EFFECT OF AGE ON SEXUAL BEHAVIOUR, SEMEN QUALITY AND FREEZABILITY OF SAHIWAL BULLS

THESIS SUBMITTED TO THE

ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE OF

MASTER OF VETERINARY SCIENCE

IN

LIVESTOCK PRODUCTION MANAGEMENT

BY

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Date: 15/9/2023

Dr. Pawan Singh
(Major Advisor & Chairman)

DEDICATED

TO ALL

MY WELL

WISHERS

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Date:

Anshul Gautam

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ABBREVIATIONS

%	:	Percentage
^{o}C	:	Degree Celcius
p<0.01	:	1 % level of significance
p<0.05	:	5 % level of significance
<	:	Less than
>	:	Greater than
Ad lib	:	Ad libitum
AI	:	Artificial Insemination
ABRC	:	Artificial Breeding Research Centre
ANOVA	:	Analysis of Variance
DPT	:	Distal Pole Temperature
e g.	:	For example
et.al	:	Co-workers
Fig.	:	Figure
ICAR	:	Indian Council of Agricultural Research
IM	:	Individual Motility (%)
IRT	:	Infrared Thermography
HOST	:	Hypo-osmotic Swelling test
MA	:	Mass Activity
Min	:	Minute
MPT	:	Middle Pole Temperature
NDRI	:	National Dairy Research Institute
RR	:	Respiration Rate
RT	:	Rectal Temperature
No.	:	Number
S.E.	:	Standard Error
SPSS	:	Statistical package software system
TDN	:	Total Digestible Nutrient

EFFECT OF AGE ON SEXUAL BEHAVIOUR, SEMEN QUALITY AND FREEZABILITY OF SAHIWAL BULLS

ABSTRACT

Age of the bull influences the sexual behaviour and quality of semen. The present study was conducted to find out the age window at which the bull produces quality semen. For this, fifteen Sahiwal bulls were selected and classified into three age groups (n=5, in each group) –G1(2-3.5), G2 (>3.5-8) and G3 (>8-12) years. The semen was collected twice a week with two ejaculates on each collection. Semen of these bulls was analysed at fortnightly interval in this study. The sexual behaviour was recorded at the time of semen collection by CCTV camera. Physiological responses,scrotal circumference (by scrotal tape) and scrotal temperature (by IRT) were taken at monthly interval. The data so obtained in the experiment was subjected to statistical analysis by one-way analysis of variance (ANOVA) using SPSS software version 26.0 to draw scientific interferences. The results revealed that the mean value for respiration rate was significantly high and scrotal circumference was significantly ($p<0.05$) low in G1 group as compared to the other two groups. No significant difference was observed among the groups for rectal temperature, scrotal surface temperature and scrotal temperature gradient. Most of the sexual behaviour parameters were significantly high in the G2 (>3.5-8) group except for the time to enter arena from preparation site and total number of mountings. Mean value of semen volume (ml) and sperm concentration (10^6/ml) was high ($p<0.05$) in the G3 group as compared to the other two groups. Mass motility was high ($p<0.05$) in the G3 group followed by G2 and G1 age group. Individual motility was high ($p<0.05$) in the G2 group followed by G3 and G1 group. Mean value of viability (%), HOST (%), Acrosome integrity (%) was non-significant ($p<0.05$) in the G2 (>3.5-8) and G3 (>8) group and low in G1 group for fresh as well as frozen semen. The post thaw motility was high ($p<0.05$) in G2 group followed by G3 and G1 age group. In incubation test the post thaw motility of the G2 group was high ($p<0.05$) at 0,90 and 120 min as compared to the other two groups. Therefore, it can be concluded from this study that sexual behaviour and semen quality of Sahiwal bulls are influenced by the age and adult (middle) aged bulls produced superior quality semen as compared to young and old groups.

साहीवाल सांडों के यौन व्यवहार, वीर्य की गुणवत्ता और हिमीकरण की क्षमता पर उम्र का प्रभाव

सारांश

वर्तमान अध्ययन साहीवाल सांडों के यौन व्यवहार, वीर्य की गुणवत्ता और हिमीकरण की क्षमता पर उम्र के प्रभाव को पता लगाने के लिए किया गया था| जिसके लिए पंद्रह साहीवाल सांडों का चयन किया गया एवं उन्हें तीन आयु समूहों (प्रत्येक समूह में n=5) -G1(2-3.5 वर्ष), G2 (>3.5-8 वर्ष) और G3 (>8-12 वर्ष) में वर्गीकृत किया गया। सप्ताह में दो बार वीर्य एकत्र किया गया और प्रत्येक संग्रहण मे दो वीर्य स्खलन एकत्रित किये गये व अध्ययन में पाक्षिक अंतराल पर इन सांडों के वीर्य का विश्लेषण किया गया। | वीर्य संग्रह के समय यौन व्यवहार को सीसीटीवी कैमरे की मदद से रिकॉर्ड किया गया । मासिक अंतराल पर शारीरिक प्रतिक्रियाएं, अंडकोश की परिधि और अंडकोश का तापमान (आईआरटी द्वारा) लिया गया। प्रयोग में प्राप्त डेटा को वैज्ञानिक हस्तक्षेप निकालने के लिए एस.पी.एस.एस. सॉफ्टवेयर संस्करण 26.0 का उपयोग कर वन वे एनोवा द्वारा सांख्यिकीय विश्लेषण किया गया । परिणामों से ज्ञात हुआ कि श्वसन दर का औसत मूल्य अन्य दो समूहों की तुलना में G1 समूह में काफी अधिक ($p < 0.05$) था और अंडकोश की परिधि का औसत मूल्य अन्य दो समूहों की तुलना में G1 समूह में काफी कम ($p < 0.05$) था। मलाशय तापमान, अंडकोश की सतह के तापमान और अंडकोश की तापमान प्रवणता के लिए समूहों के बीच कोई महत्वपूर्ण अंतर नहीं देखा गया। तैयारी स्थल से वीर्य संग्रहण प्रक्षेत्र में प्रवेश करने के समय और आरोहण की कुल संख्या को छोड़कर अधिकांश यौन व्यवहार मानदंड G2 (>3.5-8) समूह में अधिक थे। अन्य दो समूहों की तुलना में G3 समूह में आयतन (एमएल) और सांद्रता (10^6/एमएल) का औसत मूल्य अधिक (पी<0.05) था। G3 समूह में द्रव्यमान गतिशीलता उच्च (पी<0.05) थी व उसके बाद G2 और G1 आयु समूह में थी। G2 समूह में व्यक्तिगत गतिशीलता उच्च (पी<0.05) थी व उसके बाद G3 और G1 समूह में थी। जीवित शुक्राणु की संख्या (%), HOST (%), एक्रोसोम अखंडता (%) के औसत मूल्य G2 और G3 समूह में अंतर गैर-महत्वपूर्ण था और G1समूह में सबसे कम था। हिमीकृत वीर्य में पिघलने के बाद की गतिशीलता G2 समूह में उच्च ($p<0.05$) थी, इसके बाद G3 और G1 आयु समूह में थी। पिघलने के बाद ऊष्मायन परीक्षण में G2 समूह की गतिशीलता अन्य दो समूहों की तुलना में 0,90 और 120 मिनट पर उच्च ($p<0.05$) थी। इस अध्ययन से यह निष्कर्ष निकाला जा सकता है कि साहीवाल सांडों का यौन व्यवहार और वीर्य की गुणवत्ता उम्र से प्रभावित होती है एवं युवा और वृद्ध समूहों की तुलना में वयस्क (मध्यम) आयु वर्ग के सांड बेहतर गुणवत्ता वाले वीर्य का उत्पादन करते है।

CHAPTER –1

Introduction

INTRODUCTION

India has large livestock resource, which is both a significant source of revenue for the country's economy and source of livelihood particularly for landless and marginal farmers. Presently, the GVA (Gross value added) of the livestock sector has recorded an annual growth rate of 6.13% at constant prices (DADF, 2021-22). India is the largest producer of milk with 221.06 million tonnes with a growth rate of 5.29% annually (DADF, 2021-22). The per capita availability of milk is 444 gm/day. Despite having the highest milk production in the world, the productivity of our nation's cow is very low (3.36 kg/day).

The most common technique used for increasing the genetic potential of dairy animals is artificial insemination with frozen semen (Rugira *et al*., 2017). It is a matter of great significance due to large-scale use of very few genetically superior sires to cover a large female population (Stalhammar *et al*., 1988; Waldner *et al*., 2010). Along with the genetic improvement it is also beneficial in the control of venereal diseases, economical than natural service (Bearden *et al*., 2004 ; Lemma and Shemsu, 2015). The optimal use of genetically superior bulls through artificial insemination is highly dependent on precise seminal quality which allows for reasonable estimations of field fertility with normal or low-dose inseminations (Fuerst-Waltl *et al*., 2006; Christensen *et al*., 2011 and Ahmed *et al*., 2016). The reproductive capabilities of bulls are of paramount importance which are largely influenced by one or more of the following factors: a) semen quantity and quality, b) libido and mating ability c) social interactions (dominance effects) in the breeding pasture (Chenoweth, 1983). According to the government of India's breeding programme, frozen semen from top native bulls to selectively breed and upgrade the nondescript bovine population should be used. Sahiwal contributes 4.2% of the total population of indigenous cattle (Breed Report, 2020). It is India's top native milch breed and provides a sizable amount of milk for the nation's milk supply (National Cattle Breeding Policy, 1999). The sire must have extremely high reproductive performance, including sexual activity and ejaculatory performance (Rehmann *et al*., 2016), both genetic and non-genetic factors have an impact on this. Since the genetic variables are inherited characteristics, they are challenging to control in a short amount of time. Yet non-genetic elements may be controlled and managed to

improve the quality of semen produced by bulls. The most important variables include THI, season, age, testicular size, etc. (Hirwa, 2017; Ahirwar *et al.*, 2018).

It is widely acknowledged that a bull's age at semen collection has an impact on the properties of the semen (Mathevon *et al*., 1998 and Brito *et al*., 2002). Peak ejaculate volume and sperm concentrations are reached at different ages in various breeds (Snoj *et al*., 2013). In the current era of genomic selection, there is an increased demand to collect semen from genomically selected sires at a young age which hastened the genetic progress by reducing the generation interval and increased the genetic gain (Goddard and Hayes, 2007; Murphy *et al*., 2018).However, the reproductive performance of young bulls varies greatly mainly due to the large variation in the age of onset of puberty among and within breeds (Barth and Waldner, 2002) Semen obtained from adult bulls is of higher quality than that obtained from young and elderly bulls (Ahmad *et al*., 2003; Brito *et al*., 2002 and Fuerst-Waltl *et al.,* 2006). When compared to younger bulls, older bulls had larger ejaculates and more concentrated sperm (Ahmad *et al*.,2011; Bhosrekar *et al*.,1992; Murugan and Raman 2003). It was noted by Nordin *et al*. (1990), and Javed *et al*. (2000) that mature bulls exhibit more mass activity than young and elderly bulls. On the other hand Ghosh (2004) reported that age of a bull had no significant effect on ejaculate volume, sperm motility and mass activity.

The percentage of normal sperms and major sperm defects are significantly affected by the age of bulls with more abnormal sperm in the young and old age (Coulter and Foote, 1979 and Vilakazi and Webb, 2004). The undeveloped size of the testis and the compromised thermoregulatory system may be the cause of the younger bulls' poor semen qualities and high aberrant spermatozoa levels. Moreover, DNA is more prone to fragmentation due to a lack of protamination (Westfalewicz *et al*., 2013). Semen quality deteriorate with age as a result of disintegration of bodily tissues, notably testicular tissues, fat accumulation that may occur in the scrotum and alterations in seminiferous tubule degeneration. Around the scrotum, a bull may begin to accumulate fat as it ages, by decreasing the scrotal ability to radiate heat, this may have an impact on the quality of the semen (Coe, 1999 and Brito *et al*., 2002).

Good libido and proper mating ability of a breeding bull are desirable traits for a successful artificial insemination (AI) program (Ahmed *et al*., 2005). The level of sexual excitement and performance can affect the ejaculatory performance and semen quality (Pound

et al., 2002; Levis and Reicks, 2005; Kondracki *et al*., 2013). These traits are predominantly influenced by the genetic makeup of an animal. A bull's libido is a useful indicator of reproductive ability since it is demonstrated by a bull's high sperm concentration, many ejaculates, and fertility (Ahmad *et al*., 2005). Reduced libido might result in higher reaction time and subsequently, total time for successful ejaculation also increases, thus having an ultimate effect on the production of sperms productivity and reproductive functions of the animal (Soderquist *et al*., 1996 ; Vogler *et al.,* 1991). By many ejaculates in a relatively short period of time, bulls with strong libido can generate a satisfactory larger quantity of viable spermatozoa. With age, sexual experience and copulatory experience increases. When the older bulls are subjected to semen collection on a regular basis, they get accustomed to the procedure and take minimal time during semen collection (Parkinson, 2004).

Libido and semen qualities are related with the scrotum size of the bull. Scrotal circumference is an indirect but reliable indicator of onset of puberty, semen quality, total sperm production, semen volume, sperm concentration, testicular pathology, testicular weight and fertility of bulls (Ahmad *et al*., 2005; Pant *et al*., 2003 and Koonjaenak *et al*., 2007). It is significantly correlated with age and body weight in animals (Coulter and Foote, 1977). Scrotal circumference, which is positively correlated with sperm morphology (Barth and Oko,1989) increases rapidly in younger bulls, gradually in mature bulls and tended to decrease in older bulls (Nikhil *et al*., 2022). It is an essential factor as it can describe the size of testis which is correlated with the semen production, the higher the size of the testis, the high will be semen production (Perumal, 2014). This is because more than 90 percent of the contents of testes are seminiferous tubules which are spermatozoa production sites (Pant *et al*., 2003; Susilawati *et al*., 2020).

In bulls, young age is disadvantageous as leads to poor quality semen, in mid-age, the age effect becomes positive, with bulls producing high grade/quality semen, before the bulls age effect becomes negative, causing the production of abnormal and sub-standard semen, so, there is an age window at which the bull produces its maximum. Though few attempts have been made to assess the effect of age on sexual behaviour, scrotal thermoregulation, scrotal circumference, semen quality and freezability of bulls, however, how age influences reproductive performance of Sahiwal bulls has not been studied. In view of the above explanation there is a need to address this in holistic manner in Sahiwal bulls, the current study

was therefore planned to investigate the effect of age on sexual behaviour, scrotal thermoregulation, scrotal circumference, semen quality and freezability of Sahiwal bulls with the following objectives-

- To study the effect of age on sexual behavior, scrotal circumference and testicular temperature of Sahiwal bulls
- To assess the effect of age on semen quality and freezability of Sahiwal bulls.

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

Artificial insemination is playing an important role for the improvement of future progeny of non-descript livestock breeds of India. Using AI, better germplasm is multiplied more quickly since one regular semen ejaculate can be utilised for several inseminations. The role of improved male germplasm in enhancing genetic progress and influencing herd fertility remains underutilized. AI is a crucial technique for the quick improvement in milk production. The quality status of the semen used, which is further dependent on several genetic and non-genetic variables, determines whether artificial insemination will be successful or not (Fuerst-Waltl *et al*., 2006). Age is a significant factor that influences the quality of semen since it directly impacts the bulls' libido and mating capacity, scrotal circumference, scrotal heat regulation system, brain fat deposition, and reproductive system health, all of which impact the quality and amount of semen produced.

With a view to funnel the existing information available on the topic, the review of literature is being presented under relevant segments as follows:

2.1 Effect of age on sexual behaviour

Sexual behaviour of bull can be differentiated into two components: libido (sex drive) and mating ability (Anderson, 1945). Libido is defined as the willingness and eagerness of a bull to attempt mount and service while mating ability refers to the ability and competence of the bull in fulfilling this aspiration (Chenoweth, 1983;Chenoweth, 2021). The motionless rump of a female or anything comparable is the single biggest stimulus for a bull to attempt mount and service (Chenoweth *et al*., 1996). Heifers mated with high serving capacity bulls had improved first cycle pregnancy rates as compared to heifers mated with low serving capability (Blockey, 1978). Age, rearing effects, ratio of bulls to females, social influences, and genetic variations all have an impact on a bull's libido.

A research on 12 Hereford bulls was carried out to study the sexual performance of bulls from 12 to 13 months of age up to 5 years of age. Ejaculation frequency, frequency of mounts without ejaculation, and the number of mounts that results in an ejaculation showed significant age effects ($P< 0.001$). The bulls showed more mounts, fewer ejaculations, and a

lower ejaculation-to-mount ratio as yearlings compared to years 2 through 5 (Price and Borgwardt, 1994). In order to determine the impact of age on sexual behavior, an investigation was conducted by Ahmad *et al*. (2005), 21 Sahiwal bulls were taken and divided into three age categories: group 1 (up to 3 years of age), group 2 (3-5 years of age), and group 3 (more than 5 years of age). Bulls in group 3 (3.28± 0.50) had significantly ($P<0.05$) longer reaction times (min) than bulls in group 1 (2.44± 0.29) and group 2 (2.04± 0.34). Also, the libido score was significantly different ($P<0.05$) in bulls of group 2, (3.83± 0.81), group 3(3.14± 0.42) and group 1(2.80± 0.21). Study taken by Rehmann *et al*. (2016) involves 4 Sahiwal bulls from three different age groups (1-3, 3-5 and above 5 years of age). Libido was measured in terms of time (seconds) taken for entering arena (P1), time (seconds) taken from mounting the teaser until erection (P2), reaction time (seconds) (P3) and number of times a bull mounted to yield semen (P4). The results showed that older animals above five years of age exhibited the best libido (P1, P2 and P4) compared to the younger bulls. The effect of age on sexual behavior of Nili-Ravi buffalo bulls was evaluated by Javed *et al*. (1998). They took 16 buffalo bulls in total and placed them into four age groups (year): 3–4, 6-7, 8–9, and 12–15. The total means for libido score and mating behavior score were, 2.60±0.6 and 4.8±1.0, respectively. In contrast to mating behavior, which was significantly different ($P< 0.05$) among age groups and was higher in bulls of 6-7 years of age (5.19±0.11), the libido score did not change significantly among age groups. The average gap between two ejaculates measured during the study was 10.8 ±8.1 minutes, and it was consistent across all age group.

