FREQUENCY OF INSEMINATION AND TYPE OF SEMEN EFFECT ON QUANTITY AND QUALITY OF *IN VIVO* PRODUCED EMBRYOS FROM BORAN HEIFERS

AYDA MOHAMMED YUSUF

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A THESIS SUBMITTED TO THE DEPARTMENT OF APPLIED BIOLOGY

SCHOOL OF APPLIED NATURAL SCIENCE

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER'S IN APPLIED BIOLOGY (SPECIALIZATION IN BIOTECHNOLOGY)

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ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

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THESIS APPROVAL

I/we, the advisors of the thesis entitled "**Frequency of insemination and type of semen effect on quantity and quality of *in vivo* produced embryos from Boran heifers**" and developed by **Ayda Mohammed**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Ayda Mohammed** have read and evaluated the thesis entitled "**Frequency of insemination and type of semen effect on quantity and quality of *in vivo* produced embryos from Boran heifers**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in **Applied Biology (Specialization in Biotechnology)**.

Chairperson	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

FINAL APPROVAL

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

DECLARATION

I hereby declare that this Master Thesis entitled "**Frequency of insemination and type of semen effect on quantity and quality of *in vivo* produced embryos from Boran heifers**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

_____	_____	_____
Name of the student	Signature	Date

RECOMMENDATION OF ADVISORS

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "**Frequency of insemination and type of semen effect on quantity and quality of *in vivo* produced embryos from Boran heifers**" prepared under my/our guidance by **Ayda Mohammed** in partial fulfillment of the requirements for the degree of Mater's of Science in **Applied Biology (Specialization in Biotechnology)**. Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

_____	_____	_____
Major Advisor	Signature	Date
_____	_____	_____
Co advisor	Signature	Date

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TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMH	Anti Mullerian Hormone
AFC	Antral Follicle Count
AI	Artificial Insemination
BCS	Body Condition Score
BSA	Bovine Serum Albumin
BO	Bracket and Oliphant medium
CIDR	Controlled Internal Drug Release
CL	Corpus Luteum
COCs	Cumulus Oocyte Complexes
DZARC	Debre Zeit Agricultural Research Center
ET	Embryo Transfer
ETT	Embryo Transfer Technology
eCG	Equine Choronic Gonadotropin
ES	Estrus Synchronization
FSH	Follicle Stimulating Hormone
FMD	Foot and Mouth Disease
GV	Germinal Vesicle
GnRH	Gonadotropin Releasing Hormone
GH	Growth Hormone
HF	Holstein Friesian
IVC	*In vitro* Culture
IVEP	*In vitro* Embryo Production
IVF	*In vitro* fertilization
IVM	*In vitro* Maturation
IVP	In vitro Produced
IGF	Insulin Growth Factor
IETS	International Embryo Transfer Society
ILCA	International Livestock Centre for Africa
ILRI	International Livestock Research Institute

IM	Intramuscular
LH	Luteinizing Hormone
LSD	Lamb Skin Disease
MII	Meiosis two
MOET	Multiple Ovulation and Embryo Transfer
OPU	Ovum Pick Up
TVFA	Trans-Vaginal Follicular Aspiration
PGF2α	Prostaglandin F2 alpha
ZP	Zona Pellucida

ABSTRACT

In vivo embryo production is a production of progenies from genetically superior cattles using multiple ovulation and embryo transfer technology (MOET). Embryo quality affects MOET and factors that determines embryo quality are Artificial Insemination (AI) frequencies and semen type. This study was conducted from March to November 2023 to evaluate effects of AI frequency and semen type on quantity and quality of in vivo produced embryos in Boran heifers. For this purpose Randomized Experimental Design was used in which 12 donor Boran heifers were super ovulated and embryos were collected. Donors were randomly allocated to two experimental groups: Group-one-6 donors were inseminated two times (3 donors each with conventional and sexed semen) at 12 and 18 hrs interval whereas in group-two-6 donors were inseminated three times (3 donors each with conventional and sexed semen) at 12, 17 and 22 hrs interval. The results of this study showed that mean number of 7.2 ± 1.9 and 9.0 ± 2.5 for corpus luteum (CL) and 2.2 ± 0.7 and 5.7 ± 1.5 for un ovulated follicle (UOF) were counted from donors grouped under two and three times AI frequency. Mean number of CL and UOF counted from donors grouped under conventional and sexed semen treatment were 9.0 ± 2.8 and 7.2 ± 1.45; 4.8 ± 1.8 and 3.0 ± 0.8, respectively. The number of CL and UOF counted from donors under two and three times AI and conventional and sexed semen treatment were not significantly ($p > 0.05$) different. Mean number of total flush output counted from donors grouped under two and three times AI frequency were 3.5 ± 0.43 and 7.3 ± 2.2, respectively. For conventional and sexed semen treatment, mean number of total flush output counted from donors were 7.00 ± 1.93 and 3.8 ± 1.35, respectively. Mean number of transferable and non-transferable embryos counted from donors grouped under two and three times AI frequency were 0.5 ± 0.34 and 2.3 ± 1.0; 0.7 ± 0.3 and 2.3 ± 1.0, respectively. Mean number of transferable and non-transferable embryos counted from donors under conventional and sexed semen treatment were 1.7 ± 1.0 and 1.7 ± 0.65; 1.8 ± 0.98 and 1.2 ± 0.6, respectively. Mean number of unfertilized oocytes (UFO) counted from donors under two and three times AI were 2.3 ± 1.4; 2.7 ± 1.3 and Mean number of UFO counted from donors under conventional and sexed semen treatment were 3.5 ± 1.15; 1.5 ± 0.6, respectively. No significant difference was observed between insemination frequencies and semen types in terms of total flush output, number of transferable and non-transferable embryos as well as UFOs recovered from Boran heifer donors ($P>0.05$).This findings demonstrated that inseminating Boran heifers two and three times both with sexed and conventional semen did not affect embryo quality and quantity produced in vivo from donors and it was recommended that it is enough to inseminate Boran heifers two times with conventional or sexed semen for in vivo embryo production.

Key words: *Boran, donor heifers, frequency, in vivo embryos, semen type and super ovulation.*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study

Ethiopia has the biggest population of livestock in Africa with 65 million cattle, 40 million sheep, 51 million goats, 8 million camels, and 49 million poultry (CSA, 2020). Despite having the highest cattle population, the productivity and reproductive performance of cattle are poor for a variety of reasons, one of which being the limited genetic potential of the native cattle (Duguma and Janssens, 2021). Enhancing livestock genetic potential has been a top priority in Ethiopia in order to increase milk and meat production, which is currently constrained by poor reproductive performance' in cattle raised in natural conditions. By demonstrating the production potentials of cattle through the use of animal reproductive biotechnology tools like artificial insemination (AI), embryo transfer (ET), estrus synchronization (ES), germplasm cryopreservation etc., the current deficit of animal products in the country might be resolved (Daba *et al.,* 2022).

Artificial insemination is the first generation of reproductive biotechnology that has been established, based on research conducted prior to World War II. Four generations of technologies have come after this one: ET, *in vitro* fertilisation (IVF), sexing of embryos and subsequently nuclear transfer and transgenesis (Thibier, 2005). AI is the most useful and widely utilized biotechnology that has been employed in Ethiopia for more than 40 years (Juneyid *et al.,* 2017; Kassa and Wuletaw, 2018). ES is employed in big farms and ranches with hormonal techniques to manipulate bovine reproduction that address some of AI's shortcomings, such as the strict heat detection requirement and increases the reproductive effectiveness of cows and heifers (Daba *et al.,* 2022). For the past 60 years, AI using fresh or frozen semen has proven to be the most effective and successful reproductive method in animal production (Lohuis, 1995). Mammalian sperm were among the first cells to be effectively cryopreserved, and throughout the past seven decades, the livestock industry has grown to rely heavily on the use of cryopreserved semen for artificial insemination (Curry, 2007). The most significant

breakthrough occurred in 1949 when Polge, Smith, and Parkes (Polge and Smith, 1949) discovered that spermatozoa could be cryoprotected by glycerol. The early glycerol trials involved the use of fowl sperm, but after significant work, bull sperm was successfully preserved (Smith and Polge, 1950). In 1951, the first calf to be conceived by artificial insemination (AI) using frozen-thawed sperm was reported (Stewart, 1951).

Multiple Ovulation and Embryo transfer (MOET) Known as the second generation of assisted reproductive technologies (ART), which was first introduced around forty years ago, is a more sophisticated kind of reproductive biotechnology that utilizes AI techniques. The advancements made during the previous 25 years have made commercial bovine embryo transfer a significant global industry (Mapleft and Hasler, 2005; Lonergan, 2007). In Ethiopia, Multiple Ovulation and Embryo Transfer (MOET) research operations at the International Livestock Center for Africa (ILCA) facility in Bishoftu has been started in late 1990s by International Livestock Research Institute (ILRI) focusing largely on zebu cattle and the first calf was born in 1991 at the Debre Zeit Research Station and this had led to the first experimental establishment of estrus synchronization in Ethiopia and in recent years, several calves have been born using this approach at Debere Zeit Agricultural Research Center (DZARC) animal biotechnology research laboratory (Daba *et al.,* 2022).

In vitro fertilisation (IVF), oocyte recovery, and gamete and embryo sexing are parts of the third generation of ART (Bertolini and Bertolini, 2009). Other than MOET technology, it is possible to exploit the countries huge cattle population and diverse genetic resources by Ovum Pick Up (OPU) and *In vitro* Embryo production (IVEP) (Jemal *et al.,* 2022). Cattle ovarian follicle development happens in a series of waves, starting with the concurrent recruitment of a set of follicles with a diameter of 1.0–3.0 mm. Next comes the selection of a single follicle and the regression of the subordinate follicles (Ginther *et al.,* 1989). Technologies like ovum pick up (OPU) and ultrasound guided trans-vaginal follicular aspiration (TVFA) have provided a significant chance to fully utilize the extra follicular reserve that would otherwise be lost (Jemal *et al.,* 2022). OPU is the most adaptable and dependable method for producing embryos from almost any live donor, including animals with valuable genetics. By significantly reducing the generation gap, using this method on young calves can accelerate genetic gain even further (Galli *et al.,* 2001). Jemal *et al.* (2022) conducted research on native zebu breeds using

OPU/IVEP procedures and reported a total of 21.5% cleaved oocytes (embryos) at morula stage from Boran and Boran * Holstein Friesian (HF) Crossbreds. In this study it was concluded that OPU/IVF procedures could be used in local and crossbred heifers as an alternative breeders technology options in animal breeding programs to utilize the potential of genetics of both native and foreign breeds. Determination of sex of sperm and embryos was also developed. Naturally, the dairy business prefers heifer calves for milk production, whereas the beef industry favors male calves due to their greater body weights and feed efficiency. In order to regulate the sex of the livestock's progeny, techniques to ascertain the sex of sperm or embryos were created. Using a particular dye that attaches to DNA and a flow cytometer/cell sorter, sperm cells may be separated according to their DNA content since the X-bearing sperm in cattle has approximately 3.8% more DNA than the Y-bearing sperm. Through that method, several companies market sexed semen (Lonergan, 2007). The fourth generation of ART includes embryonic cloning and transgenesis (Bertolini and Bertolini, 2009). Using cells from early embryos, the first farm animal (sheep) was cloned in 1986 (Willadsen, 1986). Then, in July 1996, Dolly the sheep was born by the transfer of an adult's somatic cell nucleus (Wilmut *et al.,* 1997), which signaled the demise of a significant biological dogma: the idea that differentiated somatic cells could not be reprogrammed to a toti- or pluripotent state that would permit the formation of a new human. Later, somatic cell nuclear transfer (SCNT), or cloning by NT from adult somatic cells, was proven and replicated in a growing number of animal species. Even though it's currently not very effective, SCNT cloning has advanced research and sparked a lot of interest in a variety of related fields, along with in vitro fertilisation (Bertolini and Bertolini, 2009).

