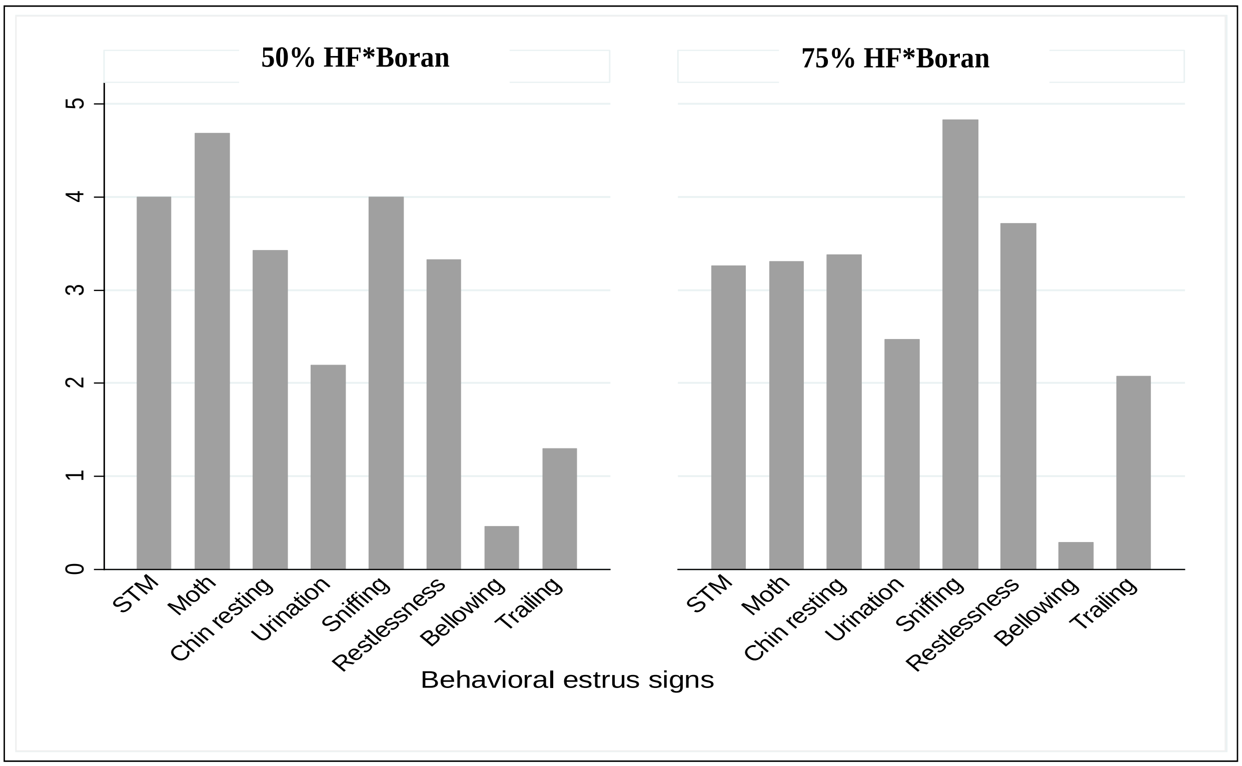


Figure 8. Types and frequency of estrus behaviors observed within and between study groups

The overall average time for onset of estrus signs after CIDR removal was 37.4 ± 1.5hrs and the duration of estrus manifestation was 28.5 ± 1.2hrs. Onset of estrus was influenced by blood level with the 50% HF*Boran commencing estrus much earlier ($p<0.05$) than the 75% HF*Boran. However, duration of estrus was not affected by blood level (Table 10).

Table 10.The time interval for onset and duration of behavioral estrus manifestation

Breed blood level*	Onset of estrus (hrs)			Duration of estrus length (hrs)		
	Mean (±SE)	Max	Range	Mean (±SE)	Max	Range
50% HF*Boran	31.6 ± 1.0	40	16	32.3 ± 1.5	44	24
75% HF*Boran	43.3 ± 1.6	60	24	24.6 ± 1.3	34	18

* - *Chi^2 ($\chi 2$) = 31;* P *= 0.009 (Onset) and $\chi 2$=21.3;* P *= 0.127 (Duration)*

Intensity of Estrus Behavior and Superovulatory Response

The overall 84.8% (n=28) of donor cows were responded to the superovulatory treatment and non-respondent cows were 15.2% (n=2, 50% cross and n=3, 75% cross dairy cows). The trend of superovulatory response (in terms of CL), total flush output and UFO count were at increasing fashion as estrus strength increasing consistently, in both blood level. But there was greater increment of UFO count as estrus strength increase in 50% HF*Boran cows (Figure 9). Fishers' chi square for POF, UFO, and fertilized embryos were $\chi 2$ = 0.023, $\chi 2$ = 0.04 and $\chi 2$ = 0.035, respectively.

