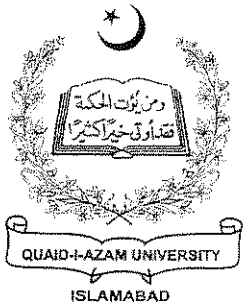
CRYOPRESERVATOIN OF EQUINE SEMEN



BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE THESIS
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CERTIFICATE

This thesis submitted by Mr. Muhammad Siddique is accepted in its present form by the department of Animal Sciences as satisfying the thesis requirement for the degree of Doctor of Philosophy in Reproductive Physiology.

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Dated : 18 Oct 2008

DEDICATED

TO

MY LOVING

MOTHER AND FATHER

In the name of Allah, the Beneficent, the Merciful.

Praise to be Allah, Lord of the Worlds,

The Beneficent, The Merciful:

Owner of the day of Judgment,

Thee (alone) we worship:

Thee (alone) we ask for help

Show us the straight path,

The path of those whom thou has favored.

Not (the path) of those who earn

thine anger,

Nor of those who go astray.

(Al-Fateha)

CONTENTS

LIST OF ABBREVIATIONS

ml	Milliliter
Tris	Hydroxyl methyl (Aminomethal)
U.S.A	United State of America
AI	Artificial Insemination
h	hour
SPU	Semen production unit
E	Estonian
T	Tori
PMS	Progressively motile sperm
SMEY	Skim Milk Egg Yolk
C	Centigrade
AV	Artificial Vagina
RD	Remount Depot
VOL	Volume
Yrs	Years
No	Number
Conc	Concentration
Gw	Grayish white
YET	Egg Yolk tris
EGB	Egg Yolk Bicarbonate
SM	Skim Milk
CUE	Cornell University Extender
CAP	Caprogen
IU	International Unit
mug	Mili Microgram
NFDMS	Nonfat dried milk solids glucose extender
GWS	Glass Wool Sephadex
EDTA	Ethylene diamine tetra acetic acid
PBS	Phosphate buffer Saline

H6	RD Poli Gold
H7	RD 8844 Aron
H8	RD Pole Rite
H9	RD 0015 Burnish rose
DF	Degree of Freedom
SS	Sum of Square
MS	Mean Square
PMOT	Progressively motile spermatozoa

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MUHAMMAD SIDDIQUE

GENERAL ABSTRACT

The present study of equine semen evaluation was carried out from January 2005 to February 2007 to perform screening test of Horse and Donkey Stallions in order to analyze their fertility state. A total of 90 horse stallions of six different breeds and 67 donkey stallions were evaluated for various semen parameters. These animals were kept under similar managemental and feeding conditions. The age of these stallions ranged between 4-22 years. All the horse and donkey stallions were divided into two groups i.e. young and old stallions. In the present data those horse and donkey stallions up to 10 years of age were included in the younger group and all those above 10 years of age were included in the older group. At least two ejaculates were taken from each stallion at an interval of 45-55 minutes for better evaluation. After sexual stimulation, ejaculates were collected from stallions by means of pre-warmed (42^0C) artificial vagina (AV). The effect of breed, age and season on, volume (ml), motility percentage, concentration ($x10^6$/ml), and dead spermatozoa of equine semen was also studied. The effect of breed on volume (ml), motility percentage, concentration ($x10^6$/ml) and dead spermatozoa was non significant of equine semen parameters. The effect of age was highly significant on all the parameters of equine semen in the case of Thoroughbred stallions but non significant in the case of other breeds (Arab, Percheron, Suffolk, Noriker, Cleveland and Donkeys). The effect of seasonal changes was also non significant on volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead spermatozoa.

In the second experiment 32 semen samples from four Thoroughbred stallions (H_6, H_7, H_8, and H_9) were preserved in liquid nitrogen at –196 °C for 24 hours to study the effect of five extenders on post thaw motility, liveability (hours) and absolute index of liveability of spermatozoa. Immediately after collection and evaluation, the semen samples were diluted with respective extenders viz. A (2.42 grams Tris), B (1.82 grams Tris + 0.52 grams Sodium citrate), C (1.21 grams Tris + 1.04 grams of Sodium citrate), D (0.60 grams Tris + 1.56 grams of Sodium citrate) and E (2.08 grams of Sodium citrate) having fructose, glucose, lactose, egg yolk, glycerol and antibiotics penicillin and dihydrostreptomycin containing as their common components. After an equilibration period of 6 hours, semen samples were frozen by fast method and stored in liquid nitrogen for 24 hours. Thawing was carried out at 37°C for 30 seconds in a water bath to observe post thaw motility percentage, liveability (hours) of spermatozoa and absolute

index of liveability (hours) for comparing the relative efficiency of the five extenders. The mean values of post thaw motility percentage were 46.87 ±2.10, 38.75 ±1.56, 50.62 ±0.94, 44.37±2.57 and 41.37±2.82 for extenders A, B, C, D and E respectively. Corresponding values for liveability at 37°C were 8.87 ± 0.39, 8.5 ± 0.26, 9.37 ±0.49, 8.37 ± 0.42 and 7.87± 0.39 hours respectively. The mean values of absolute index of liveability at 37 °C were 179.43 ± 2.31, 130.26 ± 3.09, 205.77 ± 6.23, 153.86 ± 6.59 and 141.35 ± 1.25 for extenders A, B, C, D and E respectively. The mean values of post thaw motility for four stallions (H_6, H_7, H_8, H_9) 44.87 ±2.10, 45.00 ±1.63, 36.87±1.62 and 35.62 ± 1.75 respectively. The mean values of absolute index of liveability at 37 °C for four stallions were 168.66 ±14.74, 155.88 ±15.05, and 167.52 ± 15.90 and 157.47 ± 14.83 respectively. The effect of five extenders on post thaw motility, liveability (hours) at 37 °C and absolute index of liveability was significant ($P<0.01$). The effect of four stallions on these parameters was non- significant. The extender C containing Tris 1.21 grams and Sodium citrate dihydrate 1.04 grams was the best for successful preservation of stallion semen at-196 °C. The order of merit for five extenders used was C, A, D, E and B.

In the third experiment, the comparison was carried out for the conception rate with natural service, liquid cooled semen and frozen thawed semen. This experiment was conducted in breeding season (March - August). In this study 60 mares (n = 60) were used. These mares were kept under similar conditions of management and feeding at Chenab Breeding Area in District Faisalabad and Sargodha.

These 60 mares were divided in to three groups.ie, Group 1, Group 2 and Group 3. Each group had 20 animals each. All the animals were examined for any reproductive abnormality by rectal palpation. All the mares were found free from any reproductive problems. Group 1 was bred with natural service, Group 2 was inseminated with liquid cooled semen and Group 3 was inseminated with frozen thawed semen. All the animals were examined for pregnancy diagnosis after 90 days.

After pregnancy diagnosis it was concluded that with natural service 11 mares out of 20 (11/20) were found pregnant (55 percent), with liquid cooled semen 9 mares out of 20 (9/20) found pregnant (45 percent) and with frozen thawed semen 8 mares out of 20 (8/20) were found pregnant (40 percent).

GENERAL INTRODUCTION

The equine, a great national asset with its population of 4.0 million head (Economic Survey of Pakistan, 2007) is the major source of Equestrian activities and transportation in hilly terrain in the country. Low fertility rate of equine breeding areas is an obstacle to meet the enhanced requirements of our ever-increasing human population. The existing gap of low productivity in the country can be well abridged by exploiting the reproductive potentional of the equines.

Retrospectively, Artificial insemination (AI) begins with a story about some Arabs stealing stallion semen from a rival tribe and bringing it home for breeding in a sponge. AI began its modern development in Russia during the early 20th century by Ivanov; the main object was not cattle but horses (Milovanov, 1934). In the late 1940s, Christopher Polge, working with chicken and the economically more important bull semen, pioneered the development of frozen-thawed semen technology (Polge, 1985). In 1957, Barker and Gandier reported the first foaling insemination with frozen epididymal spermatozoa (Graham et al., 1978). During the next decade a dozen more successful reports with ejaculated semen followed from Germany, Japan, Russia, and the U.S. (Crabo, 2001). During the past four decades several workers have investigated the problem of low fertility in domestic horses in the U.K. (Sanders, 1926), in the U.S.A. (Voss and Pickett, 1976) and in West Germany (Merkt et al., 1979). Breed and age of stallion, breeding season and the referral status of the stallion (suspect or normal) had significant ($p<0.001$) effects on fertility. The semen characteristics that were clearly associated with percentage pregnancies per service were total volume, gel-free volume, sperm motility percentage, sperm concentration, total number of spermatozoa, total number of live spermatozoa and dead sperms percentage (Dowsett and Pattie, 1982).

Semen evaluation

A basic method was outlined for conducting stallion semen evaluation. After removal of the gel fraction of the ejaculate, semen gel-free volume was determined, and any abnormality in appearance was noted. Concentration of sperm cells in semen could be determined with the use of either a haemocytometer or spectrophotometer after appropriate dilution of raw semen. The percentage of progressively motile sperm was

evaluated promptly after collection of semen with the use of a phase-contrast microscope. The total number of sperm and progressively motile sperm in the ejaculate were calculated. The determination of seminal pH and the classification of sperm morphologic features were additional seminal characteristics evaluated during a semen evaluation (Jasko, 1992).

Since the evaluation of seminal characteristics and gross morphology can be affected by the subjectivity and variability of the manual morphology assessment, computer automated sperm morphology analysis was developed. They use computer automated sperm morphology analysis to determine if the morphometric measurements of sperm heads from collected and dismount samples of the same ejaculate were similar. If the post-ejaculate dismount sample was representative of the entire ejaculate, this sample might be utilized in determining the fertility of the ejaculate (Gravance et al., 1997). A study was conducted to test whether volume alone affects fertility when sufficient numbers of spermatozoa are present. Semen from one stallion was collected, extended at 50×10^6/ml spermatozoa/ml, and stored in a commercial semen-cooling device for 18 to 30 h before insemination. It was concluded that insemination volumes as large as 120 ml have no adverse effect on fertility (Bedford and Hinrichs, 1994).

The semen characteristics of 168 horse stallions from 9 breeds, aged from 2 to 26 years were studied over 4 breeding seasons. Apart from breed only age, season of year and service frequency had significant effects. Horse stallions under 3 years of age had the lowest volumes and sperm concentrations which together with the highest proportion of dead spermatozoa resulted in the lowest of live spermatozoa per ejaculate. Horse stallions older than 13 years had low sperm concentrations and high percentages of dead spermatozoa but sperm morphology was normal. Arabian stallions had the lowest percentage of non-motile and dead spermatozoa while Shetlands were the only breed with greater than 30% dead, the other having values within accepted normal limits. Arabian stallions had sperm concentrations almost double those of other breed and with their relatively high volume; this resulted in total sperm numbers that were 3 times greater than those of any other (Dowsett and Pattie, 1987).

The effect of season has also been studied on other breeds of animals like bulls and data regarding several attributes of semen produced by 53 Sahiwal bulls maintained at Semen Production Unit, Qadirabed, District Sahiwal from 1974 to 1984 was analyzed and found that under the feeding and management condition of Semen Production Unit, Qadirabad,

District Sahiwal season has no affect on quantity or quality of the semen of Sahiwal bulls (Usmani et al., 1985). The effect of season on sperm movement characteristics, determined by computer-aided sperm analysis, was compared with those other seminal characteristics. The computer-aided determinations of sperm movement were more repeatable than the seminal characteristics of gel-free volume and sperm cell concentration based on coefficients of variation obtained from the analysis of multiple ejaculates from the same stallions. A significant ($P<0.05$) seasonal effect on the computer-aided movement characteristic of mean sperm linearity was observed, with a reduction in sperm linearity in the winter months (Jasko et al., 1991)

There were no differences in volume of gel- free semen but, because of higher sperm concentrations, total sperm numbers were greatest in summer and autumn. In addition, most dead spermatozoa were present in autumn and winter and these were accompanied by higher proportions of abnormal heads and tails. It seems that the timing of the Australian breeding season in spring and early summer is out phase with the period of peak semen production (Dowsett and Pattie, 1987).

Seasonal changes of semen quality parameters in Franches–Montagnes stallions were investigated and compared to the freezability of ejaculates collected in autumn and winter. Ejaculates were collected and evaluated every month during 1 year as well as cryopreserved in autumn and winter (September to February). In fresh semen the gel-free volume, concentration, motility and morphology (normal sperm, major defects, vacuoles and acrosome defects) were evaluated and in frozen-thawed semen the motility as well as the viability was assessed. To analyze seasonal differences, four periods of 3 months each were defined as autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). During the 1-year experiment all fresh semen quality parameters demonstrated a clear seasonal and individual pattern. The gel-free volume was significantly ($P < 0.05$) higher in spring and summer compared to autumn and winter while sperm concentration was significantly ($P < 0.05$) lower in spring than at any other time of the year. Total sperm number was significantly ($P < 0.05$) higher and sperm motility significantly ($P < 0.05$) lower in summer than in other seasons. Regarding sperm morphology, normal sperm was significantly ($P < 0.05$) higher in autumn than in winter and summer and major defects were lowest ($P < 0.05$) in autumn. In frozen-thawed semen motility was significantly ($P < 0.05$) improved in the ejaculates collected in autumn compared to winter, while

viability showed no obvious differences. Their results clearly demonstrate that individual and seasonal differences occurred in semen quality of Franches– Montagnes stallions. Ejaculates collected in autumn (September, October, November) demonstrated good quality, especially regarding sperm morphology, and were more suitable for cryopreservation because of better motility in frozen-thawed semen collected during autumn than in winter (Janett et al., 2003).

Sperm morphology in Estonian (E) and Tori (T) breed stallions was studied. The standard procedure for assessing the breeding potential of a stallion includes the parameter total number of spermatozoa classified as morphologically normal. Two ejaculates were examined from each stallion. An aliquot from each ejaculate was fixed in 1ml formol-saline immediately after collection and examined with phase contrast microscope at a magnification 1000x for all types of morphological abnormalities. Furthermore, smears were prepared and stained according to Williams (carbolfuchsin-eosin) for a more detailed examination of the sperm heads with light microscope at a magnification 1000x. The T stallion had on average 57.5±4.1 % and the E-stallions 74.4 ± 3.8 % morphologically normal spermatozoa ($p=0.012$). In 4 of 7 stallions and 7 of 8 stallions both ejaculates had >50% morphologically normal spermatozoa. There was a significant difference between breeds in mean percentage of proximal droplets (17.3+2.7% and 2.9+2.5% for T and E stallions respectively: $p=0.003$) (Kavak et al., 2003).

The effects of sperm number, concentration, and volume of insemination dose of chilled, stored, and transported semen on pregnancy rate in Standardbred mares were studied. It was concluded from this study that acceptable pregnancy rates could be achieved with properly prepared semen, chilled carefully to 6°C before transport, from insemination doses of 400 million progressively motile sperm (PMS) at packing (<400 million at delivery), provided the dose volume did not exceed 22 mL nor the concentration above 90 million per ml (Newcombe et al., 2005).

Cryopreservation

The era of successful cryopreservation of gametes from a variety of species was brought about by the serendipitous discovery in 1949 of the protective action of glycerol by scientists in Cambridge, England (Polge et al., 1964; Smith and Polge, 1950). The first pregnancy from frozen stallion semen was reported in 1957 by Barker and Gandier (Barker and Gandier 1957). Spermatozoa were recovered from the epididymides and frozen in heated, whole milk extender containing 10% glycerol as the cryoproptectant.

The better procedures for freezing and thawing of equine sperm were needed since variable fertility was obtained when cryopreserved sperm were used. To evaluate current methods of freezing equine sperm, spermatozoal quality was examined by means of two new techniques. These measured the integrity of plasma-acrosomal membranes by immunofluorescent analyses of binding of an antibody specific to the acrosome and evaluated eight parameters of spermatozoa motion using a fully automated computerized system (Blach et al., 1989).

The quality and freezability of stallion semen during breeding and non-breeding seasons was compared. Ejaculates were collected twice per week from four stallions during May and December. The semen was mixed with skim milk extender, centrifuged and resuspended in fresh extender. In winter, the average percentages of motile and morphologically normal sperm (67 and 74.3%, respectively) were higher than during the breeding season in May (59 and 65.9%; $P<0.05$). After freezing/thawing the proportions of vital and intact sperm decreased significantly. The number of motile sperm declined to 15 and 18% in May and December (range 5–40%), and of morphologically intact sperm to 51% in both seasons. The cryopreservation of sperm during December results in survival rates similar to those measured during the breeding season, even more important for successful preservation is the selection of suitable semen donors (Blottner, 2001).

The suitability of ejaculated and epididymal stallion spermatozoa for cooled storage (5°C) and cryopreservation was examined in 5 ejaculates from each of 6 stallions and in spermatozoa recovered from the caudal epididymidis after castration of these stallions. (Braun et al.1994).The cryopreservation of stallion spermatozoa in different extenders, correlations between laboratory assay results and sperm fertility were determined. Spermatozoa were cryopreserved in 1) a skim milk-egg yolk medium (CO); 2) a skim milk-egg yolk-sugar medium (SMEY); 3) CO after pretreatment with phosphatidylserine+cholesterol liposomes (CO + L) or 4) cooled to 5°C without cryopreservation (Wilhelm et al. 1996). The ability to ship cooled stallion semen to a facility that specializes in cryopreservation of spermatozoa would permit stallions to remain at home while their semen is cryopreserved at facilities having the equipment and expertise to freeze the semen properly (Crockett et al., 2001).

Optimization of equine sperm cryopreservation protocols required an understanding of the water permeability characteristics and volumetric shrinkage response during freezing (Devireddy et a., 2002).

Pregnancy Rate

The use of artificial insemination (AI) in equine breeding has become increasingly popular in the horse industry, offering many advantages over natural service. Some of the reasons for this include the choice of a great number of stallions, safety for the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. In addition, pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural mating (Samper et al., 1991). Artificial insemination using frozen thawed semen has several advantages i.e. the stallion can function in breeding programmes while also competing in sporting event; the stallion may be used for breeding even following temporary or permanent sterility, and even after its death; genetic material can be traded more easily among different countries and even different continents; the use of stallions of inferior genetic value can be limited and consequently the selection process is accelerated; the obstacles represented by the distance separating stallion and broodmare are eliminated; stallions are protected against infectious diseases; For example, it is possible to freeze semen from a horse which does not spread the viral arthritis virus avoiding that, if unfortunately in future the stallion becomes a shedder, the semen use for reproduction will be limited, even forbidden (Barbacini et al.,1997).

To improve reproductive efficiency when using cryopreserved semen, attention should be given to factors such as the stallion, the quality and handling of the semen, and age as well as reproductive history and management of the mare (Samper, 2000). Only poor and conflicting information is available about annual changes in semen characteristics (Pickett et al., 1976; Johnson and Thompson, 1983; Jesko et al., 1991), especially sperm morphology (Van der Host, 1975; Blottner et al., 2001) and semen freezability (Magistrini et al., 1987). Aiming to avoid transporting stallions to specialized centers, some studies developed protocols for freezing semen that cool the semen for a longer period before freezing. It was observed that cryopreservation after 24 hours of cooling reduced progressive motility, but cooling for 18 hours before freezing did not reduce fertility (Crockett et al., 2001; Backman et al., 2004). The storage of spermatozoa is associated with a reduction in cell viability and fertilizing capacity. The quality of stored semen is affected by handling procedures such as dilution, centrifugation and addition of semen extender (Bustamante-Filho et al., 2006). The efficiency of cooled semen depends

on an adequate shipment system. If insemination takes place within 12 hours after semen collection, then storage can be performed at either 20°C or 5°C. If semen storage exceeds 12 hours, slow cooling to 5°C is required. Semen storage at 4°C and 5°C resulted in higher sperm cell viability than storage at either 0°C or 2°C (Squires et al., 1999). The fertilization rate was highest in mares inseminated with frozen semen within 12 hr of ovulation. Foaling rate was improved by increasing the number of motile spermatozoa inseminated from 40 x 10^6/ml to 80 x10^6/ml but was not further improved by increasing the number to 160 x 10^6/ml or by increasing the frequency of insemination from once to twice daily (Pace and Sullivan, 1975).

Pregnancy rates in managed horse populations depended on the innate fertility of the mares and stallions involved and on the quality of breeding management. A single stallion was used to mate many mares; stallion fertility was a critical factor in the overall success of a breeding programmed (Colenbrander et al., 2003). The effects of different artificial insemination (AI) regimes on the pregnancy rate in mares inseminated with either cooled or frozen-thawed semen was investigated. In essence, the influence of three different factors on fertility was examined; namely the number of inseminations per estrus, the time interval between inseminations within an estrus, and the proximity of insemination to ovulation (Sieme et al., 2003a).

Techniques for properly handling, thawing and inseminating frozen semen were reviewed and also present fertility data collected in a commercial setting and factors that affect pregnancy rates for mares inseminated with frozen-thawed semen such as timing and frequency of insemination were examined for two separate data sets consisting of 332 and 536 mare cycles collected during the 2002 and 2003 breeding seasons (Loomis and Squires, 2005). An insemination dose of 500 x10^6/ml progressively motile sperm was recommended to maximize pregnancy rates when mares were bred with fresh semen under less than ideal conditions. Since that time, 500 x10^6/ml progressively motile sperm had been almost universally accepted as a standard insemination dose, regardless of a stallion's fertility or the refinements that had been made in mare management and semen extenders. Insemination doses for cooled-transported and frozen-thawed semen have also been extrapolated from this dose (Brinsko, 2006).

Numerous factors influence the pregnancy rate in horses bred by artificial insemination (AI); these include the inherent fertility of the mare and stallion, the type of semen used for insemination i.e. fresh, cooled-transported or frozen-thawed (Jesko et al., 1992) the number of spermatozoa in the insemination dose (Pickett et al., 1975; Leipold et al., 1998) the concentration of extended semen (Jesko et al., 1992) and the time for which liquid semen is stored prior to AI (Heiskanen et al., 1994). The effect on the pregnancy rate of the total number of inseminations per estrous cycle, the time interval between multiple inseminations during a single estrus, and the proximity of insemination to ovulation have been examined previously (Katila et al., 1996; Shore et al., 1998) but differences in the reported insemination regimes and in the evaluation criteria have made it difficult to objectively compare between studies and, therefore, to decide on an optimal insemination strategy.

The present study intended to fulfill the following objectives.