When an animal's genome contains genetic material from a different species, it is said to be transgenic. By using genetic engineering, it is possible to overcome species constraints and create genetically altered creatures with advantageous qualities that will be passed down Mendelian behavior to subsequent generations (Hansel and Godke, 1992). Pronuclear microinjection of a foreign gene produced transgenic mice, pigs, goats, sheep, and cattle. However, the effectiveness of these methods is still rather poor; typically, fewer than 5% of the cells carry the transgene (Nottle *et al.,* 2002).

MOET technologies can be accomplished with the careful selection of valuable donors in ideal gynecological environments, the use of the proper superovulation technique, the use of high-

quality semen, and recipients who are in good reproductive health. Each of these factors directly affects the success of the embryo transfer programme (Phillips and Jahnke, 2016; Bó *et al.,* 2019). In super ovulated cattle, ovulations are thought to start 24–30 hours after the onset of standing estrus. In order to ensure that there is enough sperm accessible at the location of fertilization, super ovulated cattle are frequently inseminated multiple times. Additionally, in super ovulated animals, deficiencies in sperm transport result in reduced numbers of sperm in the oviducts at the time of fertilization. This accounts for the fact that if cattle are inseminated too early, the sperm reservoirs may be depleted before all ovulations have occurred. On the contrary, if cattle treated for superovulation are inseminated too late, oocytes may be too old at the time of fertilization, resulting in degenerate embryos. (Schenk *et al.,* 2006).

A research conducted to compare sexed semen with conventional semen in terms of pregnancy rates in cattle showed that when utilized in well-managed dairy herds, sex-sorted semen has produced conception rates that are approaching those for conventional semen, being close to 90% of the level for conventional semen (Seidel, 2014). According to the results of some researches, due to the utilization of sexed semen, the yield of transferrable embryos is significantly reduced in cows, although this reduction is only moderate in heifers (Schenk *et al.,* 2006; Kaimio *et al.,* 2013). Some data show that sex sorting impacts later stages of embryonic development in addition to earlier stages since sorted semen-produced embryos had lower pregnancy rates after transferring on Day 7 *in vivo* embryos (Mikkola *et al.,* 2015). The effect of the frequency and time of insemination on *in vivo*-produced embryo quality and type of semen in super-ovulated cattle needs further investigation, and the problem is that in Ethiopia, studies on this area are limited. Therefore, this study was conducted to evaluate the effect of frequency of Artificial Insemination and type of semen on quantity and quality of *in vivo* produced Boran embryos.

1.2. Statement of the problem

In vivo embryo production is one of the most known reproductive biotechnology which improves the genetic potential of certain cattle breeds by selecting genetically superior donor female and male animals to produce a large number of calves whose genetics have been improved. However, there are a number of factors that affects the outcome of *in vivo* embryo

productions that needs to be studied (Jemal *et al.,* 2021). Problems related to semen have a significant impact on the *in vivo* embryo production rate in super ovulated cattle by affecting the quantity and quality of embryos. Type of semen (either conventional or sexed semen) and frequency of insemination (number of insemination) of super ovulated cattle after 24 hrs of the onset of standing estrus are among the factors which are related to semen that affects the quality and yield of *in vivo* produced embryos (Sartori *et al.,* 2004; Schenk *et al.,* 2006; Hayakawa *et al.,* 2009; Peioppo *et al.,* 2009; An *et al.,* 2010 and Kaimio *et al.,* 2013). Several researches have been conducted in different countries on *in vivo* embryo production which administered either a single insemination or multiple insemination (two, three and four times) (Sartori *et al.,* 2004; Schenk *et al.,* 2006 and Faizah, 2018) and compared conventional and sexed (sorted semen) in terms of embryo quality and quantity (Schenk *et al.,* 2006; Hayakawa *et al.,* 2009; An *et al.* 2010; Kaimio *et al.,* 2013 and Mikkola *et al.,* 2015).

In a research comparing sexed and unsexed semen for super ovulated heifers, the group that was only inseminated once with sexed semen had fewer available sperm than the group that was inseminated twice with the same amount of conventional or sexed semen. The same authors speculated that using sexed sperm in multiple inseminations would help to reduce retrograde losses, deliver more sperm to the site of fertilization, and possibly increase the rate of fertilization and recovery of transferable embryos to levels comparable to those achieved using conventionally frozen semen (Sartori *et al.,* 2004). It is recommended to inseminate super ovulated cattle multiple times in order to ensure that there is enough sperm accessible at the location of fertilization because there may be variation in the timing of ovulation meaning that some oocytes may be released too early and some are delayed in the cattle reproductive system (Schenk *et al.,* 2006). However, Larson *et al.* (2010) reported that increasing the frequency of inseminations (one straw at first detected estrus, two straws at 24 hrs and one straw after first detected estrus) failed to produce percentages of transferrable embryos equivalent to those of conventional controls. These factors (AI frequency and type of semen (conventional and sexed semen) needs to be further investigated. The problem is that in Ethiopia only one research was done by Babura *et al.*(2021) that compared conventional and sexed semen efficiency on quality and quantity of bovine embryos produced *in vivo* from Boran and Boran* Holstein Friesian cross breed cows and found no significant difference among the number of transferable embryos produced from donors inseminated with sexed semen and convectional semen but the number

of defective embryos tended to be higher with sexed semen and the number of unfertilized oocytes were significantly higher in crossbred cows. Nevertheless, no research had been conducted to study the effect of AI frequency on quality and quantity of bovine embryos produced *in vivo* from Boran heifers. So, this study was conducted to examine the effect of frequency of AI and types of semen on quantity and quality of *in vivo* produced Boran embryos.

1.3. Objectives

1.3.1. General objective

- To investigate the effect of frequency of artificial insemination and type semen on quantity and quality of *in vivo* produced embryo in Boran heifers

1.3.2. Specific objectives

- To compare the quality and quantity of *in vivo* produced embryo with two times and three times Artificial Insemination frequency.
- To assess the quality and quantity of *in vivo* produced embryo using sexed semen and conventional semen.

1.4. Research question/gap

This study was conducted to answer the questions:

- Does a difference in frequency of insemination has an effect on quality & quantity of *in vivo* produced Boran embryos?
- Does a difference in semen type (conventional and sexed semen) has an effect on quality & quantity of *in vivo* produced Boran embryos?

1.5. The hypotheses of the study

- Null hypothesis (Ho): Differences in Frequency of Artificial Insemination and type of semen has no effect on quality & quantity of *in vivo* produced Boran embryos.

- Alternative hypothesis (HA): Differences in Frequency of Artificial Insemination and type of semen has an effect on quality & quantity of *in vivo* produced Boran embryos.

1.6. Significance of the study

Inseminating super ovulated cattle's multiple times (increasing the frequency of insemination) with a suitable type of semen (conventional and sexed) that is capable of fertilizing oocytes is one way of ensuring that there are no oocytes left unfertilized in the ovaries because there is variation in the time when the oocytes are released from the cattle oviduct. So, if we inseminate cattle multiple times at different time intervals, we can be able to narrow the variation in ovulation time. So, in this sense it will be possible to improve the quality and quantity of embryos produced *in vivo* from super ovulated cattle's. For this reason, evaluation of the effect of Artificial insemination frequency and type of semen is necessary. Thus, the significance of this study is to recommend effective AI frequency either two times or three times insemination by investigating what effects does AI frequency had on quality and quantity of embryo and also identify the reason related to the defect of sexed semen on *in vivo* embryo quality and quantity compared to conventional semen to take practical measures to solve the problem.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Reproductive biotechnology techniques in cattle

Reproduction is the foundation of animal production and productivity. It is well acknowledged that one of the most significant reasons for financial losses in the animal industries is reproductive inefficiency. Despite the impressive progress achieved in the field of reproductive physiology in recent years, infertility caused by a low conception rate and a high embryonic death rate continues to be a significant issue. In order to produce great number of offspring from genetically superior animals or to produce offspring from infertile (or sub fertile) animals, different assisted-reproductive methods have been created and improved. Reproductive technologies such as AI, ES, MOET and *in vitro* fertilisation (IVF) have been created through generations to help attain this objective (Yonas *et al.,* 2020).

2.1.1. Artificial insemination in cattle

AI is the first generation reproductive technology which is process of obtaining male sperm, processing and storing it, and then introducing it artificially into the female reproductive system in order to conceive a child via methods other than natural insemination (Long, 2008). The use of AI as a tool to increase cattle production efficiency depends on three key premises: first, that spermatozoa can survive outside the body; second, that they can be reintroduced into the female genital tract in a way that results in an acceptable conception rate; and third, that the female can be identified during her fertile period (Holm *et al.,* 2008; Manafi, 2011). Compared to natural insemination, artificial insemination has a number of possible benefits. The most common one is that it may be used to improve genetics. Other benefits include cost-effectiveness, disease control, safety breeding, flexibility, and fertility management. Genetic progress in cattle can be increased up to 50% through the application of AI, using either extended semen that has been preserved in liquid form (fresh, or cooled to 5 °C) or deep-frozen (Hadgu and Fesseha, 2020).

2.1.2. Estrus synchronization

Synchronization of estrus refers to the modification of the estrous cycle to induce estrus in a significant portion of a group of females during a brief, preset time. Depending on the treatment regimen, synchronization can reduce the breeding time for females to fewer than five days, as opposed to 21 days (Yizengaw, 2017). The length of estrous cycle lasts 20 days for heifers and 21 days for adult cows. It has two different stages, 1. the first of which is characterized by the graafian follicle, the ovary's major structural component. Sometimes referred to as the estrogenic phase, the female body is being stimulated by oestrogen (E2). It is a time frame from the corpus luteum's regression until ovulation and is divided into pro-estrus and estrus phases. 2. The luteal phase, often known as the progesterone phase since the female is being stimulated by progesterone (P4), is characterized by Corpus Luteum (CL), the ovary's major structural component. Metestrus and diestrus phases are used to describe it. It is the time from ovulation to CL regression (Nebel *et al.,* 1994). The various approaches for controlling cycle length are administration of prostaglandin to regress the CL of the animal before the time of natural luteolysis, or administration of progesterone or more commonly synthetic progestin to temporarily suppress the ovarian activity, or a newer way of creating estrous synchrony is by using Gonadotropin releasing hormone (GnRH) or an-analogue, which causes ovulation of a large follicle (Patterson *et al.,* 2003).

2.1.3. *In vitro* embryo production in cattle

The phrase "*in vitro* production" (IVP) refers to the processes of obtaining immature oocytes from donor animals, maturing those oocytes or *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and culturing embryos in a controlled laboratory environment or *in vitro* culture (IVC) (Nandi *et al.,* 2006). Oocytes can be extracted from live animals using ultrasound-guided follicular aspiration (OPU) or from slaughterhouses (abattoir-derived ovaries) for use in IVEP (Hyttel *et al.,* 1997). One technique of obtaining oocytes from slaughtered animals is slicing technique which involves placing the ovaries in a petri dish with normal saline solution and using a blade to cut them into tiny pieces (Das *et al.,* 1996; Kumar *et al.,* 1997). Another technique is Slashing which involves repeatedly slicing the follicles' surface to remove all of the follicular fluid (Saleh, 2017). Ali *et al.* (2021) explained that the technique most frequently used with cow ovaries collected from the abattoir is aspiration of antral follicles using various instruments (pipette, syringe and needle, aspiration needle under vacuum pressure).