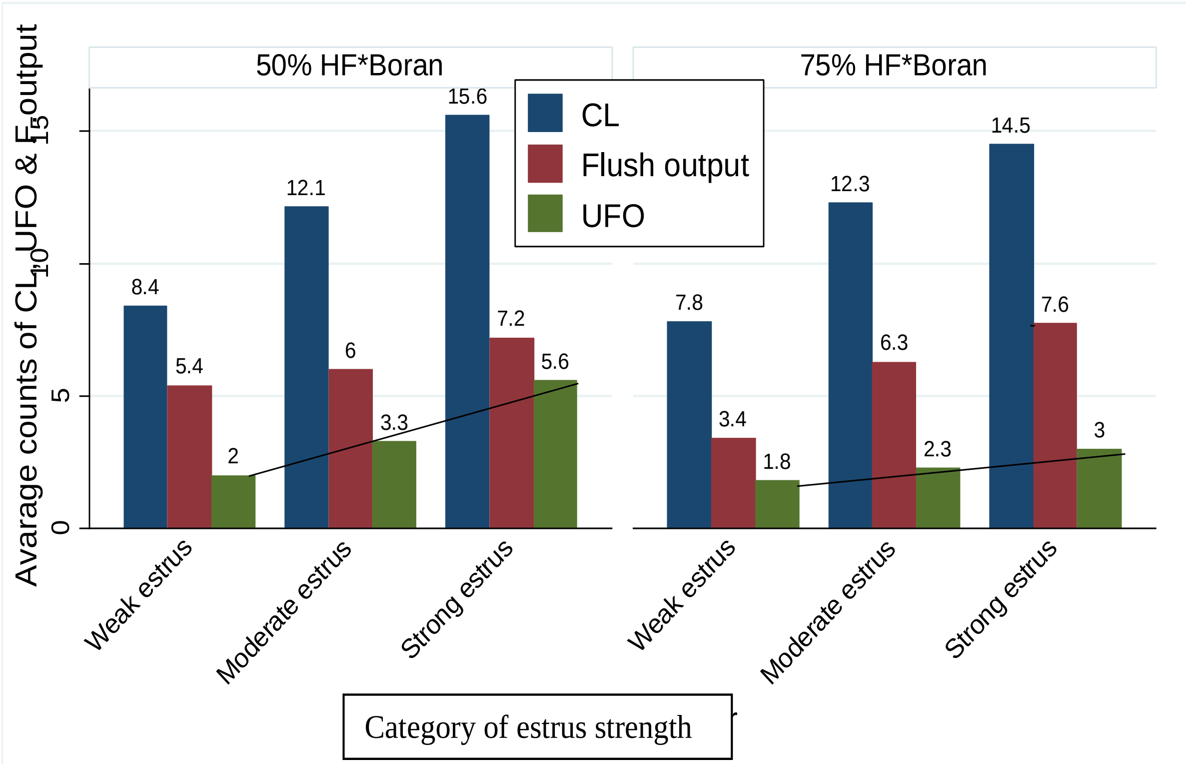


Figure 9. Association of behavioral estrus signs with pattern of the superovulatory responses

Ovulation Rate

OVR was computed for each cow after the detection of POF. When OVR was determined from the CL irrespective of the cow's blood level, 13 cows had OVR of 80-95%, 18 cows had 60-78% and 2 cows had 40-50% OVR (Figure 10).

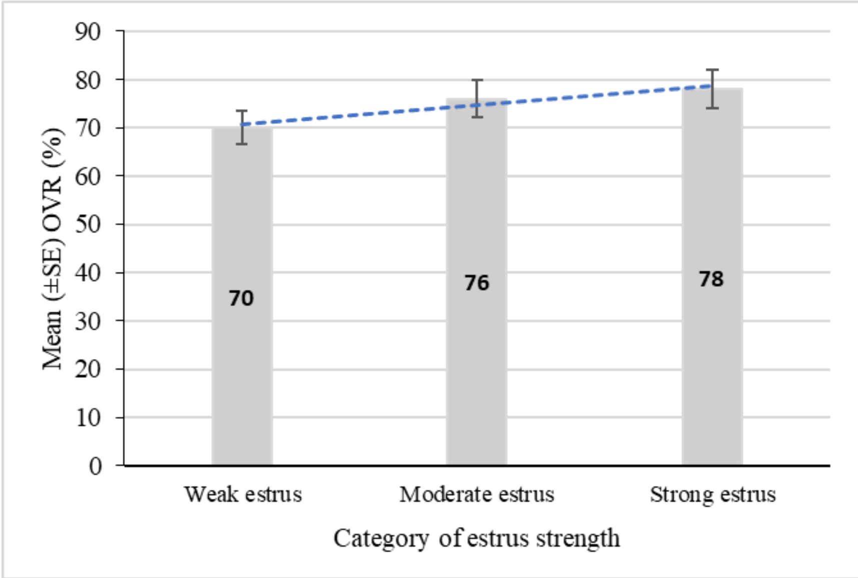


Figure 10.Trend of OVR in terms of intensity of behavioral estrus and breed blood level

When the same OVR was further assessed based on blood level, cows with higher exotic blood level and showing weak estrus had the lowest OVR as depicted in Figure 11.

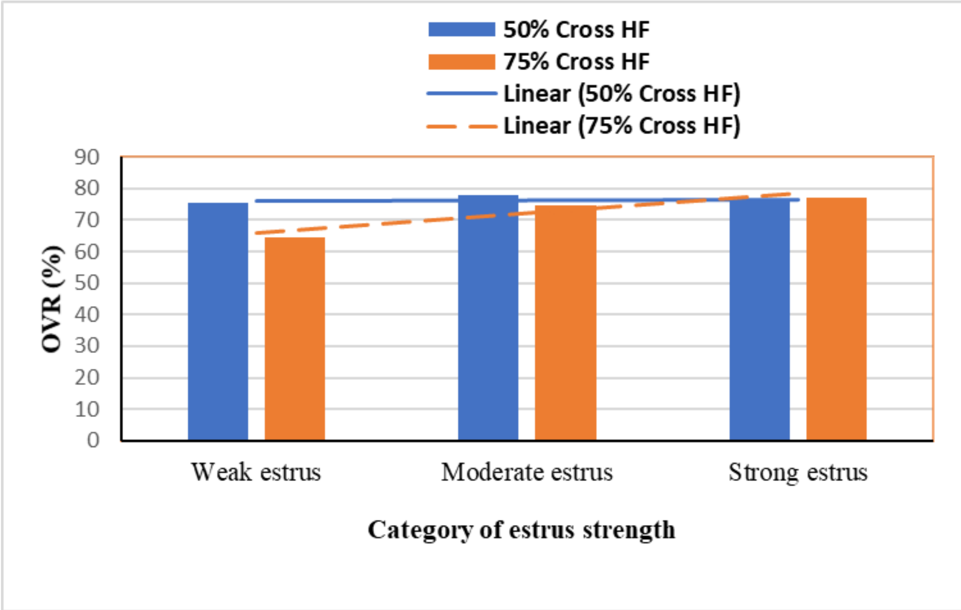


Figure 11. Effect of differences in blood level and estrus strength on OVR

On the other hand, superovulatory response was negatively influenced by over conditioning and older age and the subsequent embryo production (Figure 12, a). Incidence of UFO was higher in cows with poor body condition and older cow. As age increases by 2 years, superovulatory response decreases by 1.5 times and increases UFO by 0.6 times (Figure 12, b).