2.2 Scrotal Circumference

Scrotal circumference (SC) is a physical parameter that is easily measured and known to select superior bulls. The size of the scrotal circumference reflects the bull's ability to reproduce through the quantity and quality of semen produced (Anwar and Jiyanto, 2019). In bulls, the size of the scrotum corresponds to the size of the testicles and the amount of testosterone produced (Dasrul *et al*., 2020). Also, it is a useful predictor of puberty in bulls and is moderate to highly heritable (Bourdon and Brinks, 1986).

A study was done by Ahmad *et al*. (2005) to assess the effect of age on SC of 21 Sahiwal bulls divided into 3 age groups-group-1 (< 3 years of age), group-2 (3-5 years of age) and group-3 (>5 years of age). The results showed that scrotal circumference differed

significantly (P<0.05) from each other and was largest in group-2. Shende *et al*. (2019) studied relationship of scrotal circumference with age in Murrah bulls and they were divided into 3 age groups (months) : 18 to 24, 25 to 36, 37 to 48. The scrotal circumference showed an increasing trend with age, it increased rapidly in young bulls and gradually in mature bulls. Influence of age on scrotal circumference was assessed in Nili-Ravi bulls by Javed *et al*. (1998). Sixteen buffalo bulls were grouped into 4 age groups (years): 3-4, 6-7, 8-9 and 12-15. The SC did not differ significantly among the age groups but was relatively higher in bulls of 12-15 and 6-7 years of age compared to other two groups.

Correlation of SC with semen quality was evaluated on six adult sexually mature Murrah bulls (Kumar and Srivastava, 2017). They found out that SC was significantly and positively correlated with sperm concentration (r=0.91) and concentration/ejaculate (r=0.63). To understand relationship between scrotal circumference and semen volume, sperm concentration and number of sperm per ejaculate, 12 crossbred bulls [8 Local × Friesian, 4 Local × Sahiwal] were taken by Latif *et al*. (2009). A significant (P<0.04) positive correlation (r = 0.72) was observed between scrotal circumference and volume of semen, and between scrotal circumference and number of sperm production per ejaculate. They interpreted those crossbred bulls aged 18 months or over, with scrotal circumference more than 30 cm, yielded good quality semen. Similarly Pant *et al*. (2003) concluded in their study that there is a significant positive relationship between SC and semen volume, sperm concentration per ejaculate and total semen doses per collection. Wahyudi *et al*. (2022) conducted a meta-analysis review (38 articles) to determine the relationship between scrotal circumference and semen quality in bulls. Data analysis was performed using the CMA program using a random-effects model to estimate the correlation coefficient. The results of the meta-analysis showed that scrotal circumference was correlated ($p < 0.05$) with semen volume ($r = 0.44$), semen concentration ($r = 0.32$), and sperm motility ($r = 0.41$).

Effect of SC on semen production traits in 77 Murrah bull was examined by Sivaselvan *et al*. (2022). The SC was classified into 4 groups -<30 cm, 30 to 33 cm, 33 to 36 cm and >36 cm. Semen volume, total sperm per ejaculate, mass activity, initial motility and frozen semen doses per ejaculate showed an increasing pattern with increase in SC. The highest sperm concentration (million/ml) was found in the 30 to 33 cm group (1472.74±15.15) while lowest in <30 cm group (1210.68±20.07). Highest post thaw motility (%) in range of 30 to 36 cm

(52.44) with lowest in <30 cm group (51.93). The results indicated that bulls with larger scrotal circumference manifested better seminal values and a higher number of frozen semen doses.

2.3 Testicular Temperature Using Infrared thermography

Infrared thermography is a non-invasive remote sensing method used for taking small changes in body temperature. Thus, it can be used to measure scrotal surface temperatures and thereby helps in making interpretations regarding testicular temperature. It produces colour images corresponding to different temperature ranges.

Ahirwar *et al*. (2018) carried out an experiment to study the influence of season, age and management on scrotal thermal profile and ocular temperature in Murrah bulls using scrotal infrared digital thermography. Data of 109 bulls were used for the study. The age of bulls was classified into two groups, less than 4 years and more than 4 years. The temperature (ºC) for scrotal proximal pole, medial pole, distal pole, temperature gradient and ocular temperature of bulls <4 years of age and >4 years of age were taken. The results revealed that the age of the bulls had non-significant effect on scrotal surface temperature and ocular temperature.

2.4 Effect of bull's age on semen quality

2.4.1 Semen Volume

In a study conducted at NDRI (Karnal), Bhakat *et al.* (2011) collected and analyzed data on 46 Sahiwal bulls from 1996 to 2006.They observed the effect of age on semen volume and divided the bulls into four age groups: A1= <3 years, A2=≥ 3 to 4 years, A3= ≥4 to 5 years, and A4= ≥5 years. They discovered that the volume of the ejaculate significantly (P< 0.01) increased with the age of the bulls but decreased again in older age group. They concluded that semen production in Sahiwal bulls peaks between the age of 4 to 5 years and then starts to fall, probably as a result of the commencement of senile alterations. Fifty three Murrah buffalo bulls' data from 1996 to 2000 were examined by Ravimurgan and Sindhu (2020) to determine the impact of age on semen volume. Less than 36, 36 to 48, 48 to 60, 60 to 72, 72 to 84, 84 to 96, and over 96 months were the age categories for the bulls. It was found that the volume of semen (ml) gradually increased as the age of the bulls increases. The

lowest volume of semen was produced by bulls younger than 36 months (2.58±0.04), and the highest volume was produced by bulls older than 96 months (3.49±0.10). There was no significant increase in the volume of semen among the age groups of 36 to 48, 48 to 60, 60 to 72 and 72 to 84 months. The Bos taurus breeds (Holstein breeds, Brown swiss, Limousin, and Charolais) were the subject of a study by Snoj *et al.* (2013) to ascertain the effect of age on semen quality and quantity. They conducted an analysis of 71,983 ejaculates throughout a 31 year span of data collecting. Seven age categories for bulls were established: 12 to 24, 25 to 36, 37 to 48, 49 to 60, 61 to 72, 73 to 84 and more than 84 months. The mean ejaculate volume increased significantly with age and decreased moderately in Brown Swiss bulls after 84 months of age, as well as in Limousin and Choralais at the age of 73 to 84 months. A study conducted by Javed *et al*. (2000) examined the impact of age on the quantity and quality of semen in 16 Nili-Ravi Buffalo bulls. The bulls were classified into four age groups, three of which were considered healthy (animals younger than 5, 6-10 years old, and older than 11 years), and one of which was considered abnormal (bulls aged 6 to 10 year with poor semen quality). Although there was no statistically significant difference in semen volume (ml) among the age groups, adult bulls had the highest volume (4.96 ±0.14) followed by elderly bulls (4.77± 0.13).

2.4.2 Mass Motility

In order to find out the impact of age on semen quality, Ahmad *et al*. (2003) studied 21 Sahiwal bulls categorised into three age groups: group 1 (under 3 years old), group 2 (between 3 and 5 years old), and group 3 (beyond 5 years old). The average mass activity was 2.61±0.04 and it did not differ significantly among the age groups. Bhakat *et al*. (2011) at NDRI, Karnal, collected and analyzed data from 1996 to 2006 to assess the effect of age on semen quality. 46 Sahiwal bulls were kept into 4 age groups: A1= <3 years, A2= ≥3 to 4 years, A3=≥ 4 to 5 years, and A4= ≥5 years. The results revealed that the mass activity of semen significantly increased as the bull matured but then again decreased for older age groups (A3 and A4). Bulls in the A2 age group exhibited the highest level of mass activity, while those in the A1 and A3 age groups exhibited the lowest levels. The effect of age on semen quality was examined in a study conducted by Javed *et al*. (2000) using 16 Nili-Ravi Buffalo bulls. The bulls were split into four age groups: three of them were considered healthy (bulls less than 5, 6-10 years old, and more than 11 years old), and one was considered abnormal (bulls aged 6

to 10 with poor semen quality). The overall mass activity score was 2.65±1.03 and was lower in bulls older than 11 (2.28±0.09), although it was lower in the abnormal (1.60±0.10) than in healthy categories. Younis *et al*. (1998) studied the semen quality of young, adult and old buffalo bulls during low and peak breeding seasons.18 Nili- Ravi buffalo bulls were divided into 3 age groups (years)- young (3-4), adult (6-8) and old (12-15) with 6 bulls in each group. The mass activity of ejaculates from young, adult and old bulls was 1.93±0.13, 2.18±0.11 and 1.53±0.10 and differed significantly from one another ($P<0.05$).

2.4.3 Progressive motility

Ahmad *et al*. (2003) in a study conducted on Sahiwal bulls to investigate the effects of age on semen quality. 21 Sahiwal bulls were divided into 3 age groups- group-1 (up to 3 years), group-2 (3-5 years) and group-3 (> 5 years of age). The motility (%) was significantly higher in group-2 (65.71 ±0.34) as compared to other two groups. Asadi *et al*. (2021) carried out a study on semen characteristics and physiological changes in the testis of Jonobi bull breed, aged 2-4 years. They found that the progressive motility significantly increased with the age. Bhakat *et al*. (2011) collected and analyzed data from 1996 to 2006 to assess the effect of age on semen quality. 46 Sahiwal bulls were kept into 4 age groups: A1= <3, A2= ≥3 to 4, A3=≥ 4 to 5, and A4= ≥5 years. Percentage of progressive motile sperm was 53.99±0.05, 56.16±0.002, 54.88±0.004 and 56.85±0.005 for the four age groups, respectively. It was significantly different among the age groups and highest in A1 and A2 groups. Data on the quantity and quality of 38 Gir bulls' frozen semen were analyzed to study how age affected the characteristics of semen quality (Bhave *et al.,* 2020). The age categories (months) created were->48≤ 60, >60 ≤72,>72≤84,>84≤ 96,>96 ≤108,>108 ≤120,>120≤132,>132≤144 and>144≤156. The trend of initial motility was inconsistent but showed a slight decreasing trend with age. The influence of age on cryopreserved samples of Nellore bulls was investigated by Carreira *et al*. (2017). Three age divisions were chosen for the 44 bulls: Bulls ranged in age from 1.8 to 2 years for young, 3.5 to 7 years for adults, and 8 to 14.3 years for older bulls. The progressive motility were significantly higher in young bulls (36.93±11.28) as compared to adult (23.13±8.65) and aged (22.12±11.64). 16 Nili-Ravi Buffalo bulls were studied by Javed *et al*., 2000 to saw the effect of age and season on semen quantity and quality. The bulls were divided into 4 age groups, including three healthy (less than 5, 6-10 and more than 11-year-old animals) and one abnormal group (6–10- year-old bulls having poor semen

quality). The overall progressive motility was 56.89±0.65 % and showed no difference in healthy groups, but was lower in the abnormal group (43.23±1.35). Rehmann *et al.* (2016) assess the effect of age on semen characteristics. For the study, four mature cattle bulls from each of Friesian, Jersey, Achai, Cross (Friesian x Sahiwal) and Sahiwal in different age (1-3, 3-5 and above 5 years) were selected. For age grouping, data of the entire breed was pooled. No significant difference was observed in the 3 age groups for motility %.

2.4.4 Sperm Concentration

Six adult Cholistani breeding bulls, aged 5 to 11 years, were used in a study by Mahmood *et al.* (2014) to examine the relationship between age and semen quality. They discovered a negative association between bull's age and sperm concentration (r = -0.943). Similarly negative correlation was found between age and sperm concentration in 38 Gir bulls where the age ranges from 48 months to 156 months by Bhave *et al*. (2020). 71,983 ejaculates over the course of a 31-years period from bulls of four distinct breeds—Holstein, Brown Swiss, Limousin, and Charolais—were examined by Snoj *et al*. (2013). 12-24, 25-36, 37-48, 49-60, 61-72, 73-84, and >84 were the created age groups (months). Analysis was done on semen concentration in relation to the bulls' age. Animals less than 24 months of age had the lowest sperm concentration as compared to other groups. It's interesting to note that different breeds of animals showed higher sperm concentrations at different ages. It was noted in Brown Swiss and Choralis bulls between 73 and 84 months of age, in Limousin bulls between 61 and 72 months, and in Holstein bulls over 84 months of age. Age-related changes in Jersey bulls' semen quality were examined by Sankhi *et al*. (2019), nine bulls, ranging in age from 3 to 4, 5-7, and 8 to 9 years, were chosen for this study. The bulls with the highest sperm concentration (10^9/ml) were between the ages of 5-7 and 8–9 years.

Ahmad *et al*. (2003) in their study on 21 sahiwal bulls categorized into 3 age groups- upto 5, 3-5 and > 5 years of age. They did not find any significant difference among the age groups. Bhakat *et al*. (2011) collected and analyzed semen data from 1996-2006 of 46 Sahiwal bulls classified into 4 age groups- into A1= <3, A2= ≥3 to <4, A3= ≥4 to <5 and A4= ≥5 years. They also did not find any significant age effect on sperm concentration. Similarly no significant effect of age on sperm concentration in 12 Sahiwal bulls classified into 3 age groups of 1-3, 3-5 and above 5 years (Rehmann *et al*., 2016).

2.4.5 Hypo-Osmotic Swelling Test

Bhave *et al*. (2020) analysed data on frozen semen quantity and quality of 38 Gir bulls, available from January 2011 to December 2018 at BAIF's frozen semen station, Jind, Haryana. The effect of age on semen quality traits was studied. The age groups (months) formed were >48≤ 60, >60 ≤72,>72≤84,>84≤ 96,>96 ≤108,>108 ≤120,>120≤132,>132≤144 and>144≤156. There was a gradual increase in HOST reactive sperm up to 108 months of age after which it declined with the lowest values at 120 months of age. Age impact on semen qualities was examined in a study on Sahiwal bulls in three different age categories (1-3, 3-5, and over 5 years) (Rehmann *et al*., 2016) where the membrane integrity was significantly higher in animals older than 5 years of age (74.00±1.40) and significantly lower in animals younger than 3 years (69.79±0.94). Rafiq *et al*. (2022) carried out a study on Murrah bulls divided into 3 age groups (<3, 3-8,>8 years) to saw the effect of age on plasma membrane integrity with HOST. For fresh semen the lowest HOST value was observed for young age group while no significant difference was observed between the adult and old age group and for frozen semen significantly high value was observed for adult group, while no significant difference was found between the young and old groups.

2.4.6 Viability

In a study on the effect of age on semen characteristics and physiological alterations in the testes of Jonobi bull breed animals aged 2-4 years, results showed a significant increase in the live sperms and a decrease in the dead sperm percentage in the semen of bulls of 4 years old in comparison with 2 and 3 years old bulls (Asadi *et al*. , 2021).

Mahmood *et al.* (2014) conducted a study on 6 adult Cholistani breeding bulls, aged 5-11 years to assess correlation between age and semen viability where they found that age of bull showed negative correlation with viability (r =-0.873). Sankhi *et al*. (2019) found highest percentage of live sperm from 5-7 years age group (55.48±0.05) and lowest in 8-9 years of age (45.62±0.13) in their experiment on 9 Jersey bulls categorized into 3 age groups (3-4,5-7 and 8-9 years). Effect of age in viability revealed that highest viability (%) was found in the adult age group (3-8 year) followed by old (>8year) and young age group (<3 year) in fresh as well as frozen semen by Rafiq *et al*. (2022). For the effect of age on viability in Sahiwal bulls in 3 different age groups (1-3, 3-5, and over 5 years), no significant difference was found (Rehmann *et al*., 2016).

2.4.7 Acrosome Integrity

Mahmood *et al*. (2014) conducted a study on 6 adult Cholistani breeding bulls, aged 5-11 years to assess correlation between age and sperm acrosome integrity where they found out that age of bull showed negative correlation with acrosome integrity (r = -0.813). On the contrary, Bhave *et al*. (2020) observed a gradual improvement in acrosome integrity value with the increasing age groups in Gir bulls. Carreira *et al.* (2017) studied the effect of age on cryopreserved samples of Nellore bulls. 44 bulls were divided into 3 age groups : 1.8 to 2 years-young bulls,.3.5 to 7 years -adult bulls and 8 to 14.3 years-aged bulls. The acrosome integrity values were higher for young group (54.70± 9.49) followed by adult (46.68±10.13) and aged groups (37.53 ±9.84). Opposite results were found by Rafiq *et al*. (2022) where the lowest acrosome integrity values were found in the younger age group (<3 years) in fresh as well as frozen sample as compared to the adult (3-8 years) and old (> 8 years), while no significant difference was found in the adult and old age groups. On the other hand no significant difference was found for the effect of age on acrosome integrity in Sahiwal bulls (Abdullah, 2016) and Nili-Ravi buffalo bulls (Ahmad *et al.*, 2018).