Most of the time, the ability to produce an animal embryo in a laboratory depends on the availability of oocytes, which are currently routinely obtained from donors of cattle using transvaginal ultrasound-guided collection techniques. This technique is performed using a scanner that has a suitable endo vaginal (or vaginal use adapted) sector probe with a guided needle. Using a vacuum pump and a test tube attached to the needle, the follicular fluid and the oocyte that are inside are aspirated. A scanner with good resolution and a probe of at least 6 MHz is used in order to see follicles as small as 2-3 mm in size and to be able to see the needle during follicle aspiration (Galli *et al.,* 2001). The second stage of IVEP is in vitro maturation where an Immature antral follicles from the Germinal Vesicle (GV) stage are grown in a controlled environment until they reach (Meiosis two (MII) and are prepared for fertilization and embryonic development (Gilchrist and Thompson, 2007). The third stage of IVEP is fertilization of oocytes that involves the sperm activating the oocyte, which unites the male and female gametes and causes the development of the pro nucleus (the nucleus of sperm or egg cells) and zygote (Nandi *et al.,* 2006). The last stage of IVEP is *in vitro* culture. In this stage, Presumptive zygotes are cultivated in a specific culture medium for seven days (Samardzija *et al.,* 2015).

2.1.4. Multiple ovulation and embryo transfer

MOET is a technique of reproduction which is focused on fertilizing multiple oocytes in a shorter amount of time in order to create more viable embryos that may be transferred into the recipient. ES and hormonal stimulation of donor animals form the basis of this technique, which is then followed by AI and the harvest and transfer of viable embryos to the recipient animal (Khan *et al.,* 2022).

2.2. Procedures in MOET

2.2.1. Selection of Donor cows

The most crucial factors to consider when choosing a donor animal are its superior genetics, the breed's purity, normal physiology and health conditions, normal reproductive status, age, and the possible offspring's economic worth (Abaya *et al.,* 2019). Donor cows should be chosen for ET programmes based on the following criteria: regular heat cycles beginning at a young age, a history of no more than two breeding per conception, previous calves having been born at intervals

of about 365 days, no parturition problems or reproductive irregularities, and no conformational or detectable genetic defects (Shelton, 1982). The donor needs to be kept at a nutritional level adequate for her size and rate of milk supply. The donor cow must be in a suitable body condition score at the time of embryo transfer since both the very obese and the lean cow will have diminished fertility (Lamb, 2011).

2.2.2. Superovulation of the donor cattle

There are two widely used techniques for superovulation in cattle. One approach entails giving a single intramuscular (IM) injection of equine chorionic gonadotropin (eCG) (2,000–2,500 IU), typically on day 10 of the estrous cycle (day 0 is defined as the day cows are observed in estrus), then two injections of PGF2α (dinoprost or cloprostenol) 2-3 days later, each spaced 12–24 hours apart. FSH administration is the other widely utilized procedure. Although the super ovulatory response elicited by eCG therapy is frequently larger than that induced by FSH, FSH treatment generally results in the production of more transferable excellent quality embryos (Shelton, 1982).

2.2.3. Insemination of the cattle

The starting point for insemination treatment is when the donor is first seen in a standing oestrus. Because there is a greater than usual need to ensure that viable sperm cells reach the oviducts of the super ovulated females due to the release of numerous ova from the multiple follicles on the ovary, the estrus donor is inseminated, typically with at least two straws of semen 12 h apart, and 7 days later the embryos are flushed from the uterus (Sinclair *et al.,* 2000). In every ET programme, using high-quality semen with a high percentage of normal, motile cells is a crucial step. The uterus's body is the proper location for semen implantation. This little (1/2 to 1 inch) target is located right in front of the cervix. If ovulations are occurring at the opposite ovary, there appears to be a propensity for inseminators to pass the rod too deeply and deposit the semen into one of the uterine horns, lowering fertility (Shelton, 1982).

2.2.4. Recovery of embryo

There are methods of embryo recovery which are surgical and non-surgical method. In surgical method , a 6-inch square region located around 6 inches in front of the hip joint is shaved. The area which is present on the corpus luteum side should be prepared. At the shaved region, a

local anesthetic is administered. A 2-inch scalpel incision is made after the region has been cleaned with alcohol. By holding the uterus between the fingers of one hand, the uterus and ovaries are brought close to the incision opening. A blunt needle is used to make a tiny puncture in the exposed uterine horn. The embryo is drawn into a straw measuring 0.25 milliliters and inserted into the uterus using a tiny syringe. A few sutures are used to seal the incision, and an antibiotic solution is then injected into the stitch location to treat the infection (Abaya *et al.,* 2019).

Non-surgical methods are chosen since they may be repeated and used on a farm and do not harm the reproductive system. Palpation of the ovaries via rectum is required to determine how many corpora lutea are present. Occasionally, four or five embryos are retrieved, even in rare situations where only two or three corpora lutea are palpated by trained experts. However, if the corpora lutea are not perceptible by day 7, it is extremely uncommon to succeed in conceiving an embryo. Normal embryo collection in cattle occurs six to eight days (on average day seven) following the start of the estrus brought on by Superovulation (Kidie, 2019).

2.2.5. Embryo handling

An embryo is immediately transferred to a small Petri dish (35 × 10 mm) filled with fresh, filtered (0.22-0.45 pore size), sterile medium after being found in the searching dish. Phosphate buffered saline (PBS), which contains penicillin and 10–20 percent heat inactivated serum, is typically employed as a holding medium. The next step is to serially rinse the embryos using a fresh sterile pipette through at least three separate plates containing fresh sterile media. They are then put in a dish as they wait to be transferred or cryopreserved (Abaya *et al.,* 2019).

2.2.6. Evaluation of Embryo

According to Stringfellow. (2010), the IETS suggested codes for embryo quality ranges from "1" to "4" and for stages of embryonic development ranges from Stage code1 to stage code 9:

Embryo quality evaluation

Code 1: Excellent or good. Individual blastomeres (cells) in the symmetrical, spherical embryo mass are consistent in size, color, and density. This embryo matches the predicted developmental

stage. Smoothness and the absence of concave or flat surfaces that can encourage the embryo to stick to a Petri dish or a straw are required for the zona pellucida.

Code 2: Fair. Moderate abnormalities in the size, color, and density of individual cells, as well as the general form of the embryo mass. An intact, viable embryo mass should make up at least 50% of the cell mass.

Code 3: Poor. Significant variations in the embryo mass's form or in the size, color, and density of individual cells. An intact, viable embryo mass should make up at least 25% of the cell mass.

Code 4: Dead or degenerating. oocytes, 1-cell embryos, or degenerated embryos; non-viable. Even after freezing, exceptional and good quality embryos at the blastocyst to compact morula maturation stages often have the highest pregnancy rates. Fair and subpar embryos have a low chance of becoming pregnant after freezing and should be transplanted fresh. It is recommended to choose the embryo's stage based on the recipient's synchronization (Stringfellow, 2010).

Stages of embryonic development evaluation

1-cell (Stage code 1): It is fair to presume that any recovered object from days 6–8 that is actually only a single cell is UFO. However, differentiating between a UFO and a compact morula (stage code 4) is a difficult assignment for an untrained technician. Because not all UFOs have the same physical appearance, it can be challenging to distinguish one from the other throughout different phases of embryonic development. Numerous "normal" UFOs have a zona pellucida that is perfectly spherical, an oolemma that is also perfectly spherical (also known as the vitelline membrane), relatively uniform cytoplasmic granularity that can give the appearance of cell division or a blastocoele cavity, and extreme condensation that can be mistaken for a compact morula.

2-cell to 12- cell (Stage code 2): Blastomeres are the cells of a preimplantation embryo that have not yet undergone morphological and functional development. Any embryo retrieved between 6 and 8 days post-estrus containing 2 to 12 blastomeres is very likely dead or degenerate. In the case of a typical bovine estrous cycle, this stage of embryonic development should be present in the oviduct (not the uterus) by day 5 (day 0 = commencement of estrus). A 2 to 12-cell embryo's growth is obviously significantly delayed, and even if it were still alive, it would not be a good candidate for cryopreservation or transfer.

Early Morula (Stage code 3): A minimal number of 16 cell mass. It is challenging to identify individual blastomeres from one another. The cellular mass of the embryo takes up most of the perivitelline space. Like every other developmental stage that has been covered thus far, this stage of embryonic development is not a good candidate for cryopreservation.

Compact morula (Stage code 4): A thick mass formed by individual blastomeres. Cells are assigned to the "inside" portion of the cell ball during the compaction process. The outermost cells of the ball create a continuous ring of tight connections between them. The bulk of the embryo occupies 60 to 70 percent of the perivitelline area.

Early blastocyst (Stage code 5): an embryo that has developed into a fluid-filled cavity known as a blastocele and generally resembles a signet ring. The embryo occupies around 70–80% of the perivitelline area. At this point, it may be difficult to tell the difference between trophoblast cells and the inner cell mass, giving the impression that the embryo is of questionable quality.

Blastocyst (Stage code 6): The outer trophoblast layer may be easily distinguished from the darker, more compact core cell mass. Most of the perivitelline region is occupied by the embryo, and the blastocele is clearly visible. It is possible to visibly discern between the inner cell mass and the trophoblast at this stage of development.

Expanded blastocyst (Stage Code 7): The zona pellucida simultaneously thins to approximately one-third of its initial thickness as the embryo's overall diameter rapidly increases. At this stage of embryonic development, the capacity of the blastocoele cavity has significantly increased, therefore there is no longer any perivitelline space. It's interesting to note that enlarged blastocyst-stage embryos often seem to collapse. It is thought that this anomalous occurrence, which is defined by a partial loss of blastocoele cavity fluid, aids in the hatching of embryos. Once thinned, the zona pellucida never gets back to its previous thickness.

Hatched blastocyst (Stage code 8): At this developmental stage, the recovered embryos' zona pellucida may have completely shed or be in the process of hatching. Hatched blastocysts with a distinct blastocele might be spherical or compressed. Unless the hatched blastocysts re-expand and resurface as signet rings, it may be difficult to recognize. Because the zona pellucida is not intact,

it is not possible to wash the embryo in accordance with IETS guidelines for hygienic handling of embryos, so this stage of embryonic development is not acceptable for international commerce.

Expanded hatched blastocyst (Stage code 9): With the exception of its significantly greater diameter, this stage of embryonic development resembles a fully hatched stage coding 8. Expanded hatched blastocysts from donor female cows are rare until the flushing process is carried out more than 8 days after the commencement of estrus. Trade across international borders is not permitted at this period of embryonic development.

2.2.7. Embryo storage/cryopreservation

It is a crucial component of programmes involving ET. The fastest freezing and thawing approach was most suited for blastocysts having a multi-layered trophoblast. Bovine and sheep embryos have been successfully frozen using reliable techniques. This procedure involves adding 1.4 M glycerol as a cryoprotectant in one step, allowing the mixture to acclimatize for 20 minutes, packing it in a 0.25 ml straw, slowly chilling it (0.3 to 0.1 °C/min) to -35 °C, and then submerging it in liquid nitrogen (-196 °C). By submerging the straws in warm water, frozen embryos can be thawed (Abaya, 2019).