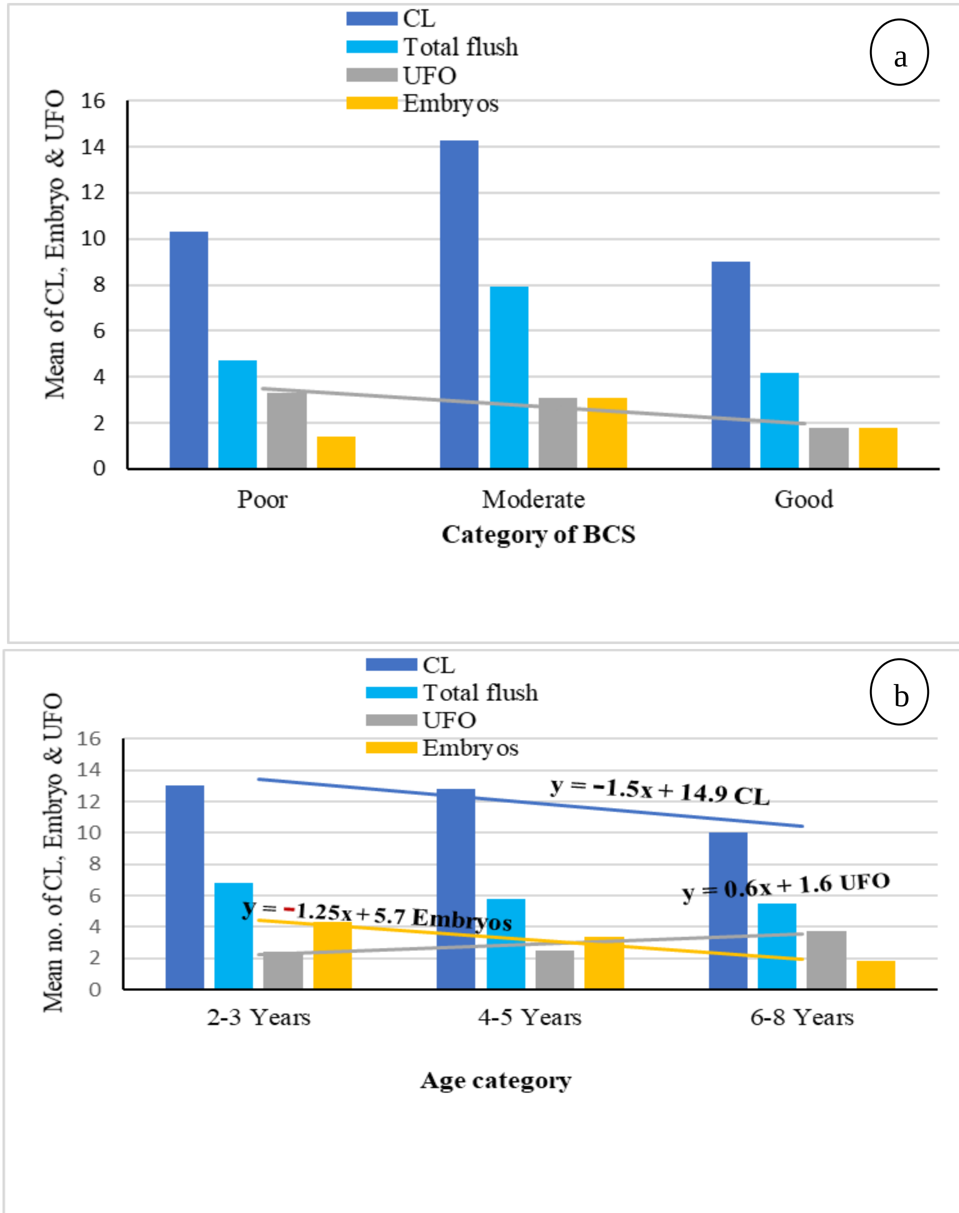


Figure 12. Effect of body condition score (a) and age (b) on performance superovulatory response and embryo recovery

4.3. Embryo Production and Quality Assessment

Overall, 88.9% (n=16) Boran and 83.3% (n=15) of HF*Boran donors were responded to the superovulatory treatment. A total of 210 (3-18/ cow) and 191 (2-15/ cow) CL were observed in Boran and HF*Boran donors. The overall recovery rate was 56.7% and 67.4% in Boran and HF*Boran, respectively. 57.6% UFO collected over the total flush output (n= 117) from Boran donor dams while 17% (n=125) was from HF*Boran. Descriptive statistics of the superovulatory responses and embryo recovery outputs were summarized in both breeds (n=36) Table 11.

Table 11. Mean (± SE) of the superovulatory response and outputs of embryo flush in Boran and HF*Boran donor cows

Parameters	Boran (n=18)	HF*Bora (n=18)
CL count	11.6±1.0 (Range= 3 - 18)	10.6±0.9 (Range= 2 - 15)
Post unovulatory follicles	1.8 ± 0.4	1.4 ± 0.3
Total flush outputs (UFO + Embryo)	6.5 ± 0.8 (Range=1- 10)	6.9 ± 0.7 (Range=2- 10)
Unfertilized ovum (UFO)	3.6 ± 0.6	1.3 ± 0.3
Total embryos	2.8 ± 0.6	5.6 ± 0.6
Transferable embryos	2.3 ± 0.6	4.7 ± 0.6

Comparison of the Dam and/ or Sire effects

Comparison of donor and/ or sire effects on quality of recovered embryos shows significant effects of donors and to lesser extent sires and statistically no significant interaction effects. Individual effects were evaluated for the validation of their significance and have shown that dam significantly affect the quality of all recovery types than sire as described Table 12.