1. Evaluation of equine semen to assess the effect of age, breed and season on different macroscopic and microscopic parameters.
2. Cryopreservation of equine semen.
3. Comparison of pregnancy rate in mares by using natural service, cooled semen and frozen-thawed semen

CHAPTER –1

Semen evaluation of horse and donkey stallion to know the effect of age, breed and season

CHAPTER 1

Semen Evaluation

Abstract

The present study of semen evaluation was carried out from January 2005 to February 2007 to perform screening tests of horse and donkey stallions in order to analyze their fertility state. A total of 90 horse and 67 donkey stallions of six different breeds (Thoroughbred, Arab, Percheron, Suffolk, Noriker, Cleveland and Donkeys) from Horse, Mule and Cattle Breeding Area, Sahiwal (79 Horse Stallions and 54 donkey stallions) and Chenab Breeding Area Faisalabad (11 horse stallions and 13 donkey stallions) were evaluated for various semen parameters. These animals were kept under similar managemental and feeding conditions. The age of these stallions ranged between 4-22 years. All the horse and donkey stallions were divided in to two groups i.e. young and old stallions. In the present study those horse and donkey stallions up to 10 years of age were included in the younger group and all those above 10 years of age were included in the older group. Each horse and donkey stallion was groomed with a brush for few minutes before collection of ejaculate to remove loose particles and dirt in order to avoid contamination of the semen. At least two ejaculates were taken from each horse and donkey stallion at an interval of 45-55 minutes for better evaluation. After sexual stimulation, ejaculates were collected from each horse and donkey stallions by means of pre-warmed (42^0C) Missouri Model artificial vagina (AV). The effects of age, breed and season on volume (ml), motility percentage, sperm concentration ($x10^6$/ml), and percentage of dead spermatozoa of equine semen was also studied. The effects for breed on volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead spermatozoa was non significant of equine semen parameters. The effects for age was highly significant on volume (ml), motility percentage, sperm concentration ($x10^6$/ml), and percentage of dead spermatozoa of equine semen in the case of Thoroughbred horse stallions but non significant in the case of other breeds of horse and donkey stallions (Arab, Percheron, Suffolk, Noriker, Cleveland and Donkeys). The effect of seasonal changes was also non significant on volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead spermatozoa.

Introduction

The equine populations of Pakistan including horses (0.3 million), donkeys (4.3 million) and mules (0.3 million) represent an important national resource (Economic Survey of Pakistan, 2007). They are bred, reared and trained for diverse purposes. On one hand they act as a mechanical force for man to draw loads, transport goods, and plough agriculture land,etc.while on the other hand they amuse mankind by providing recreation and are a source of animal sports such as polo, tent pegging, horse jumping, trick ride,etc. To carry out these manifold tasks animals with superior genetic make up are mandatory. Male and female contribute equally towards the final genetic composition of the offspring.

Factors which influence an animal to be an effective stud (mare and stallion), are physical appearance, constitution, quality, mechanical soundness, temperament but productive and reproductive health also plays a very significant role. Infertility or low fertility is a major problem in the equine family. As a routine, attention is paid to the mares to increase overall fertility state but the reproductive efficiency of stallions is totally neglected. Several pathogenic conditions influence quality and quantity of a

stallion's semen. In addition to non-pathogenic and anatomical factors including breed, age and season of the year. A lot of emphasis is placed on female infertility whereas no attention is devoted to male fertility despite the fact that an infertile stallion equally contributes towards low fertility rate. Hence there is a need to include semen evaluation in the routine for timely screening of infertile stallions with a view to overcome male infertility. After analysis stallions showing better live sperm ratio, with low defective sperms should be maintained and energy should not be wasted on low performers (Samper, 2000).

Gel-free volume of semen was determined after removal of the gel fraction of the ejaculate, and any abnormality in appearance was observed. Concentration of sperm cells in semen could be determined with the use of either a haemocytometer or spectrophotometer after appropriate dilution of raw semen. The percentage of progressively motile sperm was evaluated promptly after collection of semen with the use of a phase-contrast microscope. The total numbers of sperm and progressively motile sperm in the ejaculate were calculated. The determination of seminal pH and the classification of sperm morphologic features were additional seminal characteristics evaluated during a semen evaluation. Sperm motion characteristics could be further

evaluated with the use of computerized sperm image analysis systems and might add additional information concerning the quality of ejaculated sperm. Unfortunately, no single seminal characteristic was shown to be highly correlated with fertility, although various seminal characteristics were known to affect fertility. A complete and thorough semen evaluation must be performed to properly interpret the fertility of a semen sample (Jasko, 1992). Not all of these assays would likely be conducted on every semen sample collected. It was suggested to include determination of semen volume, sperm concentration, and an estimation of the percentage of progressively motile sperm, at a minimum in routine evaluations. Additionally, laboratory assays were particularly important to conduct on cryopreserved spermatozoa, because these cells had been exposed to substantial temperature and osmotic insults. The percentage of fully functional sperm was reduced during cryopreservation and the longevity of the cells surviving freezing was shortened. It was suggested to maintain high fertilization rates using cryopreserved sperm and use only sperm samples containing sufficient numbers of high-quality cells. Unfortunately, no single laboratory assay could estimate the fertilizing potential of a semen sample (Grahamet al., 1996).

Parlevliet et al., (1994) studied the semen characteristics of 398 horse stallions and found volume (ml) 65±26, motility percentage 53±15, sperm concentration 206.1±168.5 $x10^6$/ml and percentage of live spermatozoa was 65±16. Pickett, (1993b) studied the semen characteristics of 417 horse stallions and observed volume (ml) 45±30, motility percentage 76.43, sperm concentration 335±232 $x10^6$/ml.Dowsett and Knott, (1996) evaluated the semen characteristics of 165 horse stallions and found volume (ml) 33.7±2.13, motility percentage 76.43±15, sperm concentration 164.13±39.35 $x10^6$/ml and percentage of live spermatozoa was 82±56. Long et al. (1993) evaluated the semen characteristics of 8 horse stallions and found volume (ml) 51.6±31.5 and sperm concentration 223±148 $x10^6$/ml. Dowsett and Pattie, (1982) evaluated the semen characteristics of 47 horse stallions and found volume (ml) 45.3±30.9,motility percentage 72.1±16, sperm concentration 178.16±168.35x106/ml and percentage of live spermatozoa was 78.8. Jasko (1991) evaluated the semen characteristics of 64 horse stallions and found motility percentage 70.3±17.4.

To know the effect of age, breed and fertility a survey was conducted by using 27 Icelandic stallions covering 1590 mares within the normal Icelandic breading system

(May to September) During the season, stallions cover mares within three periods of time, each period being of a similar length (average 35.5 days). During period I, mares are covered in hand and at pasture. During periods 2 and 3 all mares are covered at pasture. An overall fertility rate for Icelandic stallions was 67.7%. The following factors were shown to have a significant effect on fertility: age of mare ($P<0.001$), training level of stallion ($P < 0.05$) and method of breeding ($P<0.05$). For some individual stallions reproductive status of the mare also had a significant ($P<-0.001$) effect. Many of these factors have been observed to affect fertility rate in other more intensively managed equine populations. However, the less dramatic detrimental effect of age and the lack of a significant effect of mare reproductive status in most stallions suggest that infertility problems are less evident in Icelandic mares, possibly due to less emphasis on selection for athletic performance and the accepted culling of sub fertile stock (Davies Moral and Gunnarsson, 2000).

Breed and age of stallion, breeding season and the referral status of the stallion had significant ($P < 0.01$) effects on fertility. A study of stallion fertility was conducted and data from 47 stallions aged 2-26 years and representing 7 breeds and 1664 mares were used to relate seminal characteristics to fertility. The semen characteristics that were clearly associated with percentage pregnancies per service were: total volume gel-free volume, sperm concentration, total number of spermatozoa and total number of live spermatozoa. Approximate threshold levels for these characteristics are presented which could provide a diagnostic basis for the classification of stallions as being suitable or doubtful for breeding purposes (Dowsett and Pattie, 1982).

The variation in semen quality in spermatozoa and behavioral characteristics of 168 stallions representing 9 breeds and ranging in age from 2 to 26 year were studied. Semen samples were collected into an artificial vagina and the number of mounts and urethral pulsations per semen sample were recorded. Semen characteristics were examined for total volume, colour, mass activity, dead spermatozoa, sperm concentration, total number of spermatozoa and semen pH. All semen characteristics with the exception of colour and urethral pulsations had significant variation due to age. Semen quality (gel-free volume, sperm concentration, total sperm numbers and sperm abnormalities) was poorest in stallions under 3 years and over 14 years of age. Significant breed variation was apparent in most characteristics except for pH, semen colour, abnormal mid pieces and urethral

pulsations. It is recommended that both the age and breed of stallion be taken into consideration when evaluating stallion semen (Dowsett and Knott, 1996).

Seasonal changes of semen quality parameters in 15 stallions from the National Stud Farm in Avenches (Switzerland) were studied. Ejaculates were collected and evaluated every month during 1 year as well as cryopreserved in autumn and winter (September to February). In fresh semen the gel-free volume, concentration, motility and morphology (normal sperm, major defects, vacuoles and acrosome defects) were evaluated and in frozen-thawed semen the motility as well as the viability were performed. To analyze seasonal differences four periods of 3 months each were defined as autumn (September, October, November), winter (December, January, February), spring (March, April, May) and summer (June, July, August). During the 1-year experiment all fresh semen quality parameters demonstrated a clear seasonal and individual pattern. The gel-free volume was significantly ($P < 0.05$) higher in spring and summer compared to autumn and winter while sperm concentration was significantly ($P < 0.05$) lower in spring than at any other time of the year. Total sperm number was significantly ($P < 0.05$) higher and sperm motility significantly ($P < 0.05$) lower in summer than in other seasons. Regarding sperm morphology, normal sperm was significantly ($P < 0.05$) higher in autumn than in winter and summer and major defects were lowest ($P < 0.05$) in autumn. In frozen-thawed semen motility was significantly ($P < 0.05$) improved in the ejaculates collected in autumn compared to winter, while viability showed no obvious differences. Our results clearly demonstrate that individual and seasonal differences occurred in semen quality of Franches– Montagnes stallions. Ejaculates collected in autumn (September, October, November) demonstrated good quality, especially regarding sperm morphology, and were more suitable for cryopreservation because of better motility in frozen-thawed semen collected during autumn than in winter (Janett et al., 2003).

The standard procedure for assessing the breeding potential of a stallion includes the parameter total number of spermatozoa classified as morphologically normal. This study investigated sperm morphology of fresh semen in randomly chosen Estonian and Tori breed stallion with proven fertility. The T stallion had on average 57.5±4.1 % and the E-stallions 74.4 ± 3.8 % morphologically normal spermatozoa (p=0.012). In 4 of 7 stallions and 7 of 8 stallions both ejaculates had >50% morphologically normal spermatozoa. There was a significant difference between breeds in mean percentage of proximal

droplets (17.3±2.7% and 2.9±2.5% for T and E stallions respectively (P=0.003) (Kavak et. al., 2003).

Neither insemination technique, volume, sperm dose, nor mare or stallion had significant effect (P>0.05) on fertility. Type of semen, breeding mares during foal heat and an interaction between insemination technique, semen parameters, and mares did have significant effects (P>0.05). In problem mares, frozen semen AI yielded significantly lower pregnancy rates than fresh semen AI (16/43, 37.2% versus 25/42, 59.5%), but this was not the case in normal mares. In normal mares, hysteroscopic AI with fresh semen gave significantly (P>0.05) better pregnancy rates than uterine body AI (27/38, 71% versus 18/38, 47.3%), whereas in problem mares this resulted in significantly.

The present study was therefore, carried out to evaluate semen of the horse and donkey stallions in order to determine the effect of breed, age and season of the year being kept in the equine breeding areas of Pakistan on the parameters of semen of volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead sperms. It was anticipated that this study would provide useful information regarding the performance of stallions and would help in proving/disproving a stallion thereby improving the overall fertility state.

Materials and Methods

In this study a total of 90 horse and 67 donkey stallions from Horse, Mule and Cattle Breeding Area Sahiwal (79 horse stallions and 54 donkey stallions) and Chenab Breeding Area Faisalabad (11 horse stallions and 13 donkey stallions) of six different breeds (Thoroughbred, Arab, Percheron, Noriker, Suffolk, Cleveland and Donkey) were evaluated for various semen parameters as described in figure given below. The age of these horse and donkey stallions ranged between 4-22 years. All the horse and donkey stallions were divided in to two groups' i.e. young and old. In the present study horse and donkey stallions in either case up to 10 years of age were included in the younger group and all those above 10 years of age were included in the older group. These animals were kept under similar managemental and feeding conditions.

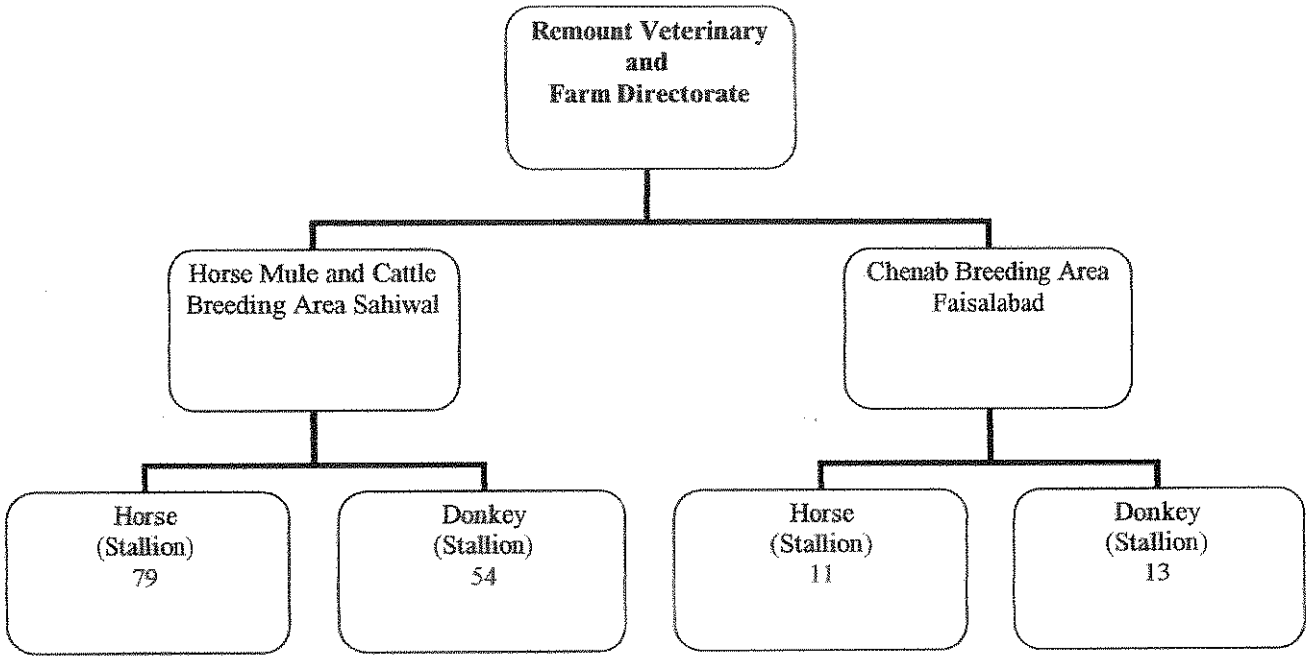

Figure-1.Organisation of Horse, Mule and Cattle Breeding Area Sahiwal and Chenab Breeding Area Faisalabad (Pakistan)

Preparation of Stallion and Semen Collection

Each horse and donkey stallion was groomed with a brush for few minutes before collection of ejaculate to remove loose particles and dirt in order to avoid contamination of the semen. The penile sheath was also cleaned. Each stallion was given enough time for sexual stimulation. One false mount was allowed for better sexual preparation before the collection of ejaculate. All the stallions were able to mount and yield semen. At least two ejaculates were taken from each stallion at an interval of 45-55 minutes for better evaluation. Each horse and donkey stallion was sexually stimulated by permitting 20 minutes restraint and one false mount. After sexual stimulation, ejaculates were collected from stallions by means of pre-warmed (42^0C) artificial vagina (AV) (Maul, 1962) and brought to semen evaluation room within one minute of collection.

Evaluation of Semen

Each ejaculate was examined both macroscopically and microscopically: -

Macroscopic Examination of Semen

Immediately after collection and removal of the gel fraction, the ejaculates were placed in a water bath maintained at 37^0C and observed for volume (ml), colour, pH and consistency. Volume of the semen was recorded directly from a graduated collection bottle/tube fitted with AV. Colour was noted by visual observation. pH was observed with a pH meter. Consistency was assessed by the flow method (Kenney et al., 1983).

Microscopic Examination of Semen

Microscopic examination was carried out for the following: -

Mass Motility

Mass motility was observed by taking a 5 mm diameter drop of semen on a clean, dry glass slide warmed at 37^0C and mass motion was observed at 40x magnification with the field diaphragm closed. Factors that affect mass motility of spermatozoa include concentration, percentage of progressively motile sperm and the speed/vigor of sperm motion. If one or more of these factors were compromised, the swirling of mass motion would be suppressed (Settergren, 1967). Descriptive assessments of mass motility for the presence of waves were evaluated (Baracaldo et al., 2006).

Description	Scale	Waves Pattern
Very Poor	0	Waves not present with immotile sperm cells
Poor	1	Waves not present with motile sperm cells
Fair	2	Barely distinguishable waves in motion
Good	3	Waves apparent with moderate motion
Very Good	4	Dark distinct waves with rapid motion

Individual Motility

This was determined by taking a 4 mm diameter drop of the semen on a clean glass slide warmed at 37^0C.One drop of sodium citrate solution was added which was then covered with a cover slip. This slide was then observed under phase contrast microscope at 400-x magnification and the percentage of sperm cells having progressive linear motion was determined. Evaluation of the type of ejaculate on the basis of rate of motility determined following :(Moris et al., 1999).

Rate of Motility	Type of Ejaculate
80 to 100	Very Good
60 to 80	Good
40 to 60	Fair
20 to 40	Poor
0 to 20	Very Poor

Spermatozoa Concentration

A haemocytometer was used after diluting the semen in concentration of 1:100 and applying methylene blue as a dye to facilitate counting. The number of sperm counted in five small squares was multiplied by 10000, which gave the concentration of sperm cells in one-milliliter volume of semen in millions (Graham, 1996).

Live/Dead Ratio

This was determined by making a semen smear after mixing a small drop of semen and 10% Eosin-Nigrosin stain on a clean glass slide. The Eosin stained the dead spermatozoa pink or red colour, live spermatozoa remained white whereas Nigrosin provided a black blue background. The sperm morphology evaluations were performed at 100x magnification on 200 sperm per sample (Dowsett et al., 1984).

Effect of Breeding and Non-breeding Season on different parameters of Equine Semen

Twenty Thoroughbred stallions between the age of 4 to 22 years from the Horse, Mule and Cattle Breeding Area Sahiwal and the Chenab Breeding Area Faisalabad were used to investigate seasonal effects on various semen parameters i.e. Volume (ml), Sperm motility percentage, Sperm concentration ($x10^6$/ml), and Dead spermatozoa percentage. The period of study was divided into two seasons: Breeding season (March-August) and Non-breeding season (September- February). Ejaculates of stallion were collected and processed as described above.

Statistical Analysis

Student's t test and analysis of variance were applied for comparison of means on the data related to semen parameters, i.e. Volume (ml), Sperm motility percentage, Sperm concentration ($x10^6$/ml), and Dead spermatozoa percentage.

RESULTS

Six types of horse breeds (Thoroughbred, Arab, Percheron, Suffolk, Noriker, Cleveland) and Donkeys were scored for the study of different semen parameters, which include volume, pH, motility percentage, sperms concentration, dead spermatozoa. All the horse and donkey stallions in each case were divided in to two groups' i.e. young and old. In the present data stallions up to 10 years of age were included in the younger group and all those above 10 years age were included in the older group. The data of semen analysis for each of the breeds are given in Table numbers1 to13.

Horse Stallions Breeds

Thoroughbred

Forty-two Thoroughbred horse stallions were scored for semen analysis. The age of the horse stallions and details of semen volume, colour, pH, consistency, motility percentage, sperm concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each horse are given in Table 1.

The age of the horse stallions ranged from 4 - 22 years. The range for semen volume was from 50 ml to 200 ml. Highest semen volume was observed in stallion numbers RD-0245 Baker's Accord, RD-9860 Decent Luck, RD-9940 Charming Prize, RD-962 Track Wind and RD-0156 Pak Pole (200 ml) while the lowest semen volume was seen in stallion number RD-8652 Diz Brow (50 ml).

The range for semen pH was from 7 to 8. In the case of Thoroughbred horses, highest semen pH was observed in stallion numbers RD-8654 Brave Bang, RD-9412 Bailiff Boy and RD-8652 Diz Brow (8.0) while the lowest semen pH was noted in stallion numbers RD-0245 Baker's Accord, RD-9860 Decent Luck, RD-9940 Charming Prize, RD-962 Track Wind and RD-0156 Pak Pole (7.0).

The range of sperm motility percentage was 10 to 80%. In the case of Thoroughbred horse stallions, the highest individual motility percentage of spermatozoa was observed in stallion numbers RD-0243 Trace Song, RD-0227 Pole Rite, RD-8844 Aron, RD-0015 Burnish, Rose, RD-9839 Poli Gold, RD-9138 Whisper Lara, RD-0255 Baker Love, RD-9152 Jambu Jet, RD-993 Baker's High, RD-992 Poly Zom, RD-933 Super Dunit, RD-987 Burning Night, RD-9922 Decent Wish, RD-9570 Maid By Blue, RD-9818 Burning Peak and RD-0145 Baker Lard (80%) while the lowest sperm motility was found in stallion numbers RD-853 Ibn-e-Tiger (10%).

The range of sperm concentration was 20 x 10^6/ml to 290 x 10^6/ml. In case of Thoroughbred horse stallions, highest sperm concentrations was observed in stallion numbers RD-8844 Aron, RD-0015 Burnish Rose, RD-9839 Poli Gold, RD-9138 Whisper Lara, RD-9152 Jambu Jet, RD-993 Baker's High, RD-992 Poly Zom, RD-933 Super Dunit, RD-987 Burning Night, RD-9570 Maid By Blue and RD-9818 Burning Peak (290 x 10^6/ml) while the lowest sperm concentrations were observed in horse stallion numbers RD-8654 Brave Bang and RD-8652 Diz Brow (20 x 10^6/ml).

The range of dead spermatozoa was 20 to 80%. In the case of Thoroughbred horse stallions the highest percentages of dead spermatozoa were found in horse stallion number RD-853 Ibn-e-Tiger and RD 8654 Brang Bang while the lowest was observed in RD-9152 Jamboo Jet.