2.4.8 Post thaw motility

Mandal *et al*. (2021) studied the effect of age on semen parameters in crossbred dairy bulls whose age ranged from 15 to 93 months. Age was divided into 5 brackets: ≤ 24 m, 25–36 m, 37–48 m, 49–60 m and > 60 m keeping 1-year interval. The results revealed that post thaw motility progressively increased with age. Highest post thaw motility was observed in the middle age group (5-7 years) in Jersey bulls as compared to the other two groups ; 3-4 and 8-9 years of age (Sankhi *et al*. 2019). Similarly, Rafiq *et al.* (2022) also observed significantly high post thaw motility in 3-8 years as compared to <3 and >8 years in Murrah bulls. Ahmad *et al*. (2003) and Bhave *et al*. (2020) in their studies did not find significant difference between the age groups for the effect of age on post thaw sperm motility in Sahiwal and Gir bulls, respectively

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

3.1 Location of the study

The study was conducted at Artificial Breeding Research Centre (ABRC) of ICAR-National Dairy Research Institute (NDRI), Karnal (Haryana) located at 29.43ºN latitude and 72.2º E longitude at an altitude of 250 meters above mean sea level. In summer, the maximum ambient temperature reaches to 45°C while in winters minimum temperature goes as low as 2°C with diurnal variation of 15-20°C. The region receives annual rainfall of 700 mm, mostly during the months of early July to mid-September. Relative humidity ranges from 41 to 85 percent.

3.2 Experimental animals

To study the effect of age on sexual behaviour, scrotal thermoregulation, scrotal circumference, fresh and frozen semen quality, fifteen Sahiwal bulls were used. The age of bulls ranged from 2-12 years of age. They were divided into three age categories: 2–3.5 years (G1), >3.5–8 years (G2), and 8–12 years (G3). The bulls selected for the research were healthy, disease-free, and had normal clinical conditions and kept at the institute's Artificial Breeding Research Centre. For the duration of the trial, the feeding and care practices for the bulls remained same. Two times each week, the bulls' semen was collected in the early morning hours. Two ejaculates were taken simultaneously after an interval of 15 to 30 minutes. All the experimental techniques and animal experimentation methodologies were approved by the Institutional animal ethics committee.

3.3 Duration of Experiment

The animals were selected and put into the experiment in the month of January, 2023 and the parameters were taken from February, 2023 to May, 2023**.**

3.4 Housing and management of bulls

Bulls were kept in individual pen (30’x 10’) under loose housing system on concrete floor with the orientation of east-west direction along its long axis. The pens were separated by solid partitions wall that restricted both direct physical and visual contact of bulls in adjacent pens. One third of the area was covered with sheet and rest was kept open. The sheds

were cleaned once daily in the morning. The bulls were fed as per ICAR feeding standard for bulls. They were offered concentrate ration (with 21% CP and 70% TDN) in the morning daily and seasonal chaffed green fodder depending on the availability was fed adlib to the animal. Bulls were having free access to clean drinking water round the clock. They were given exercise for about one hour the day prior to semen collection in the rotatory bull exerciser so as to maintain the physical health and sexual vigour of the bulls and to ensure quality semen production. Vaccination, deworming and other herd-health programme were followed as per the center schedule to ensure good health. The bulls were thoroughly washed and groomed at least half an hour before semen collection.

3.5 Cleaning and sterilization of equipment and glass wares

All the glass wares were washed and cleaned with running tap water and soaked in warm neutral detergent for at least 30 minutes. Thereafter, the items were thoroughly cleaned under running tap water using a brush. The materials were rinsed thoroughly with double distilled water to completely remove detergent residue and other impurities. After cleaning, the glass wares were dried by keeping them inverted on blotting paper. Dry glassware was wrapped in aluminium foil and then covered in clean paper before being sterilized in a hot air oven at 160 º C for 1 hour.

Rubber wares, artificial vagina (AV), buffer solutions, plastic tips, filter papers and distilled water and other such materials were sterilized by autoclaving at 15 lbs pressure at 121 º C for 20-30 minutes. After sterilization, the AV was stored in an incubator at 45 º C until their use.

3.6 Objective 1: To study the effect of age on sexual behaviour, scrotal circumference and testicular temperature of Sahiwal bulls.

3.6.1 Sexual behaviour of Sahiwal bulls

Sexual behaviour was recorded at the time of semen collection at 15 days interval. Dummy bulls were used for semen collection of Sahiwal bulls. Different dummy bulls were used on different days to minimize sexual satiation of bull from the same dummy, to provide uniform stimulus pressure and randomize dummy effects. Semen was collected twice a week as per semen collection schedule. Each bull was assigned to be handled by two experienced handlers who were familiar with the bulls. On the day of semen collection, bulls were taken

to the collection yard, where two bulls were kept as dummy. Bulls were led to a dummy and freely permitted to mount and service an artificial vagina. Each bull was sexually stimulated and two false mounts were given before semen collection. The sexual behaviour scoring was adapted as described by Anzar *et al*. (1993). The parameters recorded were-

- Time taken for entering the arena from bull preparation site (sec)-the time in which bull will come from its preparation site to the mounting site. It will show the eagerness of the bull to mount.
- Time from mounting the dummy until penile erection (sec)-time taken from mounting the dummy to the erection of penis.
- Reaction time (Sec)- It is the time taken by bull from exposure to dummy until mounting.
- Sexual aggressiveness (SA)- It is the behaviour of a bull during approach towards the dummy. It was assessed visually, and the bulls were classified as:

I. Aggressive-uncontrollable, extremely eager to mount and approached dummy with full vigour.

II. Active- approached dummy with less vigour and aggression

III. Dull-proceeded with a dull expression and took a longer time to mount than their counterparts.

IV. Shy-exhibited mild sexual interest and was reluctant to mount.

Table 3.1: Reaction time and sexual aggressiveness score for bulls

RT (sec)	Score	SA	Score
<5	6	Aggressive	4
6-15	5	Active	3
16-30	4	Dull	2
31-60	3	Shy	1
61-120	2		
121-300	1		
>300	0 (Refusal to mount)		

- Libido Score-it is calculated by using the following formula-

  Libido Score = [{(RT Score +SA Score) – 0.2 per TS}/ 10] X100

  RT - Reaction time

  SA - Sexual aggressiveness

  TS - Tactile stimulation

Tactile stimulation score -after approaching the teaser the bull exhibited certain behavioural characteristics. It consists of sniffing, bunting, licking, chin-resting, flehmen reaction, licking of sheath and urination, etc. To calculate libido score, for each tactile stimulation expression 0.2 was deducted from the total score obtained for reaction time and sexual aggressiveness. If a bull did not mount on the first attempt, then the dummy was changed. If bull did not mount the second dummy in the prescribed time (5 min) then a refusal to mount designation was noted and a 0 score was given. Reaction time and sexual aggressiveness of only successful attempt was used for libido scoring.

No of times a bull mounted to ejaculate semen-the total mounts on the dummy by the bull to ejaculate the semen.

3.6.2 Scrotal circumference

Scrotal circumference was measured as per method recommended by the Society of Theriogenology (Ball *et al*., 1983) at monthly interval. The testes were first retracted into the lower part of the scrotum for measurement of scrotal circumference. To prevent separation of the two testes, the thumb and the fingers were placed on the sides rather than on the front or back of the scrotum. Then a measuring tape (scrotal tape) was looped and placed around the greatest diameter of the scrotum and pulled snugly so that the tape was firmly in contact with the entire circumference. Scrotal circumference was measured in cm.

3.6.3 Testicular temperature

The temperature (ºC) of scrotal surface of bulls was taken by the digital IRT camera (Darvi) from a distance of 1 meter. Before taking images the scrotal area was made free from dung and manure. The thermal profile of the scrotum was measured by positioning the camera perpendicular to the scrotum. At three different points of the scrotum i.e., upper surface

/proximal pole temperature (PPT), mid surface /mid pole temperature (MPT) and lower surface/distal pole temperature (DPT) were taken for estimation of scrotal surface temperature (Menegassi *et al*., 2015). Scrotal surface temperature gradient was calculated as differences between PPT and DPT. IRT images of best quality in terms of focus and resolution were selected for analysis. A constant area of sharp and focused IRT images of particular points were selected, interpreted and analysed by Darvi tools analysis software and an average temperature of each point was taken. The IRT images were taken at monthly interval.

3.7 Objective 2: To study the effect of age on semen quality and freezability of Sahiwal bulls

The semen was collected twice a week using bull specific Artificial Vagina (IMV Technologies, France) method, with standard semen collection procedure. Semen collection was performed by well trained and experienced persons at Artificial Breeding Research Centre, NDRI, Karnal. The bulls were thoroughly washed, cleaned and dried at least 15-30 minutes before collection. The temperature of AV was maintained between 42-45 º C with sufficient pressure. A small quantity of sterilized vaseline jelly was used as a lubricant. For semen collection, a Sahiwal bull was used as a dummy. The bulls were given at least two false mounts before allowing to serve AV. Two collections were taken from a bull at an interval of atleast 15 to 30 minutes. Semen was collected in the morning hours beginning at 7:30 AM. During collection, AV was held at an angle of 55º. The semen quality was assessed at 15 days interval.

3.7.1 Assessment of semen quality

Immediately after collection, the ejaculate was brought in the laboratory and kept in water bath at 32 ºC for assessing volume, colour, mass activity, individual motility, sperm concentration, non-eosinophilic (live), hypo-osmotic swelling test, acrosome reaction and subsequent experimentation.

3.7.1.1 Ejaculate Volume

The volume of semen ejaculates was measured directly from the graduated semen collecting tube (15 mL) having 0.1 mL calibration.

3.7.1.2 Mass activity

Mass activity was evaluated as per method described by Tomar *et al*. (1966). A drop of neat semen was placed on clean, grease free slide without applying coverslip mounted on a thermal stage maintained at 37 ºC and examined under phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) at 10 X. Mass activity score was noted based on swirls activity of semen. The presence of waves and eddies throughout the whole drop was observed and based on the intensity of waves and eddies, the ejaculates were graded on a numerical scale of 0 to 5. The corresponding activity and the score are tabulated below:

3.2: Mass activity score of freshly collected semen

Semen Activity Characteristic	Mass motility score (0 to + 5 score)
Rapid waves and swirls	+5
Less rapid swirls and eddies	+4
Swirls are slowly scattered in the field	+3
Swirls and individual movement of spermatozoa are more evident from the field	+2
No wave motion observed	+1
Spermatozoa are immobile, no motion	0

3.7.1.3 Individual sperm motility

The motility was recorded as a percentage of progressively motile spermatozoa in the extended semen. This was assessed by placing a drop (6-8 µL) of diluted semen (diluted with Tris egg yolk extender) on a clean, grease free slide mounted on a stage maintained at 37 ºC and observed under phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) at 20 X after covering with a cover slip. Sperms in the slide were examined in atleast five randomly chosen microscopic areas. Percent progressive motility got assessed by two persons and its average was taken under a scale of 0-100 %, was calculated by taking the mean of progressively motile sperms in all areas.

3.7.1.4 Sperm concentration

The concentration of spermatozoa (in millions /mL) in the fresh semen was determined by a photometer (IMV, L'Aigle, France). For taking sperm concentration with photometer, 40 µL of neat semen was diluted with 3960 µL of 0.9 % sodium chloride (1:100). The optical density (OD) is determined, processed according to the dilution rate and the final concentration is expressed in millions of spermatozoa per millilitre.

3.7.1.5 Sperm viability

The method described by Bloom (1950) was followed for counting live and dead spermatozoa. Eosin-Nigrosine stain was used to determine live and dead sperm count. Dead spermatozoa can be differentiated by their ability to get stained by Eosin dye. The live spermatozoa remain colourless as they are impermeable to the Eosin stain. Nigrosine provides a blue-black background. It tells about the structural integrity of sperm cells.

Preparation of Stain

Eosin-Nigrosine was prepared by the method described by Campbell *et al*. (1953). The composition of the stain is as follows:

Table 3.3 Composition of Eosin-nigrosine stain

Eosin -Y (Water soluble)	10 gm
Nigrosine (Water soluble)	5gm
Sodium citrate buffer (2.9%, pH =6.8)	100 ml

Both solutions were prepared separately in sodium citrate buffer, boiled and cooled. Eosin and Nigrosine were mixed in the ratio of 1:4 and the mixture was kept overnight and on the next day, it was filtered through filter paper (Whatman filter paper no. 40) and stored in a dark and sealed bottle. The staining solution was brought to room temperature before using for staining. The fresh stain was prepared every 15 days to prevent precipitation.

Staining procedure:

I. A drop of semen (small for fresh semen and large for frozen-thawed semen) is taken on a clean, grease-free pre-warmed slide.

II. Eosin-Nigrosine (3-4 drops) stain, maintained at 37 ºC is placed near the semen drop.

III. Semen and stain are mixed gently using 200 µL tips and are left for 2 minutes at 32-34ºC on a warm stage.

IV. After 2 minutes, a smear is made on a clean, grease -free glass slide. The smear is allowed to dry and examined under the oil immersion objective (100 X).

V. A total of 200 spermatozoa are counted in a slide and classified as non-eosinophilic or live (unstained /colourless) and eosinophilic or dead (stained or pink).

3.7.1.6 Sperm acrosome integrity

Acrosome integrity was evaluated by using Giemsa staining method described by Hancock, 1952 and Watson, 1975. Percentage of the intact acrosome was calculated as number of acrosome-stained spermatozoa to the total number of sperms counted.

Preparation of solutions and stains

Preparation of Giemsa solution

Table 3.4: Composition of Giemsa stain (stock solution)

Chemical	Quantity
Giemsa stain	3.8g
Absolute alcohol (GR grade)	375 mL
Glycerol	125mL

Giemsa stain was grounded with absolute methanol in a pestle and mortar. To this glycerol was added. Stain mixture was stored at 37º C for one week. During this storage period, it was shaken for few minutes each day. After 7 days the stain was filtered through Whatman filter paper No.40 and stored in a dark and sealed glass bottle for further use.

Preparation of fixative

Table 3.5: Fixative for Giemsa staining

Chemical	Quantity
Sodium Chloride	10g
Sodium bicarbonate	0.5g
Formalin	125 mL
Distilled Water	Upto 1000 mL

Preparation of Sorenson's phosphate buffer

Table 3.6: Composition of Sorenson's phosphate buffer

Solution A 1.Sodium phosphate dibasic (Na2HPO4.2H2O) 2.Distilled water	11.87 g Upto 1000 mL
Solution B 1.Potassium phosphate monobasic (KH2PO4.2H2O) 2.Distilled water	9.08 g Upto 1000 mL

Sorenson phosphate buffer was prepared by adding 33 mL of solution A and 17 mL of solution B.

Preparation of working Giemsa Solution

On the day of staining Giemsa Stock Solution was diluted to final concentration of 10 % (3 mL) in 0.1 M Sorenson's buffer (2 mL) and 35 mL triple distilled water and final volume of 40 mL was made in a coplin jar.

Staining procedure

A thin smear of semen was prepared on a clean, grease -free dry slide. The smear was air-dried at room temperature for atleast 10 minutes and was fixed by immersing in fixative solution for 25-30 minutes in a coplin jar. It was washed in running tap water then with distilled water and air dried. Again, the slide was immersed in coplin jar having buffered Giemsa solution for 90 minutes and rinsed in distilled water and dried. Then dried smear was examined at 100 X oil immersion objective. After staining about 200 spermatozoa were counted for acrosome status. Giemsa stains the membrane proteins so the intact acrosome appears dark purple in colour.

3.7.1.7 Sperm plasma membrane integrity

Functional membrane integrity of spermatozoa was evaluated by hypo-osmotic swelling test HOST (Jeyendran *et al.* (1984)) and (Correa and Zavos, (1994)). The HOST was performed to assess the functional integrity of the sperm tail membrane in vitro. Hypo-osmotic solution (150 mOsmol/l) and control isotonic solution (300mOsmol/l) was prepared as follows:

Table 3.7: Composition of HOST and control solution

Chemicals	HOST solution	Control solution
Sodium citrate (g)	0.735	2.94
Fructose (g)	1.351	5.40
Millipore water upto (mL)	100	100
Osmolarity(mOsm/L)	150	300

Procedure:

One mL of hypo-osmotic solution, having an osmotic strength of 150 mOsm/Kg was mixed with 0.1 mL of semen and incubated at 37ºC for one hour. Following incubation, a drop of well -mixed solution was taken on a clean, dry glass slide and covered with a coverslip. Sperm tail curling was recorded as an effect of swelling due to the influx of water. A total of

about 200 spermatozoa were counted in different fields at 40 X magnification under phase contrast microscope. The total proportion of swollen spermatozoa was calculated by dividing the number of reacted cells by the total spermatozoa counted in the same area and multiplying the figure by 100.The proportion of swollen spermatozoa from a control sample was subtracted from HOST value. These spermatozoa were classified into four different classes according to the presence of following swelling pattern.