Table 12. Two-way ANOVA of dam and sire effect on embryo quality

Embryo quality	Dam*			Sire			Dam^sire interaction		
	MS	F	p value	MS	F	p value	MS	F	p value
UFO	2.98	11.15	0.002	0.64	2.42	0.129	1.00	3.75	0.061
Fertilized	0.55	13.20	0.001	0.25	6.12	0.018	0.02	0.65	0.413
Transferable	0.82	9.98	0.003	0.37	4.48	0.042	0.08	1.06	0.310
Freezable	0.97	4.42	0.043	0.81	3.70	0.063	0.17	0.81	0.375
Degenerated	0.44	19.86	0.000	0.00	0.24	0.625	0.01	0.69	0.425

*^ refers interactions; df=1; MS= Mean square; * significant association of dam to all outcomes than sire*

Status of embryo qualities

All embryos were sorted into grades while freezable (Grades 1&2, and Stages 4-8) and poor-quality embryos (Grades 3&4) were computed for the dam-sire combination effects. Unlike the lower fertilized embryo counts in Boran cows, there was higher count of G1 and G2 embryos from the overall recovered embryos regardless of the sire effect. Similarly, HF*Boran cows produce higher count G2 embryos than other quality grades with in the breed contest Figure 13

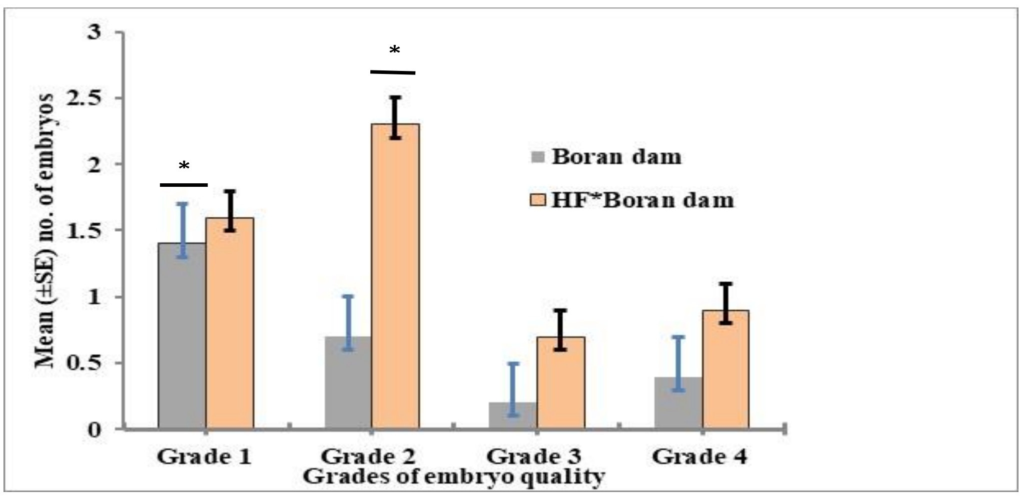


Figure 13. Overall quality of recovered embryos in both bred

**-Values are significant at 0.05, Grade 2 embryos have comparatively higher success rate due to counting of all embryos with reservation for quality Grade1 in HF*Boran*

When comparing each grade of embryo quality, HF*Boran dams inseminated with HF sire yield higher number of excellent and good embryos (Grade 1 & 2) and also had yield embryos of poor-quality grades (Grade 3 & 4) than Boran cows inseminated with Boran sire (Figure 14).

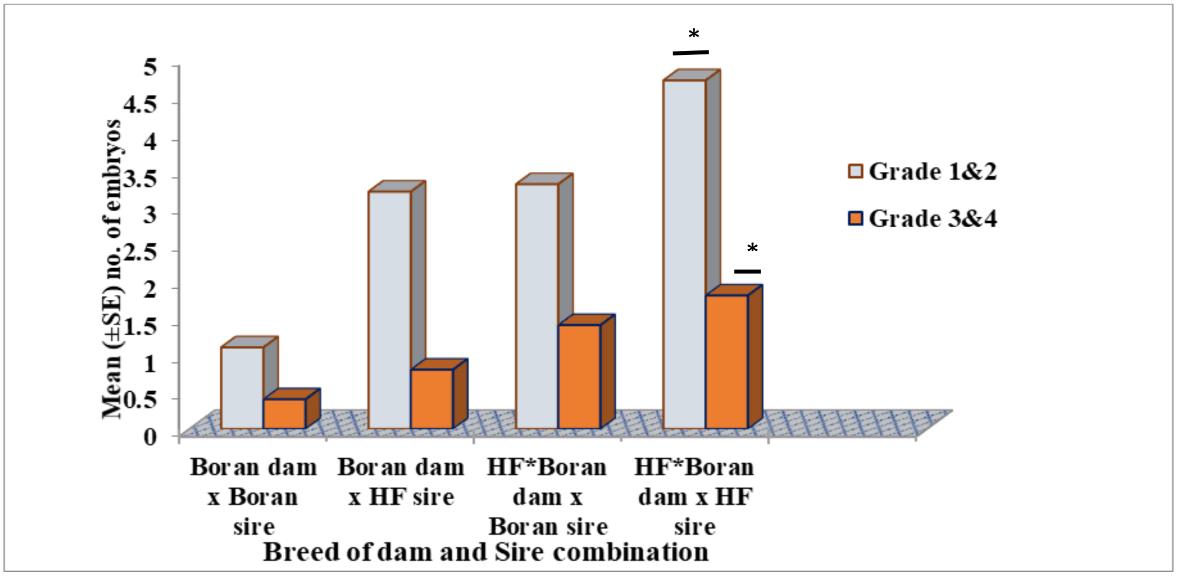


Figure 14. Classification of embryo qualities based on their dam-sire breed

""- SE bars significantly different at p<0.05*

Further analysis of different qualities of embryos recovered during alternate use of dam and sire breed showed that higher number of fertilized and transferable embryos when HF*Boran dams inseminated with either of the sires. Whereas larger number of degenerated and poor-quality embryos were found when HF*Boran donor dams inseminated with HF sires, while higher number of UFO were recovered when Boran dams inseminated with Boran sire (Table 13).

