EFFECT OF DIETARY n-3 PUFA ON OVARIAN FUNCTION, EMBRYONIC DEVELOPMENT AND SEMEN QUALITY IN HORSES

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy
(Veterinary Gynaecology and Obstetrics)

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Dedicated to....

My Beloved Parents

&

Wife Dr. Susavi

भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 29.8.2014

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Effect of dietary n-3 PUFA on ovarian function, embryonic development and semen quality in horses"** *submitted by* **Dr. Sanjay Kumar Ravi, Roll No. 1187,** *for the award of* **Doctor of Philosophy** *Degree in* **Veterinary Gynaecology and Obstetrics** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Sanjay Kumar Ravi, Roll No. 1187,** *has worked for more than 30 months in the Institute and has put in more than 300 days attendance under me from the date of registration for the Doctor of Philosophy Degree in this Deemed University, as required under the relevant ordinance.*

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(SANJAY KUMAR RAVI)

ABBREVIATIONS

%	Percent
:	Ratio
@	At the rate of
>	More than
±	Plus or minus
≤	Less than or equals to
≥	More than or equals to
µg/dl	Micro gram per deci litre
µl	Micro litre
µmol/L	Micromole per litre
^{0}C	Degree centigrade
4-AP	4-aminophenazone
acetyl-CoA	Acetyl co-enzyme A
ACOD	Acyl-CoA oxidase
ACS	Acyl-CoA synthetase
ADP	Adenosine-5-diphosphate
AI	Artificial insemination
ALA	α-linolenic acid
am	Antemeridian
ArA	Arachidonic acid
ATP	Adenosine triphosphate
AV	Artificial vagina
BW	Body weight
Ca-LCFA	Calcium salts of long chain fatty acids
CASA	Computer-assisted sperm analysis
CHE	Cholesterol esterase
CHOD	Cholesterol oxidase
EDTA	Ethylenediaminetetraacetic acid
CL	corpus luteum
D	Day
D0	Day of estrus
$D15_{PO}$	Day 15 post ovulation
D3	Third day of estrus
D6	6th day of estrus
DAP	Dihydroxyacetone phosphate
DHA	Docosahexaenoic acid
DM	Dry matter
DMF	Di-methyl formamide

DMRT	Duncan's multiple range test
D_{ov}	Day of ovulation
DPA	Docosapentaenoic acid
e.g.	Example
EED	Early embryonic deaths
ELISA	enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
et al.	Co-worker
etc	et cetera (and the rest)
FA	Fatty acids
FADS2	Fatty acid desaturase 2
FAO	Food and agriculture organization
Fig.	Figure
G3P	Glycerol-3-phosphate
GK	Glycerol kinase
gm	Gram
GnRH	Gonadotropin-releasing hormone
GPO	Glycerol phosphate dehydrogenase
Gr.	Group
H_2O_2	Hydrogen peroxide
HDL	High-density lipoproteins
HOST	Hypo osmotic swelling test
hr	hour
ICSI	Intra-cytoplasmic sperm injection
IFN-ô	Interferon tau
IL	Interleukin
IVF	In vitro fertilization
kg	Kilogram
LA	Linoleic acid
LC	Long chain
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LSD	Least significant difference
Ltd.	Limited
LTFA	Long chain trans fatty acids
MFM	Menhaden fish meal
mg	Miligram
mg/dL	Miligram per decilite
mg/kg	Miligram per kilogram
ml	mililiter
mm	Millimetre

mmol/L	Millimole per litre
MRP	Maternal recognition of pregnancy
n	Number
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
ng/mL	Nanogram per milliliter
nm	Nanometre
OA	Osteoarthritis
OD	Optical density
P<0.001	significant at 0.1% level
p<0.05	5% level of significance
PG	Prostaglandin
pg/ml	Pictograms per milliliter
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
$PGF_{2á}$	Prostaglandin $F_{2á}$
PGFM	Prostaglandin metabolite
PGHS	Prostaglandin H Synthase
pH	Hydrogen ion concentration
POD	peroxidase
POF	preovulatory follicle
PTM	Post thaw motility
PUFA	Polyunsaturated fatty acid
PVC	Poly-vinyl chloride
RIA	Radio immune assay
rpm	Rotation per minute
SD	Standard deviation
SEM.	Standard error of mean
StAR	Steroidogenic acute regulatory
TNF-α	Tumor necrosis factor - alfa
USA	Uniited States of America
VLDL	very low density lipoprotein
vs	Versus
WR	working reagent
β	Beta
µg /ml	Micro gram per millilitre

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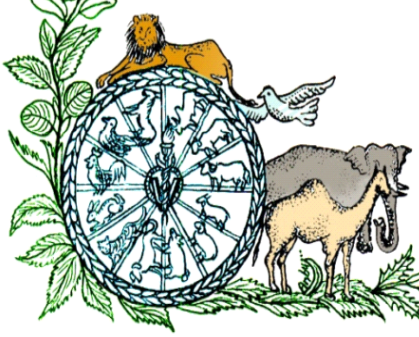
Introduction



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INTRODUCTION

Total global equine population is estimated around 59 million (FAO, 2011), whereas horses and ponies in India comprise 0.61 million out of total 1.19 million equines (Livestock census, 2007). Among more than 300 breeds of horses around the world, India has two recognized horse (Kathiawari and Marwari) and four pony (Manipuri, Spiti, Zanskari and Bhutia) breeds in different agro-climatic regions (Gupta *et al*., 2012). Worldwide, horses are being used in sports, works, leisure activities, entertainment and culture. Both, horses and ponies provide livelihood to the poor farmers and other sections of rural and semi-urban Indian society through draught and transport. Horses are part of Indian culture in marriages, used for entertainment, riding. They have special significance for providing services to the army, police, racing industry and sports. In spite of development in mechanization, horses retain great relevance especially for hill and difficult terrains where other means of transport are inaccessible. Products from horses include mare's milk and meat in many countries. Milk is used after fermentation to produce kumis by people especially with large herd. Horse meat is used for human and carnivorous animals but its consumption is taboo in many countries (http:// www.igha.org) whereas subject of controversy in others (www.sfgate.com). Byproducts from horses include hide, hooves, hair, bones and pharmaceuticals extracted from urine of pregnant mares (www.humansociety.org).

Among Indian horses, Marwari is best adopted in hot, dry and desert conditions of Marwar area of Rajasthan. The Marwari breed is known for their conformation, speed, stamina, endurance capacity and has good export potential (Gupta *et al*., 2014). Due to indiscriminate breeding, decreased demand and introduction of exotic horse breeds, the population of Indian horses have declined rapidly during the last few decades (Singhvi, 2001; Yadav *et al*., 2001; Chauhan *et al*., 2004; Gupta and Pal, 2010; Gupta *et al*., 2012). According to livestock census of India, the population of horses and ponies in between 1951 and 2007 decreased from 1.50 to 0.61 million. Though breed-

wise census of horses and ponies are not available, a maximum of around 3000 true to breed Marwari horses are available in their home tract as per information from Indian horse societies (Sharma and Kumar, 2010). Indian horses and ponies are categorized endangered because of their declining number in their respective breeding tracts (Gupta *et al*., 2012). There is an urgent need to propagate and conserve them to meet future demand of draft power in adverse conditions of hilly regions.

Higher pregnancy rate in mares is desirable to increase their number. However, not all mares get conceived and those conceived suffer sometimes with early embryonic losses. In horses, the time from breeding to day 40 of pregnancy is considered critical as most of pregnancy losses occur during this period (Villahoy *et al*., 1985; Woods *et al.*, 1987; Baker *et al.,* 1993). In mares, incidence of early embryonic deaths (EED) as reported in many studies is 8 to 15% (Roberts, 1980), 5 to 24% between 11 to 50 days post-ovulation (Ginther *et al.,* 1985) and 2.6 to 24% (prior to day 60 of gestation) with a weighted-mean 8.6% across the studies (Vanderwall, 2008). Embryonic losses that occurred between days 16 and 39 post-ovulation contributed more than 50% of the overall detected (19.11%) pregnancy losses (Sharma *et al.*,2010). Embryonic losses have been reported as increasing with advancing age (Woods *et al.,* 1987; Vanderwall *et al.,* 1989; Carnevale and Ginther, 1992; Allen *et al.,* 2007; Sharma *et al.,* 2010) and sub-fertile status of mares (Ball *et al.,* 1986, 1989; Brinsko *et al.,* 1994). The embryonic losses could be higher than actually detected, if including that occurs between fertilization to day 10 which is difficult to detect owing to non-availability of suitable technique for diagnosis. EED is recognized as major problem which causes considerable economic losses to horse breeders in terms of increased costs associated with additional breeding of mares and/or decreased foal production.

Progesterone is essential for the maintenance of pregnancy in mares as in other mammals and its only source during the embryonic period is primary corpus luteum (CL). The main function of the luteal cells is to synthesize progesterone which in turn creates a suitable uterine environment for embryonic growth and development. Inadequate progesterone due to insufficiency of primary luteal cells (Ginther *et al.,* 1985; Niswender and Nett, 1994) has often been suggested as a cause of EED. Higher concentration of plasma progesterone is associated with improved conception rate in lactating ruminants (Staples *et al.,* 1998). Continuous release of progesterone is dependent on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production from endometrium which influences

the life span of the CL. Release of $PGF_{2\alpha}$ causes lysis of CL (luteolysis), however, in the presence of a conceptus the synthesis and secretion of $PGF_{2\alpha}$ is abrogated. The conceptus plays a pivotal role in controlling the intrauterine environment, both by signaling its presence and assuring maternal recognition of pregnancy, and by contributing directly to the steroid environment of the intrauterine lumen. The equine embryo communicates by moving throughout the uterus from day 6 to 16 of gestation. Estrogen produced from an early embryo has also been postulated to be involved with maternal recognition of pregnancy. According to Sharp *et al.* (1989), there appears to be a "critical deadline" between days 9 and 14 after ovulation in which recognition of the conceptus occurs. Any restriction or delay in movement during this time fail to prevent the release of $PGF_{2\alpha}$, thus creating a uterine environment that initiates early embryonic death. Embryo loss at this time may be associated with an inability to inhibit the luteolytic action of $PGF_{2\alpha}$ during the critical period of maternal recognition of pregnancy. Strategies that can prevent release of $PGF_{2\alpha}$ and thus luteolysis are helpful for embryo survival in various species.

Among several antiluteolytic strategies (pharmacological, mechanical, and management manipulations), nutritional approach to enhance reproductive performance through fat supplementation is gaining interest (Thatcher *et al.,* 1994; Thatcher and Staples, 2000; Wathes *et al.,* 2007). Though, the objectives of previous studies remained nutritional rather than to enhance reproduction. In the past decade, feeding lipid to horses has become a common practice in equine industry. Performance horses are frequently given high-lipid diets as addition of extra fat raises the energy density of the ration. The use of high-energy diets reduces dry matter (DM) intake (Hintz *et al.,* 1978), which lowers the weight of gastro-intestinal contents, this effect being considered beneficial to performance horses (Meyers *et al.,* 1987). In addition to improved energy status, lipid supplementation has its beneficial effect on plasma progesterone concentration and embryonic survival (Thatcher and Staples, 2000).

Recently, interest is growing in dietary long chain (LC) polyunsaturated fatty acids (PUFA's) owing to their potential health benefits. The LC fatty acids with beneficial effect on reproduction are eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) omega-3 fatty acids and, arachidonic acid (ArA, 20:4 n-6), an omega-6 fatty acid. EPA and DHA are synthesized de novo from the short chain essential fatty acid α-linolenic acid (ALA, 18:3n-3) whereas the ArA is

synthesized from linoleic acid (LA, C18:2n-6) in the liver by desaturation and elongation enzyme systems common to both. There is a direct competition for the desaturation and elongation enzymes between the n-6 and n-3 fatty acids (FA's) within the de novo synthesis pathway (Lands, 1992). Both n-3 and n-6 PUFAs can influence reproductive processes through a variety of mechanisms. Dietary n-3 and n-6 FA's have been shown to improve reproduction responses in species such as dairy cattle (Ambrose *et al.*, 2003) and swine (Webel *et al.,* 2007). In particular, the ratio of omega-6 to omega-3 PUFA plays an important role in several aspects of animal health, production and reproduction (Abayasekara and Wathes, 1999). The n-3 FA's have potential benefits on immunity and reproduction, and thus reducing the dietary n-6 to n-3 FA's ratio may improve animal health, performance and reproduction. Mattos *et al.* (2000) proposed that n-3 PUFA may reduce uterine $PGF_{2\alpha}$ secretion and/or decrease the sensitivity of the CL to $PGF_{2á.}$ This may improve fertility by reducing embryonic loss associated with the inadequate suppression of $PGF_{2\alpha}$ in early pregnancy. It is also suggested that n-3 PUFA may act in concert with embryo derived interferon-tau to inhibit release of uterine produced $PGF_{2\alpha}$, thus preventing the onset of luteolysis and facilitating the establishment of pregnancy in cattle (Binelli *et al.,* 2001; Mattos *et al.*, 2003). Therefore, increased conception rates following dietary inclusion of PUFA's may be associated with improved embryonic survival (Thangavelu *et al.,* 2007).

Several researchers have studied the effects of n-3 FA's on reproduction in animals and humans (Abayasekara and Wathes, 1999; Allen and Harris, 2001; Wathes *et al*., 2007). Studies in cattle have shown that alteration of dietary PUFA content alters the number and size of pre-ovulatory follicle (POF), ovulation rate, conception rate, progesterone production by the CL, $PGF_{2\alpha}$ production and gestation length (Thatcher and Staples, 2000; Petit *et al.,* 2001; Petit *et al.,* 2002; Petit and Twagiramungu, 2006). An increase in the number of smaller follicles may reflect a greater pool of follicles available for subsequent development. In addition to the increased number of follicles, the size of the dominant follicle increased due to lipid supplementation. Several reports in dairy cattle have indicated positive association between POF diameter and subsequent conception rate (Mapletoft *et al.,* 2005; Bello *et al.,* 2006; Lopes *et al.,* 2007). At estrus, POF is a prime candidate behind endocrine imbalance because it produces substantial amounts of estradiol and a positive correlation exists between the

diameter of POF and plasma estradiol (Noseir, 2003; Perry *et al.,* 2007). Moreover, in dairy cattle, it is speculated that POF diameter is important for the subsequent development of CL and hence conception rate. A larger POF may generate a larger CL that will secrete more progesterone and hereby have a positive effect on pregnancy recognition and pregnancy rates (Busch *et al.,* 2008; Binelli *et al.,* 2009). Inclusion of dietary n-3 FA's into sow diets increased the PUFA content in plasma of both sows and piglets and also in the milk which was associated with improved pre-weaning survival in some studies (Fritsche *et al*., 1993; Rooke *et al*., 2001). Spencer *et al*. (2004) reported an increase in the number of piglets born alive when sows were fed a source of fish oil (high in n-3 FA). There are reports that both EPA and DHA can alter circulating FA's profiles, and lower serum cholesterol and plasma triglycerides in horses (O'Connor *et al.,* 2007).

For decades, research on artificial insemination (AI) has focused on cattle, but during last more than 25 years, it gained increasing acceptance in horse breeding (Aurich, 2012). Use of frozen semen added a new dimension to the horse breeding industry. Semen cryopreservation optimizes the use of stallions with superior genetics, and reduces the transport costs of animal and transmission of diseases. Semen can be preserved for unlimited time and made available for worldwide distribution. Additionally, frozen semen can be used from stallions that are in competition or are recovering from illness that would prevent them from mating, and even after death of stallions (Miller, 2008). Use of semen cryopreservation is increasing in horses due to improvement in techniques and growing interest. There is wide individual variability of stallion's semen to tolerate the freezing and thawing process (Vidament, 2005). Unfortunately, many stallions produce semen that is unable to retain acceptable sperm motility after cryopreservation (Brinsko *et al.,* 2000). The mechanisms behind the cryosensitivity of stallion sperm are yet to be elucidated. In general, semen from stallions has poor post thaw sperm motility when compared to cattle and buffalo. Cryopreservation of sperm causes damage to cells and sperm membranes are often considered to be the primary site of freezing injury (Quinn, 1985). Stallion sperm are sensitive to cold shock and membrane damage during freezing and thawing (Watson *et al*., 1987; Wilhelm *et al*., 1993). Species differences in sperm ability to resist cold shock appear to be related to their sperm membrane lipid composition (Parks and Lynch, 1992).

Major structural component of biological membranes are phospholipids that affect both biophysical properties and functions of the cell (Pratt, 1980; Stubbs and Smith, 1984). The spermatozoa of most mammals contain high concentrations of PUFA (Nissen, and Kreysel, 1983; Parks and Lynch, 1992), which provide fluidity to spermatozoa. Sperm membranes release phospholipids into the surrounding medium during cold shock. In addition, cold shock has been linked to lipid phase transitions that cause the sperm membrane to become transiently leaky (Drobnis *et al*., 1993; Arav *et al*., 2000). Semen from all domestic species contains high levels of PUFA particularly DHA and DPA (docosapentaenoic acid; 22:5 n-6, an omega-6 fatty acid). Moreover, spermatozoa from stallions and boars have a higher proportion of DPA (Parks and Lynch, 1992). Studies in the boar have shown that a high DHA to DPA ratio in semen results in enhanced fertility, while higher levels of DPA relative to DHA results in reduced fertility (Penny e*t al.*, 2000b; Maldjian e*t al.*, 2003). Thus, species differences in the susceptibility of spermatozoa to cooling, freezing, and thawing process seems to be largely attributable to the PUFA contents of sperm plasma membrane (White, 1993).