Table 1. Semen Evaluation of Thoroughbred Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examinations				Microscopic Examinations			
			Vol (ml)	Colour	pH	Consistency	Motility %age	Concentration X 10^6 /ml	Live/Dead Ratio	Dead Sperms (%)
1	RD-853 Ibn-e-Tiger, (TP)	22	80	G.W	7.8	Thin	10	30	20: 80	80
2	RD-8654 Brave Bang	20	80	G.W	8.0	Thin	20	20	20: 80	80
3	RD-0243 Trace Song	5	160	G.W	7.6	Thick	80	260	78: 22	22
4	RD-954 Bold Seek	12	90	G.W	7.6	Thick	70	220	76: 24	24
5	RD-9894 Best Burn	9	90	G.W	7.6	Thick	70	220	76: 24	24
6	RD-0245 Baker's Accord	4	200	G.W	7.0	Thick	70	200	70: 30	30
7	RD-877 Blue Lagoon	19	100	G.W	7.8	Thin	20	60	20: 80	80
8	RD-019 Baker Shade	5	180	G.W	7.2	Thick	70	220	70: 30	30
9	RD-0227 Pole Rite	4	160	G.W	7.6	Thick	80	260	78: 22	22
10	RD-8844 Aron	16	150	G.W	7.4	Thick	80	290	80: 20	20
11	RD-0015 Burnish Rose	4	150	G.W	7.4	Thick	80	290	80: 20	20
12	RD-9839 Poli Gold	9	150	G.W	7.4	Thick	80	290	80: 20	20
13	RD-9860 Decent Luck	9	200	G.W	7.0	Thick	70	200	70: 30	30
14	RD-9831 Burn Aie	9	180	G.W	7.2	Thick	70	220	70: 30	30
15	RD-9138 Whisper Lara	16	150	G.W	7.4	Thick	80	290	80: 20	20

Continued on next page

16	RD-0255 Baker Love	4	150	G.W	7.4	Thick	80	240	75: 25	25
17	RD-9152 Jambu Jet (TP)	15	150	G.W	7.4	Thick	80	290	80: 20	20
18	RD-998 Blu Burn	8	90	G.W	7.6	Thick	70	220	76: 24	24
19	RD-9940 Charming Prize	8	200	G.W	7.0	Thick	70	200	70: 30	30
20	RD-962 Track Wind	11	200	G.W	7.0	Thick	70	200	70: 30	30
21	RD-0331 Noir Hut	4	180	G.W	7.2	Thick	70	220	70: 30	30
22	RD-993 Baker's High	8	150	G.W	7.4	Thick	80	290	80: 20	20
23	RD-9510 Blu Song	12	90	G.W	7.6	Thick	70	220	76: 24	24
24	RD-0038 Decent Star	7	180	G.W	7.2	Thick	70	220	70: 30	30
25	RD-992 Poly Zom	8	150	G.W	7.4	Thick	80	290	80: 20	20
26	RD-961 Bringing Luck	11	90	G.W	7.6	Thick	70	220	76: 24	24
27	RD-933 Super Dunit	14	150	G.W	7.4	Thick	80	290	80: 20	20
28	RD-969 True Gold	10	90	G.W	7.6	Thick	70	220	76: 24	24
29	RD-0348 Baker Star	4	180	G.W	7.2	Thick	70	220	70: 30	30
30	RD-987 Burning Night	4	150	G.W	7.4	Thick	80	290	80: 20	20
31	RD-015 Lovely Creek	4	90	G.W	7.6	Thick	70	220	76: 24	24

Continued on next page

32	RD-0352 Trace Eye	6	180	G.W	7.2	Thick	70	220	70: 30	30
33	RD-9412 Bailiff Boy	12	80	G.W	8.0	Thin	20	40	75: 25	75
34	RD-9922 Decent Wish	8	150	G.W	7.4	Thick	80	240	75: 25	25
35	RD-9570 Maid By Blue	11	150	G.W	7.4	Thick	80	290	80: 20	20
36	RD-0013 Burnish Lord	9	90	G.W	7.6	Thick	70	220	76: 24	24
37	RD-9818 Burning Peak	7	150	G.W	7.4	Thick	80	290	80: 20	20
38	RD-8828 Harmon's Jewel	16	90	G.W	7.6	Thick	70	220	76: 24	24
39	RD-0145 Baker Lard	6	150	G.W	7.4	Thick	80	240	75: 25	25
40	RD-8652 Diz Brow (TP)	20	50	G.W	8.0	Thin	20	20	20: 80	80
41	RD-9925 Burn Gal	8	90	G.W	7.6	Thick	70	80	76: 24	24
42	RD-0156 Pak Pole	6	200	G.W	7.0	Thick	70	200	70: 30	30

Statistical Analysis

Mean age of Thoroughbred horse stallions studied was 9.83±6.75 years. Comparatively younger horse stallions were of 6.57±0.27 years of age and old horse stallions were 15.125±0.90 years of age. Mean semen volume was 137.85±6.60ml, mean motility percentage was 76.62±1.26, mean sperm concentration was 213.81±12.10 $x10^6$/ml, and mean percentage of dead sperms was 31.05±2.81 in all the Thoroughbred horse stallions taken together.

In young thoroughbred horse stallions mean semen volume was (156.18±6.68ml) significantly higher ($t_{(40)}$ =4.18; p=1.29-06) than in older horse stallions (108 .12±9.84 ml). Similarly, sperm motility percentage in younger horse stallions (76.15±0.99) was significantly higher ($t_{(40)}$ =3.39; p=0.0005) than in older horse stallions (56.25±6.76). Sperm concentration was also significantly higher ($t_{(40)}$ =2.45; P =0.018) in younger horse stallions (235.77±2.89 $X10^6$/ml) than in older horses (178.12±26.30 $X10^6$/ml) .However ,mean percentage of dead sperm was significantly low ($t_{(40)}$ = 2.88; p = 0.0063) in younger horse stallions (25.19 ± 0.82) compared to older horse stallions (40.56 ± 6.72).

Table 2. Mean age related changes in semen volume, motility percentage, sperm concentration and percentage of dead sperm in Thoroughbred horse stallions.

Type of Horse stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperms (%)
All Horse stallions (n=42)	9.83 ± 6.75	137.85 ± 6.60	67.62± 1.26	213.81 ± 12.10	31.05± 2.81
Young Horse stallions < 10 years (n =27)	6.57±0.27	156.15 ± 6.68	76.15 ± 0.99	235.77± 2.89	25.19±0.82
Old Horse stallions > 10 years (n=15)	15.12± 0.90	108.12±9.84	56.25 ±6.76	178.12±26.30	40.56±6.72
Young Vs Old Horse Stallions	-	$t_{(40)}$=4.18 p = 0.00129	$t_{(40)}$=3.39 p =0.0015	$t_{(40)}$=2.45 p =0.018	$t_{(40)}$=2.88 p = 0.0063

Arab

Sixteen Arab horse stallions were scored for semen analysis. The age of horse stallions and details of semen volume, colour, pH, consistency, individual motility percentage, sperm concentration, live/dead ratio of spermatozoa, percentage of dead spermatozoa of each Arab horse stallion is given in table 3. The age of the horse stallions ranged from 4-22 years.

The range for semen volume was 80 to 200 ml. In the case of Arab horse stallions, highest semen volumes were observed in horse stallion numbers RD-0158 Oriel, RD-848 Abu-el-Deepak, RD-9057 Harnaz, RD-9210 Hoffa and RD-8841 Naseem Fawaras (200 ml) while the lowest semen volume was found in stallion number RD-9372 Nimar (80 ml).

The range of pH was 7 to 8. In the case of Arab horse stallions, highest semen pH was observed in horse stallion number RD-9372 Nimar (8.0) while lowest semen pH was found in horse stallion numbers RD-0158 Oriel, RD-848 Abu-el-Deepak, RD-9057 Harnaz, RD-9210 Hoffa and RD-8841 Naseem Fawaras (7.0).

The range of individual motility percentage of sperm was 20 to 80. In case of Arab horse stallions, highest sperm motility was observed in stallion numbers RD-0149 Prince Star, RD-0247 Para Boy, RD-9176 Hakam and RD-8952 Abopu-el-Sehar (80%) while lowest sperm motility was found in horse stallion number RD-9372 Nimar (20%).

The range of sperm concentration was (30 x 10^6/ml) to (290 x 10^6/ml). In the case of Arab horse stallions, the highest sperm concentration was found in horse stallion number RD-8952 Abopu-el-Sehar (290 x 10^6/ml). While lowest sperm concentration was observed in horse stallion number RD-9372 Nimar (30x10^6/ml).

Table 3. Semen Evaluation of Arab Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examination				Microscopic Examination			
			Vol (ml)	Colour	pH	Consistency	Motility %age	Concentration $X10^6$	Live /Dead Ratio	Dead Sperms (%)
1	RD-9372 Nimar	13	80	G.W	8.0	Thin	20	30	15: 85	85
2	RD-0158 Oriel	6	200	G.W	7.0	Thick	70	200	70: 30	30
3	RD-848 Abu-el-Deepak	22	200	G.W	7.0	Thick	70	200	70: 30	30
4	RD-937 Arfa	14	90	G.W	7.6	Thick	70	220	76: 24	24
5	RD-0162 Mona Pride	7	90	G.W	7.6	Thick	70	220	76: 24	24
6	RD-9485 The Shadow	12	90	G.W	7.6	Thick	70	220	76: 24	24
7	Parragonn	15	120	GW	7.2	Thick	70	200	70: 30	30
8	RD-9057 Harnaz	16	200	G.W	7.0	Thick	70	200	70: 30	30
9	RD-0149 Prince Star (TP)	5	150	G.W	7.4	Thick	80	240	75: 25	25
10	RD-9210 Hoffa	14	200	G.W	7.0	Thick	70	200	70: 30	30
11	RD-0247 Para Boy	4	150	G.W	7.4	Thick	80	240	75: 25	25
12	RD-9176 Hakam	15	150	G.W	7.4	Thick	80	240	75: 25	25
13	RD-9676 Abu Anastazia	10	180	G.W	7.2	Thick	70	220	70: 30	30
14	RD-8841 Naseem Fawaras	18	200	G.W	7.0	Thick	70	200	70: 30	30
15	RD-9712 Gemini Boy	9	90	G.W	7.6	Thick	70	220	76: 24	24
16	RD-8952 Abopu-el-Sehar	17	150	G.W	7.4	Thick	80	290	80: 20	20

Statistical Analysis

Mean age of all the Arab horse stallions studied was12.31±1.19 years. The mean age of younger horse stallions was 6.83±1.04 years and of older horse stallions was 15.60±0.63 years. In all horse stallions taken together mean semen volume was 146.25±11.79 ml, mean sperm motility percentage was 69.37±3.47; mean sperm concentration was 208.75±13.32x10^6/ml and mean percentage of dead sperm was 30.37±3.77.

Unlike thoroughbred horse stallions, Arab horse stallions showed a non-significant age effect on the parameters studied. Mean semen volume in young horse stallions (143.33±6.14 ml) was not significantly different ($t_{(14)}$ =0.84; p= 0.41) from older horse stallions (148.00±20.9 ml). Younger horse stallions (73.33±2.11) and older horse stallions (67.00±5.38 ml) showed non-significant differences ($t_{(14)}$ =0.87; P =0.39) in sperm motility percentage.

Similarly, mean sperm concentration in younger horse stallions (223.33±6.14x 10^6/ml) compared to older horse stallions (200.00±20.92x 10^6/ml) was not significantly different from each other ($t_{(14)}$ =0.84; p =0.41).The same was the case observed in mean percentage of dead sperm in younger horse stallions (26.33±1.11) and older horse stallions (32.8±5.90), which showed a non-significant difference in mean percentage of dead sperm ($t_{(14)}$ =0.83; p = 0.42).

Table 4. Mean age related changes in semen volume, motility percentage, sperm concentration and dead sperm in Arab horse stallions.

Type of Horse Stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperms (%)
All Horse Stallions (n=16)	12.31 ±1.1	146.25 ±11.7	69.37±3.7	208.75 ± 13.3	30.37± 3.7
Young Horse Stallions<10 years (n=6)	6.83±1.0	143.33± 6.1	73.33 ±2.1	223.33±6.1	26.33±1.1
Old HorseStallions>10 years (n=10)	15.02± 0.6	148.00±20.9	67.00 ±5.3	200.00±20.9	32.80±5.9
Young Vs Old Horse stallions	-	$t_{(14)}$=0.84 p = 0.41	$t_{(14)}$=0.87 p =0.39	$t_{(14)}$=0.84 p =0.41	$t_{(14)}$=0.83 p= 0.42

Percheron

Fourteen Percheron horses were scored for semen analysis. The age of the stallions and details of semen volume, colour, pH, consistency, motility percentage, sperm concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each Percheron horse are given in table 5. The age of the horse stallions ranged from 5 to 15 years.

The range of semen volumes was from 50 to 200 ml. In the case of Percheron horse stallions, highest semen volume was observed in horse stallion numbers RD-0041 Master Hope (260 ml) while lowest semen volume was found in stallion numbers RD-9535 Panda and RD-006 Badil Joy (90 ml).

The range of semen pH was 7.0 to 7.8. In the case of Percheron horse stallions, highest semen pH was observed in horse stallion number 93/96 Bantu (7.8) while lowest semen pH was found in stallion numbers RD-90573 Prince Boy, M-99171 Precious Smart Perch and RD-9119 Marshal (7.0).

The range of motility percentage of sperm was 20 to 80. In the case of Percheron horse stallions, highest sperm motility percentage was found in stallion numbers RD-9470 Pauper, RD-9667 Jaster Jesse, RD-0061 Rockface and RD-03-27 Master Boy (80%) while lowest sperm motility percentage was observed in stallion number 93/96 Bantu (20%)

The range of semen concentration was 120 x 10^6/ml to 290 x 10^6/ml. In the case of Percheron horse stallions, highest sperm concentration was observed in horse stallion number RD-9470 Pauper (290 x 10^6/ml) while lowest sperm concentration was found in horse stallion number 93/96 Bantu (120 x 10^6/ml).

Table 5. Semen Evaluation of Percheron Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examination				Microscopic Examination			
			Vol (ml)	Colour	pH	Consistency	Motility %ge	Concentration $X10^6$/ml	Live/Dead Ratio	Dead Sperms (%)
1	RD-0213 Master Piece	5	180	G.W	7.2	Thick	70	220	70: 30	30
2	RD-9470 Pauper	12	150	G.W	7.4	Thick	80	290	80: 20	20
3	RD-9535 Panda	11	90	G.W	7.6	Thick	70	220	76: 24	24
4	RD-9667 Jaster Jesse	11	150	G.W	7.4	Thick	80	240	75: 25	25
5	RD-006 Badil Joy	6	90	G.W	7.6	Thick	70	220	76: 24	24
6	RD-90573 Prince Boy	15	200	G.W	7.0	Thick	70	200	70: 30	30
7	M-99171 Precious Smart	8	200	G.W	7.0	Thick	70	200	70: 30	30

Continued on next page

8	0127 Badil Prize	6	120	White	7.6	Thick	60	190	72: 28	28
9	RD-0061 Rockface	8	130	G.W	7.6	Thick	80	280	80: 20	20
10	RD-0215 Jaster Lad	4	150	G.W	7.2	Thick	60	180	65: 35	35
11	RD-0041 Master Hope	6	260	G.W	7.6	Thick	75	240	74: 26	26
12	RD-03-27 Master Boy	4	160	G.W	7.6	Thick	80	260	78: 22	18
13	RD-9119 Marshal	15	140	G.W	7.0	Thick	60	220	70: 30	30
14	93/96 Bantu	13	100	G.W	7.8	Thin	20	120	20: 80	80

Statistical Analysis

The mean age of all the Percheron horse stallions was 8.85±1.21 years. Among them the mean age of younger horse stallions was 5.88±0.51 years and the mean age in older ones was 12.83±1.15 years. Overall mean semen volume was 151.43±12.62 ml; sperm motility percentage was 67.50±4.15; sperm concentration was 220.4±11.53 x 10^6/ml and mean percentage of dead sperm was 30±4.05.

In younger horse stallions mean semen volume was higher (160.25±16.49ml) than in older horse stallions (138.33±19.64 ml) but this difference between the two was not statistically significant ($t_{(12)} = 0.90$; $P = 0.38$). Mean sperm motility percentage, though, was higher in younger horse stallions (71.67 ± 2.63), than in old horse stallions (60±10.49) but they did not show a significant difference ($t_{(12)} = 1.39$; $P=0.19$). The semen concentration showed no significant difference ($t_{(12)} = 0.63$; $P = 0.53$) although younger horse stallions showed higher mean semen concentration (225.55 ± 10.9 x 10^6 / ml) than the old horse stallions (210.00± 27.20 x 10^6 / ml). Similarly in the case with mean percentage of dead sperm which is higher in old horse stallions (36.80 ± 10.96) than in younger horse stallions (26.22 ± 1.75) the difference is not significant ($t_{(12)} = 1.28$; $P = 0.22$).

Table 6. Mean age related changes in semen volume, motility percentage, sperm concentration and dead sperms in Percheron horse stallions.

Type of Horse Stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperms (%)
All Horse Stallions (n=14)	8.8 ±1.2	151.4 ±12.6	67.5±4.1	220±11.5	30± 4.0
Young Horse Stallions <10years (n=8)	5.88±0.5	160.2 ±16.4	71.6 ±2.6	225.5±10.9	26.2±1.7
Old Horse Stallions >10years (n=6)	12.8±1.1	138.3 ±19.6	60±10.4	210±27.2	36.8±10.9
Young Vs Old Horse Stallions	-	$t_{(12)}$=0.92 p= 0.38	$t_{(12)}$=1.39 p=0.19	$t_{(12)}$=0.63 p =0.53	$t_{(12)}$=1.28 p = 0.22

Noriker

Six Noriker horse stallions were scored for semen analysis. The age of the horse stallions and details of semen volume, colour, pH, consistency, motility percentage, sperm concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each Noriker horse stallions are given in table 7. The range of semen volume was 90 to 200 ml. In the case of Noriker horse stallions, highest semen volume was found in horse stallion numbers RD 09833 Noble Love and RD-907 Khyber (200 ml) while lowest semen volume was observed in stallion number RD-9173 (90 ml).

The range of semen pH was 7.0 to 7.6. In the case of Noriker horse stallions, highest semen pH was observed in stallion numbers RD-0236 Noble Prize and RD-9173 (7.6)

The range of semen motility percentage was 70 to 80. In the case of Noriker horse stallions, highest sperm motility percentage was observed in stallion numbers RD-0017 Noble Law, RD-8945 Nova and RD-0236 Noble Prize (80%) while lowest sperm motility percentage was found in stallion numbers RD 09833 Noble Love, RD-9173 and RD-907 Khyber (70%)

The range of sperm concentrations was 200 x 10^6/ml to 290 x 10^6/ml. In the case of Noriker horse stallions, highest sperm concentration was found in horse stallion number RD-0017 Noble Law (290 x 10^6/ml) while lowest sperm concentration was observed in horse stallion numbers RD 09833 Noble Love and RD-907 Khyber (200 x 10^6/ml).

Table 7. Semen Evaluation of Noriker Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examination				Microscopic Examination			
			Vol (ml)	Colour	pH	Consistency	Motility %ge	Concentration $X10^6$/ml	Live/Dead Ratio	Dead Sperms (%)
1	RD-0017 Noble Law (TP)	7	150	G.W	7.4	Thick	80	290	80: 20	20
2	RD-8945 Nova	18	150	G.W	7.4	Thick	80	240	75: 25	25
3	RD 09833 Noble Love	8	200	G.W	7.0	Thick	70	200	70: 30	30
4	RD-0236 Noble Prize	5	160	G.W	7.6	Thick	80	260	78: 22	22
5	RD-9173 (TP)	15	90	G.W	7.6	Thick	70	220	76: 24	24
6	RD-907 Khyber	16	200	G.W	7.0	Thick	70	200	70: 30	30

Statistical Analysis

Mean age of Noriker horse stallions studied was 11.5 ± 2.2 years and mean semen volume was 158.3 ± 16.6 ml, mean sperm motility percentage was 75.3 ± 2.2, mean sperm concentration was 235.3 ± 14.5x 10^6 ml and mean percentage of dead spermatozoa was 25.1 ± 1.6. Age related changes in these parameters were also analyzed. The mean age in younger horse stallions was 6.3 ± 0.8 years. Mean semen volume in younger horse stallions was 170±10.8 ml and mean motility percentage of sperm was 76.6 ± 2.3. Mean sperm concentration was 250±18.7x $10^{6/}$ ml and mean percentage of dead sperm in younger horse stallions was 24.0 ± 3.0.

Mean age of old horse stallions was 16.6±0.8 years. In old horse stallions mean semen volume was (146.67 ± 31.79 ml) less than younger horse stallion, but they do not differ significantly from each other ($t_{(4)} = 0.66$; $P = 0.54$). Older horse stallions showed no difference in mean sperm concentration (73.3 ± 3.3 x 10^6 ml) ($t_{(4)} = 0.71$; $p = 0.52$) and mean percentage of dead sperms (26.33 ± 1.85; ($t_{(4)} = 0.65$; $P = 0.55$) compared to younger horse stallions. Reduction in mean sperm concentration in older horses (220.00 ± 11.54 x 10^6 ml) was not significant compared to younger horse stallions ($t_{(4)} = 1.03$; $P = 0.35$).

Table 8. Mean age related changes in semen volume, motility percentage, semen concentration and dead sperms in Noriker horse stallions.

Type of Horse Stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$/ml	Dead Sperms (%)
All Horse Stallions (n=6)	11.5 ±2.2	158.3 ±16.6	75.0±2.2	235.0 ± 14.5	25.1±1.6
Young Horse stallions <10 years (n=3)	6.6±0.8	170.0 ±10.8	76.6 ±2.3	250±18.7	24±3.0
Old Horse stallions >10 years (n=3)	16.3± 0.8	146.6±31.7	73.3 ±3.3	220±11.5	26.3±1.8
Young Vs Old Horses	-	$t_{(4)}$=0.66 P = 0.54	$t_{(4)}$=0.71 P =0.52	$t_{(4)}$=1.03 P =0.35	$t_{(4)}$=0.65 P = 0.55

Suffolk

Seven Suffolk horse stallions were scored for semen analysis. The age of the horse stallions and details of semen volume, colour, pH, consistency, individual motility percentage, sperm concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each Suffolk horse stallions are given in table 9.The range of semen volumes was 90 to 300 ml. In the case of Suffolk horse stallions, highest semen volume was found in horse stallion number RD-9233 Dragoon (300 ml) while lowest semen volume was observed in horse stallion number RD-988 Pole of Leed (90 ml)

The range of semen pH was 7.0 to 7.8.In the case of Suffolk horse stallions, highest semen pH was noted in horse stallion number RD-9233 Dragoon (7.8) while lowest semen pH was observed in horse stallion number RD-9979 Bendorf Joy (7.0). The range of sperm motility percentage was 40 to 80%. In the case of Suffolk horse stallions, highest sperm motility was observed in stallion number RD-9041 Brave Prince (80%) while lowest sperm motility percentage was found in horse stallion number RD-9233 Dragoon (40%).

The range of sperm concentration was $140x10^6$/ml to $290x10^6$/ml. In case of Suffolk horse stallions, highest sperm concentration was found in stallion number RD-9041 Brave Prince ($290x10^6$/ml) while lowest sperm concentration was observed in stallion number RD-9233 Dragoon ($140x10^6$/ml).

Table 9. Semen Evaluation of Suffolk Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examination				Microscopic Examination			
			Vol (ml)	Colour	pH	Consistency	Motility %age	Concentration X 10^6/ml	Live/Dead Ratio	Dead Sperms (%)
1	RD-9041 Brave Prince	17	150	G.W	7.4	Thick	80	290	80: 20	20
2	RD-9361 Prince Laurel (TP)	16	120	GW	7.2	Thick	70	200	70: 30	30
3	RD-9979 Bendorf Joy	7	200	G.W	7.0	Thick	70	200	70: 30	30
4	RD-988 Pole of Leed	8	90	G.W	7.6	Thick	70	220	76: 24	24
5	RD-9442 Samson	14	170	G.W	7.2	Thick	70	180	75: 25	25
6	RD-0242 Fantastic	6	100	G.W	7.4	Thick	65	220	70: 30	30
7	RD-9233 Dragoon	16	300	G.W	7.8	Thin	40	140	60: 40	40

Statistical Analysis

Mean age of Suffolk horse stallions was 12.0 ± 1.00 years and in them the mean semen volume was 161.42 ± 27.37 ml. Mean motility percentage of sperm was 66.43±4.72 and mean sperm concentration was 207.1±17.2x10^6/ml. Mean percentage of dead sperm was 28.4 ± 2.4.