Table 13. Average number of different categories of embryo qualities resulting from the four dam-sire combinations of Boran and HF*Boran cross breeds

Embryo category	Mean (±SE) number of embryos for dam sire combinations (**x**)			
	Boran dam **x** Boran sire	Boran dam **x** HF sire	HF*Boran dam **x** Boran sire	HF*Boran dam **x** HF sire
Fertilized	1.8 ± 0.8	3.8 ± 0.8	4.8 ± 0.8[a]	6.4 ± 0.8[b]
Transferable	1.2 ± 0.8	3.2 ± 0.8	4.1 ± 0.8	5.3 ± 0.8[a]
Freezable	1.2 ± 0.8	3.1 ± 0.8	3.3 ± 0.8	4.7 ± 0.8[a]
Degenerated	0.6 ± 0.8	0.7 ± 0.8	1.9 ± 0.8[a]	1.8 ± 0.8[a]
Poor quality	0.4 ± 0.3	0.8 ± 0.3	1.4 ± 0.3	1.8 ± 0.3[a]
UFO	3.9 ± 0.7[a]	3.3 ± 0.7	2.1 ± 0.7	0.6 ± 0.7

Superscripts significantly different at p<0.05 and 0.001, for the values "a" and "b", respectively

Embryo Transfer

Comparing embryo transfer performance between frozen and fresh embryos, the overall conception rate in frozen embryos was lower (38.3%) than fresh transfer (55.7%). Fresh and frozen thawed G1 and G2 quality embryos were transferred to the recipients and performance is presented in Table 14.

Table 14. Comparison of conception rate in embryo transfer

Embryo	Breed of embryos	Recipient	No. of transfer	No. of pregnant	Conception rate
Frozen embryo	HF*Boran	HF	6	1	16.6
	Boran	HF	5	3	60.0
Fresh embryo	HF*Boran	Boran	5	2	40.0
	Boran	Boran	7	5	71.4.0
Total conception			23	11	47.8

CHAPTER 5

5.DISCUSSION Pages 59-64

Determination of the Superovulatory Hormone Dose

In this experiment, a clear breed difference was observed regarding the superovulatory response. Generally crossbred cows were found sensitive to lower doses of Pluset® (650 IU) than the HF, a finding that is also supported by a previous study carried out by Degefa et al. (2018) in Boran breeds and by Afriani et al. (2018) in *Pesisir* cattle. A hyper stimulation (hypertrophy of the ovaries) was observed in HF*Boran cross breed when a higher dose of Pluset® was administered. This is known to be due to the lower FSH threshold of the breeds for ovarian stimulation during folliculogenesis (Youngs, 2007). Hyperstimulation cases the ovarian surface to be covered by the few hyperthoriphied follicles and suppress the suboirdinate follicles there by leading to atrophy. Furthermore, administration of high doses of Pluset® doesn't mean a proportional increase in response due to limited numbers of FSH receptors in the ovary (Nilchuen *et al.*, 2011). On the contrary, the HF in this study showed an increasing response at higher doses of Pluset®. The present finding confirms the presence of breed related optimal threshold for FSH treatment during superovulation while factory recommended dose of 1000 IU still works for pure HF breeds to attain maximum superovulatory responses (Youngs, 2007; Degefa *et al.,* 2016).

As a response to the superovulation, the incidence of POF found in this study was within the range of previous studies (Ireland *et al.,* 2008; Berisha *et al.,* 2019) indicating a variations of 8 to 20 POF per ovary. The CL count at the time of embryo collection was also similar with previous reports such as 14 CL at 650 IU Pluset dose (Baruselli *et al*., 2006) and 18 CL in OPU case (Surjus *et al*., 2013). Though blood level did not show significant effect for CL count in this study, response was excellent for the crossbred cows at the optimal dose (Degefa *et al.,* 2018). Since Jahnke *et al.* (2015). Similarly, Cremonesi *et al.* (2020) reported that the differences in blood level of the breed also hampers ovarian response.

Estrus Characteristics and Superovulation

Poor estrus manifestation is a common finding in superovulated cows (Rottgen et al., 2018), however, in this study the behavioural estrus in dairy cows subjected to the superovulation were well appreciated in about 85% of treated cows. Similar to Orihuela (2000) and Mehmood et al. (2012), the frequent signalling behavioural manifestations were mounting others, standing to be mounted and trailing, the three top estrus behaviours exhibited during observation under this study. Different from the finding of Rottgen et al. (2018) who reported vocalization as estrus climax, bellowing in the current study was the least recorded behaviour; and actually, bellowing was not common in grouped animals where intimacy probably eliminates the need for such communication. Behavioural estrus was intense in the evening, moderate in the morning hours, and less intense during the day which is contrary to the report by Negussie et al. (2002) where 97.8% cows were standing to be mounted. There were no significant variations in behavioural estrus based on blood level differences. But in other study by Burnett et al. (2018), behavioural estrus was more intense in crossbred than purebred. Estrus manifestation is an energy dependent process hence breeds like pure HF are known to be prone to the influence of nutritional conditions in showing overt estrus signs particularly during times of negative energy balance.

The onset and duration of estrus were apparently related to the blood level in this study. This is in agreement with Sankar and Archunan (2012) and Madureira et al. (2015) who also reported shortened duration of behavioural manifestation in exotic breeds that cause estradiol to be abolished sooner due to higher metabolism. The higher *Taurus* blood seems to be related to the shorter duration of manifestation in the 75% HF*Boran groups in this study. On the other hand, reports by Baruselli et al. (2006) and Jiménez et al. (2011) showed a shorter estrus duration in the pure *Bos indicus*. Smaller follicles were implicated by the authors for releasing lower estrogen thereby influencing the duration of estrus in *Bos indicus* breeds. Since energy level has a direct effect on estrus manifestation, the condition has probably been further confounded with genetic make-up in our finding.

On the other hand, Jiménez et al. (2011) and Degefa et al. (2016) reported that superovulated cows showed 46% more estrual activities than normally synchronized cows similar to the present finding. Cows with strong and moderate estrus intensity had those comparably higher OVR than cows with weak estrus. Cow's with strong estrus often also produce relatively larger preovulatory follicles that are likely to result in ovulation indicating both estrus intensity and size of the preovulatory follicle to play a role in the rate of ovulation (Sartori *et a*l. 2010). In the present experiment, 75% HF*Boran showed anincreasing trend of OVR as estrus intensity increases from weak to good compared to the 50% HF*Boran crosses. Large number of follicles in the *Taurine cows* that were less competent before ovulation due to the genetic differences will eventually become atretic (Gonçalves *et al.,* 2012).