2.2.8. Selection of recipient females

The selection of recipients for ET was based on breeding factors and used the following criteria: cows that display calving ease, good milking capacity, and are reproductively sound. Their estrus was coordinated, and before the embryos were transferred, the recipient cows were checked to see if the corpus luteum was present (Menta, 2023).

2.2.9. Donor-recipient synchronization

The recipient's reproductive system should closely mimic the donor's in order to maximize embryo survival in the recipient female after transfer. The donor's and the recipients' estrous cycles must be coordinated, ideally within one day of each other. Similar to how the donor cows are synchronized, the receivers might be done at the same working hours. The timing of recipient cow estrous synchronization must coincide with the donor cow's insemination in order for the recipient and the donor to have a comparable uterine environment when the transfer occurs seven days later. On recipient females who are already cycling, synchronizing products are more successful. Cows

in anestrus or those that aren't cycling who are too skinny or too short days after giving birth won't be good receivers. PGF-induced estrus will occur in recipients in 60 to 72 hours and in super ovulated donors in 36 to 48 hours, hence recipients synchronized with PGF must be treated 12 to 24 hours before donor cows (Berber *et al.,* 2002; Bó *et al.,* 2002; Hasler, 2014).

2.2.10. Transfer of embryo

Through surgical method of embryo transfer, Shaving a 6 inch square region around 6 inches in front of the hip joint gets the recipient ready for surgery. The shaved region receives an injection of local anesthesia. A 2 inch incision is made with a scalpel after the region has been cleaned with alcohol. By gripping the uterus with the fingers of a hand, the uterus and the ovaries are pulled close to the entrance of the incision. A dull needle is used to make a tiny puncture in the exposed uterine horn (Abaya, 2019). The embryo is drawn into a straw measuring 0.25 milli litres and inserted into the uterus using a tiny syringe. A few sutures are used to seal the incision, and an antibiotic solution is then injected into the stitch location to treat the infection (Stringfellow, 1998).

In non-surgical method The recipient animal receives a mild epidural anesthetic injection between the vertebrae of the rump to lessen rectal contractions. The AI gun is loaded with a 0.25 ml straw containing the embryo that will be transferred. The cervix can be felt and held by inserting the left hand into the rectum. The uterus is carefully connected to the ovary that has a corpus luteum by delicately inserting the insemination pistol via the cervix. Without applying force, the embryo should be inserted as deeply into the uterine horn as is practical (Abaya, 2019).

2.3. Factors affecting MOET

As outlined by Hasler (2001) a number of variables have been shown to influence the increase in pregnancy rates and the efficiency of MOET. Nutritional status, cycle stage, sensitivity to FSH, follicle population, and bull effect can all have an impact on MOET (Faizah *et al.,* 2018).

2.3.1. Effect of nutrition on MOET

Although the relationship between domestic ruminant females' nutritional and metabolic state and reproductive success has long been known, However, the underlying processes are still poorly understood (Ponsart *et al.,* 2014). According to authors, the release of gonadotrophins is modulated

by the interaction of central metabolic signals, such as circulating levels of insulin, growth hormone (GH), leptin, and the insulin-like growth factor system (IGF) (Garnsworthy *et al.,* 2008). The best predictor of embryo production appears to be the competency or quality of extracted oocytes and this depends on the physiological and reproductive status of the donor animal, which are affected by age, health and nutrition (Ponsart *et al.,* 2014).

Diet can affect the hypothalamus-pituitary-ovarian axis at different levels, which can then affect ovarian function. Follicular development may be impacted by modifications to the feeding plane. By causing changes in metabolites and metabolic hormones like insulin and IGF1, as well as hormones and growth factors in follicular fluid, alterations in the nutrition plan can have an impact on follicular development (Ponsart *et al.,* 2014). Early lactation can be delayed by insufficient energy intake, which can also affect the quality of the oocyte and the competency of the follicle (Butler, 2003). Contrarily, increased energy intake and body weight gain in non-lactating donor cows or heifers reduced the developmental competence of oocytes *in vitro* and the quality of the embryo *in vivo* (Kendrick *et al.,* 1999).

2.3.2. Effects related to super ovulatory treatment

Ovarian responses are influenced by a variety of factors, including breed, age, feeding, and other management considerations. Genetic variation in different breeds has an influence on response to super ovulation treatment which affects the yield and quality of embryos produced *in vivo* (Hirayama *et al.,* 2019). The Response to superovulation is also influenced by the donors' lactation state (Erdem *et al.,* 2020). According to research, lactating cows had a lower superovulation response and transferrable embryo count than nonlactating cows. Due to lactation and high milk production, dry matter intake and energy metabolism increased. This led to a reduction in the levels of estradiol and progesterone in the blood, which in turn caused persistent follicle formation, poor oocytes quality, and disruptions in embryonic development (Chebel *et al.,* 2008; Scenna *et al.,* 2009). Different doses of super ovulatory hormone (FSH) also have an effect on super ovulatory response and embryo production as explained by Karl *et al.* (2020). In this study, Anti mullerian Hormone (AMH), Antral Follicle Count (AFC), size of ovulatory follicle, estradiol concentration and number of ovulatory follicle were altered by variation in different doses of FSH, however, progesterone and number of CL was not affected by doses of FSH level.

2.3.3. Effect of Bull

The influence of the bull on herd fertility in both dairy and beef herds has been somewhat neglected because, by definition, herd fertility tends to reflect female rather than male traits (e.g., postpartum resumption of cyclicity, submission rate, pregnancy per AI, calving interval, etc.). This is because male fertility has received much less attention than female fertility (O'Callaghan *et al.,* 2021). This author mentioned that bull fertility can have a significant impact on overall reproductive success, particularly if bulls with low fertility are often employed in certain herds. The fertility of a single bull can have a significant impact on productivity and financial gains, even though it is difficult to determine the precise percentage of poor herd reproductive efficiency that can be attributed to individual bull fertility.

Significant variation in bull field fertility persists despite animal breeding facilities using strict pre- and post-thawed quality control inspections (Fair and Lonergan, 2018). Additionally, while standard laboratory tests evaluate sperm function in terms of motility and morphology, they do not distinguish between sperm transportation through the female reproductive canal, sperm fertilizing ability, or capacity to support early embryo development (O'Callaghan *et al.,* 2021). This authors assessed the contribution of sire on recovered embryo quality and quantity, fertilization failure and sperm transport using multiple ovulation and ET program with super ovulation and *in vitro* fertilization technique. The main finding of this study was differences in field fertility among AI sires were not reflected in fertilization rate however sire effect resulted variation in Embryo quality at day 7 and the proportion of conceptuses surviving to day 15 in recipient females.

2.3.4. Effect of time of insemination

Accurate timing of insemination and the number of accessory sperm present at the site of fertilization are key factors in optimizing fertilization rates in the donor cow (Dalton *et al.,* 2000). According to the research, there is some debate regarding the ideal timing to begin AI in ET programmes (Sales *et al.,* 2005). Donaldson (1985) advised inseminating super ovulated cows with a single dose of semen 12 hours after the start of estrus. However, Schiewe *et al.* (1987) suggested two doses of insemination at 24 hours. According to some reports, super ovulated cows might fertilize at a rate of 65% to 70% using different insemination regimes (Donaldson, 1983; Hawk and Tanabe, 1986; Lerner *et al.,* 1986; Saacke *et al.,* 1998). Dalton *et al.* (2000) suggested the

insemination of super ovulated cattle with one unit of semen 24 h after onset of estrus as it yielded an acceptable fertilization rate, embryo quality, and percentage of embryos with accessory sperm compared to insemination at 0 or 12 h after onset of estrus. Also, in another study carried out on the effect of different time of insemination regimen with sex sorted and conventional semen on super ovulated donor cows, it was reported that greater number of transferrable embryos were found when sex-sorted sperm was used to inseminate the animals at 18 and 30h compared to insemination at 12 and 24h. However, a greater embryo production was obtained with non-sorted sperm. This shows that variation in insemination time has an effect in the outcomes of embryo production (Soares *et al.,* 2011).

2.3.5. Effect of sex sorted semen and frequency of Artificial Insemination on *in vivo* embryo production

The quality of the transferred embryos into recipient female animal determines the rate of pregnancy in MOET program (Bo and Mapletoft, 2016). Among the factors affecting embryo quality in super ovulated donors are greater retrograde semen loss due to suboptimal timing of semen deposition, stage of the estrous cycle when the super stimulation treatment begins, and type of semen (Carvalho *et al.,* 2013). Sorting of sperm is one of the reason which has been attributed to the damage of spermatozoa caused by the sexing process. The stress on sperm cells during sorting of sperm includes dilution of the semen sample, mechanical forces including being sent through the flow cytometer at 60 miles per hour at 40 pounds per square inch, pressure from the collection process, and finally, centrifugation to purify the sample (Boneya, 2021). Spermatozoa are partially capacitated after sorting, which reduces their life duration and, as a result, their ability to fertilize (Vazquez *et al.,* 2003).

Sex-sorted semen is employed for super ovulated donors to increase the production of heifer calves from genetically superior females. Genomic breeding has made it easier to choose breeding females by favoring young heifers over cows as embryo donors, which has tipped the ratio of cows to heifers (Kaimio *et al.,* 2013). Comparing super ovulated animals inseminated with sex-sorted semen to those inseminated with conventional semen, the embryo yield is often reduced. Compared to heifers, the decline is more noticeable in cows. In general, there are two ways used in experimental designs that compare the two types of sperm. From a biological perspective,

comparing equal amounts of sperm per insemination is the sensible approach since it reveals the cause of the reduction whether it is due to decreased quality or a lower dosage (Kaimio *et al.,* 2013). Sartori *et al.* (2004) compared super-ovulated Holstein heifers with deep uterine horn AI of the same doses of sexed or conventional semen (10 million sperm/dose). When sexed semen was employed, the rate of fertilization was decreased. When compared to unsexed semen, the quantity and percentage of viable embryos retrieved from heifers inseminated with sexed semen were impaired, which suggests that sexing itself lowers the fertility potential of semen. However, Another research using equal, relatively low amounts of sperm (five million/dose) failed to find a difference between sexed and conventional semen in terms of embryo production (Hayakawa *et al.* 2009). Babura *et al.* (2021) also found no significant difference between the number of transferrable embryos in Bos indicus cows when inseminated with conventional and sexed semen. However, the number tends to be higher in cross bred cows inseminated with sexed semen. This would indicate that the sexed semen utilized in the current investigation is of excellent quality and that the insemination took place at a later period than it would have with convectional semen. As a result of probable pre capacitation of the sex-sorted spermatozoa, sex-sorted sperm has a shorter life span in the female reproductive tract and should be used for insemination closer to the period of ovulation (Maxwell *et al.,* 2004; Peippo *et al.,* 2009). Another way used to compare conventional and sexed semen is the comparison of large amount of sexed semen with a lower amount of conventional semen as reported by Schenk *et al.* (2006). In this study, inseminating large numbers of sex-sorted sperm (10 million) reduced embryo yield. But From an economic point of view, it is not rational to deposit sexed semen with comparable sperm counts to conventional semen. Therefore, using dosages typically utilized in commercial practice is appropriate in investigations comparing the two forms of semen. In the majority of investigations on sexed semen, a low dosage of sorted semen was compared with a typical AI-dose of conventional semen. Numerous studies have conclusively shown that cows inseminated with minimal amounts of sex-sorted semen had lower numbers of transferrable or high quality embryos and larger proportions of unfertilized ova (Schenk *et al.,* 2006, Peippo *et al.,* 2009, Hayakawa *et al.,* 2009 & Soares *et al.,* 2011). But Sexed semen has a mild to insignificant negative impact on heifer embryo development (Hayakawa *et al.,* 2009, Peippo *et al.* 2009, An *et al.* 2010).