The data were further analyzed to observe the effect of age on the seminal parameters. The mean age of younger horse stallions was 7.0 ± 2.0 years. In younger horse stallions mean semen volume was 95.0 ± 7.0 ml but in older horse stallions mean semen volume was (188.0 ± 30.8 ml) higher than in younger horse stallions. The difference between the two was not significant ($t_{(5)}$ = 1.79; P = 0.13). There was not much difference in mean sperm motility percentage in young horse stallions (67.50 ± 2.5) and old horse stallions (66.00 ± 6.78). Mean sperm concentration in younger horse stallions (220.00 ± 0.00) was higher than in old horse stallions (202.00 ± 24.57), but they did not differ significantly from each other ($t_{(5)}$ = 0.44 ; P = 0.67). Mean percentage of dead sperm showed not much difference in younger horse stallions (27.0±3.0) and older horse stallions (29.00 ± 3.31), which was not significantly different from each other.

Table 10. Mean age related changes in semen volume, motility percentage, sperm concentration and percentage of dead sperms in Suffolk horse stallions: -

Type of Horse stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperms (%)
All Horse stallions (n=7)	12.0 ±1.0	161.4 ±27.3	66.4 ± 4.7	207.1 ± 17.2	28.4± 2.4
Young Horse stallions <10years (n=3)	7.0 ± 2.0	95.0 ± 7.0	67.5 ±2.5	220.0± 0.0	27.0 ±3.0
Old Horse stallions >10years (n= 4)	15.7±1.0	188.0±30.8	66.0 ±6.7	202.0±24.5	29.0 ± 3.3
Young Vs Old Horse stallions	-	$t_{(5)}$=1.79 p = 0.13	$T_{(5)}$=0.13 p =0.90	$t_{(5)}$=0.44 p =0.67	$t_{(5)}$=0.34 p = 0.74

Cleveland

Five Cleveland horse stallions were scored for semen analysis. The age of the horse stallions and details of semen volume, colour, pH, consistency, motility percentage, concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each Cleveland horse stallions are given in table11.

The range of semen volume was 150 to160 ml. In the case of Cleveland horse stallions, highest semen volume was observed in horse stallion number M-9965 Nice Palms (160 ml) while lowest semen volume was found in horse stallion numbers Twin Palms Roscoe and RD-9571 Mister Royal (150 ml).

The range of semen pH was 7.4 to 7.6. In the case of Cleveland horse stallions, highest semen pH was noted in horse stallion number M-9965 Nice Palms (7.6) while lowest semen pH was observed in horse stallion numbers Twin Palms Roscoe and RD-9571 Mister Royal (7.4).

In the case of Cleveland horse stallions, highest and lowest sperm motility percentage were observed in stallion numbers Twin Palms Roscoe, M-9965 Nice Palms and RD-9571 Mister Royal (80%).

The range of sperm concentration was $260x10^6$/ml to $290x10^6$/ml. In the case of Cleveland horse stallions, highest sperm concentration was noted in stallion numbers Twin Palms Roscoe and RD-9571 Mister Royal ($290x10^6$/ml) while lowest sperm concentration was observed in horse stallion number M-9965 Nice Palms ($260x10^6$/ml).

Table 11. Semen Evaluation of Cleveland Horse Stallions

S/No	Horse Stallion No and Name	Age (Yrs)	Macroscopic Examination				Microscopic Examination			
			Vol (ml)	Colour	pH	Consistency	Motility %ge	Concentration $X10^6$/ml	Live/Dead Ratio	Dead Sperms (%)
1	Twin Palms Roscoe	16	150	G.W	7.4	Thick	80	290	80: 20	20
2	M-9965 Nice Palms	8	160	G.W	7.6	Thick	80	260	78: 22	22
3	RD-9571 Mister Royal	11	150	G.W	7.4	Thick	80	290	80: 20	20
4	RD-0226 Stirling Dot	6	150	G.W	7.4	Thick	80	240	75: 25	25
5	8750 Gyrgygous	10	70	G.W	7.8	Thin	20	70	30: 70	70

Statistical Analysis

Only five Cleveland horse stallions were available for study. Comparing this small number was difficult to categorize them in two younger and older horse stallions. Mean age was 10.2 ± 1.8 years, mean semen volume was 136.0 ± 16.6ml; mean sperm motility percentage was 68.0 ± 12.0, mean sperm concentration was 230.0 ± 41.1x10^6 / ml, and mean percentage of dead sperm were 31.4±9.6 (Table-12).

Table 12. Mean age related changes in semen volume, motility percentage, sperm concentration and percentage of dead sperm in Cleveland horse stallions: -

Type of Horse stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperms (%)
All Horse stallions (n=5)	10.2 ± 1.85	136.00 ±16.61	68.0± 12.0	230.00 ±41.11	31.4±9.69

Donkey Stallions

Sixty-Seven Donkey Stallions were scored for semen analysis. The age of the donkey stallions and details of semen volume, colour, pH, consistency, motility percentage, sperm concentration, live/dead ratio of spermatozoa and percentage of dead spermatozoa for each donkey stallion are given in table 13.

In the case of donkey stallions, highest semen volume was observed in donkey stallion numbers 15/91 Munsif, 61/93 Mace, 79/89 Mukha and 21/92 Mikran (200 ml) while lowest semen volume was found in donkey stallion number 35/97 Mezzo (60 ml).

In the case of donkey stallions, highest semen pH was observed in donkey stallion numbers 7889 Millat and 68/94 Musha (8.0) while lowest semen pH was found in donkey stallions numbers 48/97 Mego, 15/91 Munsif, 61/93 Mace, 79/89 Mukha and 21/92 Mikran (7.0).

In the case of donkey stallions, highest sperm motility percentage was noted in donkey stallion numbers 62/98 Maverick (85%) while lowest sperm motility was found in donkey stallions numbers 7889 Millat, 68/94 Musha and 35/97 Mezzo (20%).

In the case of donkeys stallions, highest sperm concentration were observed in donkey stallion numbers MSB-0277 Musnad (340 x 10^6/ml) while lowest sperm concentration was found in donkey stallion number 7889 Millat (30 x 10^6/ml).

Table 13. Semen Evaluation of Donkey Stallions

S/No	Donkey Stallion No and Name	Age (Yrs)	Macroscopic Examination					Microscopic Examination		
			Vol (ml)	Colour	pH	Consistency	Motility % age	Concentration $X10^6$/ml	Live/Dead Ratio	Dead Sperms (%)
1	19/92 Minister	15	120	GW	7.2	Thick	70	200	70: 30	30
2	14/95 Marco	13	180	G.W	7.2	Thick	70	240	75: 25	25
3	48/97 Mego	9	140	G.W	7.0	Thick	60	220	70: 30	30
4	63/98 Magilian	9	150	G.W	7.4	Thick	80	240	75: 25	25
5	42/97 Mhnful	9	150	G.W	7.4	Thick	80	240	75: 25	25
6	7889 Millat	18	80	G.W	8.0	Thin	20	30	20: 80	80
7	60/02 Marakish	7	120	GW	7.2	Thick	70	200	70: 30	30
8	60/89 Mashooq	18	80	GW	7.8	Thin	30	60	20: 80	80
9	65/98 Maharaja	9	120	GW	7.2	Thick	70	200	70: 30	30
10	61/01 Mega	6	120	GW	7.2	Thick	70	200	70: 30	30
11	2/00 Mallenum	7	120	GW	7.2	Thick	70	200	70: 30	30
12	15/91 Munsif	16	200	G.W	7.0	Thick	70	200	70: 30	30
13	48/93 Medium	14	150	G.W	7.4	Thick	80	240	75: 25	25
14	61/93 Mace	13	200	G.W	7.0	Thick	70	200	70: 30	30

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15	36/92 Modern	15	150	G.W	7.4	Thick	80	240	75: 25	25
16	24/00 Mid Stone	6	120	GW	7.2	Thick	70	200	70: 30	30
17	41/01 Mohawak	7	120	GW	7.2	Thick	70	200	70: 30	30
18	50/01 Might Star	5	120	GW	7.2	Thick	70	200	70: 30	30
19	7/95 Miffi	12	180	G.W	7.2	Thick	70	220	70: 30	30
20	9/00 Mounty	7	160	G.W	7.6	Thick	80	260	78: 22	22
21	7/00 Marry	7	120	GW	7.2	Thick	70	200	70: 30	30
22	35/01 Mona Stire	6	150	G.W	7.4	Thick	80	240	75: 25	25
23	93/95 Mehram	11	180	G.W	7.2	Thick	70	220	70: 30	30
24	5/00 Master	7	160	G.W	7.6	Thick	80	260	78: 22	22
25	68/94 Musha	13	70	GW	8.0	Thin	20	40	20: 80	80
26	52/93 Mokil	13	160	G.W	7.6	Thick	80	260	78: 22	22
27	4597 Matin	9	120	GW	7.2	Thick	70	200	70: 30	30
28	44/93 Moruel	13	160	G.W	7.6	Thick	80	260	78: 22	22
29	98/95 Motel	11	120	GW	7.2	Thick	70	200	70: 30	30
30	21/96 Meaqdow Prince	10	120	GW	7.2	Thick	70	200	70: 30	30

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31	58/97 Mountie	9	180	G.W	7.2	Thick	70	220	70: 30	30
32	69/98 Mahanama	9	160	G.W	7.6	Thick	80	260	78: 22	22
33	30/00 Mashal	8	120	GW	7.2	Thick	70	200	70: 30	30
34	64/94 Madic	12	120	GW	7.2	Thick	70	200	70: 30	30
35	15/95 Middler	11	120	GW	7.2	Thick	70	200	70: 30	30
36	76/89 Mellow	7	120	GW	7.2	Thick	70	200	70: 30	30
37	29/92 Milan	15	160	G.W	7.6	Thick	80	260	78: 22	22
38	71/94 Morsel	13	120	GW	7.2	Thick	70	200	70: 30	30
39	12/95 Mole	13	120	GW	7.2	Thick	70	200	70: 30	30
40	39/01 Manila	6	120	GW	7.2	Thick	70	200	70: 30	30
41	86/90 Madhoo	16	180	G.W	7.2	Thick	70	220	70: 30	30
42	25/00 Margala Ridge	7	180	G.W	7.2	Thick	70	220	70: 30	30
43	79/89 Mukha	17	200	G.W	7.0	Thick	70	200	70: 30	30
44	32/01 Malta	7	150	G.W	7.4	Thick	80	240	75: 25	25
45	55/93 Mayday	13	150	G.W	7.4	Thick	80	240	75: 25	25

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46	11/00 Moon Light	7	180	G.W	7.2	Thick	70	220	70: 30	25
47	60/93 Morus	14	180	G.W	7.2	Thick	70	220	70: 30	30
48	56/97 Mutt	9	180	G.W	7.2	Thick	70	220	70: 30	25
49	67/94 Mieky	13	150	G.W	7.4	Thick	80	240	75: 25	15
50	35/97 Mezzo	10	60	G.W	7.8	Thin	20	40	20: 80	80
51	38/87 Mango	19	90	G.W	7.6	Thick	70	220	76: 24	24
52	21/92 Mikran	16	200	G.W	7.0	T	70	200	70: 30	30
53	55/97 Murky	9	150	G.W	7.4	Thick	80	240	75: 25	25
54	38/97 Macho	10	70	G.W	7.2	Thick	80	200	78: 22	22
55	62/98 Maverick	9	80	G.W	7.2	Thick	85	280	86: 14	14
56	66/02 Master charge	6	80	G.W	7.4	Thick	80	280	80: 20	20
57	99/94 Match star	13	120	White	7.2	Thick	70	220	82: 16	16
58	10/03	5	80	G.W	7.4	Thick	70	240	74: 26	24
59	0277 Musnad	7	120	White	7.2	Thick	80	340	80: 20	20

Continued on next page

60	49/97 Modem	9	150	G.W	7.4	Thick	80	280	80: 20	20
61	19/2000 Marry Gold	8	90	G.W	7.6	Thick	70	220	76: 24	24
62	72/98 Matchless	7	150	G.W	7.4	Thick	80	240	75: 25	25
63	96/93 Moon Star	14	180	G.W	7.2	Thick	70	220	70: 30	25
64	24/96 Mitty	10	120	G.W	7.2	Thick	70	200	70: 30	30
65	84/98 Modish	8	180	G.W	7.2	Thick	70	240	75: 25	25
66	57/97 Myth	10	80	G.W	7.8	Thin	30	140	28: 72	72
67	50-84 Murfi	22	160	G.W	7.6	Thick	80	260	78: 22	22

Statistical Analysis

All the donkeys stallions studied were of mean 10.64±0.50 years of age. Their mean semen volume was 137.1±4.4 ml; motility percentage was 69.7±1.7; sperm concentration was 203.5±10.3x10^6/ml; and mean percentage of dead sperm was 28.8±2.0.

Mean age of old donkey stallions was 14.2±0.2 years. Mean semen volume in old donkey stallions was higher (145.8±6.8) than in younger (129.7±5.5) donkey stallions but this difference between the two was not significant ($t_{(65)}$ =1.84; p=0.07).

Old donkey stallions showed not much difference in mean sperm motility percentage as (68.06±2.79) compared to younger donkey stallions ($t_{(65)}$ =0.92; p =0.36). Mean sperm concentration (203.54±10.31x10^6/ml) in old donkey stallions was less than in younger donkey stallions and the two differ significantly in this regard ($t_{(65)}$ =1.42; p=0.16). A slight increase in mean percentage of dead sperm in old donkey stallions (31.87±2.97) was also not significantly different compared to that of younger donkey stallions ($t_{(65)}$=0.87; p =0.38).

Table 14. Mean age related changes in semen volume, motility percentage, and sperm concentrationand percentage o dead sperm in Donkey Stallions.

Type of Donkey stallions	Mean Age (Years)	Volume (ml)	Motility Percentage	Concentration $X10^6$ /ml	Dead Sperm (%)
All donkey stallions (n=67)	10.64 ±0.50	137.16 ±4.43	69.77± 1.72	203.54 ± 10.31	30.12±1.76
Young donkey stallions <10years (n = 36)	7.53±0.30	129.72 ±5.59	67.53 ± 0.30	221.67±7.82	28.80±2.05
Old donkey stallions >10years (n= 31)	14.28± 0.26	145.80±6.82	68.06 ±2.79	203.54±10.31	31,87±2.97
Young Vs Old donkeys	-	t (65)=1.48 p = 0.070	t (65)=0.92 p =0.36	t(65)=1.42 p =0.16	t(65)=0.87 p = 0.38

Effect of Breeding and Non breeding season on different parameters of equine semen

The effects of seasonal changes on volume, pH, motility percentage, sperm concentration and percentage of dead spermatozoa are shown in Table 15. Mean semen volume was 129.00 ± 9.44 ml during the breeding season while it decreased to 120.75 ± 0.00 ml during the non-breeding season but the difference between the two was not significant ($t_{(38)}$ =0.54 p= 0.06). There was no appreciable difference in mean semen pH values, which was 7.49 ± 0.06 and 7.49 ± 0.06 during the breeding and the non-breeding seasons, respectively. Mean motility percentage was higher (64.50 ±4.55) in the breeding season and lower (58.50 ± 3.97) in the non-breeding season but there were not significantly different from each other. On the same pattern, although concentration of spermatozoa was higher (196.00 ± 19.11) in the breeding season but not significantly higher from the non breeding season (185.00 ± 20.12).The percentage of dead sperm was higher (30.75 ± 4.18) during the breeding season and lower (23.15 ± 3.39) in the non-breeding season but the reduction in percentage of dead spermatozoa was not significant compared to the breeding season ($t_{(30)}$ =1.41; p =0.06).

Table 15. Mean values of semen parameters from ejaculates of 20 Thoroughbred horse stallions during the breeding and the non breeding seasons.

Semen Parameter	Breeding Season (± S.E)	Non-Breeding Season (± S.E)
Volume (ml)	129.00 ± 9.44	120.75 ±11.52
PH	7.49 ±0.06	7.49 ± 0.06
Motility (%)	64.50 ± 4.55	58.50 ± 3.97
Concentration ($x10^6$/ml)	196.00 ± 19.11	185.00 ±20.12
Dead Sperms (%)	30.75 ± 4.18	23.15 ±3.39

Effect of breeds on different parameters of equine semen

The breed wise means and standard errors of different equine semen parameters are presented in Table 16.The maximum volume was observed in the Suffolk breed (161.43 ± 27.34) while lowest volume was observed in Thoroughbred and donkey breeds (137.86 ± 6.60). The pH values were highest in Cleveland (7.47 ± 0.05) and lowest in Noriker (7.33 ± 0.11) whereas donkey stallion also showed minimum values of pH (7.33 ± 0.23). The values of individual motility percentage were highest in the Cleveland breed (80%) and lowest in the Suffolk (66.43%). The sperm concentration showed maximum values in the Cleveland (280.00±7.73) while lowest in the Suffolk (207.14±45.72). Analysis of variance was carried out to compare their parameters among different equine breeds. Analysis of variance showed non-significant difference in mean semen volume ($F_{(7,149)}$ = 0.83; p =0.56), mean motility percentage ($F_{(7,149)}$ = 0.86; p=0.53), mean dead spermatozoa ($F_{(7,149)}$ = 1.88; p =0.75) and mean sperm concentration ($x10^6$/ml) ($F_{(7,149)}$ = 0.95; p =0.46).

Table 16. Effect of breed on various equine semen parameters (Means ± S.E)

Breeds	n	Volume (ml)	pH	Motility (%)	Concentration ($X10^6$/ml)	Dead Sperms (%)
Thoroughbred	42	137.86 ± 6.60	7.44 ± 0.04	67.62 ± 2.95	213.81±12.10	31.05±2.81
Arab	16	146.25 ± 11.79	7.34 ± 0.75	69.38 ± 3.47	208.75 ± 13.32	30.37±3.77
Percheron	14	151.43 ± 12.66	7.40 ± 0.07	67.50 ± 4.15	220.00 ± 11.54	30.00±4.05
Noriker	6	158.33 ± 16.60	7.33 ± 0.11	75.00 ± 2.24	235.00 ± 14.55	25.17±1.68
Suffolk	7	161.43 ± 27.34	7.37 ± 0.10	66.43 ±4.71	207.14 ± 17.25	28.42±2.40
Cleveland	5	153.33 ±2.57	7.47 ± 0.05	80.00 ± 0.00	280.00 ± 7.73	32.4±9.69
Donkey	67	137.16 ± 4.44	7.33 ± 0.028	69.78 ± 1.72	213.28 ± 6.41	30.12±1.76

Comparison of semen characteristics between younger and older horse and donkey stallions; between breeds and between stallions; and age related changes in semen characteristics

Analysis of variance for different semen characteristics between younger and older horse and donkey stallions, between the breeds, Volume (ml), motility percentage and sperm concentration (X 10^6) showed highly significant decreases ($p < o.001$) in older horse and donkey stallions as compared to younger horse and donkey stallions. Mean percentage of dead sperm showed non-significant differences among older and younger horse and donkey stallions ($P = 0.091$).

Semen characteristics Volume (ml) ($P = 0.38$), motility percentage ($P = 0.41$) and sperm concentration ($x10^6$/ml) ($P = 0.30$) showed non-significant differences among younger and older horse and donkey stallions with the exception of mean percentage of dead sperm, which showed highly significant differences among equine breeds.

Analysis of variance for semen characteristics between breeds for volume (ml) ($P = 0.0024$), motility percentage ($P = 0.001$), sperm concentration ($x10^6$/ml) ($P< 0.0001$) and mean percentage of dead sperm ($P = 0.0001$) showed highly significant differences. The difference for semen characteristics between stallions in volume (ml) ($P = 0.35$) was not significant, but in motility percentage ($P = 0.03$), sperm concentration ($x10^6$/ml) ($P< 0.0001$) and mean percentage of dead sperm ($P = 0.03$), highly significant differences were observed.

Table.17 Analysis of variance for different semen characteristics volume (ml), motility (%), sperm Concentration ($X10^6$/ml) and dead sperm (%)between breeds and between stallions.

Semen characteristics	Between breads				Between Stallions				Remarks	
	MS	df	F	P	MS	d-f	F	P	MS	d-f
Volume (ml)	16450	6	3.84	0.0024	4840	11	1.13	0.35	4281	66
Motility (%)	33010	6	4.31	0.001	16730	10	2.18	0.03	7657	60
Concentration ($x10^6$/ml	2253000	6	608.42	<0.0001	11755000	13	317.4	<0.0001	3703	78
Dead sperm (%)	973.1	6	4.38	0.001	408	10	2.18	0.03	222.2	60

Table -18 shows coefficients of regression and standard errors for different semen characteristics in relation to age of various breeds, regression coefficients showing whether positive or negative trends or all not significantly different from zero. Negative trends with advancing age for semen volume were observed in Arab stallions, Percheron and Cleveland breeds. Advancing age showed reduction in sperms motility in Thoroughbred, Arab, Percheron, Noriker and donkey stallions. Decreased sperm concentration ($x10^6$/ml) was observed in Thoroughbred, Percheron, Noriker, Suffolk and Donkeys stallions with advancing age. Mean percentage of dead sperm showed a decrease with the increase in age only in the Cleveland breed.

Table 18. Regression analyses on age of semen characteristics volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead sperm among various equine breeds.

Characteristics	Thoroughbred (n=42)	Arab (n=16)	Percheron (n= 14)	Noriker (n=6)	Suffolk (n=7)	Cleve land (n=5)	Donkey (n=67)
Volume (ml)	0.43± 0.54	-1.35± 2.89	-1.11± 2.99	1.50± 3.64	8.75±5.53	-0.08± 5.17	1.51± 11.0
P	0.42	0.64	0.71	0.70	0.17	0.98	0.16
Motility (%)	-0.34± 0.24	-0.44± 0.75	-1.15± 0.93	-0.30± 0.47	0.17± 1.16	0.17± 3.73	-0.68± 0.42
P	0.10	0.56	0.23	0.56	0.88	0.96	0.11
Concentration ($x10^6$/ml)	-0.02± 0.95	0.62± 0.79	-1.83± 2.69	-3.11± 2.86	-0.05± 4.2	5.37± 12.00	-2.82± 1.54
P	0.48	0.44	0.51	0.34	0.99	0.69	0.07
Dead Sperms (%)	0.21± 0.20	0.40± 0.81	1.09± 0.91	0.29± 0.34	0.041± 0.59	-0.58± 2.99	0.74± 0.42
P	0.29	0.62	0.25	0.45	0.94	0.85	0.08

Discussion

In horse breeding, the stallions plays a significant role to maintain a population with desirable characters .In the past few decades breeding of mares by stallions based on semen quality of the stallions has improved the fertility of mares to some extent. If semen quality is not of optimum standard the whole breeding system will become questionable. Therefore in the modern horse breeding system semen evaluation is of paramount significance .The major parameters for semen evaluation can be subdivided into macroscopic, microscopic, biochemical and fertility trials. The macroscopic parameters include volume (ml), colour, pH, and consistency. The microscopic parameters include motility percentage, sperm concentration ($x10^6$/ml), live dead ratio and percentage of dead spermatozoa (Dowsett and Pattie, 1982).