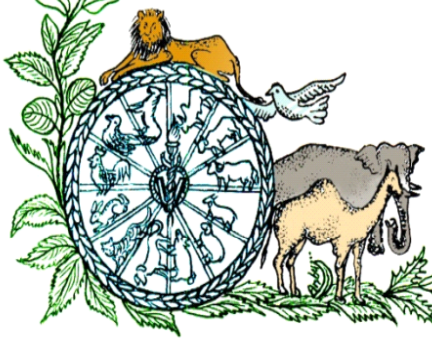
Supplementation of horses with nutraceutical rich in DHA has shown a three-fold increase in semen DHA after 14 weeks of treatment (Brinsko *et al*., 2005). After 48 h of cooled storage, increase in total and progressive sperm motility have been observed. In another study, supplementation with a nutraceutical containing greater amounts of DHA resulted in increased sperm output (Harris *et al*., 2005). This compound is reported particularly useful in those stallions having semen that cooled or froze poorly (Brinsko *et al.,* 2005). Further, studies are needed on large number of stallions to determine how consistently feeding products rich in DHA will improve variables related to semen quality of stallions. A number of studies have been conducted with nutritional approach rather than having emphasis on reproductive functions to study the effect of dietary lipid on performance of working horses (Hambleton *et al.,* 1980; Oldham *et al.,* 1990; Duren *et al.,* 1999; Pagan *et al.,* 2002). Information on the effect of PUFA supplementation on ovarian functions and embryonic development in mares is not available so far. Role of n-3 PUFA on sperm ability to withstand cooling and storage also warrant further investigations. Based on the above knowledge, the present study was designed with following objectives:

Objectives :

1. **To study the effect of dietary n-3 PUFA supplementation on follicular, luteal and embryonic development in Marwari mares.**
2. **To correlate the size of preovulatory follicle and corpus luteum with plasma estradiol and progesterone concentration.**
3. **To study the effect of dietary n-3 PUFA supplementation on semen quality of Marwari stallion.**

❐❐❐

Review of Literature



2

REVIEW OF LITERATURE

Horses are magnificent animals, commonly known for their elegance, speed, stamina and intelligence. They were historically used in warfare, kept now days not only as pets but as true companions and being used in a wide variety of sports competition and other recreational pursuits, as well as in working activities such as police work, agriculture and therapy. The horse population is either constant or decreasing in most of the countries (GHILPA, 2011) but there remained a consistent demand for quality horses. A high pregnancy rate at the end of the breeding season and subsequent birth of live foal is highly desirable for successful breeding and profitable farming. Horses are seasonally polyestrous species during April to September in North Hemisphere which is associated with increase in daylight, temperature and availability of food (Hughes *et al.,* 1975). However, in Indian subtropical conditions, this ranges from mid February to mid November in ponies (Pal and Gupta, 2005) and Marwari mares (Ravi *et al.,* 2013). During the breeding season, estrous cycle length in Indian ponies (Pal and Gupta, 2005) and Marwari mares (Arangasamy *et al*., 2008) was reported to be 25.6 days (range 25-27) and 19.16 days (range 13-29) with average duration of estrus 5 days (range 3-8) and 6.14 days (range 3-11), respectively. In mares, one of the most challenging job is getting them conceived within breeding season. Conception failure in mares breaks the consistency of "a foal every year" and increases the cost of rearing and thus decreases the profit margins at the same time. It was reported that 9.07% of coverings were empty, whereas 23.7% of covering did not results in successful pregnancy in this species (Taveira and Mota, 2007). The birth rate ranges from 59% (Taveira and Mota, 2007) to 74% (Cilek, 2009) and the higher percentages reported usually in experiments involving a small number of mares (Arangasamy, 2008).

2.1 Fertility in Mare

In mares, fertility remained lowest in comparison to females of other domesticated species, as horse breeds have been selected based on war, draft and racing abilities rather than fertility (Engelken, 1999). In race horses, pedigree and track records are highest priority for selecting best of the breed, whereas the physiological as well as managerial factors that may affect fertility are generally overlooked. Although, as in other species, fertilization rates in mares have been reported high (over 90% per cycle) in reproductively sound mares (Ball *et al.,* 1886; Ball and Little, 1989), the pregnancy resulted from first service remained 43 to 51% in Thoroughbreds and Quarter horses (www.thehorse.com). Fertility rate in terms of conception and foal born have been reported approximately 72 and 63%, respectively in Thoroughbreds (Bhuvankumar, 2007) in Indian climatic conditions at an organized farm. Morris and Allen (2002) have observed 16 to 17% loss of pregnancies that are diagnosed by ultrasound at about day 15 and 60% of them between days 15 to 35 post-ovulation. The conception during foal heat remained around 40%. Further, the conception has been reported to decrease with mare's sub-fertile status (Ball *et al.*, 1986, 1989; Brinsko *et al.*, 1994) and advancing age (Ball *et al.*, 1986; Bhuvankumar, 2007).

Use of AI in horses has been suggested to enhance the reproductive efficiency and is usually performed either with fresh, chilled or frozen semen. Semen cryopreservation is particularly important, since it facilitates the international trade of equine semen and enables the long term preservation of sperm from superior sires (Loomis *et al*., 2008). First cycle pregnancy rates with commercially available frozen semen have been reported to be 32 to 73% (Amann and Pickett, 1987; Muller, 1987; Samper, 1995; Vidament *et al*., 1997; Metcalf, 1998; Barbacini *et al*., 1999; Loomis, 1999; Vidament *et al*., 2000; Loomis, 2001). The conception rate of 72.73% has been achieved in Marwari mares using fixed time insemination with frozen semen (Arangasamy *et al.,* 2008). The fertility of frozen semen is influenced by a number of factors including stallion selection, semen quality, insemination dose, mare selection and mare management. Pal *et al.* (2011) inseminated once (in 32 mares), twice (in 59 mares), thrice (in 4 mares) and four times (in 3 mares) per cycle with frozen semen and achieved 31, 47, 75 and 100% conception, respectively and recommended at least 2 to 3 AI per cycle in mares for optimum conception. Seasonality of breeding, long estrus duration, infertility and

occurrence of reproductive tract infections in mares are other limitations which causes low reproductive performance (Hughes, 1975; Singh *et al.,* 2010). Infertility in mares is considered as a major concern, which can be categorized either as lack of estrous cyclicity, a cyclic mare which does not conceive or a cyclic mare that conceives but then suffers from early or late embryonic mortality (Purohit, 1997).

2.2 Embryonic Losses in Mare

Early embryonic loss is major cause of infertility in the mare which is generally defined as pregnancy failure that occurs between fertilization and days 40 to 60 of gestation (Ezzo *et al.,* 2011). Embryos have been reported to die at various stages of their development, starting from fertilized eggs, through morulas, blastocysts to older embryos undergoing organogenesis (Ball *et al.,*, 1986; Allen, 2000). Per-rectal palpation revealed 7 to 16% incidence of embryonic and/or fetal losses in mares between 20 and 90 days post-ovulation (Ginther, 1979). However, embryonic losses occurred frequently in the early stages of pregnancy before day 40 (Villahoy *et al*., 1985), more specifically in the first 2 weeks of gestation (Forde *et al*., 1987; Woods *et al*., 1987; Baker *et al*., 1993). The diagnosis of early embryonic loss and recognition of factors contributing to its occurrence have been improved tremendously by routine use of transrectal ultrasonography. Ultrasonographic studies revealed 13.28% embryonic death during 19 to 21 days post-ovulation in mares (Papa *et al*., 1998). Most of these losses occurred up to 21 days post-ovulation (Ginther *et al*., 1985; Woods *et al*., 1985; Ginther, 1992; Papa *et al*., 1998). Further, the embryo losses between fertilization to day 14 have been reported approximately 9% in young, reproductively sound mares and over 60% in aged, sub fertile mares (Ball *et al.,* 1986). The highest embryonic losses (20 to 30% or even higher) have been reported in mares more than 18 years of age (Woods *et al.,* 1987; Vanderwall *et al.,* 1989; Carnevale and Ginther, 1992; Allen *et al.,* 2007).

The factors responsible for occurrence of embryonic loss in the mare have been classified as intrinsic, extrinsic and embryonic (Vanderwall, 2008). Intrinsic factors include endometrial disease, progesterone insufficiency, maternal age, lactation, foal-heat breeding, time of insemination relative to ovulation, site of intrauterine fixation of the embryonic vesicle and maternal chromosomal abnormalities. Embryonic factors can be chromosomal anomalies or other inherent characteristics of the embryo and usually are linked to intrinsic (e.g. maternal age) and/or extrinsic (e.g. oocyte handling/manipulation)

factors. Alterations in progesterone levels, uterine and oviductal environment, maternal age and postpartum breeding conditions have all been implicated causing early embryonic deaths, either directly or indirectly (Ball, 1988).

The factors that are involved in successful pregnancy maintenance in brood mares are an adequate progesterone level (Bergfelt *et al*., 1992), adequate supporting uterine environment and absence of non infectious or infectious causes (Young *et al*., 2007). Pre requisites of pregnancy maintenance in the mare include progesterone secreted by the CL (Bazer *et al*., 2009) and the transitory appearance of a mucin-like glycoprotein capsule around the blastocyst (Betteridge, 2007) which favors extensive migration of the tense spherical conceptus within the uterus. Progesterone secretion is dependent upon the CL up to approximately 80 days of gestation before the feto-placental unit begins to take over as a source of progesterone. In mares suffering from early embryonic death, reduced levels of progesterone have been observed (Volkmann *et al*., 2009; Ezzo *et al.,* 2012). Lower progesterone concentrations could be related to luteal insufficiency, endometritis or failure of maternal pregnancy recognition (Bergfelt *et al*., 1992). Slight inflammatory stimulation during endometritis may initiate production of prostaglandins (PG's) in the uterus which induces luteolysis and in turn ends pregnancy (Krakowski *et al.,* 2010). PG's are important as it causes luteolysis in the non-pregnant cyclic mare during the late luteal phase (Aurich, 2011) and its release can accelerate luteolysis in pregnant mare as well. Normally, in pregnant cows, the interferon tau (IFN-τ) produced from embryo induces secretion of prostaglandin-synthesis-inhibiting fatty acid in the endometrium to inhibit $PGF_{2\alpha}$ synthesis (Thatcher *et al.*, 1995). However, in pregnant mares, inhibition of $PGF_{2\alpha}$ release during the critical period of maternal recognition of pregnancy (MRP) is achieved by continuous migration of conceptus throughout the entire uterine lumen propelled by myometrial contractions and probably stimulated by conceptus prostanoid production (Gastal *et al*., 1998; Stout and Allen, 2001). Any restriction in conceptus mobility during this period causes release of $PGF_{2\alpha}$ that result in luteolysis and subsequent decline in progesterone leading to embryonic death in the mare (Ezzo *et al.,* 2012).

The conceptus is also considered an active partner in the successful establishment and maintenance of pregnancy (Roberts *et al.,* 1996). An adequate

sized embryo by day 16 of pregnancy is necessary in order to produce sufficient IFN-τ to prevent luteolysis and achieve successful MRP in cows (Mann and Lamming, 2001). IFN-τ production by the embryo depends greatly on an appropriate pattern of maternal progesterone secretion, especially during the first week after ovulation, when the early embryo is developing (Mann *et al.,* 2006). Some conceptuses may not be of sufficient size to adequately inhibit release of $PGF_{2\alpha}$ at this stage and thus luteolysis is not prevented (Thatcher *et al.,* 1994). During the period of CL regression, concentrations of $PGF_{2\alpha}$ and progesterone are inversely related. If release of $PGF_{2\alpha}$ is inhibited, the life span of the CL and thus sustained progesterone release can be prolonged. Successful establishment of pregnancy depends on a delicate balance between luteolytic mechanisms inherent to the endometrium at the end of diestrus and the antiluteolytic mechanisms, orchestrated by the conceptus to change endometrial function and ultimately to block luteolysis (Binelli *et al.,* 2009).

2.3 Antiluteolytic Strategies

Increased concentrations of luteolytic $PGF_{2\alpha}$ resulted in reduction in the number of functional corpora lutea and lower pregnancy rates in beef cows (Hess *et al*., 2008). Therefore, strategies to reduce endometrial synthesis and secretion of the $PGF_{2\alpha}$, during early pregnancy have been suggested to be beneficial for the successful establishment of the pregnancy (Mattos *et al.*, 2000). The various antiluteolytic strategies were proposed (Binelli *et al.*, 2001; Santos *et al.*, 2004) as; a) to stimulate growth of the pre-ovulatory follicle to generate a CL with greater progesterone producing capacity; b) to stimulate CL growth rate; c) to increase plasma progesterone concentrations in the initial three weeks after insemination; d) to decrease the effects of a dominant follicle during the critical period (i.e., days 15-19 after estrus); e) to increase the antiluteolytic stimulus provided by the conceptus and f) to decrease uterine luteolytic capacity. These antiluteolytic strategies can be catogorized as pharmacological, mechanical, nutritional and managemental manipulations.

Enhanced antiluteolytic mechanisms through diet have been reported (Thatcher and Staples, 2000) and the effect is likely to occur due to an alteration in lipid status of the endometrium. Studies in cattle (Thatcher *et al.*, 1994) and sheep (Wathes *et al.*, 2007) suggested role of fatty acids that can modulate PG synthesis. Recently, there has been increased interest in n-3 PUFA (EPA and DHA) because of their apparent ability

to reduce endometrial $PGF_{2\alpha}$ secretion both in vitro (Mattos *et al.,* 2003) and in vivo (Mattos *et al.*, 2002; Mattos *et al.*, 2004). Increasing dietary PUFAs, in particular, n-3 PUFA can possibly alter the synthesis of PG's of 2 series by: partial replacement of arachidonic acid (a precursor for $PGF_{2\alpha}$ synthesis) in phospholipids pool; decreases arachidonic acid biosynthesis by inhibition of delta-6 and delta-5 desaturase enzymes that are necessary for conversion of LA to ArA; by acting as a direct competitive inhibitor with arachidonic acid for PGHS (Prostaglandin H Synthase), and directly decreasing gene expression of PGHS (Thatcher and Staples, 2000). Hence, the selective alteration in nutrient availability to enhance the antiluteolytic effect during early pregnancy appear to be a right strategy that can be combined with various reproductive technologies to enhance embryo survival.

2.4 Fat, Fatty Acids and Essential Fatty Acids

Fat, derived either from animals or plants, is an important nutrient that provides energy, fatty acids and fat-soluble vitamins. In general, fat of animal origin are high in saturated fatty acids, whereas fat of plant origin are high in unsaturated fatty acids. Fatty acids are classified either as "unsaturated" or "saturated" based on the presence or absence of "double bonds" in their chemical structure. Saturated fatty acids have no double bonds, whereas unsaturated fatty acids have double bonds in their structure. Unsaturated fatty acids are further categorized as either "monounsaturated" (one double bond) or "polyunsaturated" (more than one double bond). Again unsaturated fatty acids are classified into different classes according to the number of carbon atoms and the number of double bonds present in their structure. LA, for example, has 18 carbon atoms and two double bonds; thus, it is conventionally written as C18:2. ALA, on the other hand, has 18 carbon atoms and three double bonds, and therefore is written as C18:3. PUFAs have more than one double bond present within the molecule and depending on the location of the first double bond relative to the methyl end of the fatty acid chain, PUFA are members of either Omega-3 (n-3) family (e.g. C18:3n-3) or the Omega-6 (n-6) family (e.g. C18:2n-6) (Ambrose and Kastelic, 2003).

Most of the fatty acids can be synthesized by animals with an exception of fatty acids belonging to the n-3 and n-6 families. Since, animals must have a dietary source of these fatty acids; they are considered essential fatty acids. The small intestine is the primary site for the absorption of dietary fat and long-chain fatty acids (Meyer *et al.*, 1997). When consumed by animals, LA is desaturated (insertion of double bonds) and

elongated (addition of carbon atoms) to form arachidonic acid (ArA; C20:4n-6). In contrast, ALA, is desaturated and elongated to form two fatty acids that are unique to fish oils: EPA (C20:5n-3) and DHA (C22:n-6). Unsaturated fatty acids are largely metabolized by rumen bacteria in a process known as biohydrogenation. This is particularly important since ruminants hydrogenate PUFAs in the rumen, limiting the amount of PUFAs that are absorbed from the small intestine (Thatcher and Staples, 2007; Santos *et al*., 2008; Doreau *et al*., 2011). During biohydrogenation, unsaturated fatty acids are converted to saturated fatty acids. However, it is possible that some specific PUFAs may pass intact through reticulo-rumen and be absorbed from the small intestine. Thus, it allows an improvement of reproductive efficiency directly on the target tissue of the reproductive system of the female (autocrine or paracrine) or by an indirect effect mediated by the endocrine system (Staples and Thatcher, 2005). Two unique fatty acids, EPA and DHA (high in fish oils), are known to largely escape ruminal biohydrogenation (Ashes *et al*., 1992; Thatcher and Staples, 2007). Furthermore, the lipid supplements partially resistant to biohydrogenation in the rumen have been developed such as calcium salts of long chain fatty acids (Ca-LCFA) with the aim of increasing the amount of unsaturated FA which can be absorbed by limiting biohydrogenation (Mattos *et al*., 2000).

2.4.1 Source of essential fatty acids

The main dietary n-6 PUFA is LA which is abundant in vegetable oils such as corn, safflower, sunflower and rapeseed oils (Gurr *et al*., 2002). Most n-3 PUFAs are derived from ALA found mainly in the chloroplasts of green vegetables and in forages

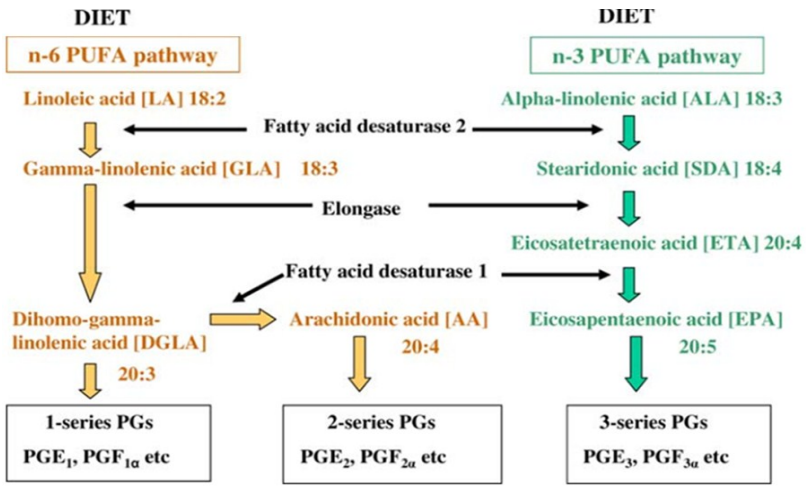


Fig. 1 : Schematic illustration for generation of 1-, 2-, and 3-series prostaglandins (PGs) from dietary polyunsaturated fatty acids (adopted from Wathes *et al.*, 2007)

(e.g. grass, legume leaves) and in some oilseeds (e.g. flaxseed, linseed). Some oils are high in omega-6, but lower in omega-3 e.g. corn oil, sunflower oil, and safflower oil, while others such as soybean and canola oils are moderately high in omega-6 FA's. The oils that contain more omega-3 than omega-6 are flaxseed and linseed oil, but the highest ratio of n-3 to n-6 FA's is found in fish oil. Both essential fatty acids LA and ALA can be converted in the liver to longer chain PUFAs (ArA, EPA, DHA) by desaturation and elongation enzyme systems common to both pathways. Fatty acid desaturase 2 (FADS2) is rate limiting (Gurr *et al*., 2002), so higher tissue concentrations of long chain PUFAs can be achieved by bypassing this step.

