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Research Article

# Effect of Insemination Frequency and Type of Semen on Quantity and Quality of *In vivo* Produced Embryos from Ethiopian Boran Heifers

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## Abstract

The use of proper superovulation technique, types of semen, frequency of artificial insemination, health condition of cattle and type of breeds are some of the common factors that directly affects the success of embryo transfer programmes. Therefore, this study was conducted to evaluate effects of AI frequency and semen type on quantity and quality of in vivo produced embryos in Ethiopian Boran heifers. In this study, 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 the study showed that mean number of 7.2 ±1.9 and 9.0 ±2.5 for CL and 2.2 ±0.7 and 5.7 ±1.5 for 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 insemination, mean number of total flush output counted from donors were 7.00 ±1.93 and 3.8 ±1.35, respectively. Mean number of 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 insemination were 3.5 ±1.15; 1.5 ±0.6, respectively. No significant ($P>0.05$) difference was observed between AI 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. The present findings demonstrated that inseminating Ethiopian Boran heifers two and three times both with sexed and conventional semen did not affect embryo quality and quantity produced in vivo from donors.

## Keywords

Boran Heifers, Insemination Frequency, *In vivo* Embryos, Semen Type, Super Ovulation

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# 1. Introduction

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 [1]. 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 [2]. Enhancing livestock genetic potential has been a top priority in Ethiopia to increase milk and meat production, which is currently constrained by poor reproductive performance in cattle raised under natural conditions. It is recommended that 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), In vitro fertilization (IVF), germplasm cryopreservation, and others, the current deficit of animal production and productivity in the country might be resolved [3].

AI is the most useful and widely utilized biotechnology which has been employed for more than 40 years [4, 5]. ES is employed in big farms and ranches with hormonal techniques to manipulate bovine reproduction that address some of AI's shortcomings, to increases the reproductive effectiveness of cows and heifers [3]. On the other hand, the advancements made during the past decades have made commercial bovine embryo transfer a significant global industry [6, 7]. In order to regulate the sex of the livestock's progeny determination of sex of sperm and embryos was also developed, that opened opportunities for several companies to market sexed semen [7]. Multiple Ovulation and Embryo Transfer (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 that are in good reproductive health. Each of these factors directly affects the success of the embryo transfer programme [8, 9].

A research conducted to compare sexed semen with conventional semen in terms of pregnancy rates in cattle showed that when utilized in well-managed exotic breeds of dairy herds, sex-sorted semen has produced conception rates that are approaching those for conventional semen [10]. According to the reports of some researchers, due to the utilization of sexed semen, the yield of transferable embryos is significantly reduced in cows, although this reduction is only moderate in heifers [11, 12]. 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 [13]. There are limited reports that investigated the effect of the frequency and time of insemination, and type of semen in super-ovulated cattle on in vivo-produced embryo quality and quantity in indigenous Ethiopian cattle breeds. Therefore, the current study was conducted to evaluate the effect of Insemination frequency and type of semen on quantity and quality of in vivo produced embryos in Boran heifers of Ethiopia.

# 2. Materials and Methods

## 2.1. Description of the Study Area

The experiment was conducted from March 2023 to December 2023 at Debre Zeit Agricultural Research Center (DZARC), animal biotechnology laboratory, Bishoftu, Ethiopia. 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 ℃ with an average annual rainfall of 757.05 mm [14].

## 2.2. Experimental Animals

A total of twelve experimental indigenous cattle (pure breed Boran heifers) were used during this study. Boran heifer donors were selected based on their good body condition, docility, health records and cyclicity. In addition, 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 such as Lumpy Skin Disease (LSD), Foot and Mouth Disease (FMD) at regular six months interval per year 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.

## 2.3. Experimental Design

Randomized experimental design was used to study the effects of frequencies of insemination and types of semen on the quality and quantity of embryos produced. In such a way experimental animals were assigned 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) 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 for three times insemination with sexed semen (treatment four) at 12, 17 and 22 hrs intervals. Flowchart for super ovulation protocol, artificial insemination, embryo flushing and evaluation for the experimental Boran heifers is described in Figure 1 below.