Effect of age and breed

In this experiment horse stallions from six different breeds namely, Thoroughbred, Arab, Percheron, Noriker, Suffolk, Cleveland, and donkey stallions were used. Their age ranged from 4-22 years. The animals up to 10 years of age were grouped as younger and above 10 years of age were grouped as older. The mean age of Thoroughbred horse stallions was 9.83 ± 6.75 years. The mean age of young and older horse stallions was 6.57±0.27 and 15.12±0.90 respectively. There was a significant ($P < 0.0063$) effect of age on volume (ml) of semen and percent motility of spermatozoa. The mean volume (ml) of semen in young horse stallions was 156.15±6.68 ml and in old horse stallions 108.12±9.84 respectively. Mean semen volume (ml) in older horse stallions is significantly low ($p<0.000001$). Mean sperm motility percentage in younger horse stallions (76.15±0.09) was significantly higher ($P < 0.001$) than in older horse stallions (56.25±6.76) respectively. The sperm concentration 235.77±2.89 $x10^6$/ml from young horse stallions were significantly higher ($p<0.018$) as compared to 178.12±26.3 $x10^6$/ml older horse stallions. Mean dead sperm percentage in the semen of younger horse stallions were 25.19±0.28 percent as compared to (40.56±6.72%) old horses. The differences indicating that old horses had significantly higher mean dead sperm percentage ($P < 0.006$). These results clearly indicate that the semen obtained from the young horses aged up to 10 years was superior compared to that from old horses. It is interesting to observe that the differences between younger and older horse stallions for

volume (ml), motility percent, sperm concentration (x10^6/ml), and dead sperms percentage was significant only in Thoroughbred horse stallions, where as other breeds did not show significant differences for these parameters.

In the present study the mean semen volume ranged from 137.16±4.4 to 161.43±2.73, mean motility percentage ranged from 66.43±4.71 to 80.00±00, mean sperm concentration ranged from 207.14±17.25 to 280.00±7.73 and mean percentage of dead sperm ranged from 25.17±1.68 to 32.4±9.69. Parlevliet et al., (1994) studied the semen characteristics of 398 horse stallions and found volume (ml) 65±26, motility percentage 53±15, sperm concentration 206.1±168.5 x10^6/ml and percentage of live spermatozoa was 65±16. Pickett, (1993b) studied the semen characteristics of 417 horse stallions and observed volume (ml) 45±30, motility percentage 76.43, sperm concentration 335±232 x10^6/ml. Dowsett and Knott, (1996) evaluated the semen characteristics of 165 horse stallions and found volume (ml) 33.7±2.13, motility percentage 76.43±15, sperm concentration 164.13±39.35 x10^6/ml and percentage of live spermatozoa was 82±56. Long et al., (1993) evaluated the semen characteristics of 8 horse stallions and found volume (ml) 51.6±31.5 and sperm concentration 223±148 x10^6/ml. Dowsett and Pattie, (1982) evaluated the semen characteristics of 47 horse stallions and found volume (ml) 45.3±30.9,motility percentage 72.1±16, sperm concentration 178.16±168.35x106/ml and percentage of live spermatozoa was 78.8. Jasko et al., (1991) evaluated the semen characteristics of 64 horse stallions and found motility percentage 70.3±17.4.In the present study the range of semen volume was (137.16±4.4ml to 161.43±2.73 ml) much higher than reported by Parlevliet et al., (1994).

The results of Thoroughbred horse stallions of the present study are also in agreement with Dowsett and Pattie, (1987) who studied the semen characteristics of 168 stallions from 9 breeds, aged from 2 to 26 years. They reported that stallions under 3 years of age had the lowest volumes and sperm concentrations that together with the highest proportion of dead spermatozoa resulted in the lowest live spermatozoa per ejaculate. Stallions older than 13 years had low sperm concentrations and high percentages of dead spermatozoa but sperm morphology was normal. Arabian stallions had the lowest percentage of non-motile and dead spermatozoa while Shetlands were the only breed with greater than 30% dead sperm, the other having values within accepted normal limits. Arabian stallions had sperm concentrations almost double those of other breed and with

their relatively high volume, this resulted in total sperm numbers that were 3 times grater than those of any other breed. They considered that poor quality of the semen was related to immature spermatogenesis in colts and testicular degeneration and abnormal epididymal function in old animals. (Skinner et al.,1979; Paufler et al., 1979 and Orgebin-Crist MC, 1969).

In the present study three Thoroughbred stallions aged 19-22 years yielded extremely poor quality semen as regards volume (ml), pH, consistency, motility percentage, sperm concentration ($x10^6$/ml) and dead spermatozoa percentage. It can be concluded that both macroscopic and microscopic examination of semen can be valuable to eliminate such animals from breeding programmes. The effect of age in the present study on the semen parameters was statistically significant ($P<0.001$) in Thoroughbred stallions only but in other breeds some stallions aged between 13 years and above also had poor quality semen based on macro and microscopic examination but the statistical significant was not apparent because of few animals in the older age group. Based on these observations stallions breeders can be advised to be vigilant when the stallion age exceeds 18 years.

The influence of age on semen characteristics has been reported by (Dowsett and Knott, 1996). They found variation in semen quality in spermatozoa and behavioral characteristics of 168 stallions representing 9 breeds and ranging the age from 2 to 26 years. Semen characteristic were examined for total volume, gel-free volume, gel volume, colour score, mass activity, non-motile spermatozoa dead spermatozoa, semen density, spermatozoa concentration, total number of spermatozoa and semen pH. All semen characteristics with the exception of color and urethral pulsations had significant variation due to age. Semen quality (gel-free volume, sperm concentration, total sperm number and sperm abnormalities) was poorest in stallions under 3 year of age and over 13 years. Significant breed variation was apparent in most characteristics except for pH, semen color, abnormal mid-pieces and urethral pulsations.

The effects of age are probably due to differences in daily sperm production and output, which, in turn, are related to factors such as immature spermatogenesis in colts, testicular degeneration due to aging and aberrant epididymal function (Bowen, 1969). While age effects on semen parameters are apparent, they have little influence on stallion fertility except in the case of very young or very old stallions. The review of literature (Skinner et. al., 1979; Squires et. al., 1999 and Voss et. al., 1976) indicates that influence of age on

semen characteristics varies depending on the parameter considered. They did not find any effect of age upon gel volume, sperm concentration, pH and sperm morphology.

Effect of Season

Twenty Thoroughbred stallions were randomly selected to see the effect of season on volume, pH, motility percentage, sperm concentration and dead spermatozoa. The animals were maintained on seasonally available green fodder and concentrate ration. Statistical analysis of the results indicates that the season had no significant effect on any of the parameters studied. It seems that the non-significant effect of season may be due to the fact that animals were capable of consuming sufficient nutritional ingredients provided in their food necessary to maintain the reproductive health. Though the summer and winter environmental temperatures differ to a great extent and summer temperature usually affects the performance of bovine bulls (Djimde and Weniger, 1984), the season has no affect on quantity as well as quality of the semen (Usmani et al., 1985). In this study semen collection from the stallions were made in the months of January- February (non breeding season) and May -June (breeding season).The environmental temperatures between the months of January- February and May-June are very different. Animals are also maintained under good managemental conditions. Therefore, no detrimental affects were observed on the parameters of semen and spermatozoa. Last, but not the least, it seems that the volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and dead spermatozoa percentage during breeding season had a tendency to be better then in the non breeding season.

The results of the present study revealed that season had no effect on the seminal parameters. Similarly (Blottner et al., 2001) did not find any effect of season on quality and freezability of stallion semen during breeding and non-breeding seasons. Ejaculates were collected twice per week from four stallions during May and December. The semen was mixed with skim milk extender, centrifuged and resuspended in fresh extender. The cryopreservation of sperm during December results in survival rates similar to those measured during the breeding season even more important for successful preservation is the selection of suitable semen donors. In the present study semen parameters including volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead sperm from stallions of age 4-18 years are within the accepted limits from fertility point of view but horse stallions older than these showed poor semen quality.

Contrary to results of the present study (Janett et al., 2003) reported that total volume, total sperm concentration and motility percentage in fresh semen were significantly higher in winter, while sperm concentration was significantly lower in summer compared to other season. This difference is probably because in Switzerland where these experiments were carried out, the winter is very severe as compared to the moderate environment of the summer.

The results of this study are also differing from those reported by (Jasko et al., 1992) who evaluated the single ejaculate of semen and records for 2 consecutive breeding seasons. However, on the basis of evaluation of a single ejaculate for each stallion, the variation in these characteristics only accounted for approximately 20% of the observed variation in fertility rate and observed significant seasonal effect on the computer assisted semen analysis movement of spermatozoa.

Conclusion

It can be concluded from the present study that horse and donkey stallions between the ages of 4 to18 years can be safely used for breeding purpose and those older than 18 years should be carefully watched for semen quality to achieve good quality results.

CHAPTER –2

Cryopreservation of Equine Semen

CHAPTER 2

Cryopreservation of Equine Semen

Abstract

Thirty-two semen samples (8 from each) from four Thoroughbred horse stallions (H_6, H_7, H_8, and H_9) were preserved in liquid nitrogen at −196 °C for 24 hours to study the effect of five extenders on post thaw motility, liveability (hours) and absolute index of liveability of spermatozoa. Immediately after collection and evaluation, the semen samples were diluted with respective extenders viz. A (2.42 grams Tris), B (1.82 grams Tris + 0.52 grams sodium citrate), C (1.21 grams Tris + 1.04 grams of sodium citrate), D (0.60 grams Tris + 1.56 grams of Sodium citrate) and E (2.08 grams of Sodium citrate) having fructose, glucose, lactose, egg yolk, glycerol and antibiotics penicillin and dihydrostreptomycin as their common components. After an equilibration period of 6 hours, semen samples were frozen by a fast method and stored in liquid nitrogen for 24 hours. Thawing was carried out at 37°C for 30 seconds in a water bath to observe post thaw motility percentage, liveability (hours) of spermatozoa and absolute index of liveability (hours) the relative efficiencies of the five extenders. The mean values of post thaw motility percentage were 46.8 ±2.1, 38.7 ±1.5, 50.6±0.9, 44.3±2.5 and 41.3±2.8 for extenders A, B, C, D and E, respectively. Corresponding values for liveability at 37°C were 8.87 ± 0.39, 8.5 ± 0.26, 9.37 ±0.49, 8.37 ± 0.42 and 7.87± 0.39 hours, respectively. The mean values of absolute index of liveability at 37 °C were 179.2± 2.3, 130.2±3.0, 205.7±6.2, 153.8± 6.5and 141.3±1.2 for extenders A, B, C, D and E, respectively. The mean values of post thaw motility for four stallions (H_6, H_7, H_8, H_9) 44.8±2.1, 45.0 ±1.6, 36.8±1.6 and 35.6 ±1.7, respectively. The mean values of absolute index of liveability at 37 °C for four stallions were 168.2±14.7, 155.8 ±15.0, and 168.0±15.9 and156.4±14.8, respectively. The effect of five extenders on post thaw motility, liveability (hours) at 37 °C and absolute index of liveability was significant ($P<0.01$). The effect of four stallions on these parameters was non- significant. The extender C was the best for successful preservation of stallion semen at-196 °C. The order of merit for five extenders used was C, A, D, E and B.

Introduction

Artificial insemination is the most important technique ever devised for the genetic improvement of livestock. The success of this technique however depends mainly on the effective preservation of spermatozoa to ensure long-term storage without damaging the fertilizing abilities. The only successful way to preserve equine semen for long periods of time, i.e. months or years is cryo-preservation or deep freezing. Different stallions tolerate the freezing and thawing process differently. In general the freezing process involves the collection of semen, evaluation of semen, dilution and centrifugation and resuspension of the sperm in the freezing extender (Pickett and Back, 1973).

The use of artificial insemination (AI) in equine breeding has become increasingly popular in the horse industry, offering many advantages over natural service. Some of the reasons for this include the choice of a great number of stallions, safety for the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. In addition, pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural mating (Samper et al., 1991). Artificial insemination using frozen thawed semen has several advantages i.e. the stallion can function in breeding programmes while also competing in sporting event; the stallion may be used for breeding even following temporary or permanent sterility, and even after its death; genetic material can be traded more easily among different countries and even different continents; the use of stallions of inferior genetic value can be limited and consequently the selection process is accelerated; the obstacles represented by the distance separating stallion and broodmare are eliminated; stallions are protected against infectious diseases; For example, it is possible to freeze semen from a horse which does not spread the viral arthritis virus avoiding that, if unfortunately in future the stallion becomes a shedder, the semen use for reproduction will be limited, even forbidden (Barbacini et al., 1997).

To improve reproductive efficiency when using cryopreserved semen, attention should be given to factors such as the stallion, the quality and handling of the semen and age as well as reproductive history and management of the mare (Samper, 2000). Only poor and conflicting information is available about annual changes in semen characteristics (Pickett et al., 1976, Johnson and Thompson, 1983; Jesko et al., 1991), especially sperm morphology (Van der Host, 1975; Blottner et al., 2001) and semen freezability (Magistrini et al., 1987). Aiming to avoid transporting stallions to specialized centers,

some studies developed protocols for freezing semen that cool the semen for a longer period before freezing. It was observed that cryopreservation after 24 hours of cooling reduced progressive motility, but cooling for 18 hours before freezing did not reduce fertility (Crockett et al., 2001; Backman et al., 2004). The storage of spermatozoa is associated with a reduction in cell viability and fertilizing capacity. The quality of stored semen is affected by handling procedures such as dilution, centrifugation and addition of semen extender (Bustamante-Filho et al., 2006). The efficiency of cooled semen depends on an adequate shipment system. If insemination takes place within 12 hours after semen collection, then storage can be performed at either 20°C or 5°C. If semen storage exceeds 12 hours, slow cooling to 5°C is required. Semen storage at 4°C and 5°C resulted in higher sperm cell viability than storage at either 0°C or 2°C (Squires et al., 1999).

Many seminal extenders have been utilized to extend and store stallion semen. The types of extender utilized include whole milk (Ebertus, 1963), cream-gelatin (Hughes and Loy, 1970), evaporated milk-glucose-glycerol (McCall and Sorensen, 1971), egg yolk-sugar-glycerol (Krause and Grove, 1967; Oshida et al; 1968), mares milk (Kamenev, 1955), egg yolk –glucose-glycerol (Nishikawa et al., 1968), skim milk –glucose (Rajamannan et al., 1968), and Tris–glucose-citric acid –egg yolk (Cranwell, 1970; Anderson, 1971). A stallion seminal extender prolongs spermatozoa survival, enhances fertility, provides a media for antibiotic treatment of semen and permits a more convenient volume to be utilized, particularly when a small number of spermatozoa is inseminated.

The pregnancy rate, spermatozoa motility and spermatozoa agglutination were significantly lower in an extender containing 0.349% Tris than in an extender utilizing 2.4% Tris. Pregnancy rates of 22.2 and 11.1% were obtained with 500 and 100 x 10^6 motile spermatozoa in a 2.4% Tris extender, and a rate of 61.1% ($P < .05$) was obtained with 500 x 10^6 motile spermatozoa in raw semen. The presence of glycerol in the Tris extenders may have been at least partially responsible for the depression of fertility. Pregnancy rates of 75.0 and 91.7% were obtained after one and three cycles, respectively, with a mean insemination volume of 1.5 ml of raw semen. Thus, a large insemination volume may not be necessary for maximum fertility in the mare (Picket, et al., 1975).

To evaluate the effects of six extenders and three glycerol levels on the motility of sperm stored at 5^0C by using a split ejaculate design, semen from 12 stallions was extended with egg-yolk-tris (YET), egg-yolk–bicarbonate (EGB), BeltsvilleF-3 (BF-3), Cornell University (CUE), Caprogen (CAP) and heated skim milk(SM) extenders. After cooling

to 5°C, additional extender containing 0% to12% glycerol was added to provide a final concentration of 0%, 3% or 6% glycerol. The SM extender was the best ($P<0.05$) for maintaining motility of equine sperm. The inclusion of 6% glycerol has no effect on the percentage of motile equine sperm. The interaction of glycerol level and extender at 5°C and SM may be satisfactory for storage of equine sperm (Province et al., 1984).

To determine the effect of various antibiotics on sperm motion characteristics following short-term exposure and during cooled storage of semen, amikacin sulfate, ticarcillin disodium, gentamicin sulfate and polymixin B sulfate were added to a nonfat, dried, skim milk - glucose seminal extender at concentrations of 1000 or 2000 mug or IU/ml. Overall, the addition of antibiotics to extender did not significantly ($P>0.05$) improve motion characteristics of spermatozoa over control samples. However, levels of gentamicin sulfate greater than 1000 mug/ml and of polymixin B sulfate equal to or greater than 1000 IU/ml should be avoided in seminal extenders used for cooled semen (Jasko et al., 1993). The effects of egg yolk and (or) glycerol added to a non-fat dried skim milk glucose extender on motion characteristics and fertility of station spermatozoa were observed by (Bedford et al., 1994) and observed that egg yolk (with seminal plasma removal) or glycerol added to non fat dried skim milk glucose extender did not depress progressive motility of cooled stallion spermatozoa but adversely affected fertility.

The stallion semen was diluted in five different extenders and evaluated for motility after cooling and storage at 5 °C for 0, 24, 48, 72 and 96 h. In conclusion INRA82 was a better extender than the other extenders tested. Inclusion of taurine (100 mM) in INRA82 and Kenney's improved sperm survival until 96 and 48 h, respectively, and sperm preincubation for 24 h in taurine containing extenders resulted in better sperm survival when washed and stored in Sp-TALP for further 12 or 24 h. (Ijaz and Ducharme, 1995).

The cooling rates and storage temperatures for stallion spermatozoa extended in caprogen (CAP). Cornell University extender (CUE), heated skim milk (SM) and a nonfat-dried milk solids glucose extender (NFDMS-glucose) were evaluated in four tubes of extended semen, either plunged into 5°C in water or cooled at a rate of -1.0°, -0.5°, or -0.2°C/min. Within each treatment, one tube of extended semen was maintained at 20°C, 15°C, 10°C or 5°C. Progressive motility was estimated immediately after dilution (0 h) and at 4, 8, 12, 24 and 36 h. Regardless of extender, all three slower cooling rates were superior ($P<0.05$) to plunging to 5°C storage temperatures of 20°C and 15°C were superior

(P<0.05) for maintaining spermatozoa motility. Semen was cooled at -1C° /min and maintained at either 20C or 15C. Spermatozoa motility was assessed as in storage at 20C or 15C resulted in similar (P>0.05) spermatozoa motility (Province et al., 1985).

The effect of semen extender on post thaw motility and filtration through a glass wool-Sephadex (GWS) filter for frozen stallion semen was studied by Alghamdi et al., (2002). Kenney extender was compared with glucose-EDTA extender by use of various dilution rates that resulted in differing concentrations of seminal plasma. Use of Kenney extender as the centrifugation extender significantly improved post-thaw motility and GWS filtration, compared with glucose-EDTA. Extending semen at a dilution of 1:3 was significantly better than 1:1 for both motility and GWS filtration. Semen with poor post-thaw quality was significantly improved in regard to motility and GWS filtration when semen was frozen with seminal plasma at a concentration of 5%, compared with semen frozen with seminal plasma at a concentration of 25%. Use of Kenney extender at a high dilution 1:3 immediately after collection of semen can improve post-thaw quality of frozen stallion semen.

To determine the efficiency of a new extender, MP-50, in improving the post-thaw semen quality of Mangalarga Marchador semen, semen samples from 15 stallions were collected and frozen with MP-50 or INRA-82 (control). After thawing, the mean percentage of total and progressive sperm motility in MP-50 was higher than INRA-82 (49 vs. 21.46% and 23.26 vs. 8.8%, respectively, P< 0.05). Thus, MP-50 is a better alternative for the improvement of sperm preservation of Managalarga Marchador stallions. (Gomes et al., 2002). Thawing at 75°C for 7 seconds is the best thawing temperature. (Snoeck et al., 2002). The effect of intrauterine infusion of skim milk extender, seminal plasma and raw semen on the endometrium and blood flow in the uterine and ovarian arteries in mares was evaluated. There was an increase in intrauterine fluid as early as 1 h after infusion on any of the substances. The infusion of skim milk semen extender had no effect on uterine blood flow. In contrast, ovarian blood flow increased only in the artery ipsilateral to the preovulatory follicle and only after the infusion of raw semen (P<0.05). In conclusion, the changes in uterine perfusion observed after intrauterine infusion may be associated with endometrial inflammation and vasodilatory components in the seminal plasma, whereas the changes seen in ovarian blood flow are possibly attributable to the interaction between sperm and oviduct (Bollwein et al., 2003).

Extender containing only mannose sugar (49 mg/ mL) displayed an immediate depression in progressive motility compared with controls (45.5% versus 62.9%, respectively; $P < 0.001$). The 37-mg/mL-mannose extender had a less dramatic decrease in motility ($P < 0.05$) and only after storage at 5°C for 12 h (48.7% versus 58.0%, respectively). Extender with 25mg/ml mannose performed no differently than the control formulation under all conditions. A semen extender containing up to 37mg/ml mannose could maintain motile spermatozoa for 12 h and 25 mg/ml mannose concentrations preserved motility during long-term cooling. Likewise, sperm extended with up to 37 mg/ml of mannose had the same fertilizing capability as sperm in tradional extender mixtures (King et al., 2005).

The motility of spermatozoa in milk protein extender was significantly higher than in PBS or skim milk extender. Velocity of spermatozoa after storage was higher in milk protein extender. Membrane integrity was significantly lower in semen diluted with PBS than in the semen diluted with both extenders. Addition of N-acetyl cystein was without effect on the examined parameters. However centrifugation further improved spermatozoa quality in the defined milk protein. (Pagl et al., 2006). The ability of three commercially available diluents to maintain the motility of cold-stored stallion semen was evaluated and it was observed that there were significant stallion and treatment effects however there were no significant interactions between stallion and treatment. (Webb and Humes, 2006).The cooling of stallion spermatozoa for 24 hours before freezing, while maintaining sperm viability and fertility was possible (Melo et al., 2007).

Among various factors affecting fertility of the frozen semen, composition of the extending medium is the most important one. Tris (hydroxy methyl) amino methane has successfully been used as an organic buffer for deep-freezing of equine semen (Picket et al., 1975). Another organic buffer, sodium citrate dihydrates has also proved very useful for the purpose (Province et al., 1984).

The present project was designed to study the effect of two buffers Tris (hydroxy methyl) amino methane and sodium citrate dihydrate, in different proportions on the cryo-preservation of equine semen.