Multiple inseminations have been implemented for super ovulated donors to offset the variation in timing of ovulation and to insure sperm cell availability for fertilization of multiple ova in the

oviducts. As a result of this presumption, AI regimens for donors requires two units of semen or more to be given three or four times at induced estrus, spaced out by 12-hour intervals. Fertilization rates are still inconsistent across donor females and within females even with these insemination schedules (Godke *et al.,* 1990). In a research comparing sexed and unsexed semen for super ovulated heifers, the group that was only inseminated once with sexed semen had fewer accessory sperm than the group that was inseminated twice with the same amount of conventional or sexed semen (Sartori *et al.,* 2004). According to Schenk *et al.* (2006), fewer transferable embryos were recovered when 2×10^6 or 10×10^6 total sexed sperm were inseminated at a fixed time (i.e., not after an observed estrus) compared with that for insemination of a total of 4×10^6 or 20×10^6 conventional sperm in two doses, 12 and 24 h after an observed estrus. The authors speculated that using sexed sperm in multiple inseminations would help to reduce retrograde losses, deliver more sperm to the site of fertilization, and possibly increase the rate of fertilization and recovery of transferable embryos to levels comparable to those achieved using conventionally frozen semen. However, increasing the number of inseminations failed to produce percentages of transferrable embryos equivalent to those of conventional controls, according to Larson *et al.* (2010).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Description of the study area

This experiment was conducted from March 2023 to December 2023 at Debre Zeit Agricultural Research Center (DZARC), animal biotechnology laboratory, Bishoftu. Bishoftu is located at 45 km southeast of Addis Ababa (8°46′13.57"N, 38°59′50.45"E) at an altitude of 1920 meters above sea level. The average annual temperature is 18.7°C with an average annual rainfall of 757.05 mm (DZARC, 2020).

3.2. Study population

The total population of study animals that are found in Debrezeit agricultural research center under Animal Biotechnology research program are 80 dairy cattles of which pure Boran breeds are around 43 and 37 are cross breeds (Boran-Holstein Friesian cross cattles). All animals are fed grass hay, a roughage mix of tef straw and supplemented with commercially prepared concentrated feed with mineral salts based on their body weight requirement. Water accessibility is adlibitum for all animals. All animals are vaccinated for Lamb Skin Disease (LSD), Foot and Mouth Disease (FMD) at regular six months interval per year.

3.3. Study/Experimental animals

A total of twelve experimental animals (pure breed Boran heifers) were used during this study. Boran heifer donors were selected based on their reproductive performance, body condition, docility, health records and cyclicity. Clinical records of all heifers were reviewed, and animals with bad temper and reproductive problems were excluded from the experiment. All donors underwent thorough reproductive examination prior to the beginning of the experiment.

Donor heifers were kept under similar management for feeding regime, housing, watering and health management conditions. All animals were fed grass hay as a basal diet, a roughage mix of tef straw and supplemented with commercially prepared concentrated feed with mineral salts.

Animals were also provided with Green Alfalfa fodder using the cut and carry method. Water was also installed constantly at the barn. All animals were vaccinated against common contagious infectious diseases and dewormed against internal and external parasites. The DZARC animal biotechnology research laboratory; with its barn and animal handling facility was made conducive working environment for subsequent *in vivo* embryo production procedures.

3.4. Experimental/research Design

Randomized experimental design was used to study the effects of frequencies of insemination and types of semen on the quality of produced bovine embryos. Experimental animals were selected purposively through gynecological examination and grouped to the types of semen and different frequencies of insemination treatment using simple random method of assignment to assign donors to each experimental groups.

The study animals were divided into two groups. In Group one, six Boran donors were used of which three Boran donors were used for two times artificial insemination with conventional semen (treatment one) and the rest three Borans (treatment two) were used for two times artificial insemination with sexed semen at 12 and 18 hrs intervals.

In group two, another six Boran donors were used of which three Boran donors were used for three times artificial insemination with conventional semen (treatment three) and the other three Borans were used for three times insemination with sexed semen (treatment four) at 12, 17 and 22 hrs intervals. The experimental design for super ovulation protocol, Artificial insemination, embryo flushing and evaluation for Boran heifers is described in Fig 1 below.

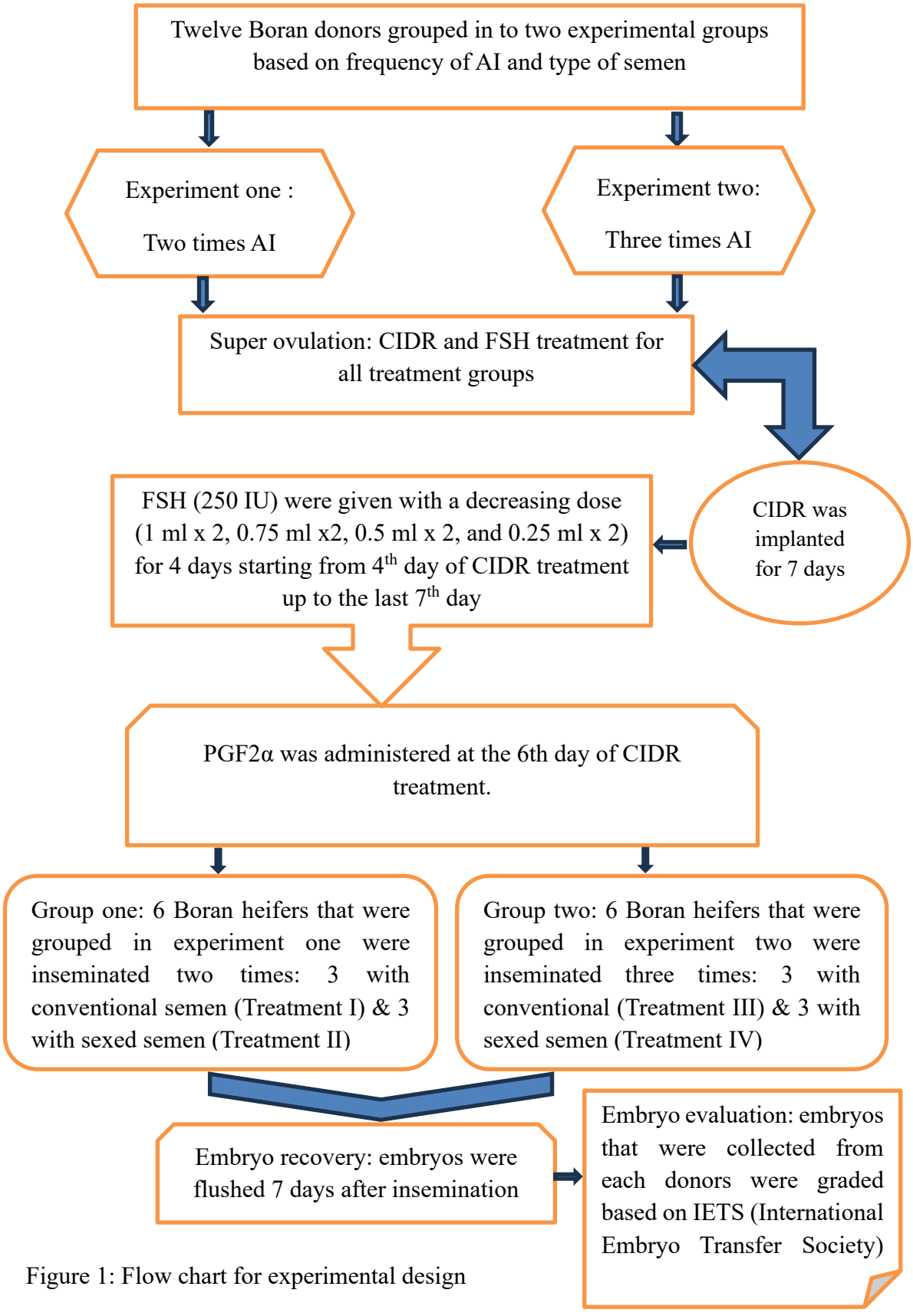

Figure 1: Flow chart for experimental design

3.4.1. Estrus synchronization of donor heifers

On day zero Control Intra Vaginal Drug release (CIDR, 1.9 mg progestogen) was installed with an applicator into the vagina of all donor animals for 7 days. Additionally, 2 ml PGF2α (Estrumate) was given on morning and afternoon schedule of AM and PM bases on 6-day post CIDR insertion.

3.4.2. Super ovulation of donor heifers

Superovulation treatment was administered using a total of 250 IU (5 ml FSH) for each Boran donors on morning and afternoon with 12 hr interval on AM and PM bases with a decreasing doses for four consecutive days (1 ml x 2, 0.75 ml x2, 0.5 ml x 2, and 0.25 ml x 2), beginning on day 4 post CIDR insertions. Finally, CIDR was removed on day 7 with an injection of the last shot of FSH in Boran heifers.

Superovulation of animals were done as previously described protocol by Degefa (2016) shown in fig 2.

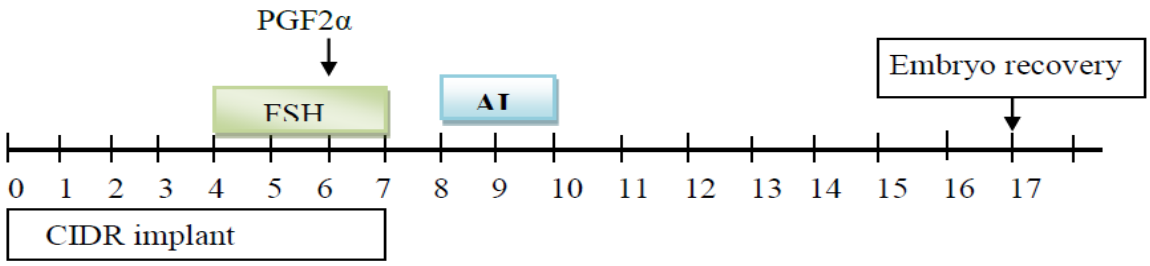

Figure 2: Super ovulation and embryo recovery protocol used in Boran heifers (Source: Degefa, 2016).

3.4.3. Heat detection of donors

After CIDR was removed from the donors, heat signs were closely observed and heat detector (ESTROTECTTM #U.S.pat. #6,467,430) was mounted on the backbone with the top of the patch roughly between the hip bones of all animals to control duration of oestrous. The heat detector helped to know the exact standing estrous time and to inseminate them at the precise duration of oestrous.

3.4.4. Insemination of the donor heifers

A straw of 12 Convectional semen with 30 x 10^6 sperm cells was used that were purchased and 18 sexed semen straws with 2 × 10^6 sperm cells was donated from Livestock development institute at Kality, Addis Ababa, Ethiopia.

The study animals that were assigned to group one, six Boran donors of which three boran donors were inseminated two times with conventional semen (treatment one) and the rest three Borans (treatment two) were inseminated two times with sexed semen at 12 and 18 hrs intervals.

In group two, another six Boran donors were used of which three Boran donors were inseminated three times with conventional semen (treatment three) and the other three Borans were inseminated three times with sexed semen (treatment four) at 12, 17 and 22 hrs intervals. Each heifers were inseminated 12 hours after they exhibit standing heat (estrous).