Observations/pattern:

a. No swelling, no membrane reaction

b. Swelling of the tip of the tail

c. Different type of hairpin-like swelling pattern

d. Complete tail swelling

Spermatozoa displaying B, C or D were considered positive for the HOST test.

3.7.2 Preparation of extender for cryopreservation

The extender is always prepared fresh, generally an hour before collection of semen so that the medium gets stabilized. Fresh eggs are procured from the market.

Table 3.8 Composition of semen extender

Buffer solution	**For 100 ml**
Tris	2.422 g
Citric acid (monohydrate)	1.36 g
Fructose (anhydrous)	1.25 g
Millipore water	60 Ml
Streptomycin	0.1 g
Benzyl penicillin	100,000 IU
Make upto	73.6 mL

The pH of the dilutor is adjusted to 6.8 with 10% NAOH.

Final Extender		
Buffer solution	73.6 mL	184 mL
Egg yolk	20 mL	50 mL
Glycerol	6.4 mL	16 mL
Total	100 mL	250 mL

3.7.3 Cryopreservation of Semen

The processing method for semen freezing is described below-

After evaluation and dilution rate the fresh semen was extended to 80 million spermatozoa/ml so that final number of sperms /straws can be 20 million.

Filling and sealing of semen straws: French Top Bull mini straws (0.25 ml,135 mm length and 2mm diameter, IMV) of orange colour were used. Automatic straw filling and sealing machine (IMV, France) was used for filling of semen into the straws and sealing it. Filling and sealing of straws was done at room temperature.

Equilibration time: The filled and sealed straws were kept on freezing racks and placed in cold handling cabinet where the temperature of straws was brought to 4 º C within 1-1.5 hours. Following achieving this temp, the straws were then kept for 4 hours.

After completion of equilibration time, the freezing rack along with spreaded straws were kept in the liquid nitrogen vapor freezing chamber of biological freezer. The temperature of semen straws reaches from 4ºC to - 140º C within 7 minutes.

Storage: The straws were then transferred into goblets with the help of cryo-gloved hand and the goblets were stored in separate canisters in the liquid nitrogen (LN2) in cryovessel. Cryovesssel was always kept to desired level of LN2 by replenishing it time to time.

Thawing of frozen semen: The frozen semen was thawed in thawing unit (IMV, France) maintained at 37º C with holding time kept as 45 seconds. The thawed semen samples were subjected to different tests i.e., progressive motility, percent live spermatozoa, HOST test and acrosome integrity etc.

3.8 Statistical analysis

The data obtained from experiments were subjected to statistical analysis by one way analysis of variance (ANOVA) using SPSS software version 26 and means were compared using Duncan's multiple range test to draw scientific interferences.

CHAPTER –4

Results and Discussion

RESULTS AND DISCUSSION

4.1 Objective 1: To study the effect of age on sexual behaviour, scrotal circumference and testicular temperature of Sahiwal bulls

4.1.1 Sexual behaviour

The results of sexual behaviour in three different age groups of Sahiwal bulls are presented in table 4.1.1 and graph 4.1.1-4.1.6.

Time to enter arena from preparation site (sec)-

The mean time (sec) taken by the bulls to come to the arena from preparation site was 2.77±0.19, 5.06±0.27 and 5.23±0.20 in G1, G2 and G3 groups, respectively. It was significantly ($p<0.05$) lower in G1 group as compared to G2 and G3 age group while it did not vary between G2 and G3 groups. The results of this trial are different from Rehmann *et al*. (2016), they found that the time taken to reach the arena from preparation site was significantly lower in old age group as compared to young and adult age group while it did not differ between the young and adult age groups. This might be due to the playful behaviour of the bulls of G1 group. Also the bulls of G2 and G3 group are more experienced so they always come to the arena calmly.

Erection time (sec)-

The erection time (sec) for G1, G2 and G3 age groups was 2.88±0.22, 2.23±0.15 and 2.46 ± 0.16, respectively. It was significantly ($p<0.05$) lower in G2 group bulls followed by G3 and G1. Rehmann *et al*. (2016) found insignificant difference between the three age groups. Testosterone modulates nearly every component involved in erectile function, from pelvic ganglions to smooth muscle and the endothelial cells of the corpora cavernosa. It also regulates the timing of the erectile process as a function of sexual desire, coordinating penile erection with sexual activity.

Reaction time and reaction time score-

The reaction time (sec) for G1, G2 and G3 age groups was 158.33±23.38, 73.90±11.59 and 141.20 ±20.62, respectively. It was significantly ($p<0.05$) lower in G2 group as compared

to G1 and G3 groups whereas no significant difference ($p<0.05$) was found between G1 and G3 groups. Ahmad and Asmat (2005) in Sahiwal bulls found significantly longer reaction time in old age group bulls as compared to young and adult age group but no difference in young and adult age group whereas Rehmann *et al*. (2016) found a shorter reaction time in old age group and it did not differ between young and adult age groups bulls. According to them, the older bulls are exposed to repeated experience of semen collection; therefore, they become habitual to the process and take little time during semen collection.

The reaction time score (0-6 scale) for G1, G2 and G3 age groups was 1.66±0.24, 2.96±0.29 and 2.33±0.30, respectively. It depends on reaction time. It was higher in G2 group followed by G3 and G1 group.

Number of mountings-

Total number of mountings taken by G1, G2 and G3 age groups was 3.88±0.29, 3.00±0.00 and 3.03±.03, respectively. It was significantly ($p<0.05$) higher in G1 group as compared to G2 and G3 groups while no significant difference was found between G2 and G3. The results are in agreement with Rehmann *et al*. (2016) who also found significantly higher no. of mounting in young age group. Price and Borgwardt (1994) also found inverse relationship between age and no. of mounting. The higher number of mountings in young bulls is due to less experience.

Sexual aggressiveness (1 to 4 scale)-

The sexual aggressiveness score in G1, G2 and G3 age groups of bull was found to be 2.44±0.16, 3.13±0.13 and 2.63±0.21, respectively. It was significantly ($p<0.05$) higher in G2 group of bulls followed by G3 and G1. It might be due to the fact that testosterone level is at its peak in adult age group.

Libido Score-

The libido score for G1, G2 and G3 age group was found to be 37.33±3.42, 56.26±4.08 and 43.80±4.86, respectively. It was significantly ($p<0.05$) higher in G2 group followed by G1 and G3. Ahmad and Asmat (2005) also found significantly higher libido score in the adult age group. Javed *et al*. (1998) found insignificant differences between the three age group. Libido score depends on reaction time score and sexual aggressiveness score.

Table 4.1.1: Sexual Behaviour in Sahiwal bulls of different age groups

Age group (years)	Time to enter arena (sec) Mean±SE	Erection time (sec) Mean±SE	Reaction time (sec) Mean±SE	Reaction time score (0-6) Mean±SE	Total mounting (no.) Mean±SE	Sexual aggressiveness (1-4) Mean±SE	Libido score (0-100) Mean±SE
G1(2-3.5)	2.77^{a}±0.19	2.88^{a}±0.22	158.33^{a}±23.38	1.66^{a}±0.24	3.88^{a}±0.29	2.44^{a}±0.16	37.33^{a}±3.42
G2(>3.5-8)	5.06^{b}±0.27	2.23^{b}±0.15	73.90^{b}±11.59	2.96^{b}±0.29	3.00^{b}±0.00	3.13^{b}±0.13	56.26^{b}±4.08
G3(8-12)	5.23^{b}±0.20	2.46^{ab}±0.16	141.20^{a}±20.62	2.33^{ab}±0.30	3.03^{b}±.03	2.63^{ab}±0.21	43.80^{ab}±4.86

Means bearing different superscripts in a column differ significantly ^{a,b} ($p<0.05$)

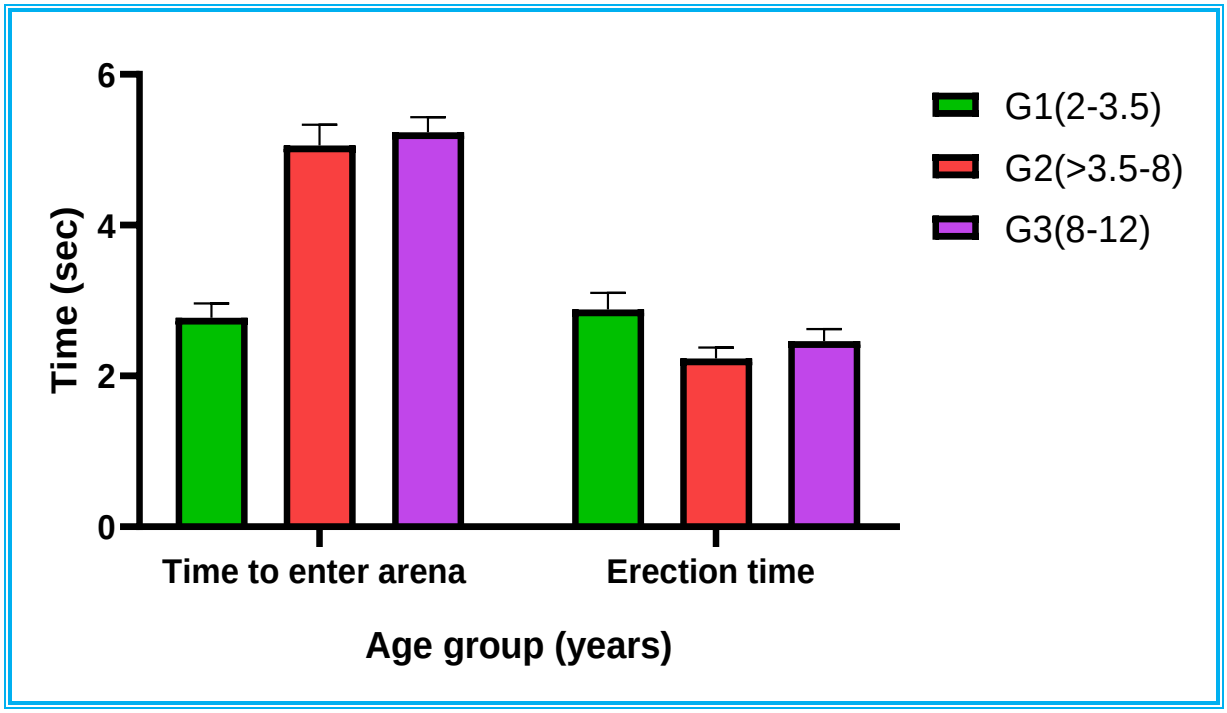

Fig 4.1.1 Time taken by the bulls to enter arena from preparation site (sec) and erection time in Sahiwal bulls of different age groups