Materials and Methods

The semen of four horse stallions H_6 (RD-9839 Poli Gold), H_7 (RD-8844 Aron), H_8 (RD-Pole Rite) and H_9 (RD-0015 Burnish Rose) of the Thoroughbred breed maintained at the Chenab Breeding Area Faisalabad, was used in this study. The stallions belonged to different age groups and were normal size. They were kept under similar managemental and feeding conditions. The age of these stallions ranged between 5-16 years.

Preparation of Stallion and Semen Collection

Prior to semen collection, horse stallions were properly cleaned and all hygienic measures were observed during semen collection. Semen was collected twice a week early in the morning with two ejaculates per collection. A Missouri Model Artificial Vagina was used to collect the semen (Lambert and McKenzie, 1940; Anderson, 1945). In order to get a complete ejaculate of good quality each stallion was given enough time for sexual stimulation and one false mount was allowed for sexual stimulation before the collection of the first ejaculate. The second ejaculate was collected 45-55 minutes following the first collection (Maul, 1962).

Fresh semen evaluation

Immediately after collection, the ejaculates were filtered (sterile gauze), the gel-fraction was removed, the remaining volume was measured in a graduated cylinder and transferred to a water bath at 37°C for quality tests i.e. volume, colour, pH, mass activity, individual motility percentage and concentration of spermatozoa (Kenney et al.,1983). pH- value was determined by using pH indicator paper. Total and progressive motility and sperm concentration were estimated as in chapter 1 (Dowsett and Knott, 1996). Sperm smears were stained with eosin nigrosine stain and the live: dead ratio was evaluated for 200 sperm according to Dowsett et al., (1984). Ejaculates showing motility percentage of at least 60- 80 per cent were pooled. A total of 32-pooled samples were available for further processing.

Experimental semen extenders

Five experimental extenders were used in this study, the compositions of which are given in Table 1. Egg yolk was separated by complete removal of albumen. Egg yolk was poured into a cylinder by puncturing the yolk membrane. The required amounts of Tris, sodium citrate dihydrate, citric acid, fructose, glucose, lactose, penicillin and streptomycin for each extender were weighed and placed in separate beakers. Redistilled water was added to dissolve these ingredients and make the final volume of 73 ml. Then the required volumes of egg yolk (20 ml) and glycerol (7 ml) were added to each solution and each extender was stirred with a separate glass rod for uniform mixing of the ingredients. The extenders were stored in 5 pre-labeled, clean and sterilized beakers, which were kept in a water bath at 37°C until used for dilution of semen samples.

Extension of semen.

Each pooled semen sample was divided into five portions, each of which was diluted in the ratio of 1:10 semen extender with one of the five experimental extenders. Extension was made by placing the semen and extenders in a water bath at 37°C. Immediately after extension, the motility percentage of spermatozoa for each extender was checked.

Filling and Sealing of Straws

French straws made of polyvinyl chloride; five different colours corresponding to five extenders were used in this experiment. The semen was drawn into straws with an automatic suction machine. The open ends of the straws were sealed with polyvinyl chloride powder.

Equilibration

The semen filled straws were stored for an equilibration period of 6 hours at 4°C in the refrigerator. Motility of spermatozoa was observed at the end of equilibration period in different extenders for the four stallions (H_6, H_7, H_8, and H_9) as were used in the case of motility percentage after extension.

TABLE - 1 COMPOSITION OF EXPERIMENTAL EXTENDER

Ingredients	Experimental Extenders				
	A	B	C	D	E
Tris (gm)	2.42	1.82	1.21	0.60	-
Citric acid (gm)	1.34	1.00	0.67	0.34	-
Sodium citrate (gm)	-	0.52	1.04	1.56	2.08
Fructose (gm)	0.25	0.25	0.25	0.25	0.25
Glucose (gm)	0.25	0.25	0.25	0.25	0.25
Lactose (gm)	1.00	1.00	1.00	1.00	1.00
Penicillin (units/ml)	100000	100000	100000	10000	100000
Streptomycin (mg/ml)	100	100	100	100	100
Glycerol (ml)	7	7	7	7	7
Egg yolk (ml)	20	20	20	20	20
Distilled water up to (ml)	100	100	100	100	100

Cryopreservation

Cryopreservation was performed in a wide mouth-freezing chamber containing liquid nitrogen. A wire net, the temperature of which was lowered down by dipping it into liquid nitrogen for a while, was used for holding the straws in the vapors of liquid nitrogen 5 cm above its surface. The cryopreservation process was completed within 10 minutes (rapid method of freezing). Then the wire net containing the straws was gradually lowered down into liquid nitrogen (Ahmed and Chaudhry, 1980).

Storage of cryopreserved semen

Small plastic goblets filled with liquid nitrogen were used to collect the straws from the freezing chamber by holding the goblet in one hand with the help of long forceps and gathering the straws with the other hand. Immediately after plunging them into plastic goblets, the straws were transferred to bigger steel goblets, having a sieve at the lower end. These steel goblets with straws were kept vertically in the storage container filled with liquid nitrogen for at least 24 hours before thawing.

Thawing of straws

Thawing of straws was carried out in a water bath at 37°C. The process of thawing was completed within 30 seconds (Pickett and Amann, 1993). Motility percentage of spermatozoa for each extender was then recorded as initial post thawing rate of motility.

Evaluation of cryopreserved semen

Thawed semen was separately procured into dry sterilized vials for each extender and was transferred to an incubator to determine livability of spermatozoa at 37°C. The livability was recorded for all extenders by recording the percentage of motile spermatozoa at hourly intervals till the death of all spermatozoa.

The rate of motility after thawing and liveability of spermatozoa at 37°C was used to compute an absolute index of liveability by using the following formula (Melovenof, 1962).

$$Ia = (T \times R)$$

Where,

Ia = is the absolute index of liveability.

T = is the time interval between observations.

R = is the average percentage motility for every two consecutive observation, starting from the time of incubation

Statistical analysis

Student's t test and analysis of variance were applied for comparison of means on the data related to semen parameters, i.e.extention of semen, Equilibration of semen, Post thaw motility percentage, Liveability (hours) of spermatozoa after thawing at 37°C and Absolute index of livability at 37^0 C.

Results

Physical characteristics

The physical characteristics of semen samples for the stallions H_6, H_7, $H_8$and H_9 are shown in Table 2. All samples were grayish white in colour. On the basis of consistency, all samples were thick in nature. The volume of the semen samples ranged from 120 to 150 ml with a mean of 134.3±4.2; 130 to150 with a mean of 136.2±3.8; 125 to 160 with a mean 142.5±5.0 and 125 to 170 with a mean of 135.0 +3.9 ml for horse stallions H_6, H_7, H_8 and H_9, respectively. Motility percentage ranged from 60 to 80 with mean of 75.6±2.8, 65.6±2.7, 70.6±2.5 and 68.4±2.6 for horse stallions H_6, H_7, and $H_8$and H_9, respectively. The mean sperm concentration for horse stallions H_6, H_7, H_8 and H_9 were 247.3±5.4, 267.5±5.9, 265±6.2 and 249.3±5.4 x10^6 /ml, while the mean percentage of dead sperms were 25.5±1.4, 22.5±1.3, 24.6±1.9 and 26.5±1.3, respectively.

Table 2. Mean values of Physical Characteristics of Semen Samples of Horse Stallions

Stallions	Number of samples	Colour	Volume	Consistency	Motility (%)	Sperm concentration ($x10^6$)	Dead sperms (%)
H_6	8	GW	134.37±4.27	Thick	75.66±2.87	247.37±5.48	25.5±1.43
H_7	8	GW	136.25±3.87	Thick	65.68±2.78	276.5±5.90	22.5±1.63
H_8	8	GW	142.5±5.00	Thick	70.62±2.58	265.00±6.27	24.6±1.34
H_9	8	GW	135.00±3.90	Thick	68.45±2.67	249.38±5.46	26.8±1.53

Evaluation of unfrozen semen

The mean motility percentages of semen samples, immediately after extension were 62.6±0.8, 62.5±0.7, 63.6±0.9, 61.8±0.9and 63.1±0.7 for horse stallions H_6, 61.7±0.8, 62.5 ± 0.9, 63.5±0.8, 63.6±0.9and 62.1±0.9 for horse stallion H_7 61.8±0.9, 62.5 ± 0.9, 64.5 ±0.9, 63.1±0.9and 63.1±0.9 for horse stallion H_8 62.5± 0.9, 61.8 ±0.9, 63.7±0.8, 63.7+0.8 and 63.6±0.7 for horse stallion H_9, respectively, in all extenders i.e. A, B, C, D and E. The statistical analysis showed that the differences between means were not significant (P>0.01).

Extension of semen.

After extension the mean motility percentage of spermatozoa in the five extenders and four stallions do not differ significantly ($F_{4,35} = 0.22$; $p = 0.92$) (Table 3)

A two-way analysis of variance was applied to see which of the two, stallions or extenders was important from the mean spermatozoa motility point of view. This showed a non-significant effect of different extenders and of different stallions on mean motility of spermatozoa (Table 4).

Table 3. Mean values of motility percentage after extension for four stallions (H_6, H_7, H_8, H_9,) in different extenders

Motility Percentage after extension					
Extenders					
Stallions	A	B	C	D	E
H6	62.6±0.8	62.5±0.7	63.6±0.9	61.8±0.9	63.1±0.7
H7	61.7±0.8	62.5±0.9	63.5±0.8	63.6±0.9	62.1±0.9
H8	61.8±0.9	62.5±0.9	64.5±0.9	63.1±0.9	63.1±0.9
H9	62.5±0.9	61.8±0.9	63.7±0.8	63.7±0.8	63.6±0.7

Table 4. Two-way analysis of variance showing comparison of motility percentage of spermatozoa after extension in relation to different extenders and stallions

Source of Variation	**Df**	**SS**	**MS**	**F**	**P**
Extender	4	2.248	0.5620	1.906	0.1741
Stallion	3	0.7838	0.2613	0.8861	0.4760
Residual	12	3.538	0.2948		

Equilibration

Motility of spermatozoa was observed at the end of equilibration period in different extenders for the four stallions (H_6, H_7, H_8, and H_9) as were used in case of motility percentage after extension. Table 5 shows the mean motility percentage for each horse stallion in different extenders. Horse stallion number H_6 shows no appreciable mean difference in motility percentage ($F_{4, 35} = 0.22$; $p = 0.92$). Similar results were obtained for stallion number H_7, ($F_{(4,35)} = 0.32$; $p = 0.86$), stallion number H_8 ($F_{(4,35)} = 0.32$; $p = 0.86$) and stallion number H_9 ($F_{(4,35)} = 0.88$; $p = 0.48$)

Two-way analysis of variance was carried out to find whether different extenders have effect on mean motility percentage or the different stallions behave differently in different extenders. Both extenders and stallions showed no significant difference in mean sperm motility percentage after equilibration period ($p > 0.04760$) Table 6.

Table 5. Mean values of motility percentage after equilibration for four stallions (H_6, H_7, H_8, H_9,) in different extende

Motility Percentage after equilibration					
Extenders					
Stallions	A	B	C	D	E
H6	62.6±0.8	62.5±0.7	63.6±0.9	61.8±0.9	63.1±0.7
H7	61.7±0.8	62.5±0.9	63.5±0.8	63.6±0.9	63.1±0.9
H8	61.8±0.9	62.5±0.9	64.5±0.9	63.1±0.9	63.1±0.9
H9	62.5±0.9	61.8±0.9	63.7±0.8	63.7±0.8	63.6±0.7

Table 6. Two-way analysis of variance showing comparison of motility percentage of spermatozoa after equilibration relation to different extenders and stallions

Source of Variation	**df**	**SS**	**M S**	**F**	**P**
Extender	4	8.75	2.1875	0.331081	0.85517141
Stallion	3	231.25	6.607143	0.8861	0.4760
Residual	12	240	0.2948		

Motility Percentage after thawing

Semen of each stallion i.e. H_6, H_7, H_8, H_9 was diluted with five extenders i.e. A, B, C, D and E. The percent initial motility of sperm after thawing is given in Table 7.

In each extender an appreciable difference in mean motility after thawing was observed. In the case of horse stallion H_6 the lowest motility percentage was observed in extender B (38.7±1.5) and the highest motility percentage was 50.6±0.9 noted in extender C. In extenders A, D and E the percentage of motility ranges were 41.8±2.8 to 46.87±2.1. Horse stallion H_7, also showed the highest percentage of motility 50.0±1.33 with extender C and the lowest motility percentage in extender B (38.12±1.87). Mean sperm motility percentage ranged from 39.3±3.0 to 45.0±1.63 in extender A, D, and E.

Horse stallion H_8 has the higher motility percentage with extender C (51.2±1.2) and the lowest motility percentage in extender A (36.8±1.7). Mean sperm motility percentage ranged from 38.7±1.5 to 41.87±2.8 in extenders B, D and E.

Similarly, the highest mean motility percentage was observed in extender C (51.2±1.5) in the case of stallion, H_9 and the lowest mean motility percentage was observed with extender D (30.2±2.2). Mean percentage motility percentage ranged from 35.6±1.7 to 40. 0±1.6 in extenders A, B and E

Single factor Analysis of Variance was applied in the case of each stallion to find out how mean percentage sperm motility varied between extenders. All the four stallions i.e., H_6 ($F_{(4,35)} = 4.39$; $p=0.005$), H7 ($F_{(4,35)} = 6.11$; $p = 0.0007$); H8 ($F_{(4,35)} = 7.67$ $p=0.00015$) and H_9 ($F_{(4,35)} = 12.08$; $p = 2.84$ E---06) showed highly significant differences in mean motility percentage after thawing.

Two-way Analysis of Variance was carried out to find out whether extenders have a significant role in the initial motility percentage or whether stallions play a significant role. The results of this study showed that extenders play a highly significant role in the initial mean motility percentage after thawing.

Table 7. Mean values of initial motility percentage after thawing for four stallions (H_6, H_7, H_8, H_9,) and different extenders

Motility Percentage					
Extenders					
Stallions	A	B	C	D	E
H6	46.87±2.10	38.75±1.56	50.62±0.94	44.37±2.57	41.87±2.82
H7	45.00±1.63	38.12±1.87	50.00±1.33	39.37±3.05	40.00±1.63
H8	36.87±1.62	38.75±1.56	51.25±1.25	41.87±2.30	41.87±2.82
H9	35.62±1.75	38.12±1.82	51.25±1.56	30.25±2.26	40.00±1.63

Table 8. Two-way analysis of variance showing comparison of motility percentage of spermatozoa after equilibration relation to different extenders and stallions

Source of Variation	df	SS	MS	F	P
Extender	4	403.9	101.0	8.706	0.0015
Stallion	3	76.00	25.33	2.185	0.148
Residual	12	139.2	11.60		

Liveability (hours) of spermatozoa after thawing at 37°C.

Mean liveability of spermatozoa of the four stallions, H_6, H_7, $H_8$and H_9 in different extenders, after thawing is given in Table 9. The highest mean liveability of spermatozoa in all the four stallions was observed in extender C. In H_6 mean liveability ranged from 7.87 to 8.87; in H_7 from 7.37 to 8.50; in H_8 from 7.87 to 8.87 and in H_9 from 7.25 to 8.5 in extenders A, B, D and E, respectively.

Single factor Analysis of Variance shows non significant ($F_{(4;\ 35)}$ =1.94; p =0.12) differences in mean liveability in the five extenders. Highly significant ($F_{(4,35)}$ =7.23; p =0.00025), difference in mean liveability of spermatozoa from H_7 in different extenders and non-significant differences for H_8 ($F_{(4;\ 35)}$ =2.56; p =0.055) were observed. Highly significant difference in mean liveability was observed in the five extenders for stallion number H_9 ($F_{(4,35)}$ =7.98; p =0.00011).

Two way Analysis of Variance was carried out to find out whether extenders or stallions play a significant role in mean liveability of spermatozoa .The results of two way Analysis of Variance showed that the more significant role in difference of mean liveability of spermatozoa is due to extenders ($F_{(4,35)}$=40.04; p =<0.0001).The four stallions also showed significance differences in mean liveability of spermatozoa ($F_{(4,35)}$ =6.57; p =0.007).

Table 9. Mean values of Liveability (hours) after thawing for four stallions (H_6, H_7, H_8, H_9,) and different extenders

Liveability (hours) after thawing					
	Extenders				
Stallions	A	B	C	D	E
H_6	8.8±0.3	8.5±0.2	9.5±0.4	8.4±0.4	7.8±0.4
H_7	8.7±0.2	7.3±0.2	9.4±0.2	8.2±0.2	7.4±0.3
H_8	8.6±0.3	7.8±0.29	9.3±0.4	8.3±0.4	7.5±0.3
H_9	8.5±0.2	7.2±0.2	9.2±0.4	8.5±0.3	7.6±0.3

Table 10. Two-way analysis of variance showing comparison of liveability of spermatozoa after thawing in relation to different extenders and stallions

Source of Variation	df	SS	MS	F	P
Extender	4	7.227	1.807	40.04	0.0001
Stallion	3	0.8901	0.2967	6.576	0.0071
Residual	12	0.5414	0.04512		

Absolute index of liveability at 37^0 C

The mean values of the absolute index of livability of spermatozoa for different extenders and stallions are presented in Table11.

Analysis of Variance revealed that the effect of extenders on the absolute index of livability of spermatozoa was highly significant ($P< 0.01$). The ranking order of the extenders was C, A, D, E and B. Analysis of Variance (Table 12) showed that the effect of stallions on the absolute index of livability of spermatozoa was significant ($P> 0.05$). Stallions H_6 and H_8 were better than $H_7$and H_9 in this respect. The interaction between extenders and stallions was, however, non significant.

Table 11. Absolute Index of Liveability (hours) after thawing at 37°C for four stallions (H_6, H_7, H_8, H_9,) in differ extenders

Absolute Index of Liveability (hours) after thawing at 37°C					
Extenders					
Stallions	A	B	C	D	E
H_6	182.8±14.8	135.6±9.8	215.2±14.9	165.3±18.4	144.2±16.4
H_7	173.1±12.7	124.9±7.33	199.6±14.3	142.4±20.4	139.3±13.6
H_8	179.0±14.1	135.6±9.8	215.1±14.9	169.2±18.5	142.5±16.4
H_9	182.8±14.8	124.8±7.3	193.0±8.9	142.4±20.3	139.2±13.6

Table 12. Two-way analysis of variance showing comparison of Absolute Index of liveability of spermatozoa after thawing at 37° C in relation to different extenders and stallions.

Source of variation	df	SS	M S	F	P
Extender	4	14881.86	3720.465	1.73E-09	0.004
Stallion	3	713.99	237.99	7.44	3.4971
Residual	12	383.8173	31.98478		

Discussion

In the present study, 32 semen samples (8 samples from each horse stallion) collected from four stallions (H_6, H_7, H_8 and H_9) and were used to investigate the effect of five experimental extenders on post thaw motility percentage, liveability of spermatozoa at 37^0C and the absolute index of liveability.

Tris (hydroxmethyl) aminomethane and sodium citrate dihydrate ($Na_3C_6H^9O_7$. $2H_2O$) are two organic buffers and are being used successfully for the preservation of equine spermatozoa (Picket et al., 1975; Province et al., 1984). Among five semen extenders used in this study, the main variation in the components of the extenders were the two organic buffers i.e. Tris (hydroxmethyl) aminomethane and sodium citrate dihydrate alone or in various proportions. Other components were the same. In addition to these major components, each extender contained citric acid, egg yolk, glycerol, streptomycin, penicillin and distilled water along with fructose, glucose and lactose in equal amounts. The extender C containing 1.21 gms Tris + 1.04 gms of sodium citrate dihydrate proved to be the best in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability of spermatozoa at 37^0C. Extender A containing 2.42 gms of Tris, extender D containing 0.06 gms Tris + 1.56 gms sodium citrate dihydrate and extender B containing 1.82 gms Tris and 0.52 gms Sodium citrate dihydrate although inferior to extender C showed better post thaw motility percentage. Extender A containing 2.42 gms Tris was inferior to extender C but superior to all other extenders and was good enough to be used for AI as has been also indicated by its being superior to all other extenders while B, D and E showed minor differences.

Different solutions of chemical compounds have their specific eutectic points (the temperature at which the whole liquid phase is converted into solid phase). It is well known that during freezing solidification of solutions adversely affects spermatozoa. However, this effect can be reduced by completing the solidification process in more than one step. This can be achieved through replacing a particular compound with other components having similar properties but different eutectic points. In this way the solidification process will take place at different temperatures and thus its harmful effects will be diluted. In the present study Tris which is commonly used as a buffer has been replaced by another buffer sodium citrate dihydrate in different proportions to have

different solidification temperatures of these solutions and thus accordingly reduce the harmful effect on the spermatozoa (Watson, 1995).

The better performance of the extender C containing 1.21 gms Tris + 1.04 gms sodium citrate dihydrate may be due to the synergistic effect of the two buffers. The difference was highly significant as far as the post thaw motility, post thaw livability of spermatozoa at 37°C and absolute index of livability are concerned.

The results of the present study revealed that the combination of Tris (hydroxymethyl) amino methane and sodium citrate dihydrate should be used as buffer for deep-freezing stallion spermatozoa. However, if only one buffer is to be used, Tris (hydroxymethyl) amino methane should be preferred over sodium citrate dihydrate. These results are similar to Foote, (1970) who reported that bovine sperm motility following freezing and thawing in Tris based extender was slightly superior to that in citrate extender but there was no difference in their fertility. The results of the present study show that extender C containing Tris 1.21 percent with other components gives better post thaw motility, liveability (in hours) and absolute index of liveability as compared to the other extenders A, B and D, respectively. The extender A containing 2.42 percent Tris showed less post thaw motility, liveability and absolute index of liveability. In agreement with the present study Picket, et al., (1975) studied that pregnancy rate, spermatozoa motility and spermatozoa agglutination were significantly lower in an extender containing 0.349% Tris than in an extender utilizing 2.4% Tris.

In the present study seven percent glycerol was used in five experimental extenders in which the quantities of Tris and sodium citrate dihydrate varied according to composition of the experimental extender A, B, C, D and E, respectively. There were no detrimental effects of glycerol on equine sperm motility. Similarly Province et al., (1984) also reported that the inclusion of 6% glycerol had no effect on the percentage of motile equine sperm. The interaction of glycerol level and extender was non significant. The inclusion of 6% glycerol has detrimental effects on the motility percentage of canine sperm. Pace and Sullivan, (1975) extended semen in a Tris based extender containing 7% glycerol, and froze it in 10-ml ampules. They concluded that fertility was maximal when insemination was 0 to 12 hours before ovulation, and that insemination 0 to 12 hours after ovulation was somewhat inferior. They also stated that insemination 12 to 36 hours before ovulation was preferable to insemination 12 to 24 hour after ovulation.