3.4.5. Embryo recovery

Super ovulatory response was determined using a real-time B-mode ultrasound with 5 MHz linear array probe (SIUI, Altay Scientific S.P.A., Italy) by counting a total number of CL and un ovulated follicles on the ovaries on day 7 immediately before embryo flushing.

Embryo recovery (flushing) was performed on day 7 after AI as shown in annex II and embryos were collected using non-surgical closed standard gravitational flushing technique with commercial flushing medium (ViGRO™, Bioniche Animal Health, USA) and using a two-way Foley catheter (18 Fr 650 mm length; MOFA®, Canada) to collect the embryos as previously described by Degefa *et al.* (2018). Briefly, all boran heifers ready for flushing were directed into the chute (a narrow metal enclosure for restraining livestock) and were given epidural anesthesia (2 to 5 ml of 2% lidocaine, JEIL.PHARMA.CO, LTD, Daegu, Korea) to prevent straining during flushing. The animals rectum was emptied, the vulva and perineal area were thoroughly cleaned with water and favlone (3% cetrimide + 3% Gluconate) and disinfected with 70% alcohol. Foley catheter was inserted into the uterine horn of the Boran heifer donors and the tip of the folly catheter was ballooned with air using sterile syringe to tie on two- third of the uterine horn to prevent lick out of the flushing media and recover an embryo. The Y tube foley catheter was connected to the flushing media in the upper side and embryo filter and the catheter had a flush

in and flush out mechanism to flush in the flushing fluid and to flush out the uterine fluid with gravity that was directed into the embryo filter that filtered unwanted fluid. The remaining uterine fluid that contained an embryo was transported into the *in vivo* embryo laboratory for evaluation.

3.4.6. Embryo evaluation

Embryos were searched and evaluated under stereo microscope as shown in annex I.

Embryos were evaluated according to IETS recommended quality and stage of development codes (Stringfellow, 2010) as follows:

Code 1: Excellent or Good.

Excellent *:* Any embryo recovered with a characteristics of Individual blastomeres inside the spherical, symmetrical bulk of the embryos that were homogeneous in size, color, and density and with at least 85% of the cellular material that has viable embryonic mass and minim irregularities were evaluated as quality grade one or excellent embryo. Embryos were evaluated as quality grade 1 or excellent embryo based on the proportion of embryonic cells that extruded material in the perivitelline area. This embryos were considered transferrable embryos.

Good: embryos that were asymmetrical which contained blastomeres that were not part of the main morula mass or were slightly retarded evaluated as quality grade one (code 1) or good embryos. This embryos were referred to as transferable or viable embryos.

Code 2: Fair. any embryos with a general form of the embryonic mass or the size, color, and density of the individual cells in the embryo that were moderately irregular with embryonic mass with at least 50% intact were evaluated as quality grade 2 (code 2) embryos. Because of this, these embryos were referred to as "transferable" embryos.

Code 3: Poor. embryos that exhibited significant inconsistencies in the size, color, and density of the individual cells as well as in the structure of the embryonic mass with at least 25% percentage of intact embryo were classified as code 3 (poor) embryos. This embryos were referred to as transferrable embryos.

Code 4: Dead or degenerating. Embryos or any unfertilized oocyte (UFO) that were non-viable with a dark cytoplasm, non-intact cell membranes and other significant defect were assigned as quality grade 4 embryos. This embryos or UFOs that were recovered on approximately day 7 of the estrus cycle and did not advance to at least the morula stage of embryonic development were categorized in quality grade four embryos.

Stages of development

1-cell (Stage code 1): any recovered object from days 6–8 that had only a single cell were evaluated as UFO.

2-cell to 12- cell (Stage code 2): Blastomeres are the cells of a preimplantation embryo that have not yet undergone morphological and functional development. Any embryo retrieved after 7 days post-estrus that contained 2 to 12 blastomeres were considered and evaluated as dead or degenerate or stage code 2 embryos. This embryos were referred to as non-viable or non-transferrable embryos.

Early Morula (Stage code 3): Any embryos with a minimal number of 16 cell mass that were difficult to identify individual blastomeres from one another and with a cellular mass of the embryo that took up most of the perivitelline space were evaluated as stage code 3 or early morula embryos. Those embryo were referred to as non-transferable or non-viable embryos.

Compact morula (Stage code 4): any embryos with a thick mass formed by individual blastomeres, the mass of the cells that were assigned to the "inside" portion of the cell ball during the compaction process, the outermost cells of the ball that created a continuous ring of tight connections between them and the bulk of the embryo that occupied 60 to 70 percent of the perivitelline area were classified as stage code 4 or compact morula embryos. This embryos were referred to as transferable or viable embryos.

Early blastocyst (Stage code 5): an embryos that had been developed into a fluid-filled cavity known as a blastocele that generally resembled a signet ring and the embryo that occupied around 70–80% of the perivitelline area were evaluated as early blastocyst or stage code 5 embryos. This embryos were considered viable or transferable embryos.

Blastocyst (Stage code 6): any embryos with an outer trophoblast layer that were easily distinguished from the darker, more compact core cell mass and most of the perivitelline region that were occupied by the embryo and the blastocele that were clearly visible were evaluated as stage code 6 or blastocyst embryos. At this stage of development, It was possible to visibly discern between the inner cell mass and the trophoblast.

Expanded blastocyst (Stage Code 7): any embryo with a zona pellucida that simultaneously thinned to approximately one-third of its initial thickness as the embryo's overall diameter rapidly increased and the capacity of the blastocoele cavity that had significantly increased that resulted with no perivitelline space were evaluated as stage code 7 or expanded blastocyst. The enlarged blastocyst-stage embryos were seen to be collapsed. This embryo were considered transferable or viable embryos.

Hatched blastocyst (Stage code 8): These Embryos' zona pellucida were considered to have completely shed or be in the process of hatching and were not intact. This Hatched blastocysts with a distinct blastocele were found to be spherical or compressed.

3.5. Data management and analysis

The following data's were collected: Number of CL counted, Number of Un Ovulated Follicle (UOF) counted, total number of transferable embryo, quality of transferable embryo recovered per each flush, total number of non-transferable embryo recovered per each flush, quality of non-transferable embryos, total number of unfertilized oocytes (UFO) and total number of recovery (embryo and unfertilized oocyte). Collected data were entered into the Microsoft excel sheet and analyzed with SAS version 9.0 (Copyright 2002 SAS Institute Inc Cary, NC 27513, USA). Descriptive statistics such as Mean number of CL, UOF, total number of transferable embryo, quality and stage of transferable embryo, total number of non-transferable embryo, quality and stage of non-transferable embryos, total number of unfertilized oocytes (UFO) and total number of recovery (embryo and unfertilized oocyte) was calculated. 2×2 factorial design (ANOVA) was done to find any significant relationship. The statistical significance of the study considered P value less than 0.05 as significant and P value greater than 0.05 as non-significant.

CHAPTER FOUR

4. RESULTS

4.1. Super ovulatory response

During the study period, CL and un ovulated follicle (UOF) were counted on day 7 immediately before Embryo flushing procedure and mean number of 8.08 ± 1.52 CL and 3.9 ± 1.00 UOF from all experimental animals (n=12) were observed. In terms of the frequency of insemination, mean number of 7.2 ± 1.9 CL and 2.2 ±0.7 UOF were counted from donors (n=6) under two times AI frequency whereas 9.00 ±2.52 CL and 5.7 ± 1.5 UOF for the three times AI.

Under conventional semen treatment, mean number of 9.0 ± 2.8 CL and 4.8 ± 1.8 UOF were counted from donors (n=6) and also Donors (n=6) grouped under sexed semen treatment produced mean number of 7.2 ± 1.45 Cl and 3.0 ± 0.8 UOF.

The number of CL and UOF counted from donors under two times AI and three times AI were not significantly ($p > 0.05$) different. Also, the number of CL and UOF scored from donors under conventional (n=6) and sexed semen (n=6) treatment were not significantly ($p > 0.05$) different. Overall mean number of CL and UOF counted from donors that were grouped under two times and three times AI frequency treatment; conventional and sexed semen treatments were summarized in table 1 and 2.

Table 1: Mean of total CL and UOF count from donors grouped under experiment one and two.

AI Frequency	Parameters	N	Mean (±SE)	Range	F-value	P-value
Two times	UOF	6	2.2 ± 0.7	0-5		
	CL	6	7.2 ± 1.9	(3-13)		
Three times	UOF	6	5.7 ± 1.5	(3-13)		
	CL	6	9.0 ± 2.5	(4-21)		
Total	UOF	12	3.9 ± 1.0	(0-13)	4.7	0.06
	CL	12	8.08 ± 1.52	(3-21)	0.3	0.6

Table 2: Mean of total CL and UOF count from donors grouped under conventional (treatment I and III) and sexed semen (treatment II and IV) treatment.

Semen type	Parameters	N	Mean (±SE)	Range	F-value	P-value
Conventional	UOF	6	4.8 ± 1.8	1-13		
	CL	6	9.0 ± 2.8	3-21		
Sexed	UOF	6	3.0 ± 0.8	0-5		
	CL	6	7.2 ± 1.45	4-13		
Total	UOF	12	3.9 ± 1.0	0-13	1.3	0.29
	CL	12	8.08 ± 1.5	3-21	0.3	0.6

4.2. Effect of AI frequency on embryo quantity

Mean number of total flush outputs or embryo recovery (embryos and UFOs) from donors under two (n=6) and three times (n=6) artificial insemination at 12 and 18 hrs; 12,17 and 22 hrs intervals regardless of semen type, the quantity of embryo were 3.5 ± 0.43 and 7.3 ± 2.2, respectively (table 3). There were not statistically significant ($p > 0.05$) difference between two times and three times AI frequency in terms of total recovery produced from boran heifers.

Table 3: The effect of AI frequency on mean of total flush output (embryo quantity).

AI frequency	Parameters	N	Mean ± SE	Range	F-value	P-value
Two times	Total flush output	6	3.5 ± 0.43	2-5		
Three times	Total flush output	6	7.3 ± 2.2	1-14		
Total	Total flush output	12	5.42 ± 1.22	1-14	3.57	0.095

4.3. Effect of semen type on embryo quantity

Mean number of total embryo recovery from donors inseminated with conventional (n=6) and sexed (n=6) semen regardless of the frequency of artificial insemination were 7.00 ± 1.93 and 3.8 ± 1.35, respectively (table 4). There was no statistically significant ($p > 0.05$) difference

between the two types of semen in terms of total embryo recovery produced from boran heifers with conventional and sexed semen.

Table 4: The effect of semen type on mean of total flush output (embryo quantity).

Semen type	Parameters	N	Mean ± SE	Range	F-value	P-value
Conventional	Total flush output	6	7.00 ± 1.93	3-14		
Sexed	Total flush output	6	3.8 ± 1.35	1-10		
Total	Total flush output	12	5.42 ± 1.22	1-14	2.44	0.157

4.4. Effect of the interaction of AI frequency with semen type on embryo quantity

Mean number of total recovery (embryos and UFOs) from donors inseminated two times with conventional (n=3) and sexed (n=3) semen at 12 and 18 hrs intervals were 3.7 ± 0.3; 3.3 ± 0.9 and three times (n=3) with conventional and sexed (n=3) semen at 12, 17 and 22 hrs interval were 10.3 ± 2.73; 4.3 ± 2.85 respectively. There were not statistically significant ($p > 0.05$) difference between two and three times AI frequency with conventional and sexed semen in terms of total recovery produced from boran heifers. The details of the effect of the interaction of AI frequency and semen type on embryo quantity are shown in table 5.