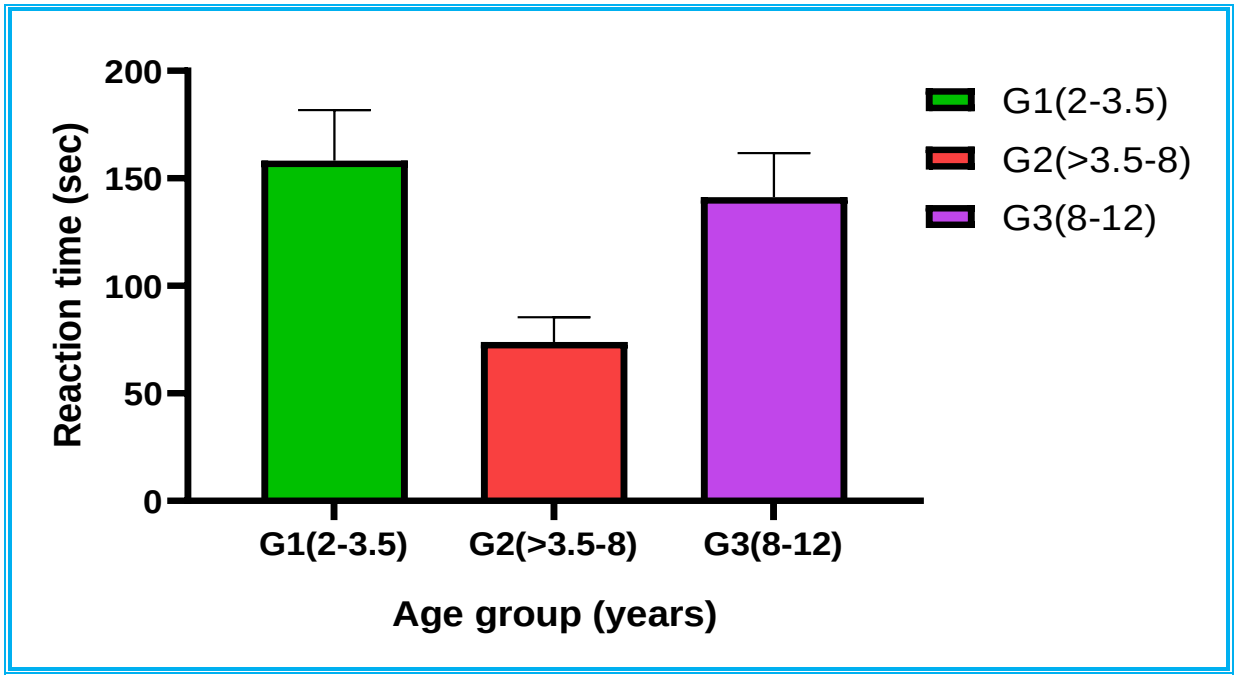

Fig 4.1.2 Reaction time (sec) in Sahiwal bulls of different age groups

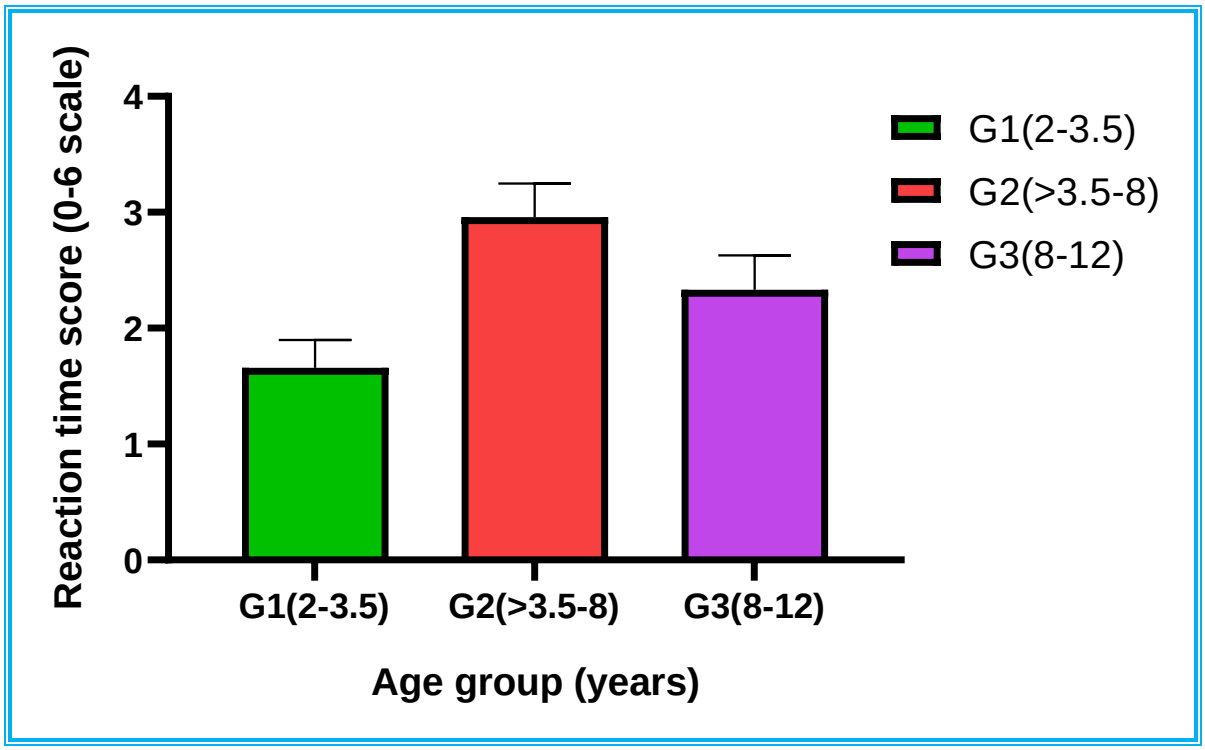

Fig 4.1.3 Reaction time score in Sahiwal bulls of different age group

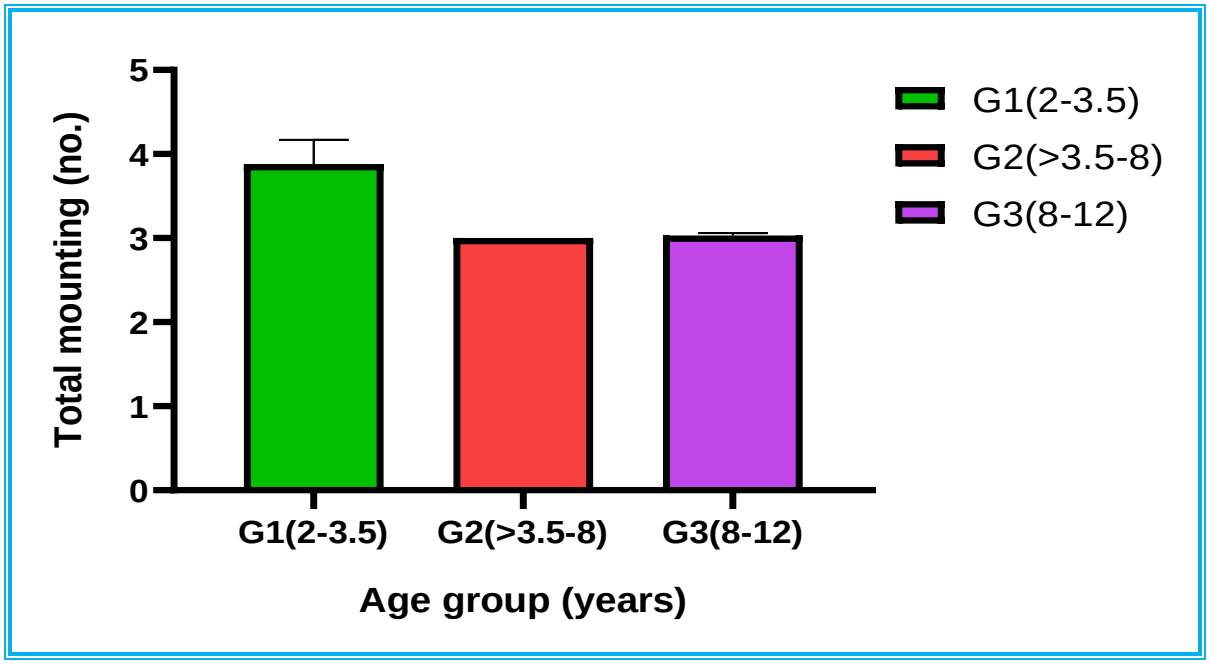

Fig 4.1.4 Total mounting (no.) in Sahiwal bulls of different age group

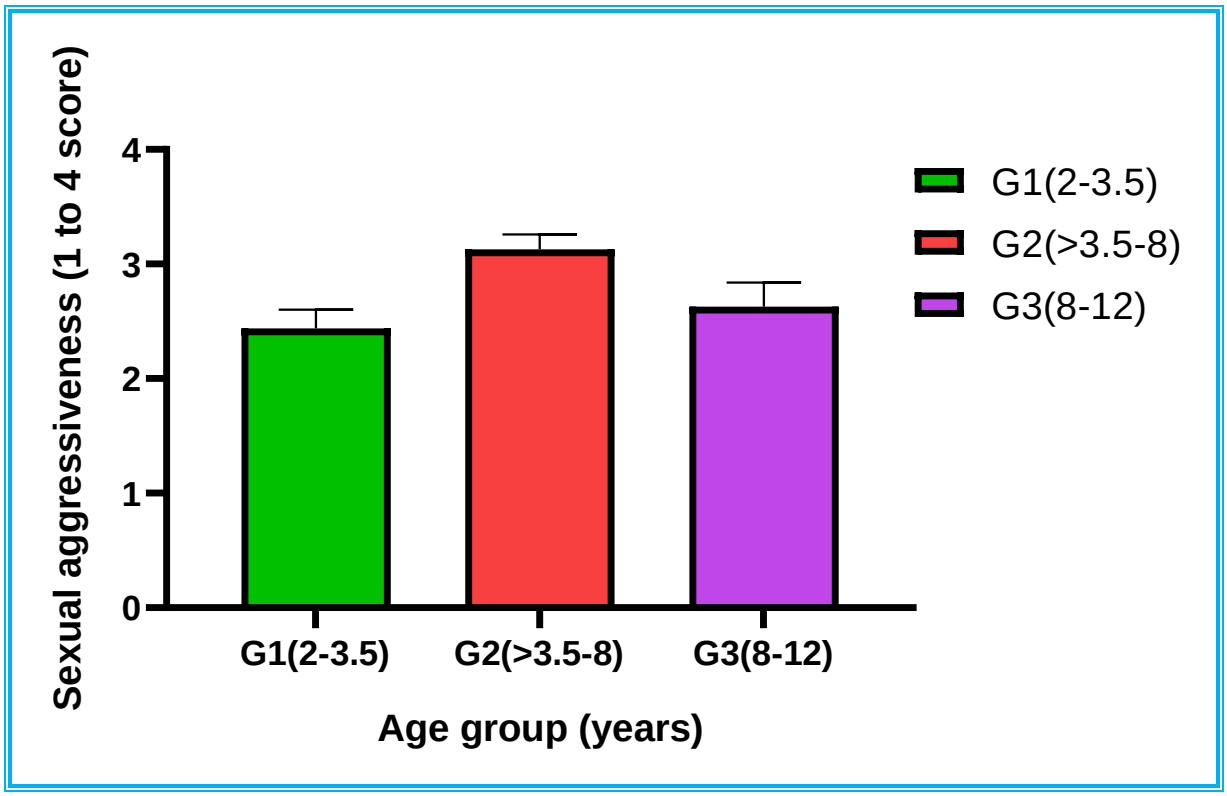

Fig 4.1.5 Sexual aggressiveness in Sahiwal bulls of different age group

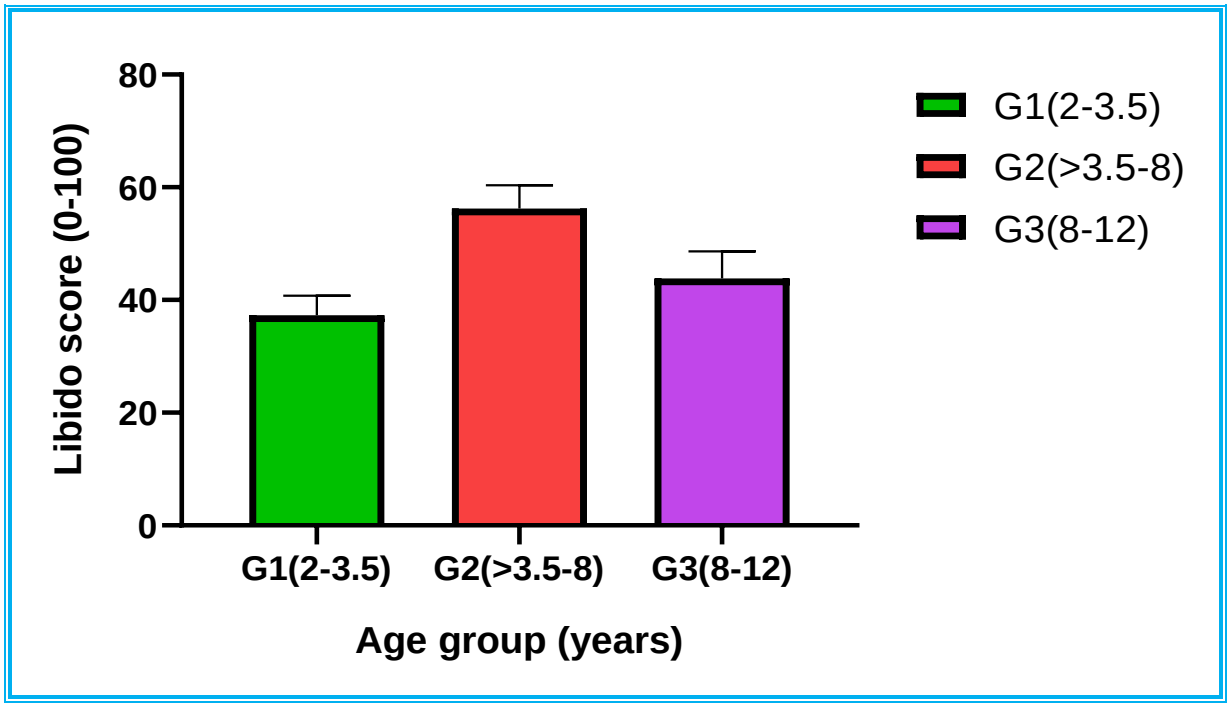

Fig 4.1.6 Libido score in Sahiwal bulls of different age group

4.1.2 Scrotal circumference

The scrotal circumference (SC) in the age group G1, G2 and G3 was found to be 31.95±0.32, 37.05±0.51 and 38.15±0.43. It was significantly ($p<0.05$) lower in G1 group as compared to G2 and G3 groups while the difference was non- significant between G2 and G3 groups. The results are in agreement with Abdullah (2016), who found lower SC values in young Sahiwal bulls than adult and old age groups of bulls. He also found out that scrotal circumference follows a curvilinear trend in bulls from 12 months to > 72 months of age. It increases rapidly in young bulls up to adult age after which it increases gradually. Results of Rafiq *et al*. (2022) in Murrah bulls also obtained similar results to our findings. Contrary to our findings, Javed *et al.* (1998) found non-significant difference among the age groups in Nili Ravi Buffalo bulls.

Table 4.1.2 Scrotal circumference (cm) in Sahiwal bulls of different age group

Age group (Years)	Scrotal circumference (cm) Mean ±SE
G1(2-3.5)	31.95^{a}±0.32
G2(>3.5-8)	37.05^{b}±0.5
G3(8-12)	38.15^{b}±0.43

Means bearing different superscripts in a column differ significantly^{a,b} ($p<0.05$)

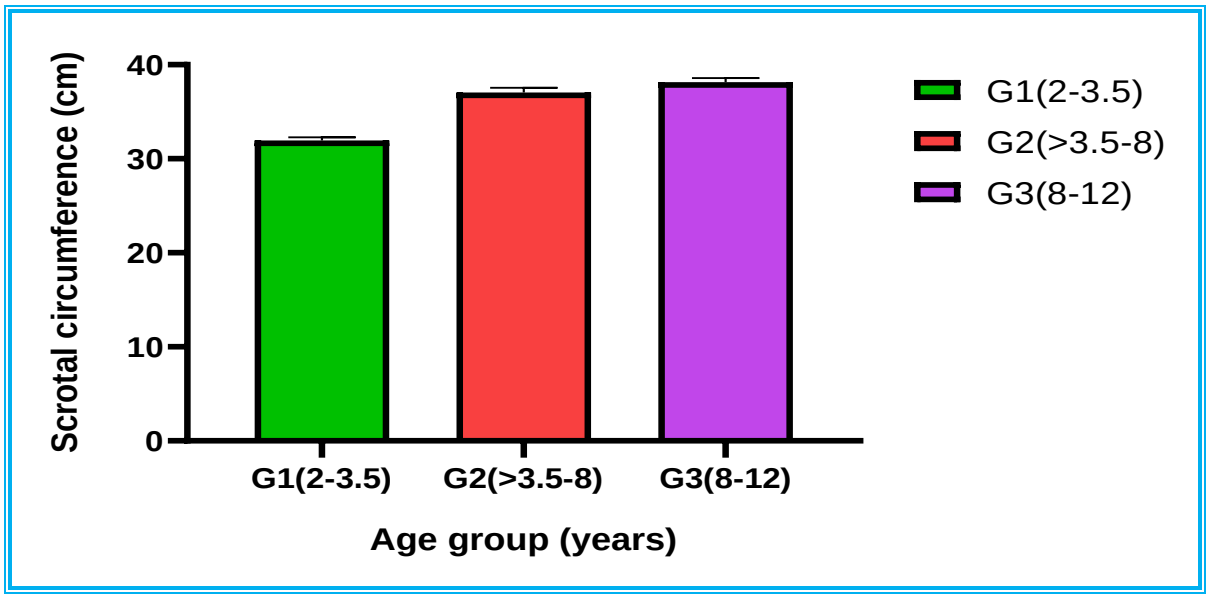

Fig 4.1.2. Scrotal Circumference in Sahiwal bulls of different age group

4.1.3 Testicular temperature

During morning time, mean value of proximal pole temperature, mid pole temperature and distal pole temperature in group G1, G2 and G3 was 32.78±0.50, 31.61±0.45, 32.25±0.37; 31.26±0.61, 30.38±0.47 , 30.89±0.54; 29.59±0.67, 29.05±0.57 and 29.23±0.58, respectively. Afternoon time, mean value of proximal pole temperature, mid pole temperature, distal pole temperature in group G1, G2 and G3 was 34.26±0.25, 33.27±0.24, 33.05±0.33; 32.87±0.29, 32.29±0.23,32.33±0.37;30.83±0.36,31.19±0.33 and 30.44±0.56, respectively. The temperature at all the three points did not vary significantly ($p<0.05$) among the age groups for morning as well as evening time.

The temperature gradient (TG) is the temperature difference between the proximal pole and distal pole. The more the difference, the better the thermoregulation of scrotum. The morning and afternoon scrotal temperature gradient for G1, G2 and G3 group was 3.19±0.26, 2.57±0.30, 3.02±0.32; 3.43±0.22, 2.07±0.21 and 2.61±0.35, respectively. No significant ($p<0.05$) difference was observed among the groups.

Not much published literature was found on effect of age on scrotal surface temperature, however one study conducted by Rafiq *et al.* (2022) on the effect of age on scrotal temperature mentioned no significant difference among the groups for scrotal surface temperature as well as scrotal gradient.

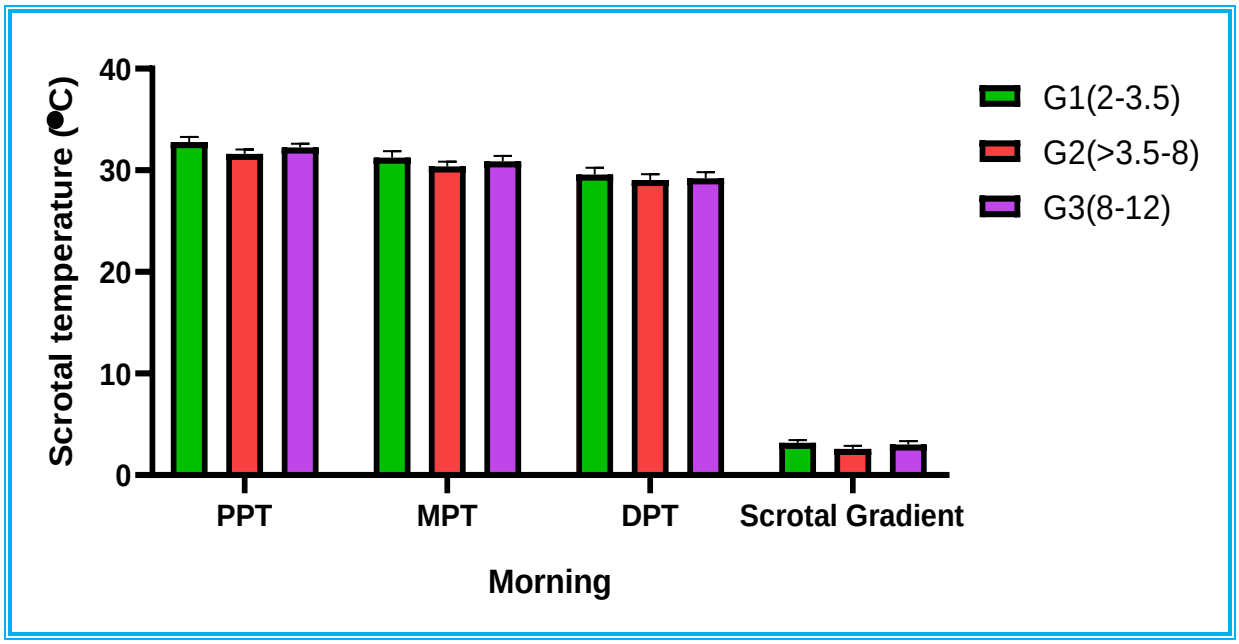

Fig 4.1.3.1 Scrotal temperature and scrotal gradient at morning time in Sahiwal bulls of different age group

Table 4.1.3 Scrotal temperature (ºC) and testicular gradient (ºC) in Sahiwal bulls of different age groups

Age group (Years)	Proximal pole (ºC) (Mean ± SE)		Medial pole (ºC) (Mean ± SE)		Distal pole (ºC) (Mean ± SE)		Scrotal temperature gradient (ºC) (Mean ± SE)	
	Morning	**Afternoon**	**Morning**	**Afternoon**	**Morning**	**Afternoon**	**Morning**	**Afternoon**
G1(2-3.5)	32.78±0.50	34.26±0.25	31.26±0.61	32.87±0.29	29.59±0.67	30.83±0.36	3.19±0.26	3.43±0.22
G2(>3.5-8)	31.61±0.45	33.27±0.24	30.38±0.47	32.29±0.23	29.05±0.57	31.19±0.33	2.57±0.30	2.07±0.21
G3(8-12)	32.25±0.37	33.05±0.33	30.89±0.54	32.33±0.37	29.23±0.58	30.44±0.56	3.02±0.32	2.61±0.35

Means bearing different superscripts in a column differ significantly^{a,b} ($p<0.05$)