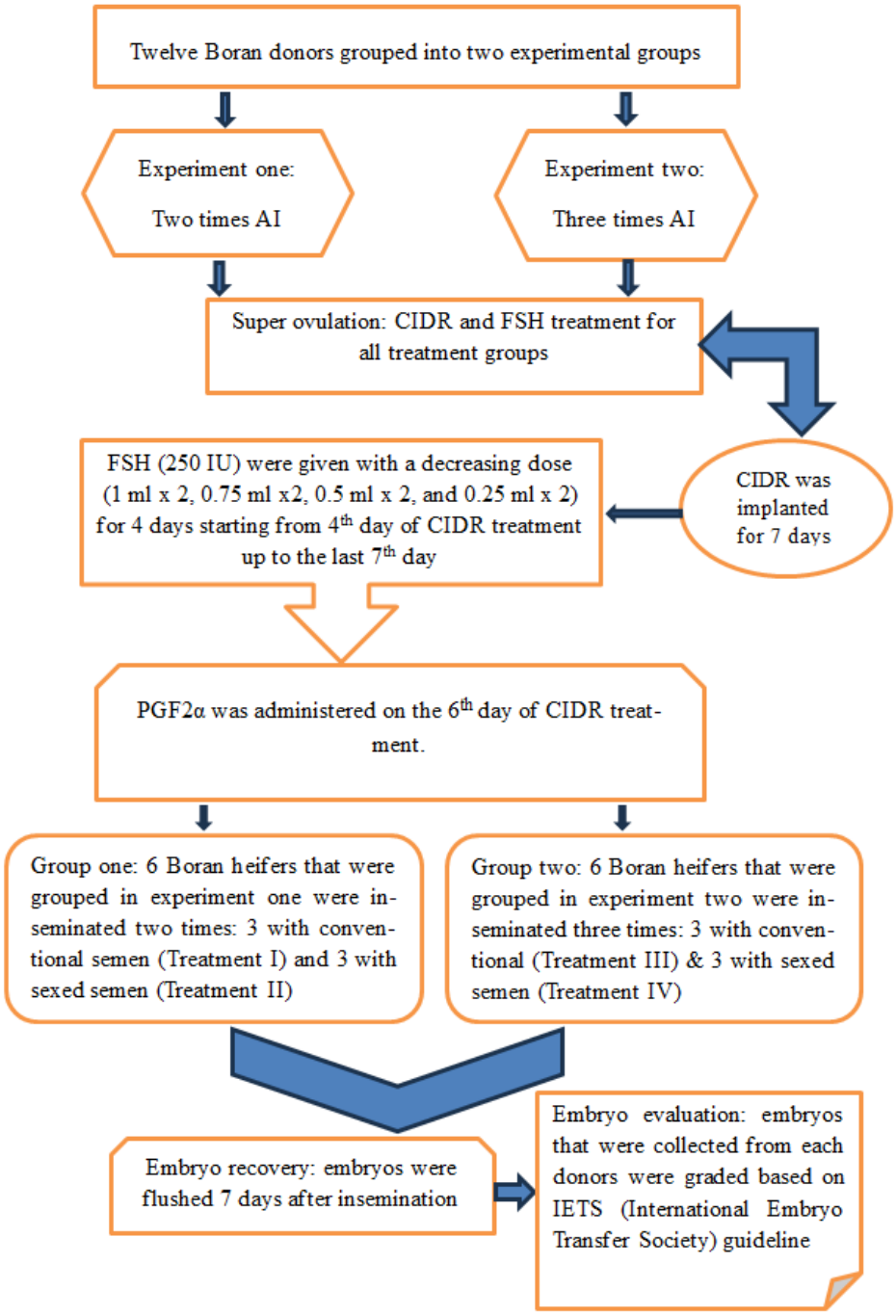


***Figure 1.*** *Flow chart for the experimental design.*

### 2.3.1. Estrus Synchronization and Super Ovulation of Donor Heifers

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

Superovulation of animals was done as previously described by Degefa [15] shown in Figure 2. Briefly, 250 IU (5

ml) FSH was administered for each Boran heifer donors on morning and afternoon with 12 hr interval on AM and PM bases with a decreasing doses for four consecutive days, 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.