2.4.2 Ratio of n-3 and n-6 PUFA

It is thought that both man and livestock species evolved on a diet with an n-6 to n-3 PUFA ratio of 1:1 (Pike and Barlow, 2000; Allen and Harris, 2001) but modern dietary trends have increased this ratio (Simopoulos, 2001). In Westernized societies, average consumption of n-6 polyunsaturated fatty acids (PUFAs) far exceeded the nutritional requirements for normal physiological functioning, predominantly as LA (Fischer, 1989). Diets fed to intensively farmed livestock have followed a similar trend (Wathes *et al*., 2007). Modern intensively farmed livestock have little, if any, access to fresh pasture. PUFA content in preserved forage are low, and animal diets are often supplemented with fat derived from oilseeds rich in LA (Pike and Barlow, 2000). Therefore, in both human and animal diets there are grounds for reducing the n-6 intake and increasing the n-3 intake to promote better health (Simopoulos, 1991; Pike and Barlow, 2000; Simopoulos, 2001). Both n-3 and n-6 PUFA provide the precursors for PG synthesis and can modulate the expression patterns of many key enzymes involved in both PG and steroid metabolism. They are essential components of all cell membranes. PUFAs act as mediators in a series of processes in several reproductive tissues, including fluidity of cell membrane, intracellular signaling and susceptibility to oxidative damage (Wathes *et al*., 2007). Changes in chain length, degree of unsaturation and position of the double bonds may have a major impact on reproductive function and play a role in livestock reproduction (Mattos *et al*., 2000).

2.4.3 Essential fatty acids and health benefits

The essential fatty acids have important functions (Sinclair, 1984); the most important is structural, contributing to the maintenance and function of cellular membranes. In addition, essential fatty acids are important in the metabolism of cholesterol and the synthesis of eicosanoids (bioactive compounds such as PG's, leukotrienes and thromboxanes, all derived from 20-carbon fatty acids). In humans, the inclusion of vegetable oils or fish oil in the diet has special interest because of the benefits regarding glucose homeostasis and insulin sensitivity (Vessby, 2000). It has been suggested that high glycaemic responses after carbohydrate intake represent a risk factor for metabolic diseases such as diabetes type II, even in healthy individuals (Ceriello *et al*., 2004). The mixing of fats, such as sunflower margarine, olive oil, soybean oil (SBO) or fish oil with a starchy meal has been shown to lower glucose and insulin responses in healthy and diabetic individuals (Cunningham and Read, 1989; MacIntosh *et al*., 2003; Gentilcore *et al*., 2006). Omega-3 fatty acid supplementation has been shown to improve insulin sensitivity in several species (Behme, 1996; Andersen *et al.,* 2008; Huag *et al.,* 2010). Fish oil supplementation is thought to reduce activity of cholesterol ester transfer protein responsible for transfer of cholesterol esters from high-density lipoproteins (HDL) to low-density lipoproteins (LDL) and is associated with reduced risk of athero-sclerosis in humans (Nestel, 2000). These effects include reducing the occurrence of lipid disorders and coronary artery disease and increasing red blood cell deformability, insulin sensitivity, and vascular compliance (Mueller and Talbert, 1988; Simopoulos, 1991). In human, application of n-3 FA's in the treatment and prevention of mental illness (Horrobin *et al.,* 1991; Stillwell and Wassall, 2003; McNamara, 2006; Clayton *et al.,* 2007; Mazza *et al.,* 2007; Song and Zhao, 2007) and prevention of several types of cancer (MacLean *et al.,* 2006; Chen *et al.,* 2007; Calviello *et al.,* 2007) has also been widely reviewed.

Studies in horses have shown positive effects of n-3 PUFA on production of inflammation mediators (McCann *et al.,* 2000; Hall *et al.,* 2004), immune function (Vineyard *et al.,* 2010), heart rate during exercise (O'Connor *et al.,* 2004), serum triglycerides (O'Connor *et al.,* 2007), and stride length (Woodward *et al.,* 2005). In horse, fish oil supplementation increased both leukotriens B4 and B5 production over that observed after feeding corn oil (Hall *et al.,* 2004). Flaxseed oil supplementation decreased endotoxin induced tumor necrosis factor (TNF-α) production (Morris *et al.,* 1991) and also decreased thromboxane B2 production as compared to unsupplemented horses (Henry *et al.,* 1990). Osteoarthritis (OA) is one of the most

significant causes of lameness in horses, leading to early retirement of equine athletes (Todhunter and Lust, 1990). Supplementation with n-3 PUFAs has been shown to mitigate inflammation in horses previously diagnosed with OA (Manhart *et al.,* 2007). Addition of vegetable oil to horse diets was associated with increased circulating cholesterol concentrations (Siciliano and Wood, 1993; Orme *et al.,* 1997) during the supplementation period but not in horses receiving the fish oil. Compared to corn oil, horses receiving fish oil had also been reported to lower serum triglycerides at day 63.

It has been suggested that n-3 PUFA alters the circulating profile of cytokine molecules (Simopoulos, 2002). Cytokines are immune cell proteins which are secreted in response to stimuli such as pathogen and stress, and assist in regulating the development of an immune or inflammatory response (Tizard, 2009). They can act on themselves (autocrine) or locally (paracrine) to elicit responses from other immune cells, and are produced by monocytes and macrophages (Tizard, 2009). n-3 PUFA can act upon intracellular signaling pathways, transcription factor activity and/or gene expression to modulate cytokine function (Simopoulos, 2002). The interactions between immune and inflammatory cells are mediated by cytokines and an appropriate amount of cytokines in response to immunologic stimuli is required for animals to remain healthy and fight off infections. TNF-α, interleukin (IL)-1, IL-6 and IL-8 are some of the primary pro-inflammatory cytokines found in the body (Webel *et al*., 1997). The n-3 PUFA are considered to be anti-inflammatory and that its dietary inclusion can lead to altered production of some of the pro-inflammatory cytokines (Wallace *et al*., 2001; Dunstan *et al*., 2004; Cotogni *et al*., 2011).

2.5 Feeding Fat to Horses

There is an increasing interest in the addition of fat to the equine diet (Harris *et al*., 1999), especially with performance horses. One of the most common reasons for supplementing fat is to increase the energy content of the ration (Meyer and Coenen, 2002). Other benefits include reduced risk of carbohydrate overload in the hindgut, reduced reactivity in horses sensitive to starch, a reduction in breathing effort due to less carbon dioxide production (Ferrante *et al*., 1993), higher resting muscle glycogen stores (Hambleton *et al*., 1980), glucose sparing effect during exercise (Treiber *et al*., 2008) and decreased heat production during exercise (Kronfeld *et al*., 1994). However, fat is not a uniform material, and the composition of fatty acids and their biological

activity can vary considerably. Most horses readily accept fat-supplemented grain mixes containing fat up to 10%. High quality vegetable oils are accepted by horses more quickly than lower-quality oils or animal fats, but mostly horses consume diets containing raw, rendered animal fat provided it is of good quality. For best consumption, fat is to be incorporated directly into horse feeds, but fat supplements can be mixed into the feed in feeding trough. When feeds contain very large amounts of fat (greater than 10% by weight), it takes longer time for horses to readily accept the diet. For best acceptability, fat can be introduced into horses' diets in small amounts, and then gradually increased over at least a week, until the desired concentration of fat has been reached (Gary, 1999).

2.6 Role of PUFA in Reproduction

PUFAs act as mediators in a series of processes in several reproductive tissues, including fluidity of cell membrane, intracellular signaling (Wathes *et al*., 2007) and play an important role in processes such as ovulation, fertilization and parturition (Abayasekara and Wathes, 1999). In cattle, dietary fatty acids were reported to influence ovarian follicular growth, CL function and progesterone production (Abayasekara and Wathes, 1999; Mattos *et al*., 2000). The cows fed diets high in EPA and DHA (supplemented fish meal) or ALA (supplemented with flaxseed) during early pregnancy have reduced $PGF_{2\alpha}$ production and increased pregnancy rates. Increased concentration of total cholesterol was associated with n-3 PUFA supplementation, suggesting a possible effect on cholesterol synthesis. Furthermore, as most of the cholesterol found in bovine blood is in the form of HDL (Staples *et al.,* 1998), increase in cholesterol recorded is likely to be at the expense of LDL. As circulating cholesterol is the primary substrate for the synthesis of progesterone (Williams and Stanko, 2000), the increase in cholesterol could be expected to have a positive effect on the concentration of progesterone (Grummer and Carrol, 1991; Hawkins *et al.,* 1995).

2.6.1 Ovarian functions: follicle and CL growth

Dietary supplementation, particularly long chain PUFAs (both n-3 and n-6) induced changes in several aspects of folliculogenesis, including both an increase in total follicular number and in the size of the dominant or pre-ovulatory follicle in cattle (Lucy *et al*., 1993; Beam and Butler, 1997; Bilby *et al*., 2006a; 2006b). The

number and size of ovulatory follicles is important to determine future potential success of ovulation rate and oocyte viability (Ambrose *et al*., 2006). The increase in size of follicles have been reported both in beef (Lammoglia *et al*., 1996; 1997) and dairy cows (Robinson *et al*., 2002) given diets supplemented with fat. The number of medium size follicles (5 to 10 mm) have been reported higher in beef cows which consumed feed with a greater content of PUFAs (Thomas *et al*., 1997) and in dairy cows which consumed a diet enriched with 5% n-3 PUFA derived from fish oil (Heravi-Moussavi *et al*., 2007). Similar results have been observed in cows fed with diets enriched with n-3 or n-6 FA's (Robinson *et al*., 2002). Staples and Thatcher (2005) observed the effect of increasing lipids in the ration on the size of the dominant follicle and found that the average size of dominant follicle was 3.2 mm greater than in females fed with some source of fat, which represented a 23% increase. In sheep, the use of Ca-LCFA from palm oil (El-Shahat and Abo-El maaty, 2010) in the feed improved both the number and size of the preovulatory follicles, as well as rate of ovulation besides the superovulatory response in Pelibuey sheep. The mechanism by which dietary fats stimulate ovarian activity is yet to be determined.

The size of the pre-ovulatory follicle is found to be positively related to improved pregnancy rate in cattle (Lopes *et al*., 2007). Vasconcelos *et al*. (2001) also reported that smaller follicles (11.5 ± 0.2 mm) resulted in development of smaller CL that secreted less progesterone when induced to ovulate as compared to larger CL from larger follicles (14.47 ± 0.39 mm) in dairy cows. A more number of lactating dairy cows ovulated when fed with diet supplemented with Ca-LCFA and had greater CL diameter than the control cows (Garcia-Bojalil, 1998a, b). Similarly, ovine follicles induced to ovulate 12 h after luteal regression had fewer granulosa cells and formed smaller CL that secreted less progesterone than follicles induced to ovulate 36 h after luteal regression (Murdoch and Van Kirk, 1998). It has also been demonstrated that POF diameter may play an important role in reproductive performance, through an influence on oocyte viability and subsequent CL function (Watson and Alziabi, 2002; Perry *et al*., 2005). Lucas *et al.* (2003) demonstrated an association between increasing follicle size and an increase in the percentage of oocytes successfully penetrated during *in vitro* fertilization (IVF) in pigs. This has similarly been shown in IVF in women (Teissier *et al*., 2000). The mean diameter of the ovulatory follicle (Ambrose *et al*., 2006; Mendoza *et al*., 2011)

and CL (Petit *et al*., 2002) reported higher when dairy cows fed diets high in n-3, while the follicle size reduced when cows fed diets high in n-6 FA (Homa and Brown, 1992). However, diets high in n- 6 FA's may also improve follicle development, as the number of medium sized follicles have been increased when beef cows supplemented with n-6 FA (from soybean) compared with long-chain n-3 FA's from fish oil or saturated fat (Thomas *et al*., 1997). However, other reports have recorded an increase in CL diameter on day 7 of the estrous cycle in response to increasing n-3 PUFA intake (Petit *et al*., 2002; Childs *et al*., 2008c).

2.6.2 Embryo development and its survival

n-3 PUFA is required for normal conception, growth, and development of an embryo (Simopoulos, 2002). A positive effect of n-3 PUFA on embryo survival has been observed due to reduced $PGF_{2\alpha}$ secretion both *in vitro* (Caldari-Torres *et al*., 2006; Mattos *et al*., 2003) and *in vivo* studies (Cerri *et al.,* 2004; Petit and Twagiramungu, 2006; Thangavelu *et al*., 2007; Childs *et al*., 2008). In human pregnancy, during the third trimester, approximately 50 to 60 mg/day of maternal DHA is reported to be transferred from body reserve to a fetus via the placenta (Clandinin *et al.,* 1980). Hammiche *et al.* (2011) demonstrated significant associations between n-3 PUFA and embryo morphology in women; and recommended twice a week dietary intake of fish for women during their reproductive years and in particular in those undergoing IVF/ICSI treatment. A diet enriched with calcium salts of LA and trans-fatty acids also enhanced embryonic development in cows with more number of total blastomeres, and better quality embryos than from cows fed calcium salts of palm oil, rich in saturated fatty acids (Cerri *et al.,* 2004). Petit and Twagiramungu (2006) have observed reduced embryo mortality in cows fed n-3 PUFA. Enhanced embryonic development has been reported in Holstein cows fed unsaturated fatty acids compared to those fed saturated fatty acids (Thangavelu *et al*., 2007). Childs *et al*. (2008b) fed cows with ruminally protected LC n-3 PUFA from fish oil that resulted in no alteration in embryo recovery rate but reduction in the number of degenerated embryos. Embryonic mortality was less for dairy cattle fed whole flaxseed (Petit and Berthiaume, 2007). Furthermore, a study with Murrah buffaloes revealed that embryos with greater development have better survived in buffalo supplemented with flaxseeds (Nazir *et al.,* 2013). They observed embryonic mortality only in two buffalo (13%; 2/15) of

supplemented group before day 42 in comparison to seven buffalo (43.7%; 7/16) in the non-supplemented group. Due to the less number of studies that have been conducted, the direct effects of n-3 on embryo development and its survival remains largely unknown and needs further investigation.

2.6.3 Conception rate

Improved conception rates have been observed in fat-fed cows along with improvement in body weight (Schneider et al., 1988; Bruckental et al., 1989). Several reviews (Grummer and Carroll, 1991; Staples *et al.,* 1998; Wathes *et al.,* 2007) have cited studies (Armstrong *et al.,* 1990; Lucy *et al.,* 1993; Carroll *et al.,* 1994; Mattos *et al.,* 2002; 2004; Bilby *et al.,* 2006b) which report putative positive effects of fat supplementation on fertility. Dairy cows fed tallow at 3% of dietary DM, had a greater pregnancy rate compared to unfed animals (Son *et al*., 1996). Lipid supplementation has been reported to improve pregnancy rates in cows (Funston, 2004); however, the specific effects of n-3 and n-6 FA on pregnancy rates in ruminants are reportedly variable.

Armstrong *et al*. (1990) observed higher conception in British Friesian cows fed fishmeal (0.8 kg/day) as compared to control (64 vs. 44%), along with a reduction in the number of services per conception (1.62 vs. 2.31). Similarly, Burke *et al*. (1997) fed menhaden fish meal (MFM) and observed a higher pregnancy rate in cow (41.3 vs 31.9%). Again, MFM fed cow had reported higher plasma progesterone concentrations (>1 ng/mL) 48 h after prostaglandin (PG) injection than cows fed a control ration. Inclusion of fish meal in diets of lactating dairy cows has been shown to improve conception rates (Staples *et al*., 1998). The conception rates and pregnancy losses have been reported higher and lower, respectively in dairy cows offered with diets high in n-3 compared with n-6 or saturated fat following artificial insemination (Ambrose *et al*., 2006; Wathes *et al*., 2007; Santos *et al*., 2008). Supplementation of diet with whole flaxseed or fish products (for 40–60 days) was found to suppress release of $PGF_{2\alpha}$, improve luteal profile and increase conception rate (Wamsley *et al*., 2005; Heravi *et al*., 2007) in dairy cattle. Fish meal supplementation for 30 days in Murrah buffaloes decreased plasma 13, 14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) and thus was reported beneficial for conception rate (Malik *et al*., 2011a, b). Similarly, the first service

conception rate on day 63 post-AI was reported higher in flaxseed supplemented in comparison to non supplemented (66.7% vs. 31.2%) buffalo (Nazir *et al.,* 2013). They also observed greater post-AI luteal phase plasma progesterone in supplemented than non supplemented group. On the other hand, pregnancy rates have been recorded lower when beef cows were offered diets high in the n-6 LA. Lower pregnancy rates can result from increased concentrations of luteolytic $PGF_{2\alpha}$ due to high n-6 diets (Hess *et al.*, 2008). Though, the mechanism(s) through which fat supplementation may affect fertility remain unknown (Wathes *et al.*, 2007), repression of $PGF_{2\alpha}$ synthesis and/or release by n-3 FA's acids may account for the improved conception rates (Staples *et al.,* 1998).

2.6.4 Mechanisms by which fats may improve fertility

Several hypotheses have been proposed by Staples *et al.* (1998) regarding the mechanism(s) by which fat supplementation improves reproductive performance including a) an amelioration of a negative energy balance (NEB) leading to an earlier return to estrus in postpartum period and thus improved fertility; b) an increase in progesterone production and secretion, and c) inhibition of $PGF_{2\alpha}$ production and/or release which influences the life span of the CL. However, later it was suggested that the effects of dietary fat may be independent of the contribution to dietary energy density (Funston, 2004) and that specific constituent fatty acids may stimulate ovarian function (Lucy *et al.,* 1992), enhance progesterone concentrations beside embryo survival. Improvement in fertility apart from improved energy status through fat supplementation have been suggested (Thatcher and Staples, 2000) and that it's positive effect is mediated through increased progesterone concentrations in plasma and thus may enhance embryo survival. Further, Petit *et al.* (2002) suggested that fatty acids may influence fertility by acting as precursors of the PG's and/or may affect steroidogenesis through increased cholesterol availability.

The circulating free FA's and those hydrolyzed from triacylglycerides are source of energy via β-oxidation (Voet *et al.*, 2008). With each step of β-oxidation pathway, a 2 carbon acetyl-CoA is generated which then enters into the tricarboxylic acid cycle.

During this cycle, each 2 carbon acetyl-CoA molecule generates reduced pyridine nucleotides which then pass into the mitochondrial electron transport chain for generation of ATP (Voet *et al*., 2008). As with any FA, n-6 and n-3 FA's can be oxidized and used as a source of energy. Additionally, there is evidence that dietary n-3 FA's affect glucose absorption (Aas *et al*., 2006; Gabler *et al*., 2007; 2009) and improve insulin-mediated glucose metabolism in animals (Borkman *et al*., 1993; Gingras *et al*., 2007), and thus energy utilization. Clarke (2001) revealed the role of n-3 FA's as 'fuel partitioners', since they can act on genes which promote FA oxidation and down regulate those involved in lipogenesis.