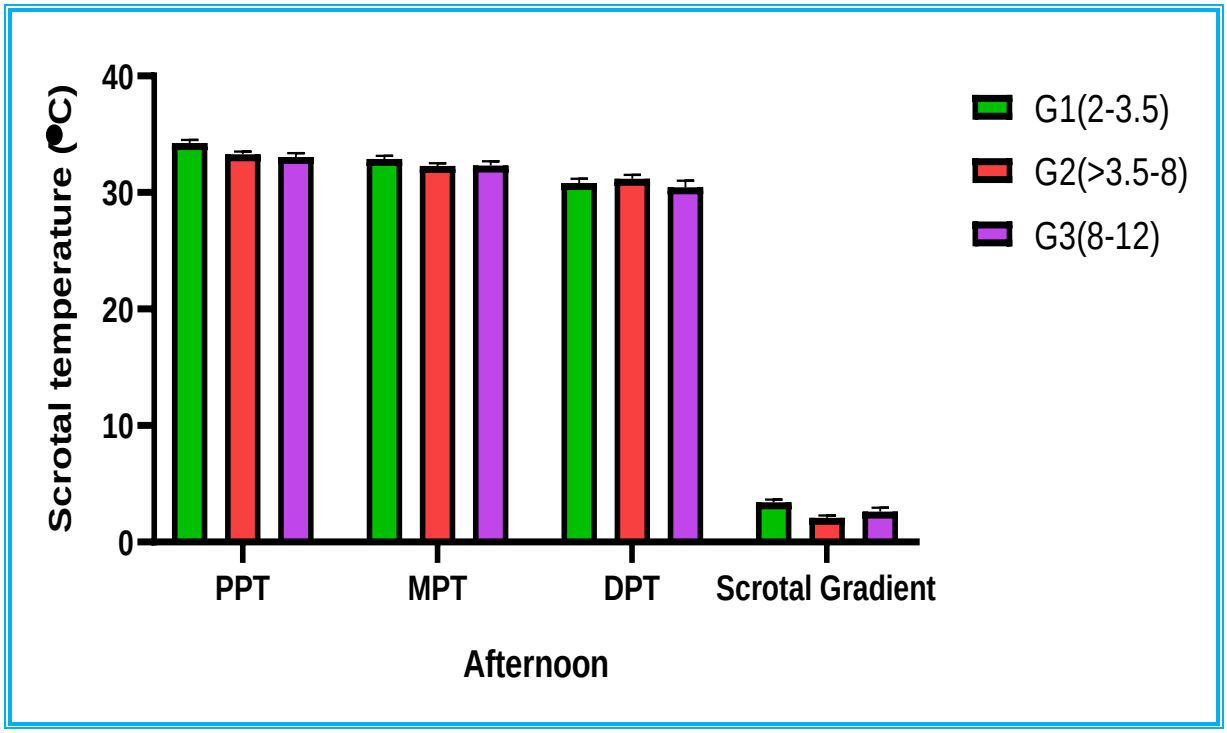

Fig 4.1.3.2 Scrotal temperature and scrotal gradient at afternoon time in Sahiwal bulls of different age groups

4.1.4 Physiological Parameters

The mean value of respiration rate (breathes/min) during morning and afternoon for the age group G1, G2 and G3 was 17.95±0.79, 13.75±0.76, 15.50±0.85 and 23.95±0.61, 20.50±0.73 and 19.55±0.67, respectively. It was significantly higher in G1 as compared to G2 and G3 while no significant difference was found for G2 and G3. The high respiration rate in the G1 age group might be due to the fact that younger animals have lower respiration rate.

The mean value of rectal temperature (ºF) in morning and afternoon for the age group G1, G2 and G3 was 100.06±0.26, 100.09±0.24, 100.18±0.23 and 101.08±0.24, 101.24±0.12 and 100.97±0.14, respectively. No significant difference was observed among the three age group in morning as well as afternoon.

Table 4.1.4 Respiration rate and Rectal temperature in Sahiwal bulls of different age groups

Age group (years)	Respiration Rate (breaths/min) (Mean±SE)		Rectal temperature (°F) (Mean±SE)	
	Morning	Afternoon	Morning	Afternoon
G1(2-3.5)	17.95[a]±0.79	23.95[a]±0.61	100.06±0.26	101.08±0.24
G2(>3.5-8)	13.75[b]±0.76	20.50[b]±0.73	100.09±0.24	101.24±0.12
G3(>8)	15.50[b]±0.85	19.55[b]±0.67	100.18±0.23	100.97±0.14

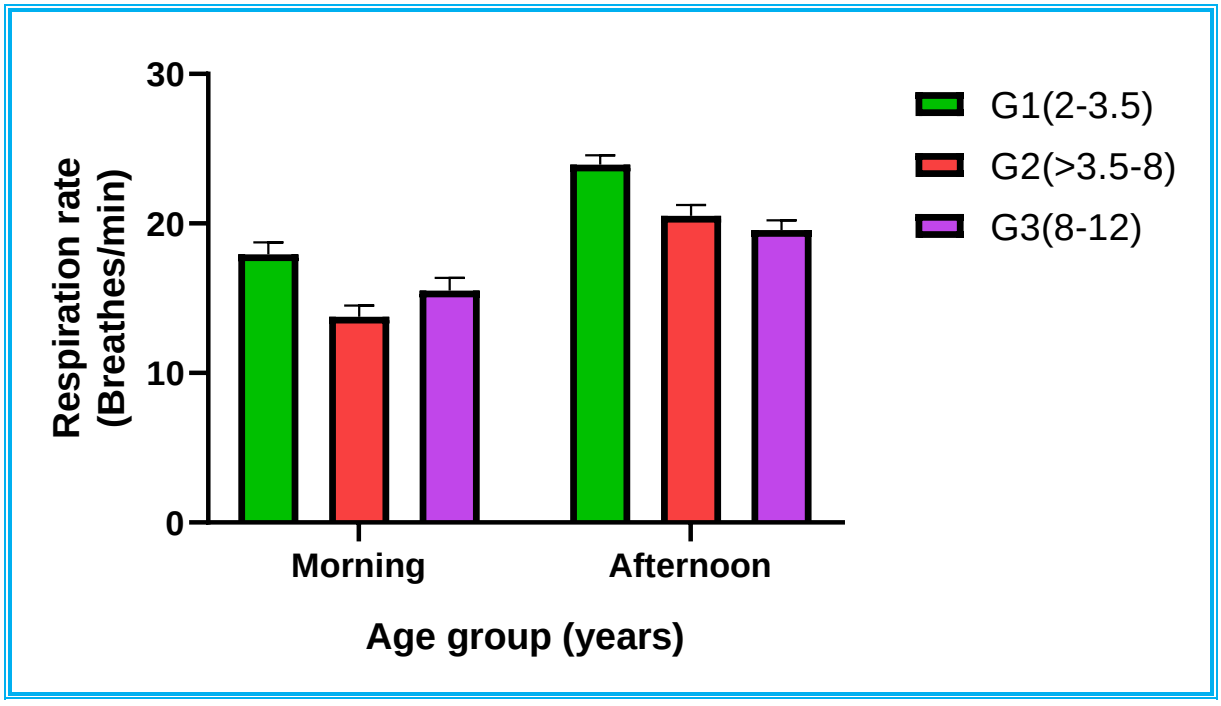

Fig 4.1.4.1 Respiration rate at morning time and afternoon time in Sahiwal bulls of different age groups

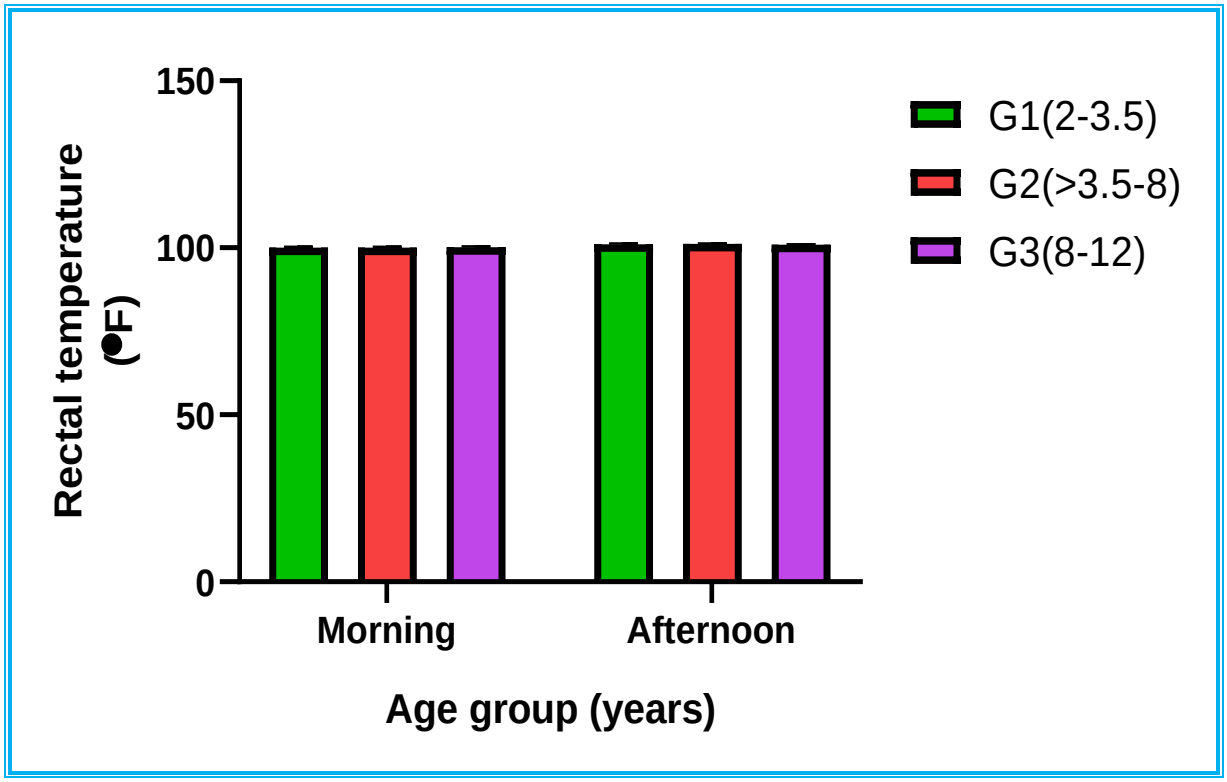

Fig 4.1.4.2 Rectal Temperature at morning time and afternoon time in Sahiwal bulls of different age groups

4.2 Objective 2: To assess the effect of age on semen quality and freezability of Sahiwal bulls

Data on seminal attributes like volume (ml), sperm concentration (million/ml), mass activity (0-5 scale) of neat semen and sperm functions parameters like percent individual motility, viability, HOST response and acrosome integrity in fresh and frozen semen were subjected to analysis and the results are discussed under each subtitles.

4.2.1 Ejaculate Volume (ml)

The mean value of ejaculate volume (ml) in G1, G2 and G3 groups of bulls was 3.74± 0.43, 4.06 ± 0.25, 5.47± 0.49, respectively (Table 4.2.1 and Fig 4.2.1). Ejaculate volume differed significantly among the groups and was found to be higher ($P<0.05$) in G3 group as compared to G1 and G2 group, whereas it did not differ between G1 and G2. Ravimurgan and Sindhu (2020) also found similar trend in Nili Ravi bulls where significantly higher ejaculate volume was obtained in older bulls. However, Bhakat *et al*. (2011) in their study found that ejaculate volume significantly increased upto a age (4-5 years) after which it begins to decline.

Abdullah (2016) in Sahiwal bulls and Javed *et al*. (2000) in Nili Ravi bulls also did not find any significant differences among the age groups. As 90% of semen is seminal plasma, which is released by accessory sex glands, the increase in ejaculate volume may be primarily caused by an increase in the size and secretions of accessory sex glands, as well as an increase in testicular size and, to some extent, spermatozoa generation.

Table 4.2.1 Ejaculate volume (ml) and sperm concentration (10^6/ml) in Sahiwal bulls of different age groups

Age group (years)	Semen ejaculate volume (ml) Mean±SE	Concentration (10^6/ml) Mean±SE
G1(2-3.5)	$3.74^a \pm 0.43$	$1289.43^a \pm 112.91$
G2(>3.5-8)	$4.06^a \pm 0.25$	$1339.43^a \pm 93.46$
G3(8-12)	$5.47^b \pm 0.49$	$1759.36^b \pm 95.07$

Means bearing different superscripts in a column differ significantly^{a,b} ($p<0.05$)

4.2.2 Sperm concentration (x 10^6 /ml)

The mean value of sperm concentration (10^6/ml) for G1, G2 and G3 groups was 1289.43 ±112.9, 1339.43 ± 93.4 and 1759.36 ±95.07, respectively. Sperm concentration differed significantly among age groups. It was high ($P<0.05$) in G3 group as compared to G1 and G2 group. It did not differ between between G1 and G2 groups. Opposite results to the present findings were found by Javed *et al*. (2000) where highest sperm concentration was found in the younger age group (<5 years old) whereas Ahmad *et al.* (2003), Bhakat *et al*. (2011) and Rehmann *et al.* (2016) found insignificant differences among the age groups. The difference between the present study and findings of Javed *et al.* (2000) may be due to the fact that the younger group formed in the present study had lower age (2-3.5 years) than the later and the study conducted by Bhakat *et al.* (2011) and Rehmann *et al.* (2016) formed groups with less difference in age and that's why they did not find any difference among the age groups in sperm concentration.

4.2.3 Mass motility (0-5 scale)

Mean value of mass motility for G1, G2 and G3 age groups was 2.69±0.18, 3.03 ±0.08 and 3.26±0.10, respectively. It was significantly (P<0.05) high in G3 group followed by G2 and G1 group. Similarly, Younis *et al*. (1998) in Nili-Ravi bulls and Bhakat *et al.* (2011) in Sahiwal bulls found significantly higher mass activity in adult bulls as compared to young bulls. Contrary to our findings, Ahmad *et al*. (2003) and Abdullah (2016) in Sahiwal bulls found no significant difference between the age groups.

Table 4.2.2 Mass motility (0-5scale) and Individual motility (%) in Sahiwal bulls of different age group

Age group (years)	Mass Motility (0-5 score) (Mean ±SE)	Individual Motility (%) Mean±SE
G1(2-3.5)	2.69^{a}±0.18	71.81 ^{a}±1.84
G2(>3.5-8)	3.03^{ab}±0.08	77.00^{b}±1.42
G3(8-12)	3.26^{b}±0.10	75.16^{ab}±1.54

Means bearing different superscripts in a column differ significantly^{a,b} (p<0.05)

4.2.4 Individual motility (%)

The mean value of percent individual sperm motility for G1, G2 and G3 groups was 71.81±1.84, 77.00±1.42 and 75.16±1.54, respectively. It was significantly (P<0.05) high in G2 group followed by G3 and G1. The results are in agreement with Ahmad *et al*. (2003) who also found significantly higher motility in adult age group as compared to other age groups. Rehmann *et al*. (2016) in Sahiwal bulls and Javed *et al*. (2000) in Nili-Ravi bulls found no significant differences between the age groups.