Cows with higher CL number exhibited a strong estrus intensity which is a result comparable to Baruselli et al. (2006). Surjus et al. (2013) also reported that strong estrus is due to increased concentration of estrogen associated with the larger number of preovulatory follicles and this is used as predictor for better superovulatory response before detecting CL at the time of embryo flushing. A higher count of CL in the 50% HF*Boran cows in this experiment is believed to have originated from the Boran genetics.

There was no significant differences for total embryo recovery in crossbred cows similar to the report by Degefa et al. (2016). However, the 75% HF*Boran had a significantly greater count of fertilized embryos than the 50% HF*Boran cows. On the other hand, the recovery of higher number of UFO and fertilized embryos increased as estrus intensity increased. Jinks et al. (2013) stated that cows with weak estrus have greater concentration of progesterone than those with strong estrus and this is believed to have a negative impact on the uterine and oviductal contractility impeding sperm and oocyte transport thereby hindering gamete fusion. Whereas cows with strong estrus have higher blood estrogen concentration that is associated with strong oviductal contractility thereby increasing the chance for fertilization and harvest of more fertilized embryos (Hunter, 2005). Other reports however, indicate that high concentration of estrogen can hinder sperm fertilization capacity (Pereira *et al.,* 2016). Therefore, the harvest of fertilized embryo relies on the balance between optimal superovulation and concentration estrogen at the time of insemination.

Cows with moderate BCS and those at younger age had a higher CL count than older cows with and those with poor BCS. Younger cows with relatively better body conditions had a profoundly better recovery of fertilized embryos which is similar to the findings by Madureira et al. (2015) and Dorice et al. (2019). It is a well-established fact that reproductive activity is a function of body condition while age influence oocyte quality and ovarian reserve.

Influence of Breed in Embryo Production and Quality

The superovulatory response was not significantly different between study breeds when sires and dams breeds were alternatively used which agrees with the previous report (Batista *et al*. 2016, Degefa *et al*. 2016 and 2018). However, other authors (Purohit *et al*., 2013; Guerreiro *et al*., 2014) showed *Bos indicus* to have an inherently low ovarian follicular population and respond less than the *Taurine* breeds. As a general consensus, *Bos indicus* are said to be more sensitive to exogenous gonadotropins and respond better to superovulation (Ferreira *et al*., 2014). This variation might indicate the presence of a more important individual variation rather than breed influence when it comes to superovulatory treatment in bovine (Curtis 2015). The average CL detection was comparable to previous studies for the HF*Boran (cross breeds) (Tadesse *et al*., 2016; Ferreira *et al*., 2014; Vieira *et al*., 2014) though fewer numbers have also been reported (Hussein *et al.*, 2014). Several other studies (Peixoto *et al.*, 2007; Vieira *et al*., 2014; Mapletoft *et al*., 2015; Perez *et al*., 2016; Perez *et al*., 2019; Vizoná *et al*., 2020) confirmed that the variations in response to superovulation and recovery rate are not only due to breed differences but also the status of donors at the time et of superovulation. In line with a study by Degefa et al. (2018) on similar animals, the Boran breed showed a comparable ovarian response as the HF*Boran crosses, however, recovery of UFO was much higher in Boran cows compared to the HF*Boran crosses. A likely cause implicated was fertilization failures that has also have been reported by several other authors (Sartori *et al*., 2010; Rasolomboahanginjatovo *et al*., 2014; Dorice *et al*., 2019) where a higher proportion of non-fertilized oocytes and degenerated embryos have been frequently recovered. Fertilization failure in superovulated cattle is generally more pronounced, averaging approximately 45%, much lower than the present finding. It is mainly associated with poor gamete transport due to hormonal imbalances or suboptimal oocyte quality which is not uncommon in zebu breeds (Dorice *et al*., 2019). Hasler (2012) in his study in Red Angus beef breeds reported a closely similar proportion of UFO and a higher proportion of degenerated embryos in superovulated cows.

The mean number of transferable embryos found in the present study was much higher compared to a previous report by Tadesse et al. (2016) from a similar HF*Boran crossbreeds. However, it was smaller than those reported for Nelore breeds of donor cows where local variability was confirmed due to farm management and donor age as the main factors (Silva *et al.*, 2009). A closer analysis of the effect of the dam-sire combination in this study confirms the insemination of Boran dam with Boran sire to be the source of the largest proportion of UFOs recovered. This finding is in agreement with Degefa et al. (2018) where a higher yield of UFO was recorded in Boran cows inseminated with a semen from Boran sire. Probable causes for the high proportion of UFO in Boran cows might be related to the breed itself or the condition of the dam including nutritional status at the time of insemination (Ferreira *et al*., 2014). Sire effects (Hasler, 2012) were also related to fertilization ability, semen quality and dose, immunogenetics, and other related factors which might contribute to the variation. However, the use of high-grade imported Boran semen in our study has not significantly altered the incidence of UFO from Degefa et al. (2018) who used both AI and natural service of indigenous Boran bulls. It is imperative to assume then the likely source of higher incidence of UFO is probably the oocyte quality than the semen.

On the other hand, a significantly larger proportion of degenerated embryos were harvested from HF*Boran donor cows in the current study. Hasler (2012) and Trigal et al. (2012) reported that male effect is more evident on variation of MOET outcomes in beef breeds and from using sex sorted semen. Contrary to this, the crossing of HF*Boran dam with semen from pure HF bulls produced a significantly higher proportion of transferable embryos relative to the Boran dams in all possible combinations. This probably shows the role of the donor dam breed which is also confirmed previously by Degefa et al. (2018) though the overall result was comparatively smaller in our study. More yield of freezable embryos was collected than is reported by Vieira et al. (2014) while the average number of fertilized embryos from HF*Boran cows is comparable. The HF*Boran donor cows produced a significantly higher number of Grade 1-3 quality embryos compared with the Boran breed in this study. Different literature (Alarcón *et al*., 2010; Trigal *et al*., 2012; Vieira *et al*., 2014; Tadesse *et al*. 2016; Chinchilla-Vargas *et al*., 2018) suggest the sources of these variations to be related to breed, reproductive behaviour, environmental factors or interaction of all.