Table 5: The effect of the interaction of AI frequency with semen type on mean of total flush output (embryo quantity).

Interaction	Parameter	N	Mean ± SE	Range	F-value	P-value
Two times AI with conventional	Total flush output	3	3.7 ± 0.3	3-4		
Two times AI with sexed	Total flush output	3	3.3 ± 0.9	2-5		
Three times AI with conventional	Total flush output	3	10.3 ± 2.73	5-14		
Three times AI with sexed	Total flush output	3	4.3 ± 2.85	1-10		
Total	Total flush output	12	5.42 ± 1.2	1-14	1.95	0.2

Note: SEM: Standard error of mean, N: no of observations

P-value: associated with the F-statistic, if P-value less than 0.05, null hypothesis is rejected, if not null hypothesis is accepted.

4.5. Effect of AI frequency on embryo quality

Mean number of total transferable and non-transferable embryos from donors inseminated with two (n=6) and three times (n=6) AI frequency at 12 and 18 hrs and 12,17 and 22 hrs intervals were 0.5 ± 0.34 and 2.3 ± 1.0; 0.7 ± 0.3 and 2.3 ± 1.0, respectively (table 6 and 7). Mean number of unfertilized oocytes were 2.3 ± 1.4 and 2.7 ± 1.3 for two and three times AI frequency (table 7). In this study there were not statistically significant ($p > 0.05$) difference between two and three times AI frequency used in insemination of Boran heifers for production of transferable and non-transferable embryos.

Table 6: The effect of AI frequency on mean of transferable embryo quality.

AI Frequency	Parameters	N	Mean (±SE)	Range	F-value	P-value
Two times	Total transferable	6	0.5 ± 0.34	0-2		
	Q1	6	0.3 ± 0.2	0-1		
	Q2	6	0.2 ± 0.2	0-1		
	S4	6	0	0		
	S5	6	0.2 ± 0.2	0-1		
	S6	6	0	0		
	S7	6	0.3 ± 0.3	0-2		
Three times	Total transferable	6	2.3 ± 1.0	0-6		
	Q1	6	2.0 ± 0.9	0-5		
	Q2	6	0.3 ± 0.2	0-1		
	S4	6	0.3 ± 0.2	0-1		
	S5	6	0.3 ± 0.3	0-2		
	S6	6	0.5 ± 0.34	0-2		
	S7	6	1.2 ± 0.9	0-6		
Total	Total transferable	12	1.42 ± 0.6	0-6	2.7	0.14
	Q1	12	1.2 ± 0.5	0-5	2.94	0.125
	Q2	12	0.25 ± 0.13	0-1	0.5	0.5
	S4	12	0.2 ± 0.11	0-1	3	0.12
	S5	12	0.25 ± 0.2	0-2	0.22	0.65
	S6	12	0.3 ± 0.2	0-2	1.98	0.6
	S7	12	0.75 ± 0.5	0-6)	0.19	0.46

Table 7: The effect of AI frequency on mean of non-transferable embryo quality.

AI frequency	Parameters	N	Mean (±SE)	Range	F-value	P-value
Two times	Total non-transferable	6	0.7 ± 0.3	0-2		
	Q4	6	0.7 ± 0.3	0-2		
	S2	6	0.3 ± 0.21	0-1		
	S3	6	0.3 ± 0.21	0-1		
	Unfertilized	6	2.3 ± 1.4	1-4		
Three times	Total non-transferable	6	2.3 ± 1.0	0-6		
	Q4	6	2.3 ± 1.0	0-6		
	S2	6	1.7 ± 0.7	0-4		

	S3	6	0.7 ± 0.3	0-2		
	Unfertilized	6	2.7 ± 1.3	0-8		
Total	Total non-transferable	12	1.5 ± 0.6	0-6	2.22	0.17
	Q4	12	1.5 ± 0.6	0-6	2.4	0.16
	S2	12	1.00 ± 0.4	0-4	3.4	0.1
	S3	12	0.5 ± 0.2	0-2	0.7	0.43
	Unfertilized	12	2.5 ± 0.7	0-8	0.06	0.8

4.6. Effect of Semen type on embryo quality

Mean number of total transferable and non-transferable embryos produced from donors that were inseminated with conventional (n=6) and sexed semen (n=6) regardless of AI frequency were 1.7 ± 1.0 and 1.7 ± 0.65; 1.8± 0.98 and 1.2 ± 0.6, respectively (table 8 and 9). Mean number of unfertilized oocytes were 3.5 ± 1.15 and 1.5 ± 0.6 for conventional and sexed semen, respectively (table 9). There were no statistically significant ($p > 0.05$) difference between conventional and sexed semen used to inseminate Boran heifer donors for production of transferable, non-transferable embryos and unfertilized oocytes.

Table 8: The effect of semen type on mean of transferable embryo quality.

Semen type	**Parameters**	**N**	**Mean (±SE)**	**Range**	**F-value**	**P-value**
Conventional	Total transferable	6	1.7 ± 1.0	0-6		
	Q1	6	1.3 ± 0.8	0-5		
	Q2	6	0.3 ± 0.2	0-1		
	S4	6	0	0		
	S5	6	0.5± 0.34	0-2		
	S6	6	0.2 ± 0.2	0-1		
	S7	6	1.0 ± 1.0	0-6		
Sexed	Total transferable	6	1.7 ± 0.65	0-4		
	Q1	6	1.00 ± 0.6	0-4		
	Q2	6	0.2 ± 0.2	0-1		
	S4	6	0.3 ± 0.2	0-1		
	S5	6	0	0		
	S6	6	0.3 ± 0.3	0-2		
	S7	6	0.5 ± 0.34	0-2		
Total	Total transferable	12	1.42 ± 0.57	0-6	0.2	0.7

	Q1	12	1.2 ± 0.49	0-5	0.12	0.74
	Q2	12	0.25 ± 0.13	0-1	0.5	0.5
	S4	12	0.2 ± 0.11	0-1	3	0.12
	S5	12	0.25 ± 0.2	0-2	1.98	0.19
	S6	12	0.25 ± 0.2	0-2	0.22	0.65
	S7	12	0.75 ± 0.5	0-6	0.21	0.654

Table 9: The effect of semen type on mean of non-transferable embryo quality.

Semen type	Parameters	N	Mean (±SE)	Range	F-value	P-value
Conventional	Total non-transferable	6	1.8± 0.98	0-6		
	Q4	6	1.8 ± 0.98	0-6		
	S2	6	1.2 ± 0.65	0-4		
	S3	6	0.7 ± 0.3	0-2		
	Unfertilized	6	3.5 ± 1.15	0-8		
Sexed	Total non-transferable	6	1.2 ± 0.6	0-4		
	Q4	6	1.2 ± 0.6	0-4		
	S2	6	0.8 ± 0.48	0-3		
	S3	6	0.3 ± 0.2	0-1		
	Unfertilized	6	1.5 ± 0.6	0-4		
Total	Total non-transferable	12	1.5 ± 0.6	0-6	0.36	0.6
	Q4	12	1.5 ± 1.9	0-6	0.38	0.55
	S2	12	1.0 ± 0.39	0-4	0.21	0.66
	S3	12	0.5 ± 0.19	0-2	0.69	0.43
	Unfertilized	12	2.5 ± 2.36	0-8	2.22	0.18

4.7. Effect of the interaction of AI frequency with semen type on embryo quality

Mean number of transferable embryos from donors inseminated two times with conventional (n=3) and sexed semen (n=3) at 12 and 18 hrs interval were 0.3 ± 0.3 and 0.7 ± 0.7 and three times with conventional (n=3) and sexed (n=3) semen at 12, 17 and 22 hrs were 3.0 ± 1.7 and 1.7 ± 1.2, respectively. Mean number of non-transferable embryos from two times AI with conventional and sexed semen were 0.7 ± 0.7 and 0.7 ± 0.3 and three times AI with conventional and sexed semen were 3.0 ± 1.7 and 1.7 ± 1.2, respectively. Mean number of unfertilized oocytes (UFOs) from two times AI with conventional and sexed semen were 2.7 ± 0.7 and 2.0 ± 1.0 and

three times AI with conventional and sexed semen were 4.3 ± 2.3 and 1 ± 0.6. There were not statistically significant ($p > 0.05$) difference between the two and three times AI frequency with conventional and sexed semen in terms of transferable, non-transferable embryo quality and unfertilized oocytes produced from boran heifers. The details of the effect of the interaction of AI frequency and semen type on the transferable and non-transferable embryo quality and developmental stages as well as unfertilized oocytes are shown in table 10,11 and figure 3, 4 and 5.

Table 10: The effect of the interaction of AI frequency with semen type on mean of transferable embryo quality.

Interaction	Parameters	N	Mean (±SE)	Range	F-value	P-value
two times AI with conventional	Total transferable	3	0.3 ± 0.3	0-1		
	Q1	3	0.3 ± 0.3	0-1		
	Q2	3	0	0		
	S4	3	0	0		
	S5	3	0.3 ± 0.3	0-1		
	S6	3	0	0		
	S7	3	0	0		
two times AI with sexed	Total transferable	3	0.7 ± 0.7	0-2		
	Q1	3	0.3 ± 0.3	0-1		
	Q2	3	0.3 ± 0.3	0-1		
	S4	3	0	0		
	S5	3	0	0		
	S6	3	0	0		
	S7	3	0.7 ± 0.7	0-2		
three times AI with conventional	Total transferable	3	3.0 ± 1.7	0-6		
	Q1	3	2.3 ± 1.45	0-5		
	Q2	3	0.7 ± 0.3	0-1		
	S4	3	0	0		
	S5	3	0.7 ± 0.7	0-2		
	S6	3	0.3 ± 0.3	0-1		
	S7	3	2.0 ± 2.0	0-6		
three times AI with sexed	Total transferable	3	1.7 ± 1.2	0-4		
	Q1	3	1.7 ± 1.2	0-4		
	Q2	3	0	0		
	S4	3	0.7 ± 0.3	0-1		
	S5	3	0	0		

	S6	3	0.7 ± 0.7	0-2		
	S7	3	0.3 ± 0.3	0-1		
Total	Total transferable	12	1.42 ± 0.6	0-6	0.56	0.5
	Q1	12	1.2 ± 0.49	0-5	0.12	0.74
	Q2	12	0.25 ± 0.13	0-1	4.5	0.07
	S4	12	0.2 ± 0.11	0-1	4.00	0.052
	S5	12	0.25 ± 0.18	0-2	0.73	0.56
	S6	12	0.25 ± 0.18	0-2	0.73	0.56
	S7	12	0.75 ± 0.5	0-6	0.67	0.59

Note: Q1 & Q2= Quality 1 & 2 or grade 1 & 2 or code 1 & 2 embryo

Table 11: The effect of the interaction of AI frequency with semen type on mean of non-transferable embryo quality.