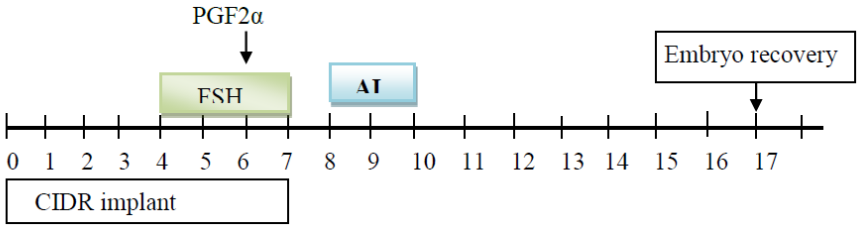


***Figure 2.*** *Super ovulation and embryo recovery protocol used in Boran heifers (Source: [6]).*

### 2.3.2. Heat Detection of Donors

After CIDR was removed from the donor heifers, 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 estrous. The heat detector helped to know the exact standing estrous time and to inseminate them at the precise duration of estrous.

### 2.3.3. Insemination of the Donor Heifers

A straw of 12 convectional semen with 30 x 106 sperm cells was used that were purchased and 18 sexed semen straws with 2 × 106 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 heifer was inseminated 12 hours after they exhibited standing heat (estrous) where finally the number of CL and UOF were counted during the study period.

### 2.3.4. Embryo Recovery and Evaluation

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 corpus luteum (CL) and un ovulated follicles (UOF) on the ovaries on day 7 immediately before embryo flushing. Embryo recovery was performed on day 7 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) as previously described by Degefa et al. [16]. Embryos were evaluated according to IETS recommended quality [quality 1 (code 1)= excellent and good to quality 4 (code 4)]= Dead or degenerating and stage of development (stage codes 1= one cell to stage code 9= expanded hatched blastocyst) [17]. Hence, the following data's were recorded: 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 (UFOs) and total number of recovery (embryo and unfertilized oocyte).

## 2.4. Data Management and Analysis

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 and non-transferable embryos, quality and stage of transferable and 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.

# 3. Results

## 3.1. Super Ovulatory Response

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 Tables 1 and 2. From all experimental animals (n=12), a mean number of 8.08 ± 1.52 CL and 3.9 ± 1.00 UOF were counted on day 7 immediately before embryo flushing procedure. 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 without showing statistically significant (p>0.05) difference. Under conventional semen inseminated donor heifers (n=6) a mean number of 9.0 ±2.8 CL and 4.8 ±1.8 UOF were counted whereas 7.2 ±1.45 CL and 3.0 ±0.8 UOF were produced from sexed semen inseminated heifers (n=6) group with no statistically significant (p>0.05) difference observation.

***Table 1.*** *Mean CL and UOF counts from donor heifers from experimental animals.*

| 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 CL and UOF count from donor heifers inseminated with conventional (treatment I and III) and sexed semen (treatment II and IV).*

| 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 |

## 3.2. Effect of AI Frequency and Semen Type on Embryo Quantity

Mean number of total embryo recovery from donor Boran heifers regardless of semen type under two (n=6) and three times (n=6) artificial insemination at 12 and 18 hrs; 12,17 and 22 hrs intervals, 3.5 ±0.43 and 7.3 ±2.2 embryo were produced, respectively (Table 3) without a statistically significant (p > 0.05) difference observation.

***Table 3.*** *Effect of AI frequency on mean total flush output (embryo quantity).*

| Parameters | N | Mean ±SE | Range | F-value | P-value |
|---|---|---|---|---|---|
| AI frequency | | | | | |
| Two times | 6 | 3.5 ±0.43 | 2-5 | | |
| Three times | 6 | 7.3 ±2.2 | 1-14 | 3.57 | 0.095 |
| Total | 12 | 5.42 ±1.22 | 1-14 | | |
| Semen type | | | | | |
| Conventional | 6 | 7.00 ±1.93 | 3-14 | | |

| Parameters | N | Mean ±SE | Range | F-value | P-value |
|---|---|---|---|---|---|
| Sexed | 6 | 3.8 ±1.35 | 1-10 | 2.44 | 0.157 |
| Total | 12 | 5.42 ±1.22 | 1-14 | | |

Mean number of total embryo recovery from Boran heifers 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 3). 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.*** *Effect of the interaction of AI frequency with semen type on mean total flush output (embryo quantity).*

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

## 3.3. Effect of the Interaction of AI Frequency with Semen Type on Embryo Quantity

Mean number of total embryos recovery from donor Boran heifers 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. The details of the effect of the interaction of AI frequency and semen type on embryo quantity were shown in Table 4.