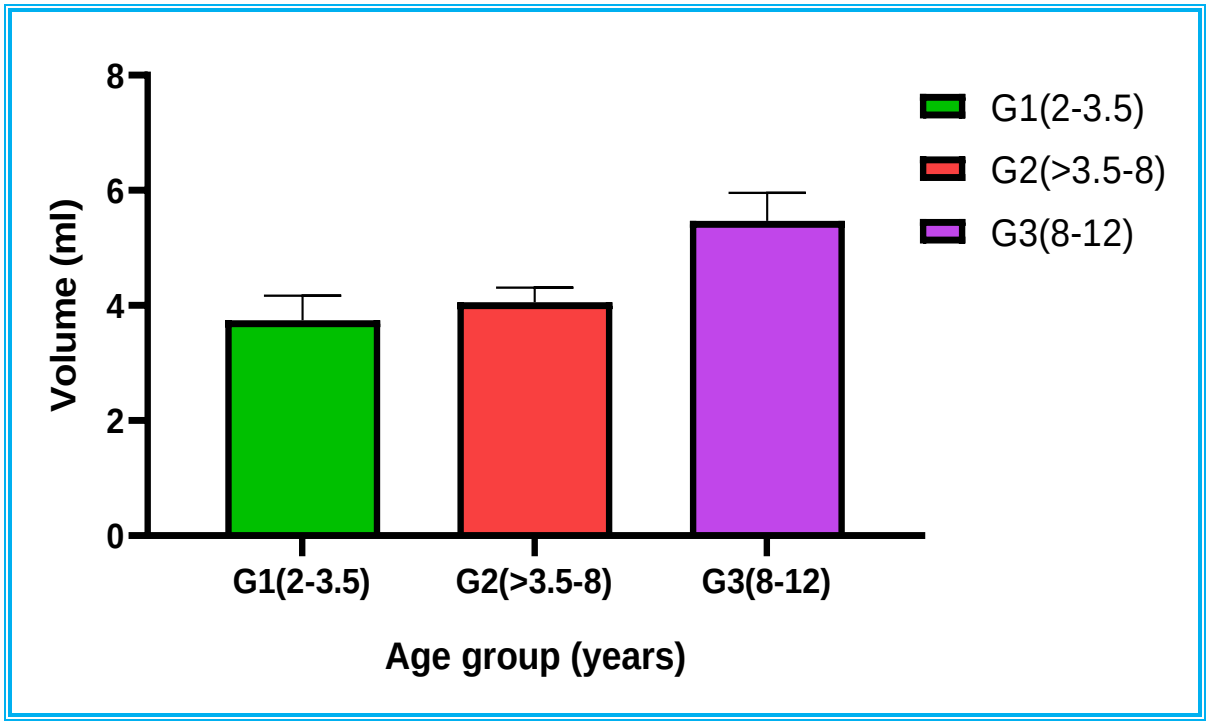

Fig 4.2.1 Ejaculate volume (ml) in Sahiwal bulls of different age groups

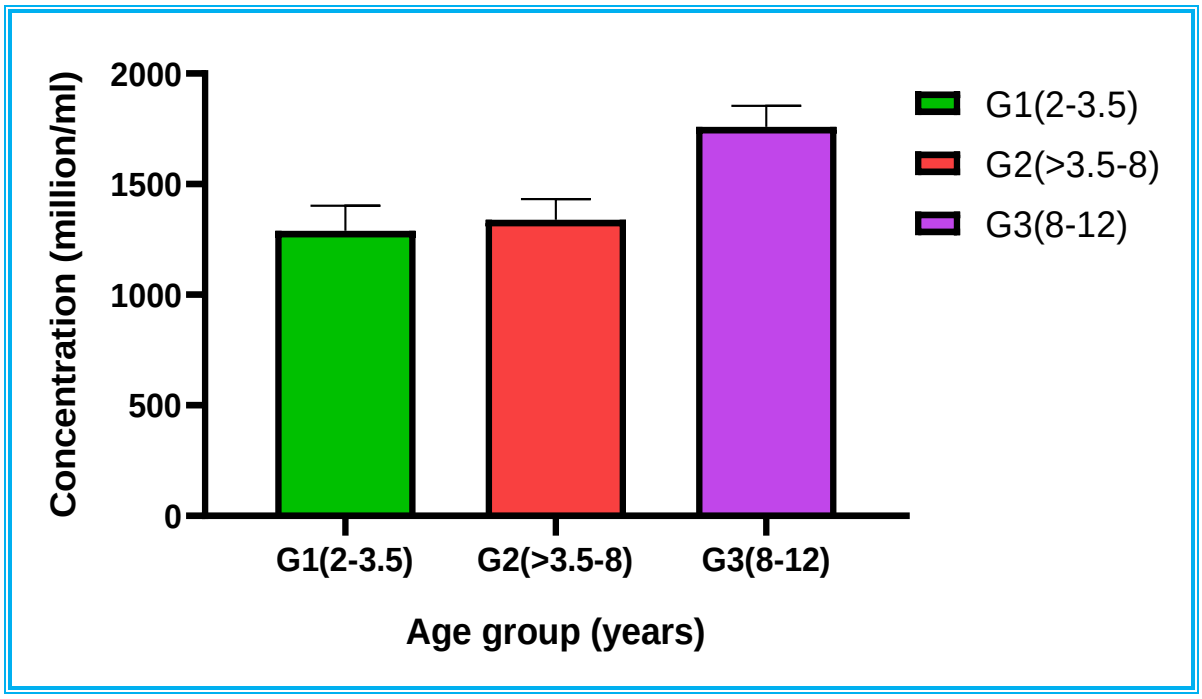

Fig 4.2.2 Concentration (10^6/ml) in Sahiwal bulls of different age groups

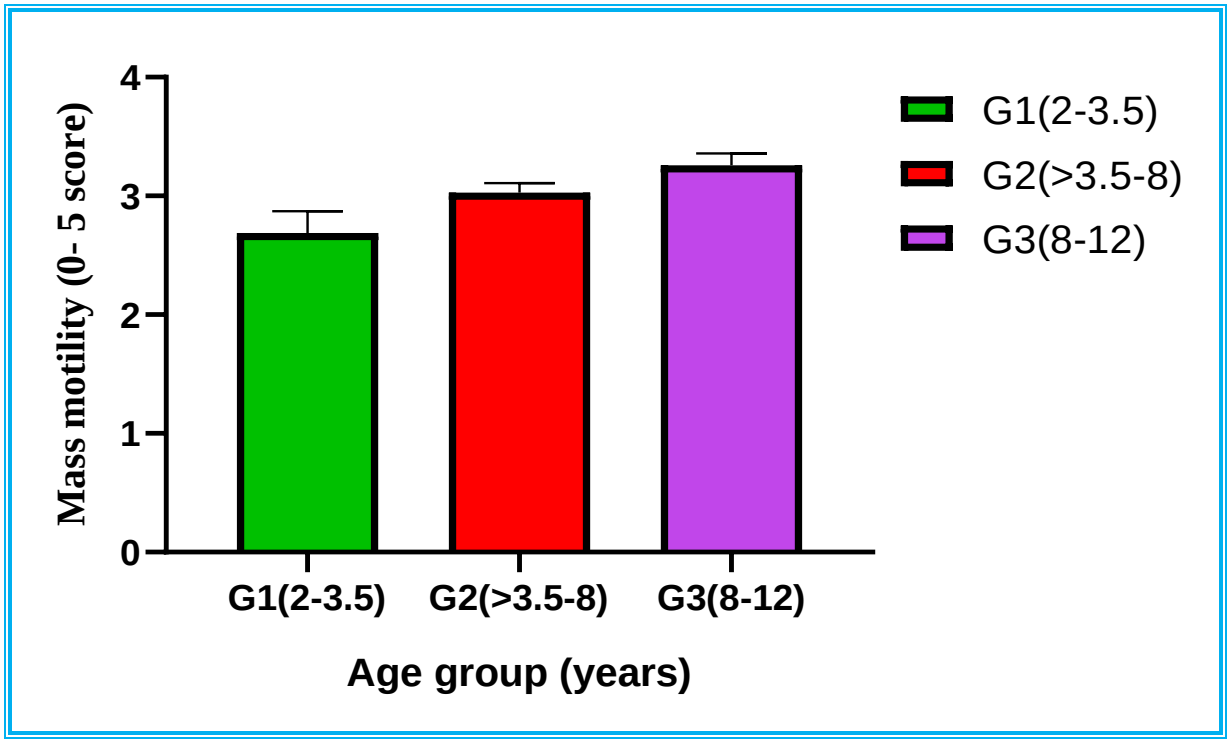

Fig 4.2.3 Mass motility (0-5 scale) in Sahiwal bulls of different age groups

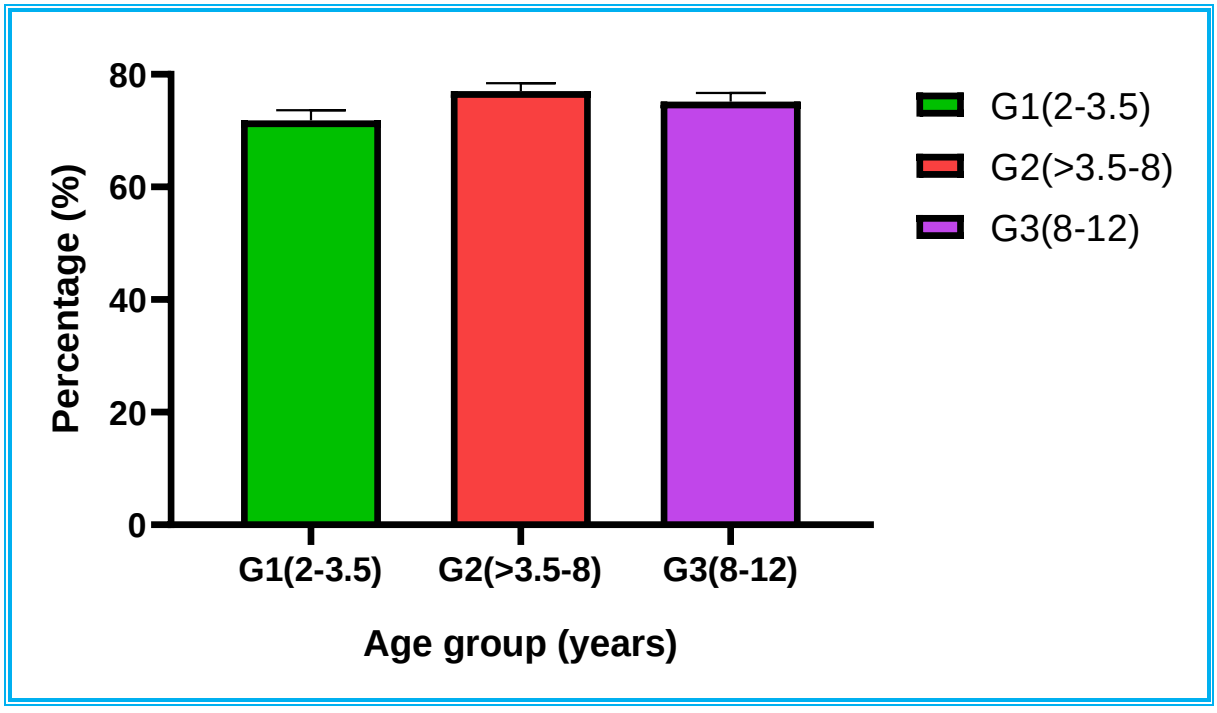

Fig 4.2.4 Individual motility (%) in Sahiwal bulls of different age groups

4.2.5.1 Viability

The mean value of viability (%) of fresh semen for G1, G2 and G3 was 72.13 ± 3.18, 78.05 ± 1.38 and 81.63 ± 1.47, respectively. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 groups while no significant difference was found between the G2 and G3 groups.

The mean values of viability (%) for extended frozen semen for G1, G2 and G3 was 45.85 ±2.97, 59.08 ±2.06 and 64.56 ±1.91, respectively. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 groups. No significant difference was found between the G2 and G3 groups.

Sankhi *et al*. (2019) found significantly high viability in the middle age group as compared to other two groups of bulls. Rehmann *et al*. (2016) and Abdullah (2016) found no significant difference between the age groups for viability in Sahiwal bulls. Low viability in the young age group in our study indicates that their sperm are more susceptible to management and environmental factors and also to cryopreservation techniques.

Table 4.2.3 Sperm functional parameters of fresh semen in Sahiwal bulls of different age groups

Age group (years)	Viability (%) Mean± SE	Host (%) Mean± SE	Acrosome Integrity (%) Mean± SE
G1(2-3.5)	72.13^{a} ± 3.18	54.50^{a} ±2.87	70.38^{a}±3.03
G2(>3.5-8)	78.05^{b} ± 1.38	64.17^{b} ±1.51	81.48^{b}±1.26
G3(8-12)	81.63^{b} ± 1.47	64.25^{b}±1.64	77.55^{b} ±1.43

Means bearing different superscripts in a column differ significantly^{a,b} (p<0.05)

4.2.5.2 Hypo-osmotic swelling test (%)

The mean values of HOST % of fresh semen for G1, G2 and G3 was 54.50 ±2.87, 64.17±1.51 and 64.25 ±1.64. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 group. No significant difference was found between the G2 and G3 age group.

The mean values of HOST % of extended frozen semen for G1, G2 and G3 was 34.05±1.99, 39.60±1.63 and 43.48±2.03. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 groups. No significant difference was found between the G2 and G3 groups.

The results are in agreement with Rehmann *et al.* (2016) and Rafiq *et al*. (2022) who found significantly lower HOST values for lower age groups. Bhave *et al*. (2020) in their study found that there was a gradual improvement in HOST reactive sperms up to a certain age after which it begins to decline. The intact functional membrane of sperm is considered as the prerequisite for sperm fertilizing ability due to successful acrosome reaction and binding to egg surface. HOST is one of the important sperm function parameter showing intactness and functionality of spermatozoa membrane.

Table 4.2.4 Sperm functional parameters of frozen semen in Sahiwal bulls of different age groups.

Age group (years)	Post thaw motility (%) Mean± SE	Viability (%) Mean± SE	HOST (%) Mean± SE	Acrosome integrity (%) Mean± SE
G1(2-3.5)	52.25^{a}±1.86	45.85^{a}±2.97	34.05^{a}±1.99	60.07^{a}±2.84
G2(>3.5-8)	57.00^{b}±0.93	59.08^{b}±2.06	39.60^{b}±1.63	68.23^{b}±1.51
G3(8-12)	53.83^{ab}±1.84	64.56^{b}±1.91	43.48^{b}±2.03	68.53^{b}±1.80

Means bearing different superscripts in a column differ significantly^{a,b} (p<0.05)

4.2.5.3 Acrosome integrity (%)

The mean values of Acrosome integrity (%) of fresh semen for G1, G2 and G3 group was 70.38 ±3.03, 81.48±1.26 and 77.55 ±1.43, respectively. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 groups. No significant difference was found between the G2 and G3 age groups.

The mean values of Acrosome integrity of frozen extended semen for G1, G2 and G3 group was 60.07±2.84, 68.23±1.51 and 68.53±1.80, respectively. It was significantly (P<0.05) lower in G1 group as compared to G2 and G3 groups. No significant difference was found

between the G2 and G3 groups. These finding are similar to Rafiq *et al*. (2022) in Murrah bulls, Gupta *et al.* (1978) in Surti bulls and Sekharan and Rao (1986) in Murrah bulls. Ahmed *et al*. (2018) in Nili Ravi buffalo bulls and Abdullah (2016) in Sahiwal bulls found no significant difference in acrosome integrity between the different age groups.

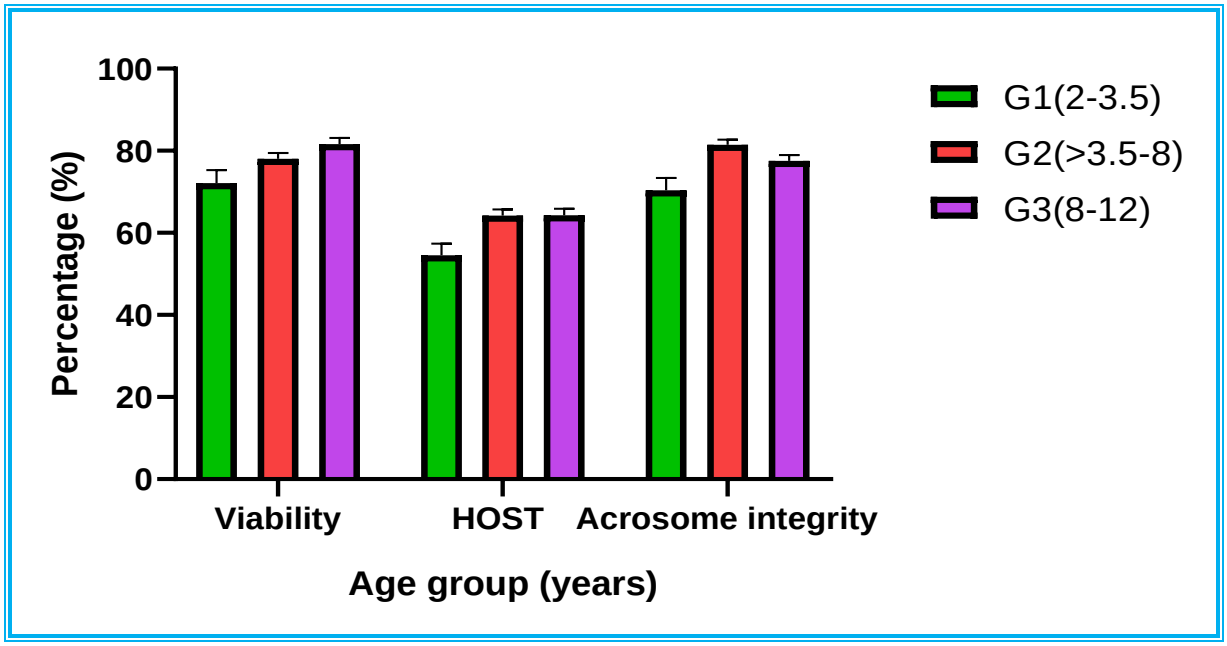

Fig 4.2.5 Sperm functional parameters of fresh semen in Sahiwal bulls of different age group

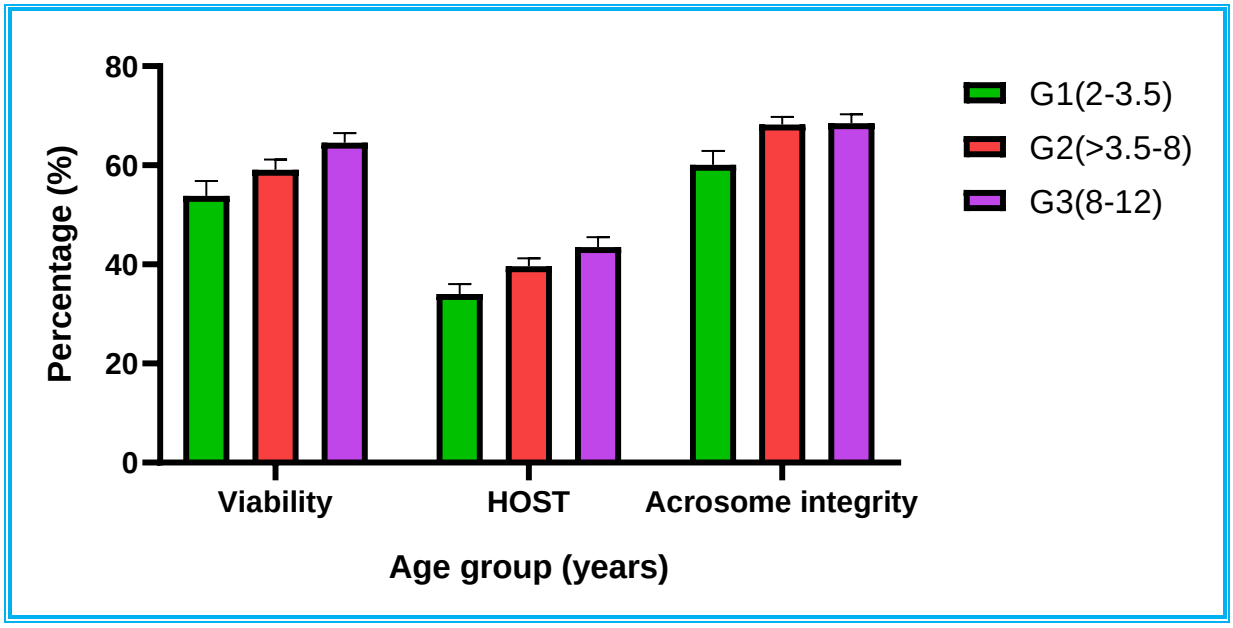

Fig 4.2.6 Sperm functional parameters of frozen semen in Sahiwal bulls of different age group

4.2.5.4 Post thaw motility

Mean value of post thaw motility (%) for G1, G2 and G3 group was 52.25±1.86, 57.00±0.93 and 53.83±1.84, respectively. It was significantly (P<0.05) high for G2 group followed by G3 and G1. The results are in agreement with Sankhi *et al*. (2019). Mandal *et al*. (2021) found significant increase in the post thaw motility as the age increases. Ahmad *et al*. (2003) in Sahiwal bulls and Bhave *et al.* (2020) in Gir bulls did not found any significant difference for post thaw motility among the age groups.