Interaction	**Parameters**	**N**	**Mean (±SE)**	**Range**	**F-value**	**P-value**
two times AI with conventional	Total non-transferable	3	0.7 ± 0.7	0-2		
	Q4	3	0.7 ± 0.7	0-2		
	S2	3	0.3 ± 0.3	0-1		
	S3	3	0.3 ± 0.3	0-1		
	Unfertilized	3	2.7 ± 0.7	2-4		
two times AI with sexed	Total non-transferable	3	0.7 ± 0.3	0-1		
	Q4	3	0.7 ± 0.3	0-1		
	S2	3	0.3 ± 0.3	0-1		
	S3	3	0.3 ± 0.3	0-1		
	Unfertilized	3	2.0 ± 1.0	1-4		
three times AI with conventional	Total non-transferable	3	3.0 ± 1.7	0-6		
	Q4	3	3.0 ± 1.7	0-6		
	S2	3	2.0 ± 1.2	0-4		
	S3	3	1.0 ± 0.6	0-2		
	Unfertilized	3	4.3 ± 2.3	0-8		
three times AI with sexed	Total non-transferable	3	1.7 ± 1.2	0-4		
	Q4	3	1.7 ± 1.2	0-4		
	S2	3	1.3 ± 0.9	0-3		
	S3	3	0.3 ± 0.3	0-1		
	Unfertilized	3	1.0 ± 0.6	0-2		
Total	Total non-transferable	12	1.5 ± 0.6	0-6	0.36	0.6

Q4	12	1.5 ± 0.6	0-6	0.98	0.45
S2	12	1.0 ± 0.4	0-4	1.14	0.4
S3	12	0.5 ± 0.19	0-2	0.67	0.45
Unfertilized	12	2.5 ± 0.7	0-8	0.98	0.35

Note: Q4= Quality or grade 4 or code 4 embryo.

S2 and S3= Stage 2 and 3 or Stage code 2 and 3 embryos.

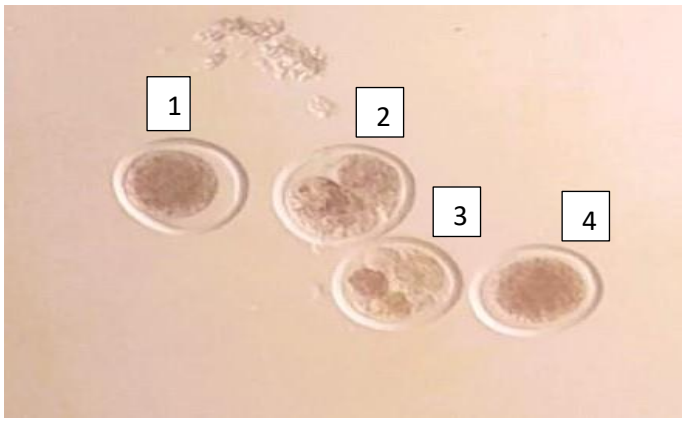

Figure 3: Embryos and UFOs collected per one flush from donor inseminated three times with sexed semen.: 1= Unfertilized oocyte (UFO); 2= Q4 (code 4) & S2 (stage code 2), 3= Q4 (code4) & S3(stage code3) and 4= Unfertilized oocyte (UFO).

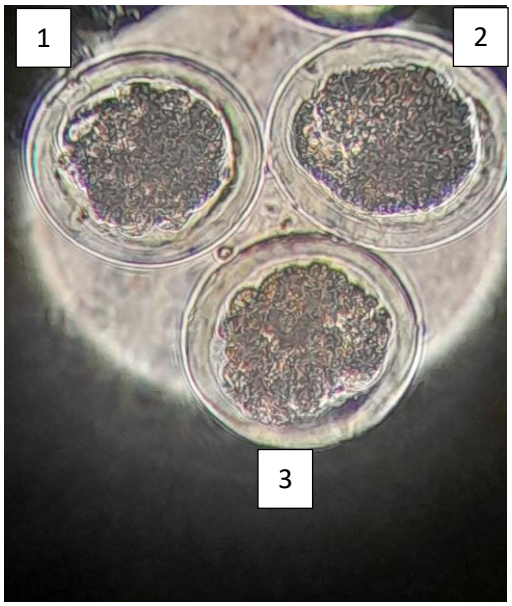

Figure 4: Transferable embryos collected per one flush from donors inseminated three times with sexed semen**:** 1 & 2=Q1 (code 1) & S5 (Stage code 5), 3= Q1 (code 1) & S4 (Stage code 4).

Figure 5: Transferable and non-transferable embryos collected per one flush from one donor inseminated three times with conventional semen: 1=Q1 (code 1) & S6 (Stage code 6), 2 & 3= Q4 (code4) or degenerated embryo.

CHAPTER FIVE

5. DISCUSSIONS

The current study was performed with the objectives of evaluating the effect of artificial insemination frequency (two and three times) and semen type (convectional and sexed semen) on quality and quantity of *in vivo* produced embryos from Boran heifers. The super ovulatory response was not significantly different between the treatment groups ($P > 0.05$) which coincide with the findings of Schenk *et al.* (2006) which is conducted on the investigation of the effect of sorted (X-bearing gamete) and non-sorted semen on fertilization rate, embryo quality and numbers of transferable embryos in super ovulated heifers and cows which were inseminated in different time intervals and artificial insemination frequencies (insemination of some heifers and cows two times at 12 and 24 h after observed standing estrus grouped under trial 1 and single timed insemination of heifers and cows grouped under trial 2). The mean number of counted CL after the super ovulatory treatment in this study was not significantly different ($P > 0.05$) between treatment groups which was similar with Sartori *et al.* (2004) and Babura *et al.* (2021) even though the super ovulatory response according to Babura *et al.* (2021) in terms of un ovulated follicles among treatment groups ($P > 0.05$) was different ($p<0.02$).

The importance of insemination of super ovulated cattle multiple times is to ensure that there is enough sperm accessible at the location of fertilization because there may be variation in the timing of ovulation meaning that some oocytes may be released too early and some are delayed in the cattle reproductive system. The result of this experiment showed that there was no significant ($p > 0.05$) difference between artificial insemination frequencies (two and three times) on produced embryo quality and quantity from Boran heifers. The result of this study was in line with the report of Sartori *et al.* (2004) with regard to the effect of artificial insemination frequencies on transferable (viable) and non-transferable (degenerate) embryos as there is no significance difference between different frequency of insemination ($p > 0.05$). Nevertheless, the result of this study contradicts with the report of Faizah *et al.* (2018) with respect to the effect of different artificial insemination frequencies on number of total recovery (embryos and UFOs), viable (transferable) embryos and unfertilized oocytes. In addition, the results of this

study was different from that of Schenk *et al.* (2006) on the effect of different artificial insemination frequencies on embryo quality in terms of the number of transferable embryos and non-transferable embryos (degenerate embryos).

Schenk *et al.* (2006) reported fewer transferable embryos from heifers breed with a single (one time) fixed-time insemination with sexed sperm compared to multiple times inseminated heifers (at 12 and 24 hrs interval) with unsexed sperm. The authors speculated that the reason for the lower transferable embryos collected from heifers breed with fixed time insemination was may be due to heifers not receiving sperm at the optimal time, adversely affecting transferable embryo production and this can be improved by inseminating more sexed sperm (optimal dosage yet to be identified) in to super ovulated heifers, probably in the form of multiple insemination overtime that would help to counter retrograde semen lose, deliver more sperm to the site of fertilization and may result in comparable fertilization and recovery rate of transferable embryo relative to the unsexed control inseminates.

A key strategy for genetic breeding improvements in dairy breeds and subsequently higher milk production is the use of sex-sorted semen in embryo development through donor cattle superovulation. The results of the experiments of this study showed that there was no significant ($p > 0.05$) difference between conventional and sexed semen on produced embryo quality in terms of transferable and non-viable embryo as well as unfertilized oocytes (UFOs). This agreed with the findings of Babura *et al.* (2021). However, previous studies have demonstrated that employing sorted semen reduces the rate of fertilization compared to using non-sorted semen in super ovulated donor cows and heifers (Sartori *et al.,* 2004; Larson *et al.,* 2010; Soares *et al.,* 2011; Kaimio *et al.,* 2013). As Sartori *et al.* (2004) and Schenk *et al.* (2009) reported, the lower fertilization rate could be attributed to the low sperm concentration and the sorting process may have caused damage in the sperm that compromised fertilization as well as subsequent embryonic development in super ovulated heifers.

In this study, the number of transferable embryos and unfertilized oocytes recovered after inseminating donor heifers with sexed semen and convectional semen did not show statistically significantly ($p > 0.05$) difference. This disagrees with the findings of Larson *et al.* (2010) where lower percentage of transferable embryos were retrieved from donor heifers and cows using sex-sorted semen than with conventional semen. This discrepancy might be due to shorter life span

and compromised fertilization rate of sex-sorted spermatozoa due to the pressure during the sorting process of sperm. This study also in opposition with the results of Peippo *et al.* (2009) where greater proportion of transferable female embryos were recovered from heifers and cows after inseminations with X-sorted spermatozoa (sexed semen) than with unsorted semen (conventional semen). However, this author reported that the use of X-sorted spermatozoa likely did not increase the proportion of transferable female embryos produced in cows on commercial dairy farms to the extent to which it occurred at the research farm due to severely compromised X-sorted spermatozoa fertilization rates in cows. Peippo *et al.* (2009) also reported greater proportions of unfertilized oocytes in all of the X-sorted spermatozoa groups when compared to the unsorted semen groups in cows ($p < 0.001$) which is in contradiction with the current study as there was no significant difference ($p > 0.05$) between sexed and conventional semen in terms of unfertilized oocytes in heifers. Nevertheless, no significant difference was observed by Peippo *et al.* (2009) between semen type in terms of unfertilized oocytes in heifers which is similar with this study.

In the present study, there was no significant difference between semen type (conventional and sexed) in the number of quality grade 1 and grade 2 transferable embryos. This is similar with the findings of Peippo *et al.* (2009). The results of this study is the same with Babura *et al.* (2021) as there was no significant difference between conventional and sexed semen in terms of grade 1 embryos. However, Babura *et al.* (2021) reported higher number of grade 2 embryos with sexed semen. The mean number of transferable embryos that recovered from heifers inseminated with sexed semen was 1.7 which is in the range of previous findings of 1.1 and 2.3 transferable embryos per recovery reported by Kaimio *et al.* (2013) and Sartori *et al.* (2004), respectively.

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATIONS

The results of this study showed that the numbers of transferable and non-transferable embryos as well as unfertilized oocytes produced from Boran heifers that were inseminated two and three times at 12 and 18 hrs and 12, 17 and 22 hrs time interval were not statistically significant. This may be due to moderate variation in the timing of ovulation in super ovulated Boran heifers (the time interval or the gap between multiple oocyte ovulation is proximate). Moreover, no significant difference was observed between the numbers of transferable embryos, non-transferable embryos and unfertilized oocytes recovered from Boran heifers inseminated with conventional and sexed semen. The reason for this may be due to good quality sexed semen used in this study. Similarly, the results of the interaction of the effects of artificial insemination frequencies and semen type on transferable and non-transferable embryos along with unfertilized oocytes produced from Boran heifers showed no significant difference.

Based on the above conclusion the following recommendation is forwarded.

- Farmers can use both conventional and sexed semen of Holstein Friesian Bull for Boran heifers to produce good quality and yield of embryo (quantity) for *in vivo* embryo production.
- it is possible to inseminate Boran heifers either two or three times a day 12 hours after the onset of standing estrus to produce good quality and yield of embryo (quantity) for *in vivo* embryo production.
- As the two types of frequency of insemination (two and three times insemination) produced good quality and yield of embryo (quantity) for *in vivo* embryo production, it is enough to inseminate Boran heifers two times at 12 and 18 hrs time interval to produce embryos *in vivo*.

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8. ANNEX

ANNEX I: Embryo searching and evaluation

ANNEX II: Embryo flushing or collection of embryos