## 3.4. Effect of AI Frequency and Semen Type on Embryo Quality

Mean number of total transferable and non-transferable embryos from donor Boran heifers inseminated two (n=6) and three times (n=6) 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 (Tables 5 and 6). 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 5.*** *Effect of AI frequency on mean 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 | | |

| AI Frequency | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | S7 | 6 | 0.3 ±0.3 | 0-2 | | |
| | 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 | | |
| Three times | 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 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 |
| Total | 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 6.*** *Effect of AI frequency on mean non-transferable embryo quality.*

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

Mean number of total transferable and non-transferable embryos produced from donor Boran heifers 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 (Tables 7 and 8). Mean number of unfertilized oocytes were 3.5 ±1.15 and 1.5 ±0.6

for conventional and sexed semen, respectively (Table 8). 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 7.*** *Effect of semen type on mean transferable embryo quality.*

| Semen type | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | 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 | | |
| Conventional | 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 | | |
| | 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 | | |
| Sexed | 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 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 |
| Total | 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 8.*** *Effect of semen type on mean non-transferable embryo quality.*

| Semen type | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | Total non-transferable | 6 | 1.8±0.98 | 0-6 | | |
| | Q4 | 6 | 1.8 ±0.98 | 0-6 | | |
| Conventional | S2 | 6 | 1.2 ±0.65 | 0-4 | | |
| | S3 | 6 | 0.7 ±0.3 | 0-2 | | |
| | Unfertilized | 6 | 3.5 ±1.15 | 0-8 | | |
| | Total non-transferable | 6 | 1.2 ±0.6 | 0-4 | | |
| Sexed | Q4 | 6 | 1.2 ±0.6 | 0-4 | | |
| | S2 | 6 | 0.8 ±0.48 | 0-3 | | |

| Semen type | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | S3 | 6 | 0.3 ± 0.2 | 0-1 | | |
| | Unfertilized | 6 | 1.5 ± 0.6 | 0-4 | | |
| | 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 |
| Total | 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 |

## 3.5. Effect of the Interaction of AI Frequency with Semen Type on Embryo Quality

Mean number of transferable embryos from donor Boran heifers 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. 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 below (Tables 9 and 10; Figures 3 and 4). There were no 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.

***Table 9.*** *Effect of the interaction of AI frequency with semen type on mean transferable embryo quality.*

| Interaction | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | Total transferable | 3 | 0.3 ± 0.3 | 0-1 | | |
| | Q1 | 3 | 0.3 ± 0.3 | 0-1 | | |
| | Q2 | 3 | 0 | 0 | | |
| Two times AI with conventional | S4 | 3 | 0 | 0 | | |
| | S5 | 3 | 0.3 ± 0.3 | 0-1 | | |
| | S6 | 3 | 0 | 0 | | |
| | S7 | 3 | 0 | 0 | | |
| | 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 | | |
| Two times AI with sexed | 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 | | |

| Interaction | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | 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 | | |
| | Total transferable | 3 | 1.7 ±1.2 | 0-4 | | |
| | Q1 | 3 | 1.7 ±1.2 | 0-4 | | |
| | Q2 | 3 | 0 | 0 | | |
| Three times AI with sexed | 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 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 |
| Total | 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 |

Q1 & Q2= Quality 1 & 2 or grade 1 & 2 or code 1 & 2 embryo.