Cooling rate has received little attention, perhaps because with pelleted semen it is difficult to measure or control, and for semen frozen in glass ampules, 0.5, 1.0 or 4.0 ml straws, freezing in vapor above liquid nitrogen was convenient and had proven to be successful with bull spermatozoa. For semen frozen in ampules, two reports are divergent in their recommendations; Schafer and Baum, (1994) recommended cooling from 6°C to –79°C in 10 minutes (possibly 8 to 10°C/minute), whereas Choa and Chang (1964) recommended a slower cooling rate of about 0.5°C /minute to –20°C and then 3°C/minute. Semen frozen as 0.1 ml pellets presumably cools very rapidly, probably in <4 minute. Cochran et al., (1984) compared post-thaw motility of spermatozoa cooled at about 60°C /minute by placing 0.5 ml straws horizontally in liquid nitrogen vapor at –160°C or at a controlled rate of 10°C/minute from +20 to –15°C and 25°C/minute. The percentage of motile spermatozoa after thawing was not influenced by cooling rate. The effect of thermal properties of the plastic or wall thickness on cooling rate of 0.5 ml straws has not been studied. Cochran et al., (1983) using 0.5ml straws semen was thawed in 37°C water for 30 seconds. Province et al., (1985) reported that CAP and NFDMS-glucose extenders were superior ($P<0.05$) to SM for maintenance of spermatozoa motility storage at 20C or 15C resulted in similar ($P>0.05$) spermatozoa motility.

In the present study 100000-units/ ml of penicillin and 100 mg/ml of streptomycin were used in five experimental extenders for the reduction of growth of microbial agents. The results of the present study clearly indicated that the performance of five experimental extenders was better in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability at 37°C of spermatozoa. Contrary to the results of the present study Jasko et al., (1993) who added amikacin sulfate, ticarcillin disodium, gentamicin sulfate and polymixin B sulfate to a nonfat, dried, skim milk - glucose seminal extender at concentrations of 1000 or 2000 mug or IU/ml. After 24 h of storage at 5°C, 2000 mug/ml of gentamicin and levels equal to and greater than 1000 IU/ml of polymixin B in seminal extender resulted in significant ($P<0.05$) reductions in the percentages of motile and progressively motile spermatozoa. After 48 h of cooled storage, a level of 1000 mug/ml of gentamicin sulfate resulted in significant ($P<0.05$) reductions in the percentages of motile and progressively motile spermatozoa. Levels equal to or greater than 1000 IU/ml of polymixin B sulfate also resulted in a significant ($P<0.05$) reduction in mean velocity. Levels up to 2000 mug/ml of amikacin sulfate and ticarcillin disodium had no significant effect on sperm motion characteristics during short-term incubation at

23°C or storage for 24 h at 5 °C. Overall, the addition of antibiotics to extender did not significantly (P>0.05) improve motion characteristics of spermatozoa over control samples. However, levels of gentamicin sulfate greater than 1000 mug/ml and of polymixin B sulfate equal to or greater than 1000 IU/ml should be avoided in seminal extenders used for cooled semen.

In the present study, 20% egg yolk was used in five experimental extender and the results of the present study were better in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability at 37°C of spermatozoa. Similarly. Martin et al., (1980) diluted equine semen in seminal extenders in which 20% egg yolk was used. Cochran et al., (1984) used a lactose based extender with 20 % egg yolk and found that post-thaw motility percentage of the spermatozoa was not affected significantly. It was suggested that the use of 20% egg yolk was a better approach for the freezing of the seminal extenders. In another study, Cristanelli et al., (1985) also confirmed that the percentage of progressive motile spermatozoa after freezing and thawing was higher for extenders with 20 % egg yolk. Bedford et al., (1994) conducted three experiments to evaluate the effects of egg yolk and (or) glycerol added to a non-fat dried skim milk glucose (NDSMG) extender on motion characteristics and fertility of stallion spermatozoa and reported that the use of 4 % egg yolk in NDSMG extender did not depress the motility percentage of the spermatozoa but adversely affected the fertility.

Another objective of the present study was to compare the semen of four stallions (H_6, H_7, H_8 and H_9). It was observed that the stallions H_6 and H_8 showed better results in terms of the absolute index of livability of spermatozoa than stallions H_7 and H_9. The average post thaws motility percentage and livability of spermatozoa at 37^0C, however, varied non-significantly among four stallions.

CHAPTER –3

Conception Rate in Mares using Natural Service, Liquid Cooled Semen and Frozen thawed Semen

CHAPTER 3

Conception Rate in Mares using Natural Service, Liquid Cooled Semen and Frozen-thawed Semen

Abstract

In this experiment the comparison was carried out for the conception rate in mares with natural service, liquid cooled semen and frozen- thawed semen. This experiment was conducted at the Horse, Mule and Cattle Breeding Area Sahiwal and the Chenab Breeding Area Faisalabad in the breeding season (March - August). In this study 60 mares (n = 60) were used which were free from reproductive diseases and disorders. These mares were kept under similar conditions of management and feeding. All the selected animals were divided into three groups i.e., Group 1, Group 2 and Group 3. Each group was comprises of 20 animals each. All the animals were examined for any reproductive abnormality by rectal pulpation. All the mares were found to be free from any reproductive problems. Group 1 was bred with natural service, Group 2 was inseminated with liquid cooled semen and Group 3 was inseminated with frozen- thawed semen. The mares were inseminated with dose a of 0.5 ml semen containing 500 x 10^6 progressive motile spermatozoa. All the animals were examined for pregnancy diagnosis after 90 days. After pregnancy diagnosis it was concluded that with natural service 11 mares out of 20 (11/20) were found pregnant (55 percent), with liquid cooled semen 9 mares out of 20 (9/20) were found pregnant (45 percent) and with frozen thawed semen 8 mares out of 20 (8/20) were found pregnant (40 percent). The conception rate was higher (55%) in mares inseminated with natural service while lowest rates (40%) were observed in the case of frozen thawed semen. The liquid cooled semen showed intermediate (45%) results.

Introduction

Artificial insemination is the most important technique ever devised for the genetic improvement of livestock (Hafiz, 1987). The use of artificial insemination (AI) in equine breeding has become increasingly popular in the horse industry, offering many advantages over natural service. Some of the reasons for this include the choice of a great number of stallions, safety for the mare and the stallion, reduced risk of infectious disease transmission, and transport inconvenience. In addition, pregnancy rates have been shown to be equal or even higher after AI with fresh or chilled semen compared to natural mating (Samper et al., 1991).

Artificial insemination using frozen thawed semen has several advantages i.e. the stallion can function in breeding programmes while also competing in sporting events; the stallion may be used for breeding even following temporary or permanent sterility, and even after its death; genetic material can be traded more easily among different countries; the use of stallions of inferior genetic value can be limited and consequently the selection process is accelerated; the obstacles represented by the distance separating stallion and broodmare are eliminated; stallions are protected against infectious diseases; For example, it is possible to freeze semen from a horse which does not spread the viral arthritis virus avoiding that, if unfortunately in future the stallion becomes a shedder, the semen use for reproduction will be limited, even forbidden (Barbacini et al., 1997).

Pace and Sullivan (1975) observed that fertilization rate was highest in mares inseminated with frozen semen within 12 hours of ovulation. Foaling rate was improved ($P < 0.05$) by increasing the number of motile spermatozoa inseminated from 40×10^6 to 80×10^6. The fertilizing capacity of frozen spermatozoa in one of the hydrogen ion extenders studied was dependent upon relative osmotic pressure and method of freezing (ampoules or pellets). Adjusting glycerol concentration to 2%, addition of glycerol 15 min before freezing and freezing 2 hr after extension all enhanced the fertilizing capacity of spermatozoa. Although wide variability in the fertilizing capacity of spermatozoa from individual stallions was observed when semen was extended and frozen, pregnancy rate was depressed in all stallions. It was concluded, therefore, that hydrogen ion extenders depress fertilizing capacity of stallion spermatozoa immediately after extension and show little promise as semen extenders.

To improve reproductive efficiency when using cryopreserved semen, attention should be given to factors such as the stallion, the quality and handling of the semen, and stallion age as well as reproductive history and management of the mare (Samper, 2000). Only poor and conflicting information is available about annual changes in equine semen characteristics (Pickett et al., 1976; Johnson and Thompson 1983; Jesko et al., 1991), especially sperm morphology (Van der Host, 1975; Blottner et al., 2001) and semen freezability (Magistrini et al., 1987). Aiming to avoid transporting stallions to specialized centers, some studies developed protocols for freezing semen that cool the semen for a longer period before freezing. It was observed that cryopreservation after 24 hours of cooling reduced progressive motility, but cooling for 18 hours before freezing did not reduce fertility (Crockett et al., 2001; Backman et al., 2004). The storage of spermatozoa is associated with a reduction in cell viability and fertilizing capacity. The quality of stored semen is affected by handling procedures such as dilution, centrifugation and addition of semen extender (Bustamante-Filho et al., 2006). The efficiency of cooled semen depends on an adequate shipment system. If insemination takes place within 12 hours after semen collection, then storage can be performed at either 20°C or 5°C. If semen storage exceeds 12 hours, slow cooling to 5°C is required. Semen storage at 4°C and 5°C resulted in higher sperm cell viability than storage at either 0°C or 2°C (Squires et al., 1999).

Hughes and Loy (1969) carried out a comparison of natural breeding and artificial insemination of mares using semen from three Thoroughbreds and three Quarter horse stallions. A total of 218 mares were bred by artificial insemination and 199 by natural breeding. One hundred and forty seven (67.4 percent) of the mares bred by artificial insemination conceived and 157 (78.9 percent) of the mares bred by natural breeding conceived. Twenty-seven out of 37 (73.0 percent) mares inseminated with diluted semen stored at 0 to 5 ^{0}C for 24 to 96 hours conceived. Out of these 37 mares, 10 out of 14 conceived with semen inseminated after 24 hours storage, 12 out of 14 after 48 hour, storage, 3 out of 6 after 72 hours storage, and 2out of 3 after 96 hours storage. The semen extender used was skim milk and cream gelatin.

Pickett et al., (1975) and Sieme et al., (2003a) investigated the effects of different artificial insemination (AI) regimes on the pregnancy rate in mares inseminated with either cooled or frozen-thawed semen.

Zidane, (1991) showed that the fertility of extended equine semen can be maintained at 20°C for several hours depending on the stallion, but for overnight delivery it must be cooled further to maintain its viability. Semen cooled from 20°C to 5°C at 2° per hour and then stored maintained satisfactory fertility i.e. 54 % after direct insemination, vs 58 % when stored at 5°C for 2 hours, vs 69 % when stored 2 hours at 20°C. After 48 hours, pregnancy rates were reduced to 7 % and 45 % for storage at 20°C and 5°C, respectively.

Samper and Morris, (1998) showed that various factors affected the success of AI with frozen-thawed semen in horses. Stallion variability was thought to be one of the major factors, but semen processing and evaluation techniques, thawing protocols, packaging systems and timing of insemination were far from standardized among laboratories. A survey was conducted on current methods for stallion semen cryopreservation used commercially around the world. From the answers to the questions in the survey, they attempted to provide an overview of procedures that were standard as well as those that were used by only few laboratories and to review critically the efficacy of these procedures. Twenty-five questionnaires were sent to individuals or laboratories in 14 countries that were involved in freezing stallion semen for commercial purposes. Questionnaires were returned from 10/14 countries with 21/25 (84%) of the addresses responding. From the responses, it became evident that most of prefreezing, freezing and thawing and post-thawing processing procedures were far from standardized. The great variety of procedures made it difficult to accept any of them as reliable. In order to increase the credibility of AI technology in the horse, laboratories need to standardize processing methods as well as the record-keeping systems. In addition, it was evident that no group of research mares was large enough to provide meaningful fertility data. It was therefore imperative to have muilticentered collaborative studies to record and disseminate information about methods and the corresponding fertility rate to gain valuable information and be able to compare different protocols.

Gahne et al., (1998) tried to find out if it was possible to decrease the insemination dose from 500×10^6 progressively motile spermatozoa to 300×10^6 progressively motile spermatozoa and still maintain an acceptable pregnancy rate when using extended fresh semen. They reported that with an insemination dose of 300×10^6 progressively motile spermatozoa the pregnancy rate per cycle was 64% and with an insemination dose of 500×10^6 progressively motile spermatozoa the pregnancy rate per cycle was 75%. There was no significant difference in the pregnancy rate between the 2 insemination doses

(P=0.341). It was concluded that when using fresh extended semen, an insemination dose of 300×10^6 progressively motile spermatozoa would yield a lower pregnancy rate than a dose of 500×10^6 progressively motile spermatozoa if stallions with good quality semen were selected.

The mares inseminated with cooled stallion semen once during an estrus had pregnancy rates comparable to those attained in mares inseminated on two (48/85, 56.5%) or three (20/28, 71.4%) occasions at 24 h intervals, as long as insemination was performed between 24 h before and 12 h after ovulation (78/140, 55.7%). Similarly, a single frozen-thawed semen insemination between 12 h before (31/75, 41.3%) and 12 h after (24/48, 50%) ovulation produced similar pregnancy rates to those attained when mares were inseminated either two (31/62, 50%) or three (3/9, 33.3%) times at 24 h intervals (Sieme et al., 2003a). The pregnancy rates in managed horse populations depended on the innate fertility of the mares and stallions involved and on the quality of breeding management. A single stallion was used to mate many mares so stallion fertility was a critical factor in the overall success of a breeding programme. Little data exist to compare the effect of natural service, liquid semen and frozen-thawed semen on conception rate of equine semen (Colenbrander et al., 2003). Satisfactory pregnancy and foaling rates were obtained from mares inseminated with good quality semen and maintained under well controlled management. Other authors however reported that animals maintained at different locations had varying pregnancy rates (Rota et al., 2004). Stallion cryopreservation despite its impact on horse industry is not an established technology because of the poor semen quality and fertility after freezing and thawing (Alveranga et al., 2005). However post thaw percent yield bears a linear relationship with prefreez quality of semen. Subnormal semen samples, otherwise acceptable, did not withstand freezing, and thus resulted in poor conception results. Different extenders used in cryopreservation showed varying impacts on post thaw semen quality. Amides were a good option for freezing semen as an alternate for glycerol, which is known to be contraceptive (Rath et al 2004). There were no differences observed in pregnancy rates for mares inseminated once or multiple times in a given cycle (51.5% versus 51.7% for data set 1 and 47.1% versus 46.1% for data set 2). Mares inseminated twice on a cycle, once before and once after ovulation, became pregnant at a rate similar to mares inseminated once within 6 h post-ovulation (48.1% versus 47.3%) Loomis and Squires, (2005).

Vidament, (2005) presented the results on procedures of freezing stallion's semen and subsequent fertility over 20 years. The freezing protocol was based on dilution in milk, centrifugation and addition of freezing extender (INRA-82) + egg yolk (2%V/V) + glycerol (2.5% V/V) at 22°C at a moderate cooling rate to 4°C and freezing in 0.5 ml straws. The pregnancy rate per cycle was 45-48% and foaling rate was 64%. The recommended glycerol concentration ratio should be maintained at 2-3.5% for good pregnancy results.

Over 30 years ago, an insemination dose of 500 X 10^6 progressively motile sperm (PMS) was recommended to maximize pregnancy rates when mares were bred with fresh semen under less than ideal conditions. Since that time, 500 X 10^6 progressively motile sperm had been almost universally accepted as a standard insemination dose, regardless of a stallion's fertility or the refinements that had been made in mare management and semen extenders. Insemination doses for cooled-transported and frozen-thawed semen have also been extrapolated from this dose (Brinsko, 2006). The standard cryopreservation technique decreases activity of these enzymes during freezing procedures. However freezing procedure did not affect the residual enzyme activity in extended semen (Bustamante-Filho et al., 2006).

The present project was designed to compare the effect of natural service, liquid cooled semen and frozen-thawed semen on the conception rate of stallion semen in mares.

Selection of the stallions

The four stallions (H_6, H_7, H_8 andH_9) used in this study were of the Thoroughbred breed. These horse stallions were from the Chenab Breeding Area Faisalabad (Pakistan). The age of these stallions ranged between 5-16 years. The semen of these horse stallions was evaluated before the start of the breeding season and during breeding season. The reproductive classification of the stallions was based on their health, condition, libido and volume of ejaculate combined with macroscopic and microscopic examination of semen for volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percent of dead sperm.

Selection of the mares

In this study 60 mares were selected. All the mares were subjected to regular teasing and rectal palpation of ovarian follicles to determine optimum time of breeding. A clinical evaluation of the reproductive health of the genitalia was made by examination of the cervix and vagina using a sterile glass speculum and by rectal palpation of the uterus. Cultures were taken from the cervices and uteri of mares in estrus. Clinical examinations were used for tests for the possibility of an infection. Only mares considered to be clinically normal and free of pathogens in the reproductive tract were bred naturally by the stallions. Stallions and mares were brought to the breeding area and their external genitalia and surrounding skin were washed and wiped dry with a clean towel. Mares had their tails bandaged and were restrained by any of the conventional methods before being bred by the stallion or by artificial insemination. Collections of stallion semen were made using the artificial vagina. Semen was diluted immediately after collection.

Preparation of semen extenders

Tris (hydroxyl methyl) amino methane and sodium citrate dihydrate based extenders were used in this study, the compositions of which are given in Table 1 of Chapter 2. Egg yolk was separated by complete removal of albumen. Egg yolk was poured into a cylinder by puncturing the yolk membrane. The required amount of Tris, sodium citrate dihydrate, citric acid, fructose, glucose, lactose, penicillin and streptomycin for each extender were weighed and placed in separate beakers. Redistilled water was added to

dissolve these ingredients and make the final volume of 73 ml. Then the required volumes of egg yolk (20 ml) and glycerol (7 ml) were added to the extender and stirred with a separate glass rod for uniform mixing of the ingredients. The extender was stored in pre-labeled, clean and sterilized beakers, which were kept in a water bath at 37°C until used for dilution of semen samples. Semen samples were diluted in the ratio of 1 part of semen with 10 parts of extenders. Extension was made by placing the semen and extenders in a water bath at 37°C. French straws made of polyvinyl chloride; were used in this experiment. The semen was drawn into straws with an automatic suction machine. The open ends of the straws were sealed with polyvinyl chloride powder. The straws were cooled and stored at 5°C.

Preparation of Liquid cooled and Frozen thawed semen

The extender C containing Tris (hydroxyl methyl) amino methane 1.21 grams and sodium citrate dihydrate 1.04 grams along with other ingredients as given in composition Table 1 of Chapter 2 showed the best performance in terms of post-thaw motility percentage, liveability (hours) and absolute index of liveability at 37°C.

The extender C was prepared in advance and stored in a freezer until used. The extender was warmed to 37°C before adding the semen and 0.5 ml volume straws were filled with automatic suction machine to inseminate the mare. Semen was diluted at the rate of 1part of semen with 10 parts of extender, cooled to 4°C for 6 hours and frozen thawed at – 196°C for 24 hours. Cooled semen was warmed to 37°C before it was deposited in the mare's uterus. Frozen semen was thawed at 37°C for 30 seconds.

Preparation of mares for insemination

The mares were adequately restrained with their tail wrapped and held to the side. The perineal areas were thoroughly scrubbed and rinsed, paying particular attention to the vulva. Any dirt or fecal material within the caudal vestibule was removed during the washing process to prevent contamination of the anterior reproductive tract during insemination. The perineal and vulvar areas were thoroughly dried prior to breeding.

Insemination Technique

Artificial insemination was carried out by inserting a sterile glass speculum into the vagina, and a sterile Chambers catheter was then passed through the speculum and in to the uterus by way of the cervix. A sterile 0.5 ml syringe filled with diluted semen was

attached to the Chambers catheter and the mixture was deposited into the uterus. An alternate method was to direct the Chambers catheter through the cervix by inserting a hand encased in a sterile glove into the vagina.

Grouping of animals

Sixty mares were divided into 3 equal groups of 20 animals in each group: -

Group – I 20 Mares were bred with natural service

Group – II 20 Mares were inseminated with liquid cooled semen at 4^0C for 6 hour.

Group - III 20 Mares were inseminated with semen frozen at -196°C for 24 hours and thawed at 37°C.

Pregnancy diagnosis

All the mares of the three groups were checked for pregnancy diagnosis through rectal palpation 90 days after insemination. All the mares were adequately restrained with their tail wrapped and held to the side. The perineal areas were thoroughly scrubbed and rinsed, paying particular attention to the vulva. Any dirt or fecal material within the caudal vestibule was removed during the washing process to prevent contamination of the anterior reproductive tract. The perineal and vulvular region were thoroughly dried prior to rectal palpation. The left hand of the technician was covered with a sterile plastic glove, lubricated with liquid paraffin and inserted into the rectum. Pregnancy was diagnosed by palpating the uterine horns.

All mares were subjected regular monitoring until the termination of pregnancy. At the termination of pregnancy all mares were checked for normal delivery or otherwise. All mares were delivered normally; no case of dystokia, stillbirth and abortion was recorded.

Statistical analysis

Test of homogeneity of ratio (Chi-square) was applied to analyze the data on fertility (Gomes and Gomez, 1984).

Results

The four stallions (H_6, H_7, H_8 andH_9) used in this study were of the Thoroughbred breed. These horse stallions belonged to the Chenab Breeding Area Faisalabad (Pakistan). The age of these stallions ranged between 5-16 years.

In this study 60 mares were selected. All the mares were subjected to regular teasing and rectal palpation of ovarian follicles to determine the optimum time of breeding.

Tris based extender C was prepared in advance and stored in a freezer until used. The extender was warmed to 37°C before adding the semen and 0.5 ml volume straws were filled with an automatic suction machine to inseminate the mare. Semen was diluted at the rate of 1 part of semen with 10 parts of extender cooled to 4°C for 6 hours and frozen thawed at –196°C for 24 hours. Cooled semen was warmed to 37°C before it was deposited in the mare's uterus. Frozen semen was thawed at 37°C for 30 seconds.

A total of 20 mares were bred by natural service, 20 by liquid cooled semen and 20 by frozen thawed semen. Eleven (55 percent) of mares were conceived with natural service, 9 (45percent) of mares were conceived with liquid cooled semen and 8 (40 percent) of mares were conceived with frozen thawed semen.

Conception rates in mares by using natural semen, liquid semen and frozen-thawed semen are given in Table 1. The conception rate was higher (55%)in mares bred with natural service while lowest rates (40%) were observed in case of frozen thawed semen. The insemination with liquid cooled semen showed intermediate (45%) results.