4.2.6 Post thaw incubation test

The mean value for sperm motility % at 0,30,60,90,120 min for G1, G2 and G3 age group was 52.25±1.86, 57.00±0.93 and 53.83±1.84; 51.75±1.85, 56.16±0.79 and 53.33±1.87; 51.00±2.05, 55.16±0.70 and 51.66±2.14; 46.50^{a}±2.10, 53.33^{b}±0.86 and 47.66^{a}±2.29; 43.02±2.93, 52.33±1.18 and 45.00±2.87 .It was significantly (P<0.05) high in G2 age group as compared to G1 and for G3 group at 0,90 and 120 min. At 30 and 60 min no significant difference was observed among the groups.

No published literature is available for the effect of age on incubation test of sperms in animals.

Table 4.2.5 Post thaw incubation tests in Sahiwal bulls of different age groups

Age group (Years)	Motility (%) Mean± SE				
	Time (min)				
	0	30	60	90	120
G1(2-3.5)	52.25^{a}±1.86	51.75±1.85	51.00±2.05	46.50^{a}±2.10	43.02^{a}±2.93
G2(>3.5-8)	57.00^{b}±0.93	56.16±0.79	55.16±0.70	53.33^{b}±0.86	52.33^{b}±1.18
G3(8-12)	53.83^{ab}±1.84	53.33±1.87	51.66±2.14	47.66^{a}±2.29	45.00^{a}±2.87

Means bearing different superscripts in a column differ significantly^{a,b} (p<0.05)

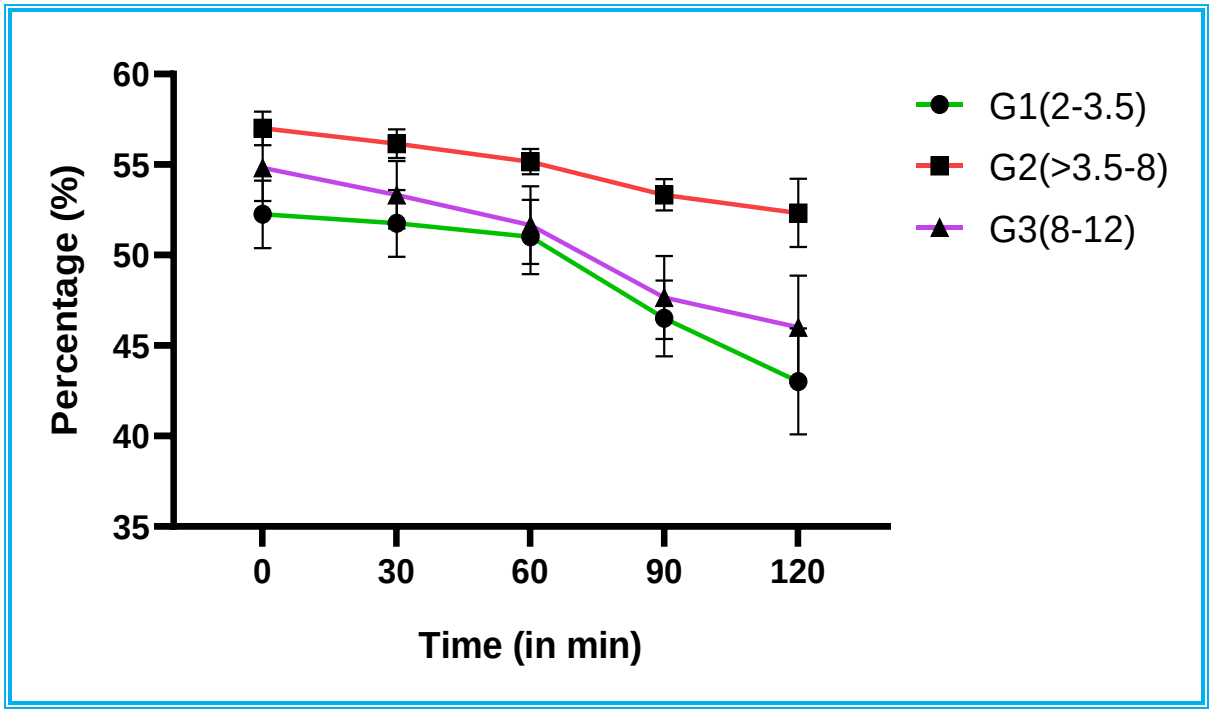

Fig 4.2.7 Post thaw incubation test in Sahiwal bulls of different age groups

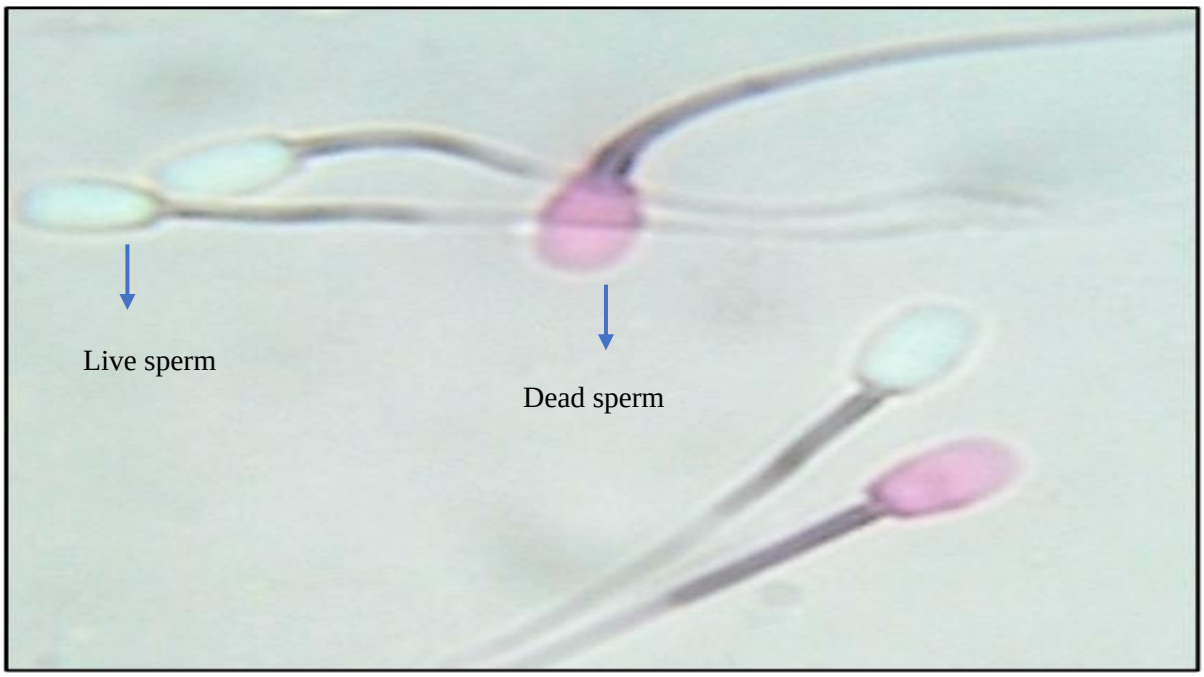

Plate no.1-Live and dead spermatozoa in Eosin-Nigrosine stain

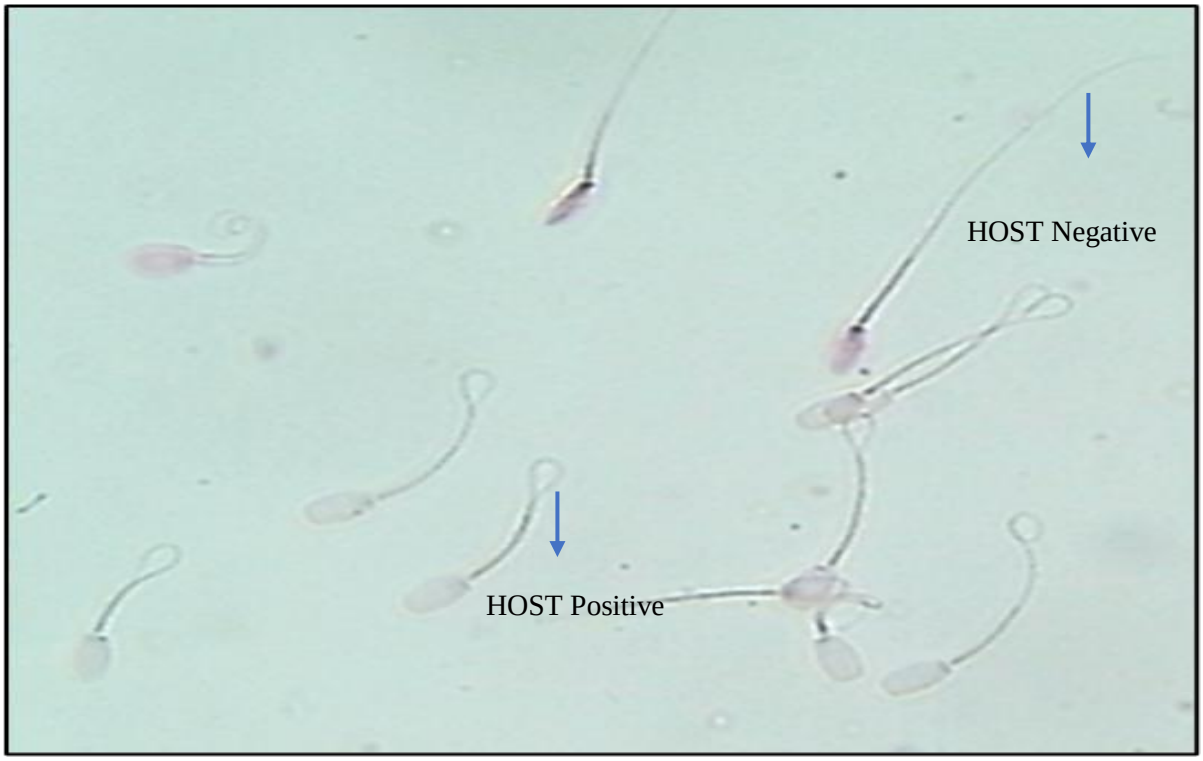

Plate no.2 -Host positive and negative spermatozoa in HOST solution

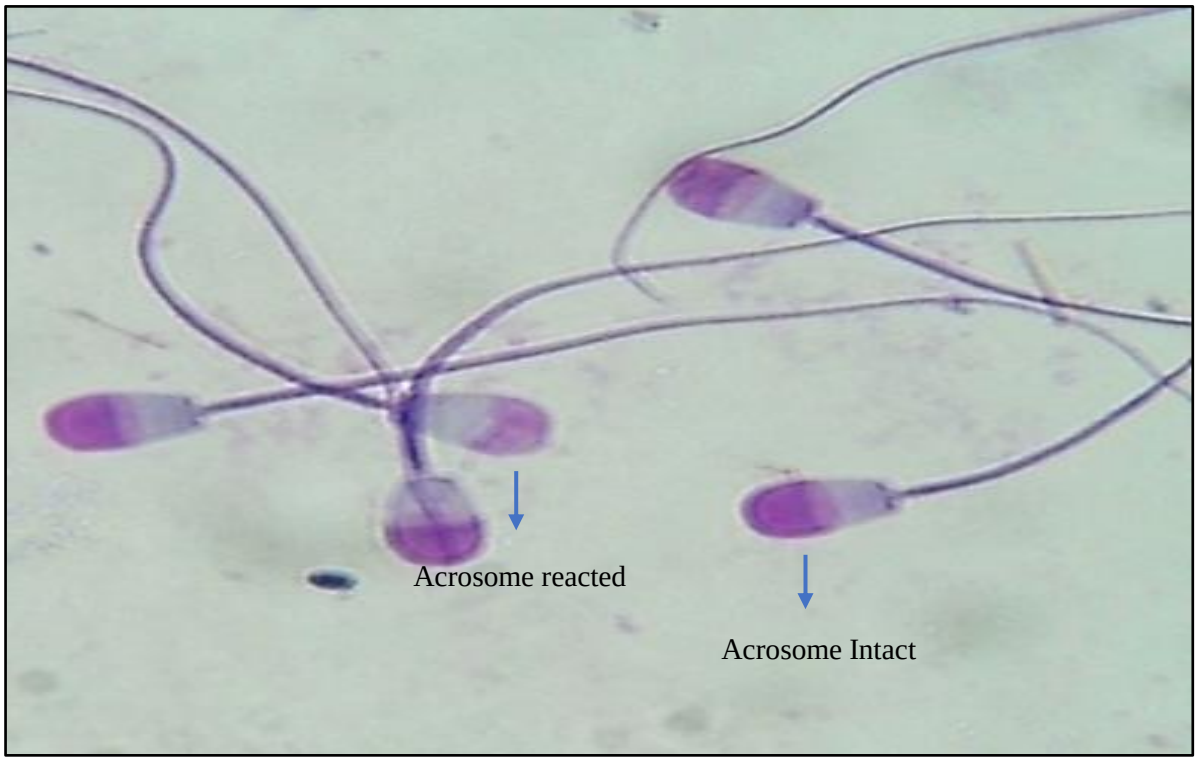

Plate no.3 -Acrosome intact and acrosome reacted sperm in Giemsa Stain

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

- Mean value of time to enter arena from preparation site was significantly ($p<0.05$) higher in the G2 (>3.5-8 year) and G3 (>8 year) age group as compared to the G1 (2-3.5 years) group.
- Mean value of erection time was significantly ($p<0.05$) higher in the G1 (2-3.5 years) group followed by G3 (>8 year) and G2 (>3.5-8 year) age group.
- The reaction time was significantly ($p<0.05$) lower in G2 (>3.5-8 year) age group as compared to the other two groups.
- The reaction time score was significantly higher in G2 (>3.5-8 year) age group followed by G3 (>8 year) and G1 (2-3.5 years) group.
- Total no. of mounting was significantly ($p<0.05$) higher in the G1 (2-3.5 years) age group as compared to the other two groups.
- Sexual aggressiveness and libido score were significantly ($p<0.05$) higher in the G2 (>3.5-8 year) age group followed by G3 (>8 year) and G1 (2-3.5 years) group.
- The mean value for respiration rate was significantly ($p<0.05$) higher in G1 (2-3.5 years) age group as compared to the other two groups.
- The mean value for scrotal circumference was significantly ($p<0.05$) lower in G1 (2-3.5 years) age group as compared to the other two groups.
- No significant ($p<0.05$) difference was observed among the groups for rectal temperature, scrotal surface temperature and scrotal temperature gradient.
- Mean value of volume (ml) and concentration (10^6/ml) were significantly ($p<0.05$) higher in the G3 (>8 year) age group as compared to the other two groups.
- Mass motility was significantly ($p<0.05$) higher in the G3 (>8 year) age group followed by G2 (>3.5-8years) and G1 (2-3.5years) age group.
- Individual motility was significantly ($p<0.05$) higher in the G2 (>3.5-8years) age group followed by G3 (>8 year) and G1 (2-3.5years) age group.

- Mean value of viability (%), HOST (%), Acrosome integrity (%) was significantly ($p<0.05$) lower in G1(2-3.5years) age group as compared to the other two groups for fresh as well as frozen semen.
- The post thaw motility was significantly ($p<0.05$) higher in G2 (>3.5-8years) age group followed by G3 (>8 year) and G1 (2-3.5years) age group.
- In post thaw incubation test the motility of the G2 (>3.5-8years) age group was significantly ($p<0.05$) high at 0, 90 and 120 min as compared to the other two groups.

Conclusions-

- Age has significant influence on sexual behaviour of Sahiwal bulls, being superior in G2 (>3.5-8 years) age group, except for the time to enter arena from preparation site.
- Scrotal circumference size varied among the age groups, being highest in G2 (>3.5-8 years) and G3 (>8 years) group ; however, significant influence of age on scrotal surface temperature and scrotal temperature gradient was not evident.
- Age of Sahiwal bulls significantly influenced the semen quality with better quality observed in bulls of G2 (>3.5-8 years) group followed by bulls of G3 (>8 years) group and least in G1 (2-3.5 years) group bulls.

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