Progesterone prepares the uterus for implantation of the embryo and also helps to maintain pregnancy by providing nourishment to the conceptus. Increased concentrations of plasma progesterone have been associated with improved conception rates of lactating ruminants (Staples *et al.,* 1998). The main precursor for the synthesis of progesterone is cholesterol. The circulating concentration of cholesterol is increased consistently by fat supplementation (Grummer and Carroll, 1991). Hawkins *et al*. (1995) suggested that increase in plasma progesterone in cows supplemented with fat in diets may not be due to increased synthesis but rather reduced clearance of progesterone from circulation.

Cis-linoleic acid (C18:2), an n-6 fatty acid commonly found in natural fat sources can serve as a substrate for the synthesis of $PGF_{2\alpha}$ by desaturation and elongation to form ArA which serves as an immediate precursor for $PGF_{2\alpha}$. Key regulatory enzymes for these conversions include Δ6-desaturase and cyclooxygenase. The n-3 FA's inhibit PG synthesis by competitive inhibition with these key enzymes. Competition between n-3 and n-6 FA's for the binding site on prostaglandin synthetase means that increasing the concentration of n-3 FA will decrease production of dienoic PG's (Barnouin and Chassagne, 1991). The amount of particular fatty acids reaching the target tissues is likely to influence whether PG's synthesis is stimulated or inhibited. ALA has been shown to be an inhibitor of PG synthesis by the endometrium (Danet-Desnoyers, 1993; Thatcher *et al.*, 1994). The incorporation of the n-3 PUFA; EPA and DHA into uterine endometrial cells can influence the synthesis and secretion of PG through displacement of arachidonic

acid, the precursor of the luteolytic $PGF_{2\alpha}$, and/or competing for the enzymes necessary for $PGF_{2\alpha}$ biosynthesis (Mattos et *al.,* 2003). Because these fatty acids are also known to decrease $PGF_{2\alpha}$ secretion (Sinclair, 1984), feeding fishmeal to cattle has been reported to enhance fertility (Thatcher *et al*., 1997).

2.6.5 PUFA and steroidogenesis

Endocrine imbalance, particularly of progesterone and estradiol, is considered as major reason underlying early embryonic mortality (Stevenson *et al.*, 1990; Morgan and Lean, 1993; Diskin and Morris, 2008). At the time of estrus, the POF is known for increased circulating concentrations of estradiol. Again, a positive correlation has been reported between POF diameter and plasma estradiol concentration in dairy cattle (Lopes *et al.*, 2007; Lynch *et al.*, 2010). A positive correlation was observed between diameter of POF and size of CL as well as plasma progesterone concentration on day 5 post-ovulation in both cattle (Lopes *et al.*, 2007; Pfeifer *et al.*, 2009) and buffalo (Pandey *et al.,* 2011). It is often predicted that a large CL releases more progesterone in the circulation (Binelli *et al.*, 2009), but the results are not always consistent (Childs *et al.*, 2008). Additionally, the dietary fat has been reported to increase the level of cholesterol and progesterone synthesis (Staples *et al.*, 1998); or reduce the rate of its clearance from the blood (Hawkins *et al.,* 1995) and thus increased lifespan of induced corpora lutea in cattle (Williams and Stanko, 2000). Both n-3 and n-6 PUFA may affect a number of factors associated with the synthesis and metabolism of important reproductive hormones such as progesterone and estradiol. Diets high in n-3 PUFA were found to be associated with lower plasma cholesterol concentrations (Robinson *et al.*, 2002), which may lead to reduced steroid hormone synthesis, as cholesterol is a precursor for both progesterone and estradiol (Staples *et al.*, 1998). However, inhibition of $PGF_{2\alpha}$ by high n-3 PUFA feeding may prevent regression of the corpus luteum (CL) and resulting in sustained progesterone release (McCracken *et al.*, 1972). Increased plasma concentrations of EPA have also been associated with reduced progesterone clearance (Galbreath *et al.*, 2008). Conversely, diets high in n-6 FA found associated with higher cholesterol (Robinson *et al.*, 2002), steroidogenic acute regulatory (StAR) protein (Wang

et al., 1999) and PGE_2 (Marsh, 1970), which may stimulate progesterone production (Wathes *et al.*, 2007). Although progesterone production appears to be lower with n-3 and higher with n-6 FA's, the exact mechanism through which n-3 and n-6 FA's modulate progesterone and estradiol is unclear. While n-3 and n-6 FA's may alter the availability of cholesterol as a substrate for steroid synthesis, the most significant effects of n-3 and n-6 FA's are likely to be mediated through their effects on the synthesis of series-2 and series-3 PG's and the subsequent effects of PG on progesterone and estradiol. The interaction of n-3 FA with steroid hormone production and metabolism may be further complicated by negative feedback, as estradiol may reduce the activity of Δ6-desaturase (Gonzalez *et al.*, 1986), thereby reducing the availability of LC n-3 PUFA substrates for synthesis of series-3 eicosanoids. More studies have been suggested to evaluate PUFA supplementation on luteal cell stimulation of progesterone.

2.7 Semen Cryopreservation in Horses

Birth of foal using frozen-thawed semen was first reported in 1957 (Barker and Gandier, 1957). Since then, several scientific advancements have made cryopreservation of equine semen an international success. Despite considerable challenges, more and more number of mares are now being bred with cryopreserved semen worldwide. Recent increase in interest for using AI in equines is due to acceptance of foals born through AI by the major horse breed registration societies, realization of advantages of semen cryopreservation and AI (Aurich and Aurich, 2006). AI in horses allows dissemination of superior germplasm at a faster rate and increased number of mares inseminated with semen from an elite stallion especially when he is heavily booked during breeding season, busy in competition, sick or deceased (Loomis *et al.*, 2008). Influenced by economic benefits, higher quality foals are now being produced from high valued quality stallions. Stallion owners also utilize frozen semen to market their stallions over wider range of mare owners while offering more flexibility to breeders with regard to mares' global location and ovulation schedule.

On the other hand, during semen cryopreservation the quality of the semen is severely compromised. Many stallions produce semen that is unable to provide acceptable

motility after undergoing freezing-thawing process (Brinsko, 2000). Lower fertility has been reported in mares inseminated with frozen semen (Samper, 2000; 2001). Cryopreserved semen of good quality is imperative in order to achieve an acceptable non-return and/or conception rates (Hoflack *et al*., 2006). Further, the use of equine frozen semen is limited in part by the variability between stallions and between ejaculates, ability of the spermatozoa to tolerate the freezing-thawing process and thus subsequent variable reduction in fertility (Amann and Pickett, 1987; Pal *et al.,* 2011). Vidament (2005) reported that 30% of stallions had semen with a freezability rating "good," 40% of stallions had semen that freezes "satisfactorily," and 30% of stallions had semen that freezes "very poorly". This variability of spermatozoa from individual stallions to withstand freezing remained a puzzle.

2.8 Cryodamage to Sperm Cells

Cryopreservation is reported to cause serious damage to the spermatozoa (Hammerstedt *et al.,* 1990). During cryopreservation cells are exposed to low temperatures and therefore are vulnerable to chilling injury (Watson and Morris, 1987). In many studies (Quinn and White, 1966; Morris, 1987; Arav *et al.,* 1996), it has been suggested that chilling injury, is the major limitation for successful cryopreservation of gametes. During cryopreservation up to 50% of sperm viability is lost (Watson, 1995) and this loss is attributed mainly to the cryo-injuries (Mazur, 1980). Sperm cryopreservation induces the formation of intracellular ice crystals, osmotic and chilling injury that causes sperm cell damage, cytoplasm fracture, and could even effect the cytoskeleton or genome structures (Parks and Graham, 1992; Isachenko, 2003). These events are accompanied by a loss of motility, viability, and fertilizing capacity of sperm, a phenomenon commonly referred to as "cold shock" (Watson, 2000).

The cell membranes have been revealed as primary site for structural and functional chilling injury in sperm (De Leeuw *et al.,* 1991, Drobnis *et al.,* 1993). The damage to plasma membrane is of varying magnitude among species, during freezing and thawing (Tuli *et al.*, 1981; Holt *et al.*, 1992).One possible cause of chilling injury is the membrane damage that is associated with the thermotropic lipid phase transition (Drobnis *et al.,* 1993). De Leeuw *et al*. (1991) examined the ultrastructural changes in different sperm

plasma membranes and have shown that at low temperatures the intra-membranous components get aggregated and redistributed in the head and principal piece of the sperm. This led to phenomenon known as lateral phase separation. Drobnis *et al*. (1993) emphasized this hypothesis with results showing that spermatozoa chilling injury is associated with the membrane lipid phase transition and suggested lateral phase separation to be the main cause of damage to the membrane. Neild *et al*. (2003) have used various fluorochromes to evaluate membrane damage during the freeze-thaw process for equine sperm. The most pronounced damage to sperm occurrs after thawing. Differences in the ability of sperm from various animals to resist cold shock appear to be related with their sperm membrane lipid composition (Parks and Lynch, 1992). The lipid composition of sperm membranes not only influences the response of sperm to cooling and freezing, but also plays a major role in the physiologic changes leading to fertilization (Langlais and Roberts, 1985; Ladha, 1998).

2.9 Role of PUFA in Semen Quality

Phospholipid FA's are major structural component of biological membranes and known to affect both biophysical properties and functions of the cell (Pratt, 1980; Stubbs and Smith, 1984). Additionally, chilling injury is dependent on the biochemical, and biophysical properties of sperm membranes (Stubbs and Smith, 1984; Watson and Morris, 1987). In mammalian spermatozoa, n-3 PUFA, in particular DHA, is predominant (Jain *et al*., 1976; Poulos *et al*., 1986; Kelso *et al*., 1997; Zalata *et al.*, 1998; Conquer *et al.*, 1999; Robinson *et al*., 2006). However, the phospholipid bilayer has a distinctive PUFA in its composition that varies somewhat from species to species. Spermatozoal plasma membranes in most mammals have a higher proportion of DHA than DPA, but the spermatozoa from stallions and boars have a higher proportion of DPA (Parks and Lynch, 1992). The species differences in the susceptibility of spermatozoa to cooling, freezing, and thawing process seems to be largely attributable to the PUFA contents of sperm plasma-membrane (White, 1993). Bulls and roosters produce sperm that are very resistant to cold shock and freeze well, whereas sperm from boars and stallions have very low tolerance to cold shock and in general, freeze

poorly. Major differences in the lipid content of bull sperm compared to those of boars and stallions are the relative amounts of DHA and DPA.

Since animals are unable to synthesize PUFAs, in particular DHA and DPA from saturated or monounsaturated fatty acids, they must acquire them from precursor PUFAs in their diet. Fatty acids in the diets are known to affect fatty acid composition of sperm in human (Conquer *et al*., 2000) and in variety of farm animals (Kelso *et al*., 1997; Zaniboni *et al*., 2006; Paulenz *et al*., 1999; Rooke *et al*., 2001; Brinsko *et al*., 2005; Harris *et al*., 2005). These fatty acids are important for sperm membrane integrity, sperm motility and viability, as well as cold sensitivity (Robinson *et al*., 2006). Fatty acids in diets have been demonstrated to be effective in altering the lipid composition as well as the structure and function of the sperm plasma membrane of mammals (Penny *et al*., 2000a; Conquer *et al*., 2000; Drokin *et al*., 1999; Brenner, 1984) and fowl (Blesbois *et al*., 1997). Spermatozoa use lipid metabolic pathways for the production of a part of their energy (Lenzi *et al*., 1996). Unfortunately, most proprietary horse feeds are very high in precursors for n-6 FA's while the precursors for n-3 FA's, such as DHA, are very low. Since high n-6 to n-3 FA ratios in semen have been associated with reduced sperm quality and fertility (Penny *et al*., 2000b; Maldjian *et al*., 2003), typical equine diets low in DHA but high in DPA could have a negative impact on quality of some stallion's semen and its tolerance to cooling and freezing. Further, the proportions of PUFA have been reported to decrease significantly after freezing and thawing (Cereloni *et al*., 2006). Supplementing the stallion's diet with precursors of n-3 FA's, e.g., cod liver oil, fish oil or flaxseed oil, can increase the overall level of n-3 FA in semen.

Fish oil supplemented diets modified the fatty acid profile of blood plasma and sperm, in particular increased the proportion of DHA (Castellano *et al*., 2010). The transfer of dietary PUFAs to sperm has been shown to be effective in a number of species (Penny *et al*., 2000b; Conquer *et al*., 2000; Blesbois *et al*., 1997; Drokin *et al*., 1999). Brinsko *et al*. (2007) suggested that ingestion of n-3 FA's may alter the cholesterol: phospholipids ratio of the sperm plasma membrane. Manipulation of this ratio may render greater fluidity of the membrane and more resilience to oxidative stress. *In vitro* studies supported this hypothesis; as loading spermatozoa with cholesterol

dextrin appeared to increase motility and viability of both stallion (Moore *et al.*, 2005) and jack cryopreserved semen (Alvarez *et al.*, 2006). Purdy and Graham (2004) studied the addition of cholesterol-loaded cyclodextrins to egg yolk diluents and observed higher percentages of motile and viable sperm after thawing. Brinsko *et al.* (2005) supplemented the diet of stallions with commercially available nutraceutical for boars containing DHA and observed no change in sperm motion characteristics in fresh semen but cooled semen stored for 48 h showed improved motility parameters. Moreover, improvements in semen quality after 24 h cooled-storage have been recorded in stallions with poor semen quality (Elhordoy *et al.*, 2008). Dietary supplementation of fish oil rich in n-3 FA's, EPA and DHA, appears to have beneficial effects in horses, including improvement in semen quality (Huang *et al.*, 2010). Manipulation of dietary PUFAs has also been shown to change the PUFA components of spermatozoa in fish (Labbe *et al.*, 1995; Pustowska *et al.*, 2000), boar (Penny *et al.*, 2000b) and chicken (Blesbois *et al.*, 1997; Kelso *et al.*, 1997) with and without consequences on reproductive capacity. There are evidences that there is a positive correlation between DHA and the proportion of motile spermatozoa in human (Conquer *et al.*, 1999, Conquer *et al.*, 2000), boar (Rooke *et al.*, 2001), goat (Dolatpanah *et al.*, 2008), sheep (Towhidi *et al.*, 2008) and chicken (Cerolini *et al.*, 1997) semen.

Studies with boars (Penny *et al*., 2000b; Rooke *et al*., 2001; Maldijian *et al*., 2003) and humans (Conquer *et al*., 1999; Nissen and Kreysel, 1983) revealed that a high DHA to DPA ratio in semen resulted in enhanced fertility, while higher DPA levels resulted in reduced fertility. In asthenozoospermic men, the level of DHA in seminal plasma and the ratio of n-3 to n-6 FA's in sperm found to be lower than in normozoospermic men (Conquer *et al*., 1999). Feeding a nutriceutical compound rich in n-3 FA's have increased sperm production, motility characteristics, morphology and fertility of some stallions (Brinsko *et al.*, 2005; Harris *et al.*, 2005). In stallions, feeding DHA had a positive influence on motility of cool-stored and frozen-thawed semen (Harris *et al*., 2005; Brinsko *et al*., 2005). In bulls, significant reductions with age in their sperm concentrations of the PUFA mainly DHA have been reported (Kelso *et al*., 1997). This may be of interest in such case to use dietary fish oil supplements. Dietary supplementation of PUFA improves semen quality in a variety of species, but studies on

this aspect are less in the horse. Enhancement of the reproductive efficiency of breeding stallion can be achieved by improving the quality of frozen semen by making the sperm plasma membrane more robust so that it can withstand cryopreservation stress.

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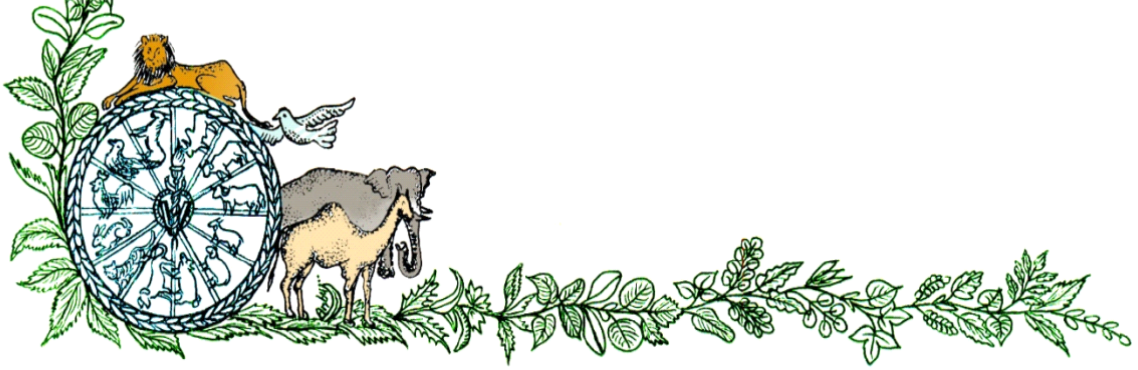
Materials and Methods



3

MATERIALS AND METHODS

3.1 Location of the Experiments

The present study was conducted in Marwari mares and stallions maintained at National Research Centre on Equines, Equine Production Campus, Bikaner (Rajasthan) to investigate the effect of dietary n-3 PUFA on ovarian function, embryonic development and semen quality. Bikaner is situated in middle of the Thar desert at an altitude of 238 m above the mean sea level between latitude of $27^0$11' to $29^0$3' north and longitude of $71^0$54' to $74^0$12' east. The temperature in this arid region register its extreme, rising above 48ºC in summer and coming down to near 4ºC during winter. The average day lengths during summer and winter remain approximately 13 and 11 hr, respectively. The study was carried out during breeding season between March to September.