***Table 10.*** *Effect of the interaction of AI frequency with semen type on mean non-transferable embryo quality.*

| Interaction | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| | Total non-transferable | 3 | 0.7 ±0.7 | 0-2 | | |
| | Q4 | 3 | 0.7 ±0.7 | 0-2 | | |
| Two times AI with conventional | S2 | 3 | 0.3 ±0.3 | 0-1 | | |
| | S3 | 3 | 0.3 ±0.3 | 0-1 | | |
| | Unfertilized | 3 | 2.7 ±0.7 | 2-4 | | |
| | Total non-transferable | 3 | 0.7 ±0.3 | 0-1 | | |
| | Q4 | 3 | 0.7 ±0.3 | 0-1 | | |
| Two times AI with sexed | S2 | 3 | 0.3 ±0.3 | 0-1 | | |
| | S3 | 3 | 0.3 ±0.3 | 0-1 | | |
| | Unfertilized | 3 | 2.0 ±1.0 | 1-4 | | |
| | Total non-transferable | 3 | 3.0 ±1.7 | 0-6 | | |
| | Q4 | 3 | 3.0 ±1.7 | 0-6 | | |
| Three times AI with conventional | S2 | 3 | 2.0 ±1.2 | 0-4 | | |
| | S3 | 3 | 1.0 ±0.6 | 0-2 | | |

| Interaction | Parameters | N | Mean (±SE) | Range | F-value | P-value |
|---|---|---|---|---|---|---|
| Three times AI with sexed | Unfertilized | 3 | 4.3 ±2.3 | 0-8 | | |
| | 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 | | |
| Total | Unfertilized | 3 | 1.0 ±0.6 | 0-2 | | |
| | 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 |

Q4= Quality or grade 4 or code 4 embryo; S2 & S3= Stage 2 & 3 or Stage code 2 & 3 embryos

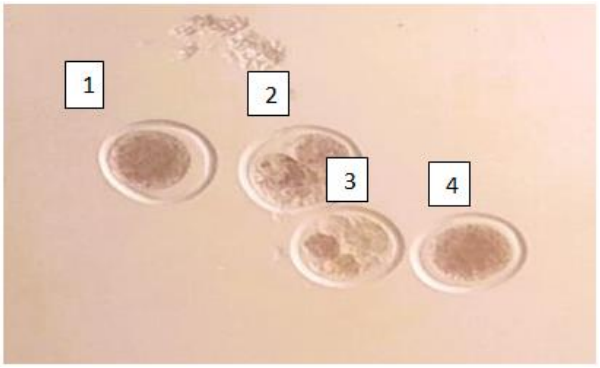


***Figure 3.*** *Embryos and UFOs collected per one flush from donor Boran heifers inseminated three times with sexed semen. 1= Unfertilized oocyte (UFO); 2= Q4 (code 4) & S2 (stage code 2), 3= Q4 (code4) & S3 (stage code3) and 4= Unfertilized oocyte (UFO).*

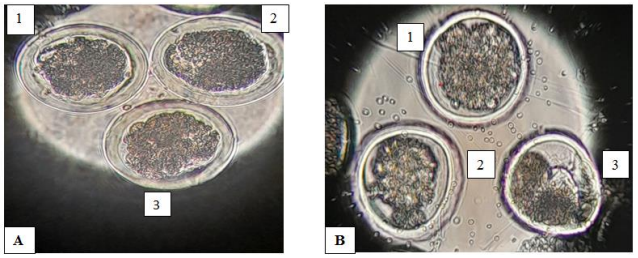


***Figure 4.*** *Collected embryos per one flush from donor Boran heifers inseminated at different frequencies with different semen types. A) Transferable embryos collected per one flush from donor Boran heifers inseminated three times with sexed semen. 1 & 2=Q1 (code 1) & S5 (Stage code 5), 3= Q1 (code 1) & S4 (Stage code 4). B) Transferable and non-transferable embryos collected per one flush from one donor Boran heifers inseminated three times with conventional semen. 1=Q1 (code 1) & S6 (Stage code 6), 2 & 3= Q4 (code4) or degenerated embryo.*

## 4. Discussions

The current study was performed with the objectives of evaluating the effects of insemination frequency (two and three times) and semen type (convectional and sexed semen) on quality and quantity of in vivo produced embryos from Ethiopian 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. [11] 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 at different time intervals and insemination frequencies. The mean number of counted CL after the super ovulatory treatment in present study did not show statistically significant difference ($P > 0.05$) between treatment groups which was similar with the findings of Sartori et al. [18] in Holstein heifers.