Embryo Transfer

The overall embryo transfer performance was greater compared with the research done by Tadesse et al. (2016) which is around 20%. However, pregnancy rate in this study was lower in frozen embryos than when freshly transferred which was lower than another study reported by Montiell et al. (2006). Similar to this finding, Ongubo et al. (2015) reported that freshly transferred embryos had a greater conception rate than frozen. Conception rate from freshly transferred embryos was greater by 17.4% compared with those from frozen embryos Montiell et al. (2006) also reported that pregnancy rates from frozen-thawed embryos is 10 to 15% lower than rates for fresh embryos transfers. Freezing of embryo is known to cause subtle damage to embryonic structures thereby lowering their survival at post-thaw and transfer. Especially in this study, prefreezing qualities of the embryos recovered from HF*Boran donors were poor (partially degenerated) which is believed to have originated from breed influence subsequently contributing to the overall lower conception rate.

CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

This study has demonstrated the role of breed or exotic blood level in superovulatory response, estrus manifestation and *in vivo* embryo production. Although doses recommended for HF breeds may work, optimized lower doses have been found to give better supeovulatory response as the indigenous blood level increase or conversely, increasing the dose of FSH as the exotic blood level increase is known to result in a better response hence increasing the likelihood of production of more good quality embryos.

- The highest superovulatory response recorded was at FSH dose of 650 IU in the crossbred cows that also subsequently produced higher number of transferrable embryos. However, increasing the dosage of the FSH did not bring an increased superovulatory response but subsequently resulted in lower recovery of good quality embryos.
- Evaluation of estrus behavior was very informative and each of the behavior was indicative to follow the animal status, and assists inseminators to make decision for insemination. It was also noted that estrus intensity at the time of supeovulation has an important role in the quality of embryos produced in superovulated animals. The variability in estrus behavior was found to be a critical risk factor for the superovulatory outcome.
- Further, estrus intensity has also been known to be influenced by exotic blood level further suggesting the need for due consideration of this during *in vivo* embryo production.
- This study has also demonstrated neither the breed of the sire nor the dam influenced the overall outcome of *in vivo* embryo production, however, the quality of embryos was highly dependent on breed.
- A higher incidence of UFO was evident in Boran cows irrespective of the sire breeds while higher number of degenerated embryos were produced by HF*Boran cows and

comparatively, more transferable embryos were recovered when semen from HF sire was used in both breeds of the dams. Further, higher UFO counts were evident in Boran and 50% HF*Boran cows than the other group of 75% HF*Boran and HF donor cows showing the role of exotic blood level in embryo quality.

- It is therefore, plausible to assume that in all these findings, a number of other unstudied confounding factors such as quality of oocytes, quality of sperm, reproductive stage at the time of embryo production, nutritional status of donor animals may also play a role either individually or in combination in the overall success of *in vivo* bovine embryo production.
- In any case, it is obvious that breed is an important outstanding factor to carefully consider during selection of donor cows.

Based on the above facts and findings the following recommendations are given as the way forward:

- MOET is an expensive procedure mainly because of the cost of FSH used in superovlation. Based on this study, donor animals have shown response to lower dose of FSH (650 IU) suggesting the need for further dose optimization experiment in other crossbred donor cows
- It has been most challenging to find out the magnitude of the contribution of individual factors in the overall outcome of *in vivo* embryo production. Therefore, studies are mandatory to find out the role of nutrition or nutritional status, reproductive stage, oocyte quality, and quality of sperm for the donor breed selected during *in vivo* embryo production
- The Boran breeds currently available in Ethiopia are not improved breeds. Although the semen utilized was drawn either from pure breeds or proven sires, comparative studies for qualities of oocytes including competency for improved and unimproved breeds is important
- MOET as biotechnology tool, is capital intensive and hence, require government attention for grant and delivery of research facilities for research institutions. If the stereotyping in this new but very useful technology is overcome, studies should be expounded to other indigenous breeds.

CHAPTER 7

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8. ANNEXES

a. Pictorial demonstrations of experimental procedures

ET crew moving to private farm CIDR application in Nordeli private Farm

Embryo flushing session

Demonstration of Pluset®/ FSH and and Estrumate hormones

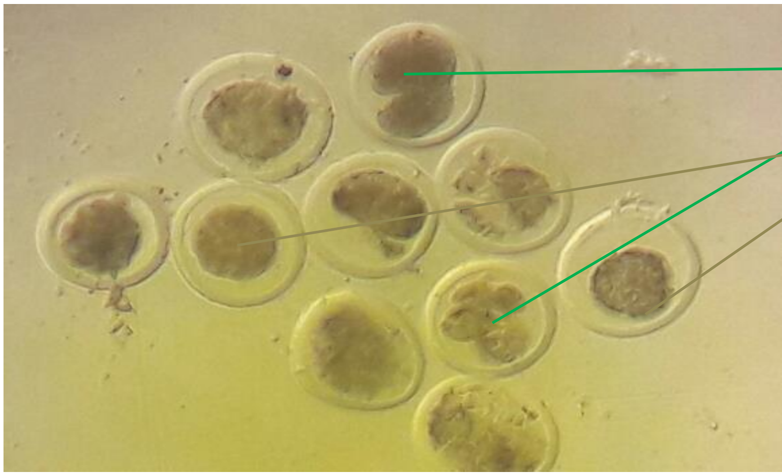
Embryo searching using stereomicroscope

Freezable embryos selected as G1 and G2

Donor*Sire semen	Mean (±SE)	
	Grade1	Grade2
Boran#Boran Semen	0.4 ± 0.5	0.8 ± 0.7
Boran#HF Semen	2.4 ± 0.5	0.7 ± 0.7
Holstein*Boran#Boran Semen	1.3 ± 0.5	2.0 ± 0.7
Holstein*Boran#HF Semen	1.9 ± 0.5	2.8 ± 0.7