3.2 Selection of Experimental Horses and Management Practices

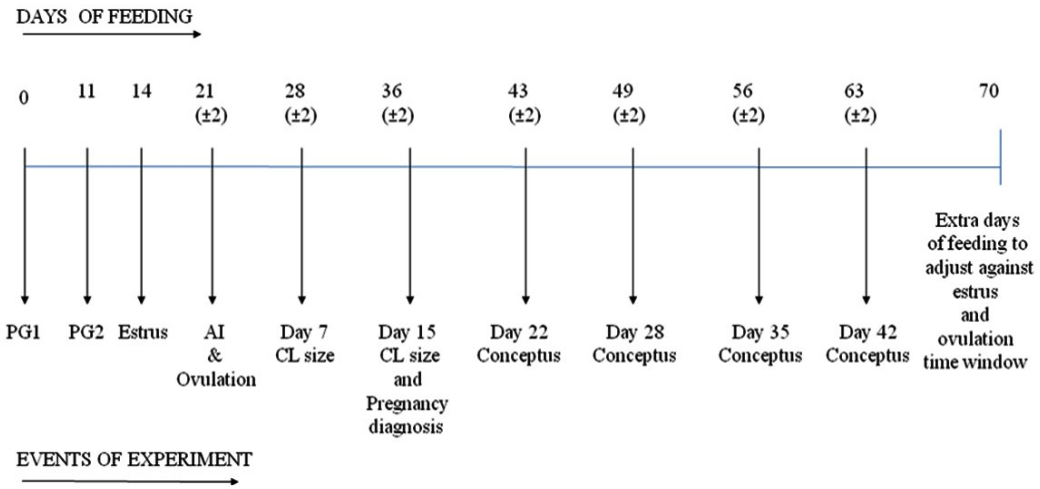
Two experiments have been conducted in present study. Experiment I comprised of twenty clinically healthy, normal cycling breedable Marwari mares having body condition score 6 to 7. The age and body weight of the experimental mares varied between 4 to 11 years and 310 to 410 kg, respectively before start of the experiment. The history of mares was thoroughly examined to avoid any reproductive disorders like abortion, dystocia, retention of fetal membrane, endometritis, metritis or pyometra. Ultrasonography assisted examination of mares was performed before initiation of experiment for functional ovaries, normal uterine horns and patent cervix. For experiment II, six clinically healthy, fertile Marwari stallions having body condition score 6 to 7 were selected. The stallion's age and body weight was varied between 4.5 to 11 years and 300 to 350 kg, respectively before start of the experiment. All the experimental mares and stallions were kept under uniform conditions of feeding and managemental practices. The concentrate mixture fed to animals included oat or barley (40%), gram

(30%) and wheat bran (27%) plus mineral mixture and common salt (3%). Green fodder fed to animals was either one or combination of Lucerne, burseem, sorghum, millet depending on availability. Sewan grass (dry and chaffed), wheat or oat straw (chaffed), groundnut haulm was fed as dry fodder. Animals were given concentrate in the morning, green fodder around noon and dry fodder in the evening every day. Fresh drinking water was allowed ad lib in the water trough in each animal shed. Each animal shed had ample ventilation, roof to protect animals from inclement weather and open paddock for free movement of the mare and stallions.

3.3 Experimental Design

Experiment I : To study the effect of dietary supplementation with fish oil rich in n-3 PUFA on follicular, luteal and embryonic development in Marwari mares

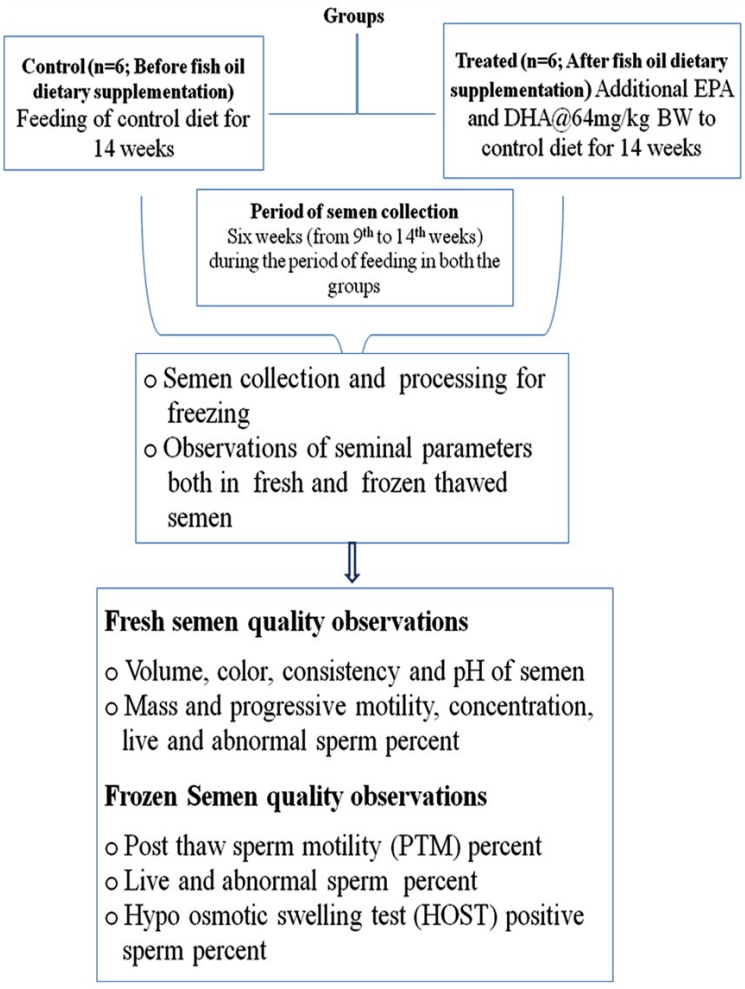
In experiment I, twenty mares were first blocked within group according to their age, body weight and then allocated randomly either in group A (Control, n=10) or group B (Treatment, n=10). The mares in group A were offered routine diet including 3 kg concentrate with mineral mixture, common salt and 9 kg fodder of green: dry in ratio 3:1. The mares in group B received additional fish oil (Avestia Pharma, Mumbai, India) in the diet having 15.28% EPA and 10.41% DHA at a combined rate of 64 mg/kg (~0.25 ml/kg) BW and mixed thoroughly into the concentrate feed. Mares of both group A and B continued to receive respective diets for 70 days including period of experiment. The schedule has been detail below.



Schematic diagram of experiment I

Experiment II : To study the effect of dietary fish oil rich in n-3 PUFA supplementation on semen quality of Marwari stallions

In experiment II, six stallions were used first without fish oil supplementation as control, offered routine diet including 3 kg concentrate with mineral mixture, common salt and 9 kg fodder of green: dry in ratio 3:1 for a period of fourteen weeks. In treated group later on, the same six stallions were offered fish oil supplementation having 15.28% EPA and 10.41% DHA at a combined rate of 64 mg/kg (~0.25 ml/kg) BW additional to the diet fed to control stallions for a period of fourteen weeks. Semen was collected from control (n=6) and fish oil supplemented (n=6) stallions at weekly interval starting from 9th week up to 14th week (six weeks period) from start of experimentation. Six ejaculates from each stallions was collected from both control and fish oil supplemented stallions to had a total of thirty six ejaculates in each group as shown below.



Schematic Diagram of Experiment II

3.4 Estrus Detection in Experimental Mares

Mares of both group A and B were given 2 ml (total 10 mg) intramuscular injection of Dianoprost Tromethamine (Lutalyse®, Pfizer Ltd, Mumbai, India), first at the initiation of feeding trial and subsequently on 11 days apart for estrus induction. Observations for typical estrus symptoms like receptivity towards stallion, squatting position, raised tail, frequent urination and winking were made in the morning every day before feeding by parading stallion near the experimental mares. The mare not in heat clearly rejected the stallion with typical behavior seen in the form of tail switching, squealing, striking, biting and/or kicking. The mare detected in estrus at first is then confirmed through ultrasonography (Kontron Medical, Type Vetson Pro, France) assisted examination of ovaries for presence of developing follicle in either side and characteristic "cart wheel" pattern of uterine mucosal folds (Photo plate 1).

3.5 Monitoring of Follicular Development, Ovulation and CL size

Follicular development was monitored using trans-rectal ultrasonography in mares of both the group A and B which responded to estrus synchronization treatment and observations were taken in their next immediate estrous cycle following estrus synchronization treatment (Plate 2). The examination of antral follicle development was started with onset of estrus (Day 0; day of estrus) followed by every alternate day till the follicular diameter reaching to 35 ±1 mm in diameter and then recorded daily until ovulation. Day of ovulation was considered as the day when the largest developing follicle was not observed following previous days of its presence. Diameter of CL was measured on days 7 and 15 following ovulation.

3.6 AI, Pregnancy Diagnosis and Monitoring of Embryonic Development

Estrus mares of both the groups A and B were inseminated artificially in uterine body with frozen semen, first when follicle diameter measured $\geq$35 mm in diameter and then repeatedly at 24 hr interval till ovulation or up to 6 hr post ovulation. Eight straws of 0.5 ml capacity straws (IMV Technologies, France) were cut after thawing in water bath (ACMAS Technocracy, Pvt. Ltd., Delhi, India) at 37°C for 30 second to make an inseminate volume of 4 ml in a sterile 5 ml syringe. Frozen semen from proven stallion with post thaw motility $\geq$35% and having approximately $300x10^6$ progressive motile

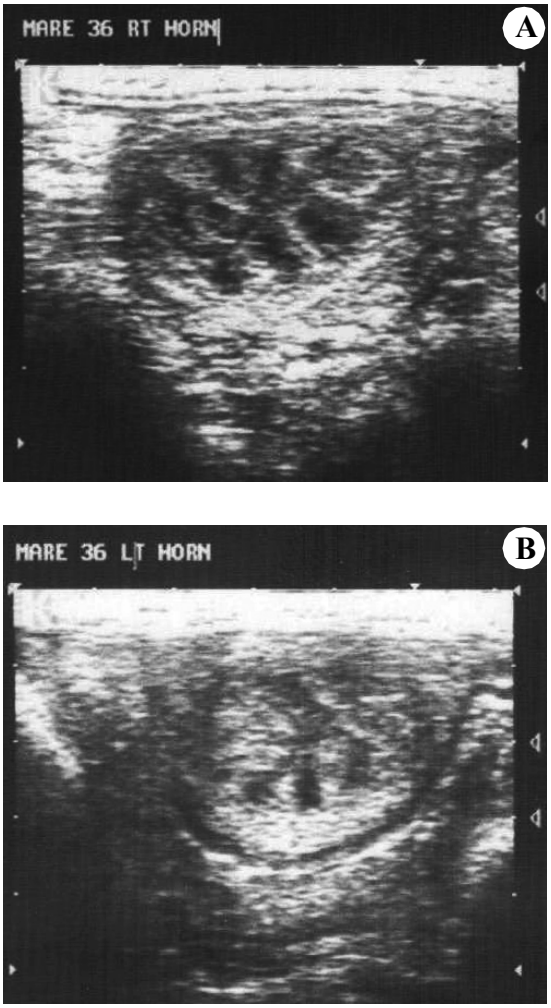


Plate 1 : Ultrasonic appearance of characteristic "cart wheel" pattern of uterine mucosal folds of right (A) and left (B) horn during estrus in mare

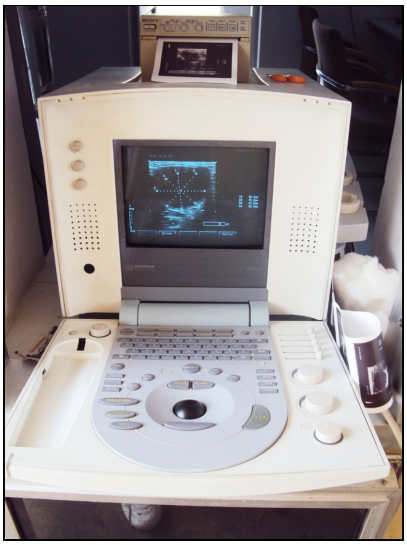


Plate 2 : Ultrasonographic examination of mare for ovarian status

sperm was used for insemination. Before AI mare was first restrained properly inside travis, perineum was washed thoroughly with soap and mild antiseptic solution in water. AI was performed per vagina using sterile equine AI catheter (IMV Technologies, France) which was placed in uterine body with lubricated gloved hand and then semen was dispensed from loaded syringe connected to other end of AI catheter. Pregnancy diagnosis in mares of both the groups was done at first on day 15 post-ovulation using trans-rectal ultrasonography. Pregnancy was again monitored across the days on 22, 28, 35 and 42 post-ovulation. Embryonic vesicle diameter was measured between the days 15 to 42 on day 15, 22, 28, 35 and 42 post-ovulation. The length and width of the embryo was also recorded on day 28, 35 and 42 post-ovulation.

3.7 Collection of Blood Plasma Samples

About 5 to 6ml blood samples were taken from jugular vein using needle attached with 9 ml collection tube with EDTA as anticoagulant (Vacutte®,Griner Bio-one Gmbh, Austria) from mares of both the groups as per schedule (Table 1). Blood plasma was separated by centrifugation at 3000 rpm for 15 minutes soon after blood collection. Two aliquots containing 1.5 ml plasma of each sample were stored in 2 ml micro centrifuge tubes at -20^{0}C until analysis.

Table 1: Blood sampling schedule

Sample No.	**1**	**2**	**3**	**4**	**5**	**6**
Day of Blood sampling	Day of estrus **(D0)**	Day 3rd of estrus **(D3)**	Day 6th of estrus **(D6)**	Day of ovulation **(D_{OV})**	Day 7th **($D7_{PO}$)**	**Day 15th ($D15_{PO}$)**
	Before ovulation					**Post-ovulation**

3.8 Hormone Estimation

The concentration of progesterone and estradiol was estimated in blood plasma using RIA kits (Immunotech®, France).

3.8.1 Progesterone estimation

3.8.1.1 Assay principle

The RIA of progesterone is a competitive assay. Samples and calibrators are incubated with ^{125}I-labelled progesterone as tracer, in respective antibody coated tubes

for estimation of progesterone. After incubation, the content of tubes is aspirated to remove any unbound material and then, bound radioactivity is measured from tubes. The amount of labelled hormone bound to solid-phase antibody is inversely related to the amount of unlabelled hormone present in the standard or samples. A calibration curve is established with known value of standards provided and unknown values of samples are determined by interpolation from the curve.

3.8.1.2 Assay procedure

All reagents were brought to the room temperature (20-25°C) before start of the assay procedure. Reagents were dispensed in the bottom of the antibody coated tubes and the procedure was operated sequentially as per the following scheme:

1. Samples and calibrators were dispensed @50µl each into antibody coated tubes.
2. Tracer (^{125}I-labelled progesterone) was added @500µl each antibody-coated tube.
3. Two additional non-coated tubes were prepared for total activity computation containing only 500µl tracer and was set-aside until counting.
4. The contents of the tubes were mixed with a vortex and incubated for 1 hr while continuous with shaking (300-350 rpm).
5. The incubation mixture was carefully aspirated while aspirator tip touched the bottom of the antibody coated tube as that all the liquid was removed.
6. The bound radioactivity was measured with the help of automatic Gamma Counter (Cobra series, Packard Biosystem, USA).

3.8.1.3 Calculation of the results

The mean net counts for each group of tubes were computed. The binding ability was evaluated as follows :

$$B/T\% = \frac{\text{Zero standard mean counts}}{\text{Total activity mean counts}} \times 100$$

The mean net counts for each calibrator and unknown sample were expressed as percentage of zero standard mean counts.

$$B/B\% = \frac{\text{Standard or sample mean counts}}{\text{Zero standard mean counts}} \times 100$$

The percent values of each calibrator on the ordinate (Y axis) versus the hormone amount expressed in ng/ml on the abscissa (X axis) is plotted in linear to obtain a calibration curve. Directly from the calibration curve hormone concentration of each sample expressed in ng/ml for progesterone can be read. However, in present study, bound radioactivity was measured on fully automatic gamma counter and has given calculated values of progesterone concentration. Analytical sensitivity for detection of progesterone was <0.05 ng/ml. Coefficients of intra and inter assay variation were ≤6.5% and ≤7.2% for serum samples.

3.8.2 Estradiol estimation

The principle of estradiol assay, assay procedure and calculation of results was same as mentioned under progesterone assay except the volume of samples and calibrators @100µl to each antibody coated tubes. Estradiol concentration (pg/ml) in unknown samples was obtained by interpolation from standard curve expressed in pg/ml. Analytical sensitivity for detection of estradiol was <6pg/ml. Coefficients of intra and inter assay variation were ≤12.1% and ≤11.2% for serum samples.

3.9 Estimation of Blood Biochemical Parameters

The blood biochemical parameters viz. cholesterol, triglycerides and non-esterified fatty acids (NEFA) were estimated using kit as per the manufacturer's instruction.

3.9.1 Cholesterol estimation

Cholesterol in plasma was estimated by biochemical cholesterol assay kit (Span diagnostic Ltd. Surat, India).

3.9.1.1 Assay principle

Cholesterol esters are hydrolyzed by cholesterol esterase (CHE) to release free cholesterol and fatty acids. In subsequent reaction, cholesterol oxidase (CHOD) oxidizes the 3-OH group of free cholesterol to liberate cholestenona and hydrogen peroxide. In presence of peroxidase (POD), hydrogen peroxide couple with 4-aminophenazone (4-AP) and phenol to produce red coloured quinonimine.

Absorbance of coloured dye is measured at 505 nm and the intensity of color formed is proportional to amount of total cholesterol concentration in the sample.

Reagents for cholesterol estimation

Buffer (R1)	PIPES pH 6.9	90 mmol/L
	Phenol 9	26 mmol/L
Enzymes (R2)	Cholesterol esterase	300U/L
	Cholesterol oxidase	300U/L
	Peroxidase	1250U/L
	4-Aminophenazone	0.4 mmol/L
	Cholesterol Standard	200 mg/dL

3.9.1.2 Assay procedure

i. Preparation of working reagent (WR)

Content of one vial of R2 was emptied into one bottle of R1. To dissolve the contents in R1, bottle was capped and mixed gently and kept protected from direct sunlight.

ii. Protocol for total cholesterol estimation

Cleaned and sterilized glass tubes marked as blank, standard and samples were dispensed in with WR, standard and samples in respective tubes as per the following scheme:

Scheme for addition of reagents and samples for cholesterol estimation

Tubes marked	Blank	Standard	Samples
WR (ml)	1.0	1.0	1.0
Standard (µl)	-	10	-
Samples (µl)	-	-	10

iii. Contents in the glass tubes were mixed properly and incubated for 10 minutes at room temperature (20-25^{o}C).

iv. Absorbance of tubes marked standard and samples were taken against blank from automatic biochemistry analyzer (RMS India, Chandigarh, India).

v. Calculations: total concentration of cholesterol were calculated as per the formula given:

$$\text{Cholesterol concentration in sample (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

3.9.2 Triglyceride estimation

Triglyceride in plasma was estimated by biochemical triglyceride assay kit (Span diagnostic Ltd. Surat, India).

3.9.2.1 Assay principle

Samples tested for triglyceride concentration are incubated with lipoprotein lipase (LPL) that results in liberation of glycerol and free fatty acids. In subsequent reaction, glycerol is converted to glycerol-3-phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase (GK) and ATP. G3P is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). In the last reaction, H_2O_2 reacts with 4-Aminophenazone (4-AP) and p-chlorophenol in presence of peroxidase (POD) to give a red colored dye. The intensity of color formed is proportional to amount of triglyceride concentration in the sample.