The importance of inseminating super ovulated cattle for multiple times is to ensure that there is enough sperm reach at the site 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 the present experiment showed that there was no significant ($p > 0.05$) difference between insemination frequencies (two and three times) on the produced embryo quality and quantity Ethiopian Boran heifers. The result of this study was in line with the report of Sartori et al. [18] on Holstein heifers with regard to the effect of 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 the present study contradicts with the report of Faizah et al. [19] with respect to the

effect of different artificial insemination frequencies on total number of viable (transferable) embryos and unfertilized oocytes recovery in Holsten Friesian breed.

Schenk et al. [11] reported fewer transferable embryos from heifer exotic 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) 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 [20]. The results of the present experiment 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 embryos as well as unfertilized oocytes (UFOs) which is in agreement with the findings of Babura et al. [21] in Boran and Holstein-Boran cross bred cattle. 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 of Holstein [18], Angus [22], Nelore and Holstein [23], Holstein and Finnish Ayrshire [12] breeds. As Sartori et al. [18] and Schenk et al. [11] 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 Boran 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. [22] where lower percentage of transferable embryos were retrieved from donor Angus heifers and cows using sex-sorted semen than with conventional semen. This discrepancy might be due to breed difference or shorter life span and compromised fertilization rate of sex-sorted spermatozoa due to the pressure during the sorting process of sperm. The present study also didn't agree with the results of Peippo et al. [24] where greater proportion of transferable female embryos were recovered from Holstein-Friesian dairy heifers and cows after inseminations with X-sorted spermatozoa (sexed semen) than with unsorted semen (conventional semen). However, the same 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. [24] also reported greater proportions of unfertilized oocytes in all of the X-sorted spermatozoa groups when compared to the unsorted semen groups in Holstein-Friesian dairy 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 Boran heifers. Nevertheless, no significant difference was observed by Peippo et al. [24] between semen types in terms of unfertilized oocytes in heifers which are similar with our present experiment.

In the present study, there was no significant difference between semen type (conventional and sexed) in the number of quality grade one and grade two transferable embryos. This is similar with the findings of Peippo et al. [24]. The results of this study is the same with Babura et al. [21] as there was no significant difference between conventional and sexed semen in terms of grade one embryos. However, Babura et al. [21] reported higher number of grade two embryos with sexed semen. In the present study, the mean number of transferable embryos recovered from Boran heifers inseminated with sexed semen was 1.7 which is in the range of the previous findings of 1.1 and 2.3 transferable embryos per recovery reported by Kaimio et al. [12] in Holstein and Finnish Ayrshire breeds and Sartori et al. [18] in Holstein heifers, respectively.

## 5. Conclusions

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 might be due to good quality sexed semen used in the present 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. Therefore, one 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. 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. However, for other studies that includes large number of experimental animals, the results of the quality and quantity of produced embryos may vary due to the huge difference in the timing of ovulation which is the gap between the first and last ovulation in some animals. For this reason, the timing and frequency of insemination using either conventional and sexed semen need to be studied thoroughly to improve the outcome of in vivo embryo production in Boran cattle for the future.

## Abbreviations

| | |
|---|---|
| AI | Artificial Insemination |
| CIDR | Control Intra Vaginal Drug Release |
| CL | Corpus Luteum |
| DZARC | Debre Zeit Agricultural Research Center |
| ES | Estrus Synchronization |
| ET | Embryo Transfer |
| FMD | Foot and Mouth Disease |
| FSH | Follicule Stimulating Hormone |
| IETS | International Embryo Transfer Society |
| IVF | *In vitro* Fertilization |
| LSD | Lumpy Skin Disease |
| MOET | Multiple Ovulation and Embryo Transfer |
| PGF2α | Prostaglandin F2α |
| UFOs | Unfertilized Oocytes |
| UOF | Un Ovulated Follicles |

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## Author Contributions

**Ayda Mohammed:** Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft

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## Ethics Approval for Experimental Animals

The procedures involving animal handling and sample collection followed the recommendation of directive 2010/63/EU and ethical approval of the study was obtained from Institutional Ethical Review Board of Adama Science and Technology University (ASTU), certificate reference number RECSoANS/BIO/03/2023.

## Availability of Data and Material

All data collected for this research are used and included in this manuscript with the submission.

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## Conflicts of Interests

The authors declare no conflicts of interest.

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