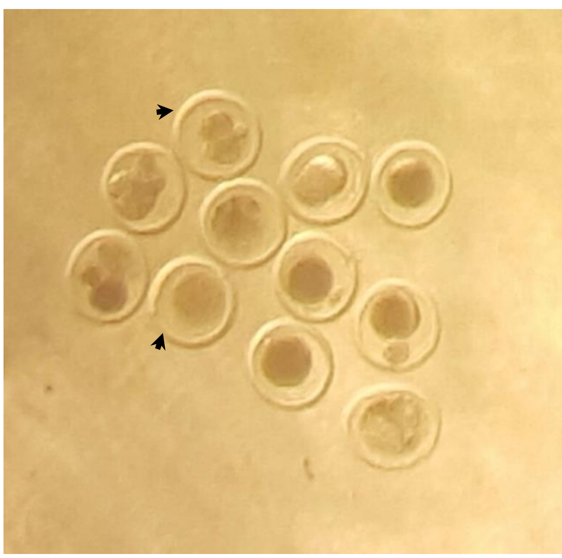
*- *insemination with*

b. Some findings of laboratory assessment for embryos quality

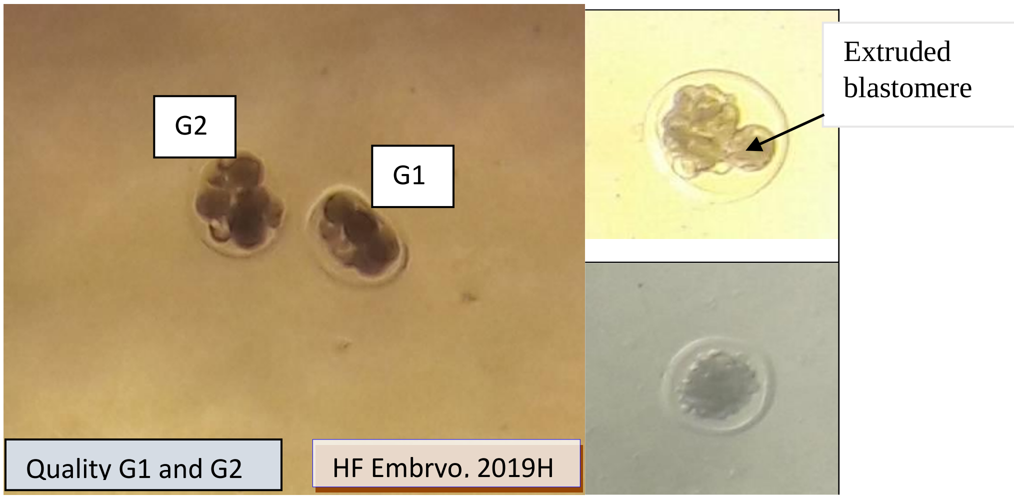


Degenerated embryos and

Unfertilized ovum from HF*Boran cross cow



Unfertilized group of embryos from Boran at single flush (Arrow = UFO)



c. Evaluation Fact Sheet for Farms

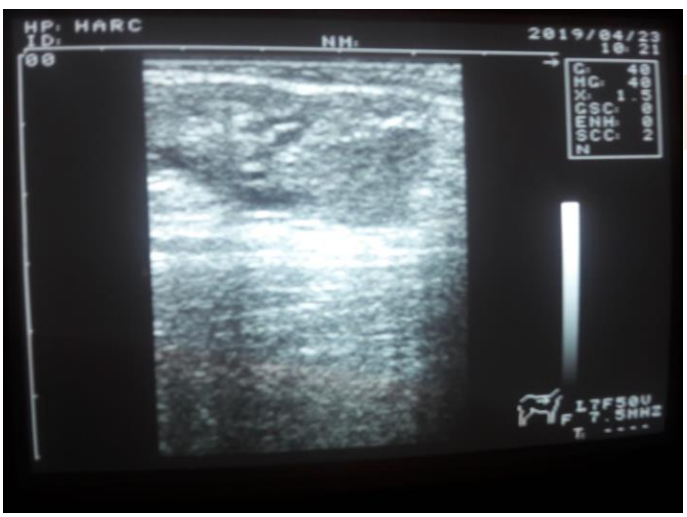
no.	Parameters*	yes	No	Remark
1	Good barn condition with sufficient space			
2	Good restraining crutch			
3	Sufficient animal walking space			
4	Attendants (feeding, milking and cleaning)			
5	Veterinarian			
6	Sufficient daily water provision			
7	Sufficient concentrate provision			
8	Green fodder			
9	Source of income			
10	Sustainable Budget allocation			
11	Veterinary drugs and equipment			
12	Sufficient number of stocks			
13	Choice of breed			
14	History of postpartum disorder and diseases			
15	Recording			

**Points were recorded and subjectively used for determination of level*

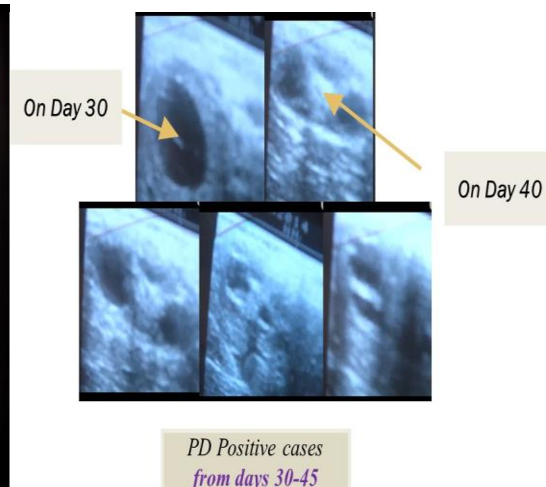
d. Manifesting estrus behaviors

Mounting others

Mucus discharging



Ultrasound demonstration of CL



Early PD test Results

Standing to be mounted

The first heifer calf born… 2019

e. Pictorial presentation of stages of embryonic development of bovine embryos

The developmental stage and quality of bovine embryos

Source: Bó and Mapletoft (2013) and Jahnke et al. (2015)