Reagents for triglyceride estimation

	GOOD pH 6.3	50 mmol/L
	p-Chlorophenol	2 mmol/L
	Lipoprotein lipase	150000 U/L
R	Glycerol kinase	500 U/L
	Glycerol phosphate dehydrogenase	3500 U/L
	4-Aminophenazone	0.1 mmol/L
	ATP	0.1 mmol/L
Triglyceride standard		200 mg/dL

3.9.2.2 Assay procedure:

i. Reagent and standard were ready to use and brought to room temperature.

ii. Protocol for total triglyceride estimation

Cleaned and sterilized glass tubes marked as blank, standard and samples were dispensed in with reagent, standard and samples in respective tubes as per the following scheme:

Scheme for addition of reagents and samples for triglyceride estimation

Tubes marked	Blank	Standard	Samples
R (ml)	1.0	1.0	1.0
Standard (µl)	-	10	-
Samples (µl)	-	-	10

iii. Contents in the glass tubes were mixed properly and incubated for 10 minutes at room temperature (20-25°C).

iv. Absorbance of tubes marked standard and samples were taken against blank from automatic biochemistry analyzer (RMS India, Chandigarh).

v. Calculations: Triglyceride concentration in samples were calculated as per the formula given:

$$\text{Triglyceride concentration in sample (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

3.9.3 Estimation of NEFA

The NEFA was estimated using 96-well flat clear bottom plate using Free Fatty Acid Quantification Kit (Sigma Aldrich, St. Louis, USA).

3.9.3.1 Assay principle

Assessment of free fatty acids (C8 or longer) is based on coupled enzyme assay which results in a colorimetric product. The initial step carried out by acyl-CoA synthetase (ACS), produces fatty acyl-CoA thiol esters from the NEFA, ATP, Mg and CoA in the reaction. The acyl-CoA derivatives react with oxygen in the presence of acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which forms a purple product that absorbs light at 570 nm which was recorded as optical density (OD)with an ELISA reader. The values of each standard expressed in µg/ml for NEFA on the ordinate (Y axis) versus the respective OD plotted in linear to obtain a calibration curve. Directly from the calibration curve concentration of NEFA in each sample expressed in µg /ml was read.

3.9.3.2 Assay procedure

Fatty acid assay buffer and fatty acid probe were brought to room temperature prior to use. ACS reagent and the enzyme mix provided were reconstituted each with 220 µl of fatty acid assay buffer and then mixed for use.

1. For preparation of standard curve with palmitic acid provided by the manufacture; 0, 2, 4, 6, 8 and 10 µl were added in duplicate to a 96 well plate.
2. 5 µl of plasma test samples were directly added to other wells.
3. Fatty acid assay buffer was added to each well to bring the volume to 50 µl.
4. 2 µl of ACS reagent was added to each well with standards or samples.
5. Plate was incubated for 30 minutes at 37^{O}C.
6. 50 µl of master reaction mix (containing 44 µl fatty acid assay buffer + 2 µl fatty acid probe + 2 µl enzyme mix + 2 µl enhancer) was added to each of the wells with standards or samples.
7. Contents in the wells were mixed well using horizontal shaker and then incubated protected from light for 30 minute at 37^{0}C.
8. Absorbance was measured from each well in the plate using ELISA reader at 570 nm.
9. Concentrations of NEFA in samples were read directly from interpolation against calibration curve obtained with standard concentration expressed in µg /ml.

3.10 Semen Collection, Processing and Evaluation

3.10.1 Sterilization of glassware and plastic ware

Glassware and plastic ware before and after use were dipped in 2% solution of laboratory detergent (Extran®, Merck Specialities private Ltd, Mumbai, India) in plastic water tub for one hour and then cleaned thoroughly using tap water and soft brush to make them grease and stain free. All were then rinsed with double distilled water and dried inside incubator. Openings of glassware and plastic ware were closed with piece of paper using thread before sterilization. Glass wares (Conical flasks, measuring cylinders) were sterilized in hot air oven at 180^{O}C for 2 hr.

3.10.2 Preparation of extender

Both primary (for initial dilution and sperm washing) and secondary extender (cryoprotective media for final dilution) were prepared in fresh in the laboratory as per the following composition:

Composition of primary extender (Citrate- EDTA) : For 100 ml volume

(i) Glucose	0.15 g
(ii) Sodium citrate dehydrate	2.60 g
(iii) Di-sodium EDTA	0.37 g
(iv) Sod-bicarbonate	0.12 g
(v) Streptomycin	0.10 g
(vi) Benzyl penicillin	0.10 g

Preparation : All above constituents were dissolved in a conical flask (250 ml capacity) by addition of double distilled water and the final volume made up to 100 ml.

Composition of secondary extender : Solution A + Solution B + egg yolk + cryoprotectant.

Solution A (EDTA- glucose)

(i) Glucose	6.00 g
(ii) Sodium citrate dehydrate	0.37 g
(iii) Di-sodium EDTA	0.37 g
(iv) Sod-bicarbonate	0.12 g
(v) Streptomycin	0.10 g
(vi) Benzyl penicillin	0.10 g

Preparation: Above all constituents were dissolved in conical flask with addition of double distilled water and the final volume made up to 100 ml.

Solution B (Lactose)

(i) Lactose	11.00 g
(ii) Streptomycin	0.08 g
(iii) Benzyl penicillin	0. 08 g

Preparation: Above all were dissolved in a conical flask with addition of double distilled water and the final volume made up to 100 ml.

Collection of egg yolk

Fresh eggs were first cleaned with cotton and wiped with 70% alcohol. Eggs were broken from its narrow end by light blow of forceps with caution not to disturb yolk. Albumin was discarded and yolk was placed cautiously over sterilized piece of blotting paper to avoid rupture of yolk membrane. Adapter of a 20 ml syringe without needle was pierced through the yolk membrane and yolk was aspirated as much as possible by pulling its plunger. Syringe was then emptied in a 100 ml measuring cylinder, ready to mix with solution A and B in order to collect 20ml egg yolk, 4 to 6 eggs were required.

Preparation of secondary extender : Lactose- Glucose -EDTA-egg yolk

Solution A and B were taken in 50 and 100 ml volume, respectively in a 500 ml capacity measuring cylinder. Then, 40 ml of egg yolk was added to this, covered with aluminium foil and mixed well by shaking the measuring cylinder. The mixed solution was then dispensed in to 50 ml centrifugation plastic tubes and centrifuged at 3000 rpm for 30 minutes in refrigerated centrifuge machine (Remi Instruments, Mumbai, India) maintaining temperature between 10 to 12°C. Tubes after centrifugation were taken out and supernatant was filtered through sterilized cotton gauze leaving the remaining egg yolk. Di-methyl formamide (DMF) was added as cryoprotectant @ 2% to total volume of supernatant collected in a measuring cylinder, mixed and kept ready for use.

3.10.3 Preparation of artificial vagina

The artificial vagina (AV) was prepared by assembling its parts putting inner rubber liner inside hard cylinder, folded and tightened with rubber bands over both ends of the hard cylinder. A soft disposable plastic liner was put over inner rubber liner; the one end of which is folded and tightened over hard cylinder with rubber band; to other end connected to pre-warmed graduated collection bottle of 250 ml capacity covered with thermo-jacket. Hot water was poured within the space between inner rubber liner and hard cylinder through nozzle with help of funnel. The optimum temperature of AV was maintained 39 to 42°C with warm water and lubricated well with liquid paraffin before use. The pressure inside AV was kept such that the stallion was able to penetrate penis with ease.

3.10.4 Semen collection

Semen was collected near to semen laboratory using Colorado model AV (Animal Reproduction Systems, California, USA). An estrus mare was used for mounting and semen collection from stallions (Plate 3). The mare was restrained with rope, her tail bandaged and perineal area was cleaned before mount. The operator holding AV directed the erected penis with other gloved hand in to AV while stallion mounted the mare. Ejaculation occurred into the lumen of the AV lined with disposable plastic liner and was collected into graduated collection bottle. The penis of the stallion was washed with lukewarm water before mounting. Soon after dismounting, penis was again given flush of mild betadine solution (0.5%). The collected semen in collection bottle covered with thermo jacket was rushed immediately to laboratory for its evaluation and processing for freezing.

3.10.5 Semen evaluation

Seminal parameters were recorded from a total of thirty six ejaculates in each group before and after supplementation of fish oil. The seminal parameters included color and consistency of semen, semen volume (total, gel and gel free), pH, total and progressive motility, sperm concentration, live and abnormal sperm percent in fresh and frozen semen, PTM and HOST percent in frozen semen samples. The observations were performed as per following methods:

3.10.5.1 Color and consistency

This was recorded by visual observation. A good sample appears milky white in color, evenly turbid and without any clots. Abnormal ejaculates may be yellow (urine contamination, white blood cells) or pink (hemorrhage). Clots or flakes in the semen may be indicative of pus that comes from tubular tract or accessory glands. Consistency may range from watery to creamy depending upon the spermatozoa concentration within the sample. Creamy appearance of an ejaculate is indicative of concentrated while a watery ejaculate often has fewer sperm cells.

3.10.5.2 Semen volume

Total ejaculate volume was recorded directly from the graduated collection bottle, soon after collection. Then, it was filtered through thin sterilized gauze to retain gel fraction and gel free semen volume is recorded from other graduated collection bottle

Plate 3 : Semen collection using artificial vagina in a breeding stallion

that was used to collect filtrate. The gauze with the trapped gel fraction was discarded and its volume was noted by subtracting gel free semen from the total ejaculate.

3.10.5.3 Seminal fluid pH

This was noted by putting a drop of semen on pH strip paper (Neutralit®, Merck Specialities private Ltd, Mumbai, India) and comparing with colour indicators.

3.10.5.4 Sperm motility

Sperm motility was assessed visually under microscope (Olympus Medical Systems India Private Ltd., Gurgaon) at 40 X from gel-free semen on a pre-warmed glass slide. Total motility was observed including sperm with oscillatory or progressive motility. Progressive motility was observed as number of progressive mortile spermatozoa out of 10 sperm cells/high power field (40X) in order to accurately estimate the number of cells that are progressively moving across the field. This method is highly subjective and very dependent on the experience of the examiner.

3.10.5.5 Sperm concentration

The sperm concentration in semen samples mixed with diluting fluid (1:20) was estimated by hemocytometer. The hemocytometer have two counting chambers each divide into 9 primary squares. The central square has triple lines around it. Inside the triple lines are 25 (5x5) secondary squares also bounded by triple lines. Within each of the 25 secondary squares are 16 (4x4) tertiary squares.

Preparation of diluting fluid

0.05 gm Eosin Y (water soluble) and 1 gm Sodium chloride (NaCl) was dissolved in 100ml distilled water.

Manual method for dilution

1. 50 µl of semen sample was extended in 1 ml of diluting fluid in a micro centrifuge tube to make a 1:20 dilution.

Sperm count technique

The diluted semen sample (1:20) was dispensed cautiously to avoid spillage and from the side of cover slip placed over the counting chambers in a hemocytometer chamber. The semen sample was allowed to settle for 2 minutes and all the sperm heads in the middle big square (the square with 25 secondary squares) were counted. Sperm concentration was calculated as per the following formula:

Sperm concentration/ml = (Dilution factor) (Count in 5 squares) (0.05×10^6)

3.10.5.6 Live sperm count

Live sperm in both fresh and frozen semen samples were counted using eosin-nigrosin stained smear of semen sample under microscope at 100 X (Nikon Instech Co. Ltd., Kanagawa, Japan). To prepare smear, one drop of semen was placed on clean, grease free, pre warmed glass slide and mixed with one drop of eosin-nigrosin stain using blunt fine glass rod. After a minute, a thin smear from the mixture is prepared on glass slide and air dried. A total of 200 sperm were counted from each smear for live sperm percent. The stained sperm appeared pink in color was counted as dead sperm and whereas unstained sperm appeared clear/color less were considered live. Partially stained sperm were considered as dead.

Preparation of Eosin Nigrosin stain for sperm live-dead count and morphology

1. 3 gm of sodium citrate (dihydrate) was dissolved in 100ml distilled water to make 3% solution.
2. 1 gm eosin B and 5 gm nigrosin were taken to dissolve in 100 ml 3% sodium citrate (dihydrate).
3. pH of the stain was adjusted to 7.0 by adding a few drops of 0.1 M NaH_2PO_4 and filtered. This mixture was kept stored for use.

3.10.5.7 Sperm morphology

Spermatozoa morphology was assessed in both fresh and frozen semen samples by microscopic examination (100 X magnification) with addition of immersion oil. Preparation of smear from semen samples was same as used for live sperm count. A total of 200 spermatozoa were examined for abnormalities such as abnormal head, detached head, proximal or distal cytoplasmic droplet, abnormal mid piece, bent or coiled tail, premature sperm cells etc.

3.10.5.8 Sperm post thaw motility (PTM)

Sperm PTM% was recorded from frozen semen samples before storage for future use. Two frozen semen straws from each semen samples were taken out and dipped for 30 seconds in water bath maintained at 37°C. Then straws were wiped with tissue paper and cut with scissor to dispense its contents into a separate micro

centrifuge tubes. Progressive motility was observed as number of progressive mortile spermatozoa out of 10 sperm cells/high power field (40X) from both the straws in order to accurately estimate the number of cells that are progressively motile, recorded as PTM%.

3.10.5.9 Hypo osmotic swelling test (HOST)

HOST positive sperm percent was recorded from frozen semen samples incubated with HOST solution kept in micro centrifuge tube. The micro centrifuge tubes were dipped in water bath maintained at 37^0C for 1 hr. After incubation period, one drop of semen sample was taken on clean, grease free, pre warmed glass slide, covered with cover slip and counted for HOST reacted sperm. A total of 200 sperm were counted from each sample. The HOST reacted sperm had shown curled, swollen tip of the tail.

Preparation of HOST solution

1.351 gm of fructose and 0.735 gm of sodium citrate was dissolved in 100 ml distilled water and pH of solution was maintained to 7.9 using 0.1 M NaH_2PO_4.

3.10.6 Processing of Semen for freezing

The semen samples having progressive sperm motility more than 60% in fresh was processed further for freezing.

Primary dilution and removal of seminal plasma

Gel free semen was mixed with an equal volume of primary extender soon after record of sperm motility, then filled in 50 ml tubes and centrifuged in refrigerated centrifuge to get soft sperm pellet at the bottom of tubes. Addition of primary extender prior to centrifugation makes the spermatozoa more resistant to cold stress and damage while centrifugation. Centrifugation was done at 2000 rpm for 3 minutes. After centrifugation, the supernatant (primary extender with semen plasma) was aspirated to discard leaving a small portion (approximately 5%) of the seminal plasma with sperm pellet to preserve viability.

Calculation of dilution rate and secondary dilution

Calculation of dilution rate: Dilution rate of semen volume was calculated as per the following formula:

$$\text{Dilution rate} = \frac{\text{No. of progressive motile sperm per ejaculate (ml)}}{\text{Required sperm concentration per ml}}$$

The sperm pellet was re-suspended using sufficient secondary extender to achieve a final concentration of 150 to 200x10^6 progressively motile sperm per ml, considering the 50% damage that occur to sperm while freezing-thawing and the volume (4 ml) of frozen semen required for AI. After final dilution, semen samples were kept in cooling cabinet before being packaged in to the straws.

3.10.7 Cooling and equilibration of diluted semen

Diluted semen was kept in cooling cabinet (IMV Technologies, France) maintained at 4-5°C for 2 hours to allow cooling of semen sample and sperm cells to get adjusted with the dilutor so that loss of sperms can be minimized. The pre-freeze motility was recorded after equilibration and before freezing process.

3.10.8 Packaging of diluted semen

The diluted semen after equilibration was filled and sealed into straws with automatic filling-sealing machine (IMV Technologies, France). Poly-vinyl chloride (PVC) straws of 0.5 ml capacity and different colors were used for packaging.

3.10.9 Method of semen freezing

Semen was frozen into straws using vapor freeze technique. The straws filled with diluted semen were laid horizontally onto a wired net and lowered into a styrofoam box that contained two inch level of liquid nitrogen. The wired net with straws was held 3 cm above the surface of liquid nitrogen for 12 minutes. The straws were turned around once or twice over wired net held in position during period of exposure to liquid nitrogen vapor; then dipped into liquid nitrogen (-196°C).

3.11 Statistical Analysis

Data were tested for normality by Shapiro-Wilk test and transformed when indicated. Assumption of homoscadesticity of the data was ascertained by Levene's test ($P>0.05$). Outliers were identified and removed accordingly. In experiment-I, data were analyzed for the main effect of group, G [Non-supplemeted (Group A) vs supplemented (Group B)], main effect of day of sampling, D and, the interaction of group-by-day (GxD) using the General Linear Model Univariate of SPSS (Version 16,

USA) with the following model: $Y_{ijk} = \mu + a_i + b_J + (ab)_{ij} + \alpha_{ijk}$; Where, $Y_{ijk} = k^{th}$ data value of j^{th} level of factor b (days) and i^{th} level of factor a (group), μ = grand mean, a_i = i^{th} level of factor a, $b_j = j^{th}$ level of factor b, $(ab)_k = k^{th}$ interaction, α_{ijk} = residual error with respect to Y_{ijk}. A significant interaction between rows and columns is when the difference between rows is not the same at each column, equivalent to variations between columns that is not the same at each row. Within group and within day differences were analyzed by Duncan's multiple range test (DMRT) followed by least significant difference (LSD) test. In experiment II, the seminal parameters were analyzed using independent sample t-test. A probability value of $p \leq 0.05$ indicated that the difference was statistically significant while a probability of $p > 0.05$ to $p \leq 0.1$ indicated that significance was approached. Data are presented as mean±SEM.

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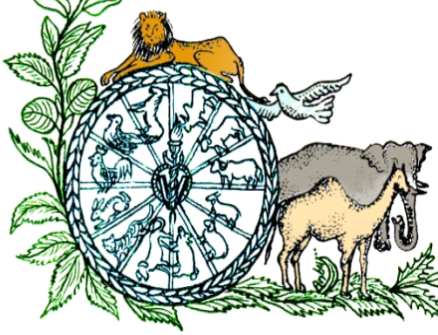
Results



4

RESULTS

Present study was conducted in two phases as experiment I and II in Marwari horses:

4.1 Experiment I : To study the effect of dietary fish oil supplementation on follicular, luteal and embryonic development in Marwari mares

For experiment-I, twenty cycling mares were selected and divided randomly into two groups (n=10 each) viz. group A (Gr. A; routine diet) and group B (Gr. B; diet supplemented with fish oil @0.25 ml/kg BW daily). Animals were fed respective diets for 70 days and observations were taken from second estrous cycle after start of experiment.