Table 1. Conception rate in mare using natural semen, liquid semen and frozen-thawed semen

Type of insemination	Number of Mares		Conception Rate (%)
	Inseminated	Conceived	
Natural Service	20	11	55
Liquid Cooled semen insemination	20	9	45
Frozen-thawed semen insemination	20	8	40

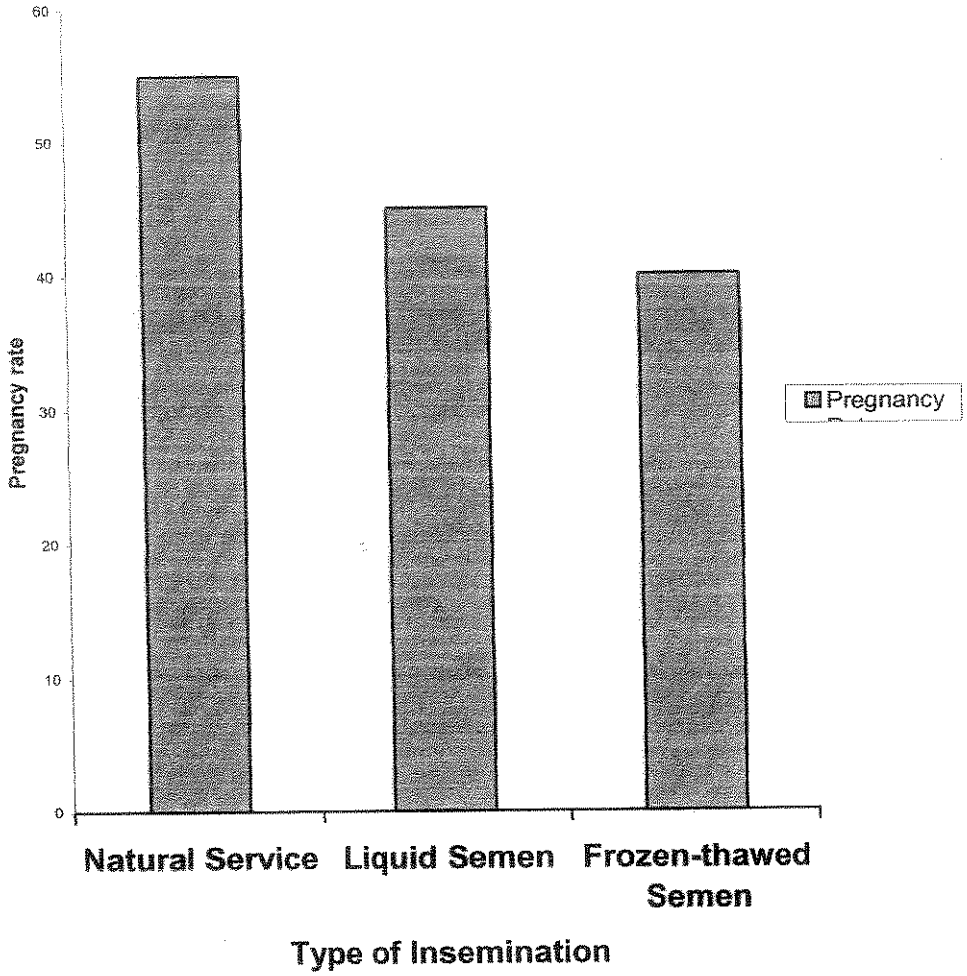

Figure-1: Pregnancy rate in mares using natural service, liquid cooled semen and frozen thawed semen.

Discussion

The fertility of the female depends upon two major factors: (1) innate properties of the mares and stallions (Colenbrander et al., 2003), (2) quality of the semen (Van der Host, 1975, Blottner et al., 2001). In any fertility programme the history of the mare contributes significantly. History includes the pre- breeding record and recent past cyclic activity, based on these records 60 mares maintained by different studs were selected for breeding which were divided into three groups of 20 animals each.

Group 1 of the mares was bred with natural service whereas the other two groups were inseminated with liquid cooled semen and frozen thawed semen. The mares were inseminated with a dose of 0.5 ml semen containing 500 x 10^6 spermatozoa. This dose agreed with the earlier recommendation of Pace and Sullivan (1975). Brinsko (2006) also confirmed that 500x 10^6 progressively motile spermatozoa is a standard dose for successful inseminations. The pregnancy results were based on rectal examination 90 days post breeding. The natural service seems to be superior followed by cooled liquid semen and frozen thawed semen respectively. The results of the present study were expected because with natural service minimum or no damage to the sperm should give best pregnancy results. In natural service the male delivers the semen directly into the uterus. Use of the extended semen, despite all precautions, every possibility of some damage during processing. The apparently lower pregnancy rate can also be associated with the single service in the cycle. The lowest conception rate with frozen thawed semen could persist because during the freezing and thawing process some sperm are damaged. The other factors, which can affect conception rate include the efficiency of the technician performing the insemination, and the stage of the estrus cycle based on correct judging ovulation time of the female (Metcalf, 1998).

The conception rates obtained from the present study are comparable to those of Hughes and Loy (1969) who carried out a comparison of natural breeding and artificial insemination of mares using liquid cooled semen from three Thoroughbred and three Quarter horse Stallions. The conception rates obtained by Hughes and Loy (1969) were 67.4 percent by artificial insemination and 78.9 percent by natural breeding.

Average pregnancy rates per cycle of mares bred with frozen thawed semen obtained by Samper (2001) were between 30 and 40 percent with a wide range between sires. Those results are in agreement with the results of the present study. He reported that stallion and

mare status were major factors in determining the success of frozen semen insemination and in order to maximize fertility with frozen semen, a careful selection of the stallions and mares, with proper client communication was critical. Dedication and commitment of the mare owner and inseminator would have the most significant impact on the pregnancy rate.

The number of progressively motile spermatozoa plays an important role in achieving better pregnancy rates in equines. In the present study the dose of semen used was 500 × 10^6 progressively motile spermatozoa, which is in agreement with Gahne et al., (1998) who tried to find out if it was possible to decrease the insemination dose from 500×10^6 progressively motile spermatozoa to 300×10^6 progressively motile spermatozoa and still maintain an acceptable pregnancy rate when using extended fresh semen. They reported that with an insemination dose of 300×10^6 progressively motile spermatozoa the pregnancy rate per cycle was 64% and with an insemination dose of 500 × 10^6 progressively motile spermatozoa the pregnancy rate per cycle was 75%.However there was no significant difference in the pregnancy rate between the 2 insemination doses (P=0.341). Therefore, they concluded that when using fresh extended semen, an insemination dose of 300 × 10^6 progressively motile spermatozoa would yield a lower pregnancy rate than a dose of 500 ×10^6 progressively motile spermatozoa if stallions with good quality semen were selected. Brinsko, (2006) reviewed the origin of the recommended insemination doses for use with fresh, liquid cooled and frozen thawed semen. He reported that over 30 years ago, an insemination dose of 500 X 10^6 progressively motile sperm (PMS) was recommended to maximize pregnancy rates when mares were bred with fresh cooled semen under less than ideal conditions. Since that time, 500 X 10^6 progressively motile sperm had been almost universally accepted as a standard insemination dose, regardless of a stallion's fertility or the refinements that had been made in mare management and semen extenders. Insemination doses for cooled-transported and frozen-thawed semen have also been extrapolated from this dose. He also presented data from a number of studies, which demonstrated the feasibility and rationale of reducing sperm numbers used to breed mares with fresh, cooled and frozen-thawed semen, including the use of deep-horn insemination techniques.

The number of inseminations per cycle also influences pregnancy rate in equines.

Loomis and Squires, (2005) reviewed two separate data sets consisting of 332 and 536 mare cycles collected during the 2002 and 2003 breeding seasons. There were no differences observed in pregnancy rates for mares inseminated once or multiple times in a given cycle (51.5% versus 51.7% for data set 1 and 47.1% versus 46.1% for data set 2). Mares inseminated twice in a cycle, once before and once after ovulation, became pregnant at a rate similar to mares inseminated once within 6 h post-ovulation (48.1% versus 47.3%). Therefore, in the present study the mares were bred with a single insemination in order to achieve better pregnancy rate in a simple way.

The glycerol concentration ratio used in the present study was 7%, which is not in agreement with Vidament, (2005) who recommended that the glycerol concentration ratio should be maintained at 2-3.5% for good pregnancy results. The cooling rate was 4°C per minute and semen volume was 0.5-ml/ straws. The pregnancy rate per cycle was 45-48% and foaling rate 64 %. The similar pregnancy rates achieved in the present study in spite of using 7% glycerol concentration ratio, preliminary shows that glycerol concentration ratio between 2-7% has no effect on pregnancy rate.

Conclusion

The present study revealed that the conception rates in mares were highest with natural service (55 percent) followed by liquid cooled semen (45 percent) and frozen-thawed semen (40 percent), respectively. There were a limited number of animals available for the present study. So the detailed studies involving larger data sets were suggested for validation of these results.

GENERAL DISCUSSION

These results of the present study clearly indicate that the semen obtained from the younger horse and donkey stallions aged up to 10 years was superior to that from older horse and donkey stallions. It is interesting to observe that the difference between younger and older horse stallions for volume (ml), motility percentage, sperm concentration ($x10^6$/ml), and mean percentage of dead sperms was significant only in Thoroughbred horse stallions, whereas other breeds did not show significant differences for these parameters.

Dowsett and Pattie, (1987) studied the semen characteristics of 168 stallions from 9 breeds, aged 2 to 26 years, over 4 breading seasons. Stallions <3 years of age had the lowest semen volume and sperm concentrations which together with the highest proportion of dead spermatozoa resulted in the lowest of live spermatozoa per ejaculate. Stallions older than 13 years had low sperm concentrations and high percentages of dead spermatozoa but sperm morphology was normal.

Arabian stallions had the lowest percentage of non-motile and dead spermatozoa while Shetlands were the only breed with greater than 30% dead, the others having values within accepted normal limits. Arabian stallions had sperm concentrations almost double those of other breeds and with relatively high volume. This resulted in total sperm numbers that were 3 times greater than those of any other breeds. There were significant differences between breeds in most morphological characteristics of spermatozoa but they were usually within the range considered normal. Thoroughbreds had considerably higher percentages of protoplasmic droplets than other breeds. Most breed differences could be considered normal but they should be taken into account when evaluating semen.

There were no differences in volume of gel- free semen but because of higher sperm concentrations, total sperm numbers were greatest in summer and autumn. In addition, most dead spermatozoa were present in autumn and winter and these were accompanied by higher proportions of abnormal heads and tails. It seems that the timing of the Australian breeding season in spring and early summer is out phase with the period of

peak semen production. They considered that poor quality of the semen was related to immature spermatogenesis in colts and testicular degeneration and abnormal epididymal function in old animals. This is also supported by Skinner et al., (1968); Paufler et al., (1979) and Orgebin-Crist MC (1969).

In present study three Thoroughbred stallions aged 20-22 years yielded extremely poor quality semen as regards volume (ml), pH, consistency, motility percentage, sperm concentration ($x10^6$/ml) and dead spermatozoa percentage. It can be concluded that both macroscopic and microscopic examination of semen can be valuable to eliminate such animals from breeding programmes. The effect of age in the present study on the semen parameters was statistically significant ($P<0.001$) in Thoroughbred stallions only but in other breeds some stallions aged between 13 years and elder also had poor quality semen based on macro and microscopic examination but the statistical significant was not apparent because of small number of older animals. Based on these observations stallions breeders can be advised to be vigilant when the stallion age exceed 18 years.

The influence of age on semen characteristics has also been reported by Dowsett and Knott, (1996). They found variation in semen quality and in spermatozoal and behavioral characteristics of 168 stallions representing 9 breeds and ranging age from 2 to 26 years. All semen characteristics with the exception of color and urethral pulsations had significant variations due to age. Semen quality (gel-free volume, sperm concentration, total sperm number and sperm abnormalities) was poorest in stallions under 3 year of age and over 11 years. Significant breed variation was apparent in most characteristics except for pH, semen color, abnormal mid-pieces and urethral pulsations.

The effects of age are probably due to differences in daily sperm production and output, which, in turn, are related to factors such as immature spermatogenesis in colts, testicular degeneration due to aging and aberrant epididymal function (Bowen, 1969). While age effects on semen parameters are apparent, they have little influence on stallion fertility except in the case of very young or very old stallions. The literature review (Skinner et al., 1968; Squires et al., 1979; Voss et al., 1979) indicates that influences of age on semen characteristics varies depending on which parameter is considered. They did not observe any effect of age upon parameters such as gel volume, sperm concentration, pH and sperm morphology.

The result of the present study indicates that season had no significant effect of any of the parameters studied. It seems that the non-significant effect of season is because the

animals were capable of consuming sufficient nutritional ingredients necessary to maintain their reproductive health. Though the summer and winter environmental temperatures differ to a great extent and summer temperature usually affects the performance of bovine bulls (Djimde and Weniger, 1984), the season has no affect on quantity or quality of the semen (Usmani et al., 1985). The semen collections from the stallions were made in the months of January- February (non breeding season) and May-June (breeding season). The environmental temperature difference between the months of January- February and May-June are very different and animals are also maintained under good managemental conditions, therefore, no detrimental affects were observed on the parameters of semen and spermatozoa. Last but not the least, it seems that the volume (ml), motility percentage, sperm concentration ($x10^6$/ml) and percentage of dead spermatozoa during breeding season had a tendency to be better then in the non breeding season.

Contrary to results of the present study Janett et al., (2003) reported that total volume; total sperm concentration and motility percentage in fresh semen were significantly higher in summer than in winter, while sperm concentration was significantly lower in summer compared to other season. This difference is probably because in Switzerland where these experiments were carried out, the winter is very severe as compared to the moderate summer environment.

The results of present study also differ from those reported by Jasko et al., (1992) who consider the evaluation of single ejaculates of semen and records for 2 consecutive breeding seasons. On the basis of evaluation of a single ejaculate for each stallion, the variation in these characteristics only accounted for approximately 20% of the observed variation in fertility rate and observed significant seasonal effect on the Casa movement of spermatozoa.

Tris (hydroxmethylaminomethane) and sodium citrate dihydrate ($Na_3C_6H^9O_7$. $2H_2O$) are two organic buffers being used successfully for the preservation of equine spermatozoa. Among five semen extenders used in this study, the main variation in the components of the extenders was the two organic buffers i.e. Tris and sodium citrate dihydrate alone or in various proportions. Other components were the same. The extender C containing 1.21 gms Tris + 1.04 gms of sodium citrate dihydrate proved to be the best in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability of spermatozoa at 37^0C. Extender A containing 2.42 gms of tris, extender D containing

0.060 gms Tris + 1.56 gms sodium citrate dihydrate and extender B containing 1.82 gms Tris and 0.52 gms Sodium citrate dihydrate although inferior to extender C showed better post thaw motility percentage. Extender A containing 2.42 gms Tris which was merit wise inferior to extender C but superior to all other extenders while B, D and E showed differences.

Different solutions of chemical compounds have their specific eutectic points (the temperature at which the whole liquid phase is converted into solid phase). It is well known that during freezing solidification of solutions adversely affects the spermatozoa. However, this effect can be reduced by completing the solidification process in more than one step. This can be achieved through replacing a particular compound with other components having similar properties but different eutectic points. In this way the solidification process will take place at different temperatures and thus its harmful effects will be diluted. In the present study Tris, which is commonly used as a buffer was replaced by another buffer, sodium citrate dihydrate in different proportions to have different solidification temperatures of these solution and thus accordingly reduce the harmful effects on the spermatozoa.

The better performance of the extender C containing 1.21 gms Tris + 1.04 gms sodium citrate dihydrate may be due to the synergistic effect of the two buffers. The difference was highly significant as far as the post thaw motility, post thaw livability of spermatozoa at 37^{0}C and absolute index of livability are concerned.

The results of the present study revealed that the combination of Tris (hydroxymethyl) amino methane and sodium citrate dihydrate should be used as buffer for deep-freezing stallion spermatozoa. However, if only one buffer is to be used, Tris should be preferred over sodium citrate dihydrate. These results are similar to those reported by other workers.

Bomstein and Steberi (1959) reported that Tris (hydroxymethyl) aminomethane was useful in prolonging survival of washed bovine spermatozoa stored at room temperature. It is virtually impossible to draw valid conclusion by comparing data from two or more reports because of confounding influences of stallion, packaging system, cooling and warming rates and the subjective nature of visual evaluations of post-thaw motility of spermatozoa (Amann and Pickett, 1987). The results of the present study revealed that the combination of Tris (hydroxymethyl) amino methane and sodium citrate dihydrate should be used as buffer for deep-freezing of stallion spermatozoa. However, if only one

buffer is to be used, Tris (hydroxymethyl) amino methane should be preferred over sodium citrate dihydrate. These results are similar to Foote, (1970) who reported that bovine sperm motility following freezing and thawing in Tris based extender was slightly superior to that in citrate extender but there was no difference in their fertility. The results of present study demonstrate that extender C containing Tris 1.21 percent with other components showed better post thaw motility, liveability (in hours) and absolute index of liveability compared to extenders A, B and D, respectively. The extender A containing Tris 2.42 percent showed less post thaw motility, liveability and absolute index of liveability. In agreement with the present study Picket et al., (1975) showed that pregnancy rate, spermatozoa motility and spermatozoa agglutination were significantly lower in an extender containing 0.349% Tris than in an extender utilizing 2.4% Tris.

In the present study 7% glycerol was used in five experimental extenders in which the quantities of Tris and sodium citrate dihydrate varied according to composition of the experimental extender A, B, C, D and E respectively. There were no detrimental effects of glycerol on equine sperm motility. Similarly Province et al., (1984) also reported that the inclusion of 6% glycerol had no effect on the percentage of motile equine sperm. The interaction of glycerol level and extender was non significant. The inclusion of 6% glycerol has detrimental effects on the motility percentage of canine sperm. Pace and Sullivan, (1975) extended semen in a Tris based extender containing 7% glycerol, and froze it in 10-ml ampules. They concluded that fertility was maximal when insemination was 0 to 12 hours before ovulation, and that insemination 0 to 12 hours after ovulation was somewhat inferior. They also stated that insemination 12 to 36 hours before ovulation was preferable to insemination 12 to 24 hours after ovulation.

Cooling rate has received little attention, perhaps because with pelleted semen it is difficult to measure or control, and for semen frozen in glass ampules, 0.5, 1.0 or 4.0 ml straws, freezing in vapor above liquid nitrogen was convenient and has proven to be successful with bull spermatozoa. For semen frozen in ampules, two reports are divergent in their recommendations; Schafer and Baum, (1964) recommended cooling from 6°C to –79°C in 10 minutes (possibly 8 to 10°C/minute), whereas Choa and Chang (1965) recommended a slower cooling rate of about 0.5°C /minute to –20°C and then 3°C/minute. Semen frozen as 0.1 ml pellets presumably cools very rapidly, probably in <4 minutes. Cochran et al., (1984) compared post-thaw motility of spermatozoa cooled at about 60°C /minute by placing 0.5 ml straws horizontally in liquid nitrogen vapor at –

160°C or at a controlled rate of 10°C/minute from +20 to –15°C and 25°C/minute.. The percentage of motile spermatozoa after thawing was not influenced by cooling rate. The effect of thermal properties of the plastic or wall thickness on cooling rate of 0.5 ml straws has not been studied. Cochran et al., (1983) used 0.5ml straws and semen was thawed in 37°C water for 30 seconds. Province et al., (1985) reported that CAP and NFDMS-glucose extenders were superior (P<0.05) to SM for maintenance of spermatozoa motility storage at 20C or 15C resulted in similar (P>0.05) spermatozoa motility.

In the present study 100000-units/ ml of Penicillin and 100 mg/ml of Streptomycin were used in five experimental extenders for the reduction of growth of microbial agents. The results of the present study clearly indicated that the performance of five experimental extenders was better in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability at 37°C of spermatozoa. Contrary to the results of the present study, Jasko et. al. (1993) added amikacin sulfate, ticarcillin disodium, gentamicin sulfate and polymixin B sulfate to a nonfat, dried, skim milk - glucose seminal extender at concentrations of 1000 or 2000 mug or IU/ml. After 24 h of storage at 5 degrees C, 2000 mug/ml of gentamicin and levels equal to and greater than 1000 IU/ml of polymixin B in seminal extender resulted in significant (P<0.05) reductions in the percentages of motile and progressively motile spermatozoa. After 48 h of cooled storage, a level of 1000 mug/ml of gentamicin sulfate resulted in significant (P<0.05) reductions in the percentages of motile and progressively motile spermatozoa. Levels equal to or greater than 1000 IU/ml of polymixin B sulfate also resulted in a significant (P<0.05) reduction in mean velocity. Levels up to 2000 mug/ml of amikacin sulfate and ticarcillin disodium had no significant effect on sperm motion characteristics during short-term incubation at 23 degrees C or storage for 24 h at 5 °C. Overall, the addition of antibiotics to extender did not significantly (P>0.05) improve motion characteristics of spermatozoa over control samples. However, levels of gentamicin sulfate greater than 1000 mug/ml and of polymixin B sulfate equal to or greater than 1000 IU/ml should be avoided in seminal extenders used for cooled semen.

In the present study 20% egg yolk was used in five experimental extenders and the results of the present study were better in terms of post thaw motility percentage, liveability (hours) and absolute index of liveability of spermatozoa at 37°C. Similarly, Martin et al., (1980) diluted equine semen in seminal extenders in which 20% egg yolk was used.

Cochran et al. (1984) used a lactose based extender with 20 % egg yolk and found that post-thaw motility percentage of the spermatozoa was not affected significantly. It was suggested that the use of 20% egg yolk was a better approach for the freezing of the seminal extenders. In another study Cristanelli et al., (1985) also confirmed that percentage of progressive motile spermatozoa after freezing and thawing was higher for extenders with 20 % egg yolk. Bedford et al., (1994) conducted three experiments to evaluate the effects of egg yolk and (or) glycerol added to a non-fat dried skim milk glucose (NDSMG) extender on motion characteristics and fertility of station spermatozoa and reported that the use of 4 % egg yolk in NDSMG extender did not depress the motility percentage of the spermatozoa but adversely affected the fertility.

The fertility of females depends upon two major factors; (1) Innate properties of the mares and stallions (Colenbrander et al., 2003), (2) quality of the semen (Van der Host 1975; Blottner et al., 2001). In any fertility programmed the history of the mare contributes significantly. History includes the pre- breeding record and recent past cyclic activity, based on these records, 60 mares maintained by different studs were selected for breeding which were divided into three groups of 20 animals each.

Group 1 of the mares was bred with natural service whereas the other two groups were inseminated with liquid cooled semen and frozen thawed semen. The mares were inseminated with a dose of 0.5 ml semen containing 500 x 10^6 spermatozoa. This dose agrees with earlier recommendation of Pace and Sullivan, (1975). Brinsko, (2006) also confirmed that 500x 10^6 progressively motile spermatozoa is a standard dose for successful inseminations. The pregnancy results were based on rectal examination 90 days post breeding. The natural service superior followed by liquid cooled and frozen thawed semen, respectively. These results were expected because with natural service minimum or no damage to spermatozoa occurred and best pregnancy results were obtained. In natural service the male delivers the semen directly in to the uterus. With the use of the extended semen, in spite of all precautions, there is every possibility of some damage to the spermatozoa during processing. The apparently lower pregnancy rate can also be associated with the single service during the cycle. The lowest conception rate with frozen thawed semen could result because during the freezing and thawing process some sperm are damaged. The other factors, which can affect conception rate, include the efficiency of the technician performing the insemination, and the stage of the estrus cycle as well as, correct judging ovulation time of the female (Metcalf, 1998).

The conception rate was higher (55%) in mares bred with natural service while the lowest rates (40%) were observed in case the of frozen thawed semen. The liquid cooled semen showed intermediate (45%) results. The lowest conceptions in mares inseminated with frozen thawed semen could be due to some damage to sperm during the processing, cooling, freezing and thawing stages.

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