4.1.1 Estrus duration and estrous cycle length

Estrus duration in mares of Gr. B (7.45±0.2; range 6 to 9 days) decreased significantly (P<0.05) than Gr. A (8.26±0.20; range 6 to 10 days). However, length of estrous cycle did not vary significantly between Gr. B (25.87±0.58 days) and Gr. A (24.93±0.67 days). The estrous cycle length ranged from 24 to 28 and 20 to 29 days in Gr. B and A, respectively (Fig.2).

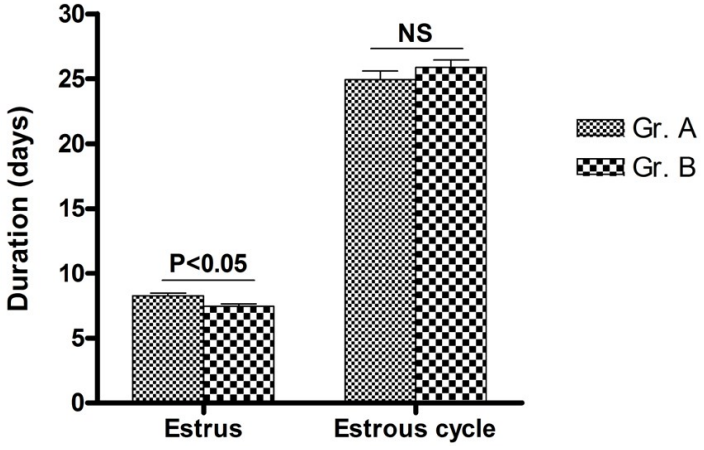


Fig. 2 : Bar diagram depicting estrus duration and estrous cycle length in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B)

4.1.2 Follicular development

Development of follicle from the day of estrus to ovulatory size is shown in plate 4. Diameter of the largest follicle on the day of estrus (D0), day 2 (D2), day 4 (D4), day 5 (D5) of estrus and one day before ovulation (D_{ov}-1) was compared between Gr. A and B. Diameter of the largest follicle was significantly greater in Gr. B than Gr. A on D0 (26.27± 0.32 vs. 24.08± 0.87 mm; P<0.05), D2 (30.14±0.54 vs. 27.08±0.70 mm; P<0.01), D4 (33.92±0.57 vs. 31.08±0.77 mm; P<0.01), D5 (40.00±0.40 vs. 37.44±0.60 mm; P<0.01) and D_{ov}-1 (48.03±1.43 vs. 42.42±1.12 mm; P<0.01) and presented in Fig. 3.

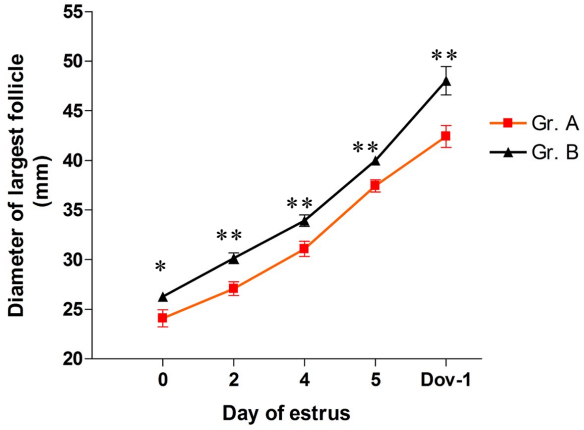


Fig. 3 : Line diagram depicting the diameter of largest follicle at different days of estrus in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [*P<0.05; **P<0.01]

Number of small (6-10 mm), medium (10.1-25 mm) and large (>25 mm) sized follicles was counted on the first day of estrus (D0). There was no significant difference between different follicle size categories in mares of Gr. A and B [small (1.70±0.61 vs. 0.70±0.43); medium (2.20±0.47 vs. 2.20±0.20); large (0.30±0.19 vs. 0.50±0.21)]; however, Gr. B tended to have lesser number of small and greater number of large sized follicle than Gr. A (Fig. 4).

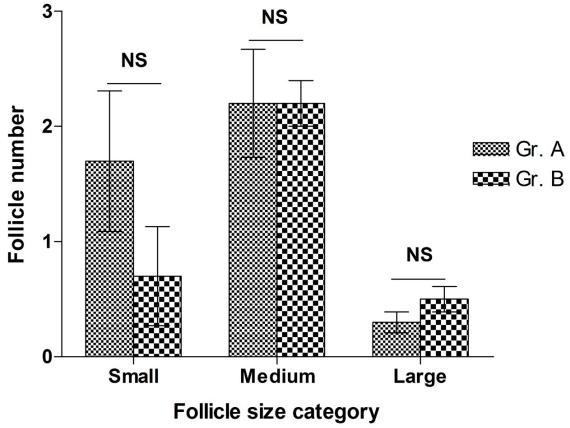


Fig. 4 : Bar diagram depicting follicle number in different follicle size categories on first day of estrus in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B)

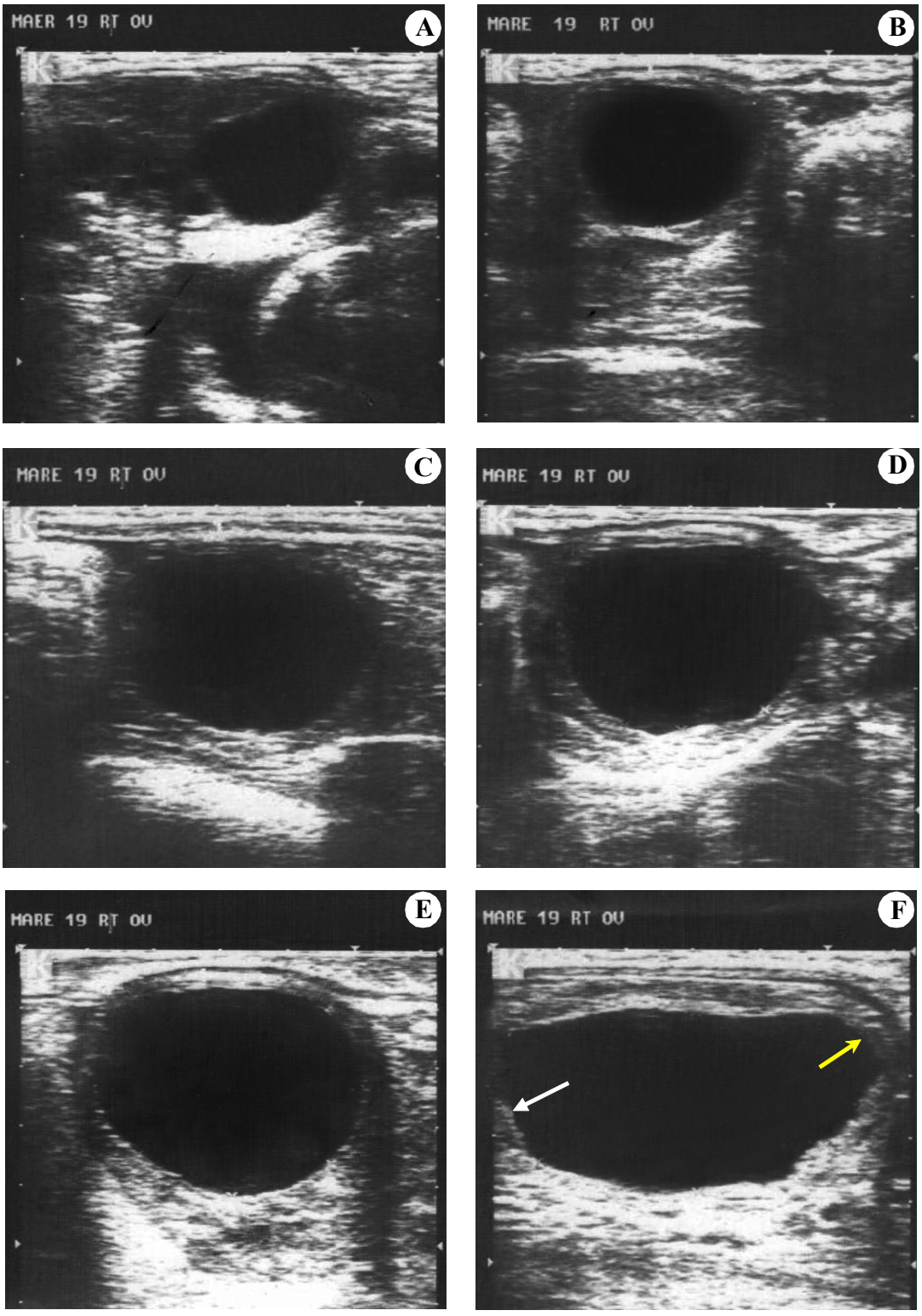


Plate 4 : Representative images of ultrasonic appearance of largest follicle in fish oil suplemented mare

(A) Day 0 : 17 x 22 mm
(B) Day 2 : 23 x 27 mm
(C) Day 4 : 29 x 35 mm
(D) Day 5 : 35 x 35 mm
(E) Day 6 : 41 x 41 mm
(F) Day 8 : 44 x 31.7 mm (**Note :** The change of shape to tear drop (yellow arrow) and serrated granulosa opposite the future ovulatory site (white arrow)

The total number of follicles irrespective of class size also did not differ significantly (P>0.05) between Gr. A (4.20±0.51) and Gr. B (3.40±0.45).

4.1.3 Corpus luteum (CL) development

The effect of fish oil supplementation on size of CL on day 7 post-ovulation ($D7_{PO}$) and day 15 post-ovulation ($D15_{PO}$) is presented in Fig. 5. The mares of Gr. B had significantly (P<0.01) larger CL size than Gr. A both at $D7_{PO}$ (31.01±0.8 vs. 26.76±0.8 mm) and $D15_{PO}$ (35.48±0.86 vs. 23.73±1.0 mm). There was significant (P<0.01) increase in CL size from $D7_{PO}$ to $D15_{PO}$ (31.01±0.8 vs. 35.48±0.86 mm) in mares of Gr. B; however in Gr. A, CL size did not differ significantly (P>0.05) from $D7_{PO}$ to $D15_{PO}$ (26.76±0.8 vs. 23.73±1.0 mm). Overall, the effect of fish oil supplementation was significant (P<0.001), however, effect of days was not significant (P>0.05) on CL development from $D7_{PO}$ to $D15_{PO}$. The interaction between group and day of CL size measurement was significant (P<0.001).

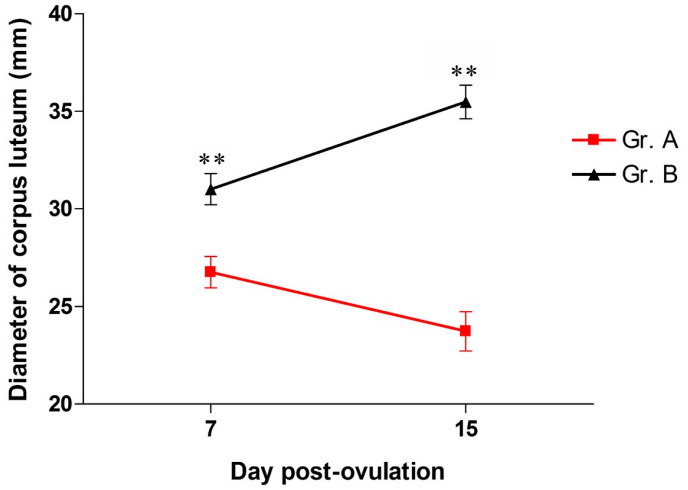


Fig. 5 : Line diagram depicting the diameter of CL in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [**P<0.01]

4.1.4 Embryonic development

Embryonic development from day 15 to 42 post-ovulation is shown in plate 5. Diameter of embryonic vesicle was compared between mares of both the groups on day 15, 22, 28, 35 and 42 post-ovulation and is presented in Fig 6. Diameter of embryonic vesicle was significantly (P<0.01) greater on day 15 post-ovulation in mares of Gr. B than A (21.41±0.71 vs. 15.27±1.25 mm); however, it was not significantly different on days 22 (27.20±1.32 vs. 26.99±1.29 mm), 28 (31.67±0.8 vs. 30.33±0.54 mm) 35 (44.04±0.8 vs. 43.31±1.41 mm) and 42 (57.77±1.4 vs. 55.90±1.8 mm) post-

ovulation. In Gr. B, embryonic vesicle diameter increased significantly (P<0.01) across the days 15, 22, 28, 35 and 42 post-ovulation. Significant (P<0.01) increase in embryonic vesicle diameter was also observed in Gr. A except from day 22 to 28 post-ovulation (P>0.05). Effect of days post-ovulation on diameter of embryonic vesicle was significant (P<0.001). However, the effect of group and the two-way interaction (group X day) failed to achieve significance (P>0.05).

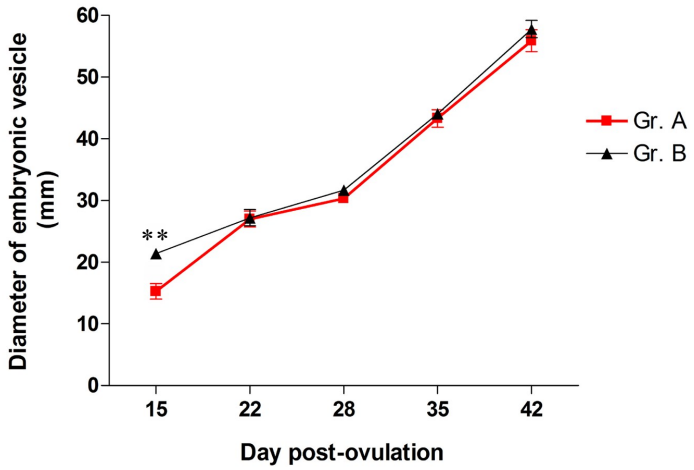


Fig. 6 : Line diagram depicting the diameter of embryonic vesicle in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [**P<0.01]

Length and width of embryo proper were recorded on days 28, 35 and 42 post-ovulation (Fig. 7 and 8). Length and width of embryo proper had a similar trend and was significantly (P<0.05) higher in mares of Gr. B (15.77±0.88 and 7.76±0.29 mm) than Gr. A (12.93±1.08 and 6.03±0.34 mm) on day 28 post-ovulation. There was significant (P<0.01) increase in length of embryo proper across the days 28, 35 and 42 post-ovulation in both Gr. A (12.93±1.08; 20.89±1.56; 30.11±1.68 mm, respectively) and Gr. B (15.77±0.88; 22.40±1.16; 35.15±2.10 mm, respectively). Similarly, the width of embryo increased significantly across the days 28, 35 and 42 post-ovulation in both Gr. A (6.03±0.34; 10.87±1.04; 16.40±0.92 mm, respectively) and Gr. B (7.76±0.29; 11.46±0.33; 18.45±1.16 mm, respectively). A significant (P<0.01) effect of fish oil supplementation was observed on length as well as width of embryo proper. Similarly, effect of days post-ovulation (28, 35 and 42 post-ovulation) on length and width of embryo proper was significant (P<0.001). However, the group X day interaction related to length and width of embryo was not significant (P>0.05).

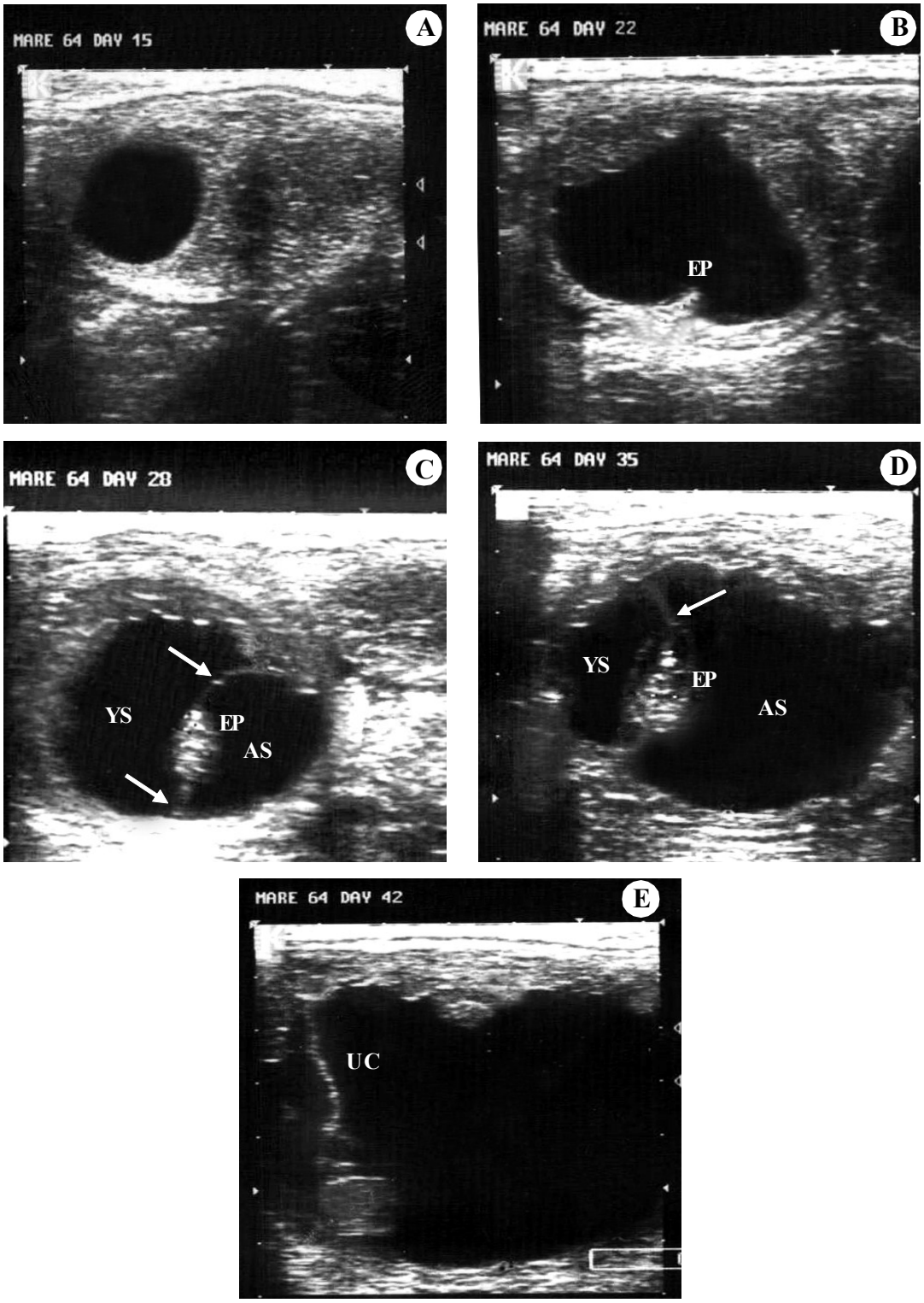


Plate 5 : Representative images depicting ultrasonic appearance of embryonic vesicle, embryo proper, placental sac and early fetus in fish oil supplemented mare

(A) Empty vesicle at day day 15

(B) A small echogenic spot on curved ventral aspect of vesicle. The image of vesicle is guitar-pick-shaped with the apex oriented dorsally

(C&D) The allanoic sac (vertical) and yolk sac (dorsal) are seperated by an echogenic line. The embryo is enlarged hyperechogenic nodule on the the seperating line**.**

(E) Fetus seen in cross-section and umblical cord is begining to form. Note that umblical cord remaining attached to dorsal pole of vesicle.

EP : Embryo proper; **YS :** Yolk sac; **AS :** Allantoic sac; **white arrow** indicates separting line.

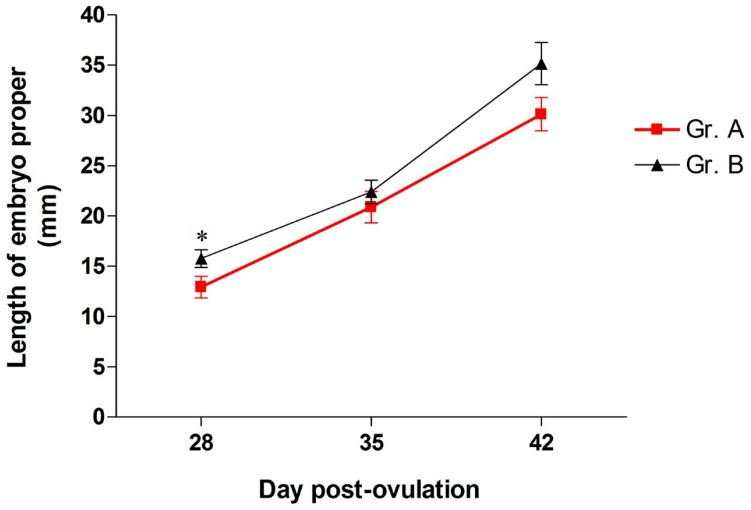


Fig. 7 : Line diagram depicting the length of embryo proper in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [*P<0.05]

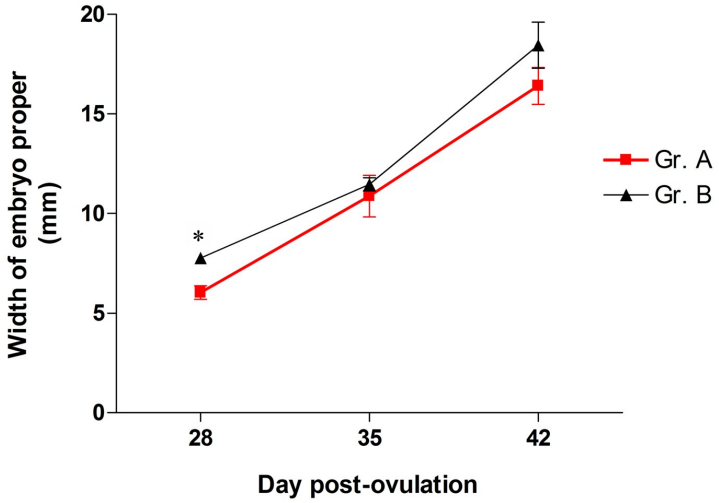


Fig. 8 : Line diagram depicting the width of embryo proper in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [*P<0.05]

4.1.5 Plasma estradiol and progesterone concentrations

Plasma estradiol concentration was estimated in mares of both the groups on D0, D3, D_{ov} (day of ovulation) and $D7_{PO}$ as presented in Fig. 9. Mean plasma estradiol concentration was significantly higher in mares of Gr. B than that of Gr. A on D0 (27.63±3.42 vs. 17.76±2.38 pg/ml; P<0.05) and D3 (27.87±3.42 vs. 15.68±2.92 pg/ml; P<0.01). However, the difference tended to approach significance (P=0.059) and was higher on D_{ov} in Gr. B than A (31.30±4.08 vs. 22.92±3.5 pg/ml). In contrast, the estradiol concentration on $D7_{PO}$ did not differ between Gr. B and A (15.81±0.75 vs. 19.85±3.25 pg/ml, respectively). In mares of Gr. A, the estradiol concentration remained

consistent and did not vary significantly across the days whereas, in Gr. B, the concentration was significantly (P<0.05) lower at $D7_{PO}$ than that of D0, D3 and $D_{ov.}$ A significant effect of group (P<0.01), effect of day (P<0.05) as well as the group X day interaction (P<0.05) on estradiol concentration was observed.

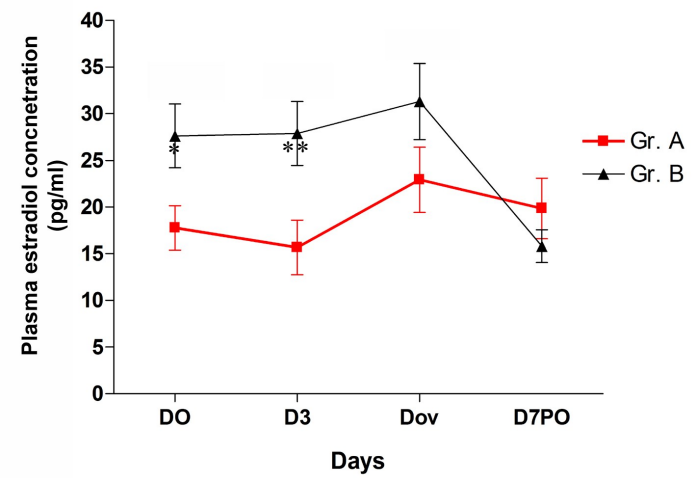


Fig. 9 : Line diagram depicting estradiol concentration on different days of estrus (D0; D3; D_{ov}) and post-ovulation ($D7_{PO}$) in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [*P<0.05; **P<0.01]

Plasma progesterone concentration was assayed in both the groups on D0, D6 (6th day of estrus), $D7_{PO}$ and $D15_{PO}$ as the mean values are depicted in Fig. 10. Progesterone concentration was significantly (P<0.01) higher in mares of Gr. B than that of Gr. A on D6 (0.51±0.1 vs. 0.11±0.01 ng/ml) and $D15_{PO}$ (4.50±0.4 vs. 2.04±0.7 ng/ml), whereas, the concentration did not differ significantly on D0 (0.94±0.65 vs. 0.42±0.19 ng/ml) and $D7_{PO}$ (4.79±0.99 vs. 4.57±0.58 ng/ml) between two groups, respectively. The progesterone concentration remained significantly higher (P<0.05) on $D7_{PO}$ (4.79±0.99 and 4.57±0.58 ng/ml) and $D15_{PO}$ (4.50±0.4 and 2.04±0.7 ng/ml) than D0 (0.94±0.65 vs. 0.42±0.19ng/ml) and D6 (0.51±0.1 vs. 0.11±0.01 ng/ml) in both Gr. B and A, respectively. In mares of Gr. A, progesterone concentration decreased significantly (P<0.05) on $D15_{PO}$ than $D7_{PO}$ (2.04±0.7 vs. 4.57±0.58 ng/ml) and the progesterone level was also declined at 6th day than day 0 of estrus (0.11±0.01 vs. 0.42±0.19 ng/ml); however, similar pattern was not observed on respective days in Gr. B (4.50±0.42 vs. 4.79±0.99 ng/ml and 0.51±0.14 vs. 0.94±0.65 ng/ml). There was significant (P<0.001) effect of group and day on progesterone concentration; however, group X day interaction was not observed (P>0.05).

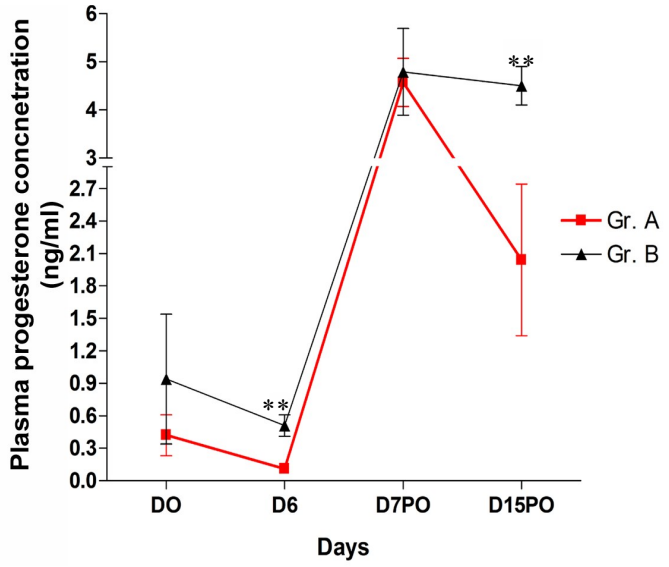


Fig. 10 : Line diagram depicting progesterone concentration on different days of estrus (Do; D6) and post-ovulation ($D7_{PO}$; $D15_{PO}$) in Marwari mares either fed a routine (Gr. A) or fish oil supplemented diet (Gr. B) [**P<0.01]

4.1.5 Correlation of largest follicle diameter and plasma estradiol concentration

Correlation between the largest follicle diameter one day before ovulation (D_{ov-1}) and plasma estradiol concentration on D_{ov} (day of ovulation) is presented in Table 2. The strong positive correlation (r=0.958; P<0.001) was found in mares of Gr. B, whereas, the correlation was moderate and tended to approach significance in Gr. A (r=0.531; P=0.057).

Table 2 : Correlation between largest follicle diameter on D_{ov-1} and plasma estradiol concentration on day of ovulation in Marwari mares fed either a routine (Gr. A) or fish oil supplemented diet (Gr. B)

	Plasma estradiol concentration (pg/ml) on D_{ov}	
	Group A	Group B
Follicle diameter at D_{ov}-1 (mm)	r = 0.531; P = 0.057	r = 0.958; P<0.001

4.1.6 Correlation of CL diameter and plasma progesterone concentration

Correlation of progesterone concentration with CL size on $D7_{PO}$-and $D15_{PO}$ was analyzed and found non-significant in Gr. A. However, the correlation was moderate and tended to approach significance on $D7_{PO}$ (r=0.706, P=0.058) but was significant on $D15_{PO}$ (r=574; P<0.05) in Gr. B as given in Table 3.

Table 3 : Correlation between CL diameter and plasma progesterone concentration in Marwari mares fed either a routine (Gr. A) or fish oil supplemented diet (Gr. B)

CL diameter (mm)	Plasma progesterone concentration (ng/ml)			
	$D7_{PO}$		$D15_{PO}$	
	Gr. A	Gr. B	Gr. A	Gr. B
$D7_{PO}$	r = 0.036 P> 0.05	r = 0.706 P= 0.058	-	-
$D15_{PO}$	-	-	r = 0.206 P> 0.05	r = 0.574 P< 0.05

4.1.7 Correlation between Follicle diameter on D_{ov}-1 and CL size

In mares of both Gr. A and B, there was no correlation (P>0.05) between follicle diameter on D_{ov}-1 and CL size ($D7_{PO}$ and $D15_{PO}$) as presented in Fig. 11.

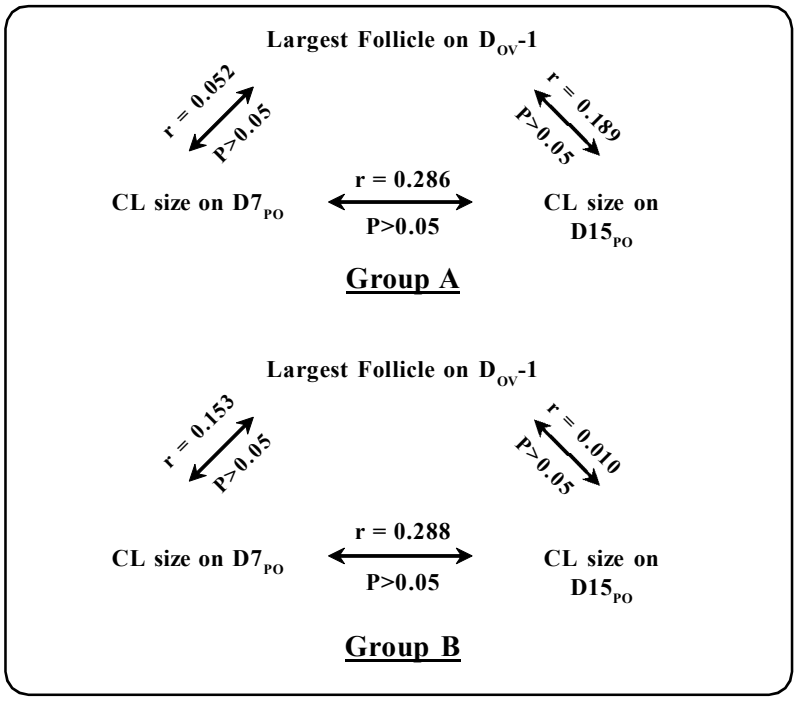


Fig. 11 : Schematic diagram showing correlation between follicle size on Dov-1, CL size on $D7_{PO}$ and $D15_{PO}$

4.1.8 Effect of fish oil supplementation on conception

Higher conception rate was observed with fish oil supplementation in Gr. B mares than Gr. A (83.33% vs 31.81%; P<0.01). Number of AI per conception was also significantly (P<0.05) lower in Gr. B than A (Table 4).

Table 4 : Conception rate and number (Median±SD) of AI per conception in Marwari mares fed either a routine (Gr. A) or fish oil supplemented diet (Gr. B)

Group	Total No. of inseminations (a)	No of pregnant mares (b)	Conception rate (b/a) x 100	Number of AI per conception (Median±SD)
Gr. A (n=10)	22	7	31.81%	2.00 ± 0.75^{B}
Gr. B (n=10)	12	10	83.33%	1.00 ± 0.32^{A}

Note: Superscripts (A, B) denote significance (P<0.05)

4.1.9 Effect of diameter of largest follicle, plasma estradiol concentration on day of ovulation, diameter of corpus luteum and plasma progesterone concentration on pregnancy in fish oil supplemented and non supplemented mares

Mares of both groups were retrospectively studied for the effect of diameter of largest follicle, CL size on $D7_{PO}$ and $D15_{PO}$, estradiol concentration at D_{ov}, progesterone concentration at $D7_{PO}$ and $D15_{PO}$ on the pregnancy. In Gr. A, the size of CL at $D7_{PO}$ and $D15_{PO}$ as well as progesterone concentration at $D7_{PO}$ had a significant (P<0.05, P=0.05; P<0.01, respectively) effect on the conception. Mares that conceived had a significantly larger size of CL on $D7_{PO}$ (31.09±2.05 vs. 24.99±0.21 mm) and $D15_{PO}$ (23.61±2.43 vs. 14.27±0.59 mm) as well as higher progesterone concentration at $D7_{PO}$ (4.88±0.69 vs. 2.83±0.59 ng/ml) as compared to mares that did not conceive (Fig. 12). The similar comparison was not possible in Gr. B because of smaller number of not conceived than conceived mares.

4.1.10 Effect of fish oil supplementation on biochemical parameters

Plasma metabolite concentration such as cholesterol, triglyceride and NEFA were measured in mares fed a routine or fish oil supplemented diet.

4.1.10.1 Cholesterol concentration

The mean concentration of cholesterol is given in Table 5. Cholesterol concentration did not differ significantly (P>0.05) between the groups at different days of estrus (D0, D3, D6, $D_{ov,}$) and post-ovulation ($D7_{PO}$ and $D15_{PO}$). In Gr. B, cholesterol concentration was significantly (P<0.05) higher on D0, D3 and D6 than that of the concentration on $D15_{PO}$ but not significant with respect to concentration on D_{ov} and

$D7_{PO}$. In Gr. A, the difference of cholesterol concentration was non-significant (P>0.05) at different days of sampling (D0, D3, D6, D_{ov}, $D7_{PO}$ and $D15_{PO}$). The effect of group and day of sampling on plasma cholesterol concentration was non-significant (P>0.05). Similarly, group-by-day interaction on plasma cholesterol concentration was non-significant (P>0.05).

4.1.10.2 Triglyceride concentration

Plasma concentration of triglyceride in mares of Gr. A and B is presented in Table 5. Triglyceride concentration was significantly (P<0.05) lower in mares of Gr. B than that of Gr. A at D3 (46.62±4.24 vs. 60.83±3.76 mg/dl), D6 (32.34±3.65 vs. 52.82±3.72 mg/dl) and D_{ov} (47.43±3.23 vs. 56.26±2.17 mg/dL), whereas, the same was not significant (P>0.05) on D0, $D7_{PO}$ and $D15_{PO}$. Within group comparison revealed significantly (P<0.05) lower triglyceride concentration on D6 than D0, D3, D_{ov} and $D15_{PO}$ but not with respect to $D7_{PO}$ in Gr. B. Significantly (P<0.05) lower concentration of triglyceride was found on $D7_{PO}$ than that of D3 and not significantly (P>0.05) different with respective values on D0, D6, D_{ov} and $D15_{PO}$ in mares of Gr. A. Effect of group was significant (P<0.001) and triglyceride concentration in mares of Gr. B (41.98±1.50 mg/dl) was lower as compared to Gr. A (55.08±1.99 mg/dl). Similarly, effect of day was found to be significant (P<0.01), however, the group X day interaction was non-significant (P>0.05).

4.1.10.3 Non esterified fatty acids (NEFA) concentration

Mean concentration of NEFA is presented in Table 5. NEFA concentration was significantly (P<0.05) lower in mares of Gr. B than that of Gr. A on D3 (318.20±7.70 vs. 349.68±10.47 µg/dl), D6 (339.74±9.78 vs. 383.19±18.47 µg/dl) and $D7_{PO}$ (323.90±7.85 vs. 351.74±10.77 µg/dL), respectively but not on D0, D_{ov} and $D15_{PO}$. The concentration of NEFA was significantly (P<0.05) higher on D6 than D_{ov} and $D15_{PO}$ but did not vary significantly on D0, D3, $D7_{PO}$ in Gr. A. However, in mares of Gr. B, NEFA concentration was significantly (P<0.05) higher on D_{ov} than D0, D3, D6 and $D7_{PO}$. Effect of group was not significant (P>0.05) with respect to NEFA concentration in mares of Gr. A (353.21±5.62 µg/dl) as compared to Gr. B (340.95±5.37 µg/dl). Similarly, the overall effect of day was not significant (P>0.05). However, the group X day interaction was significant (P<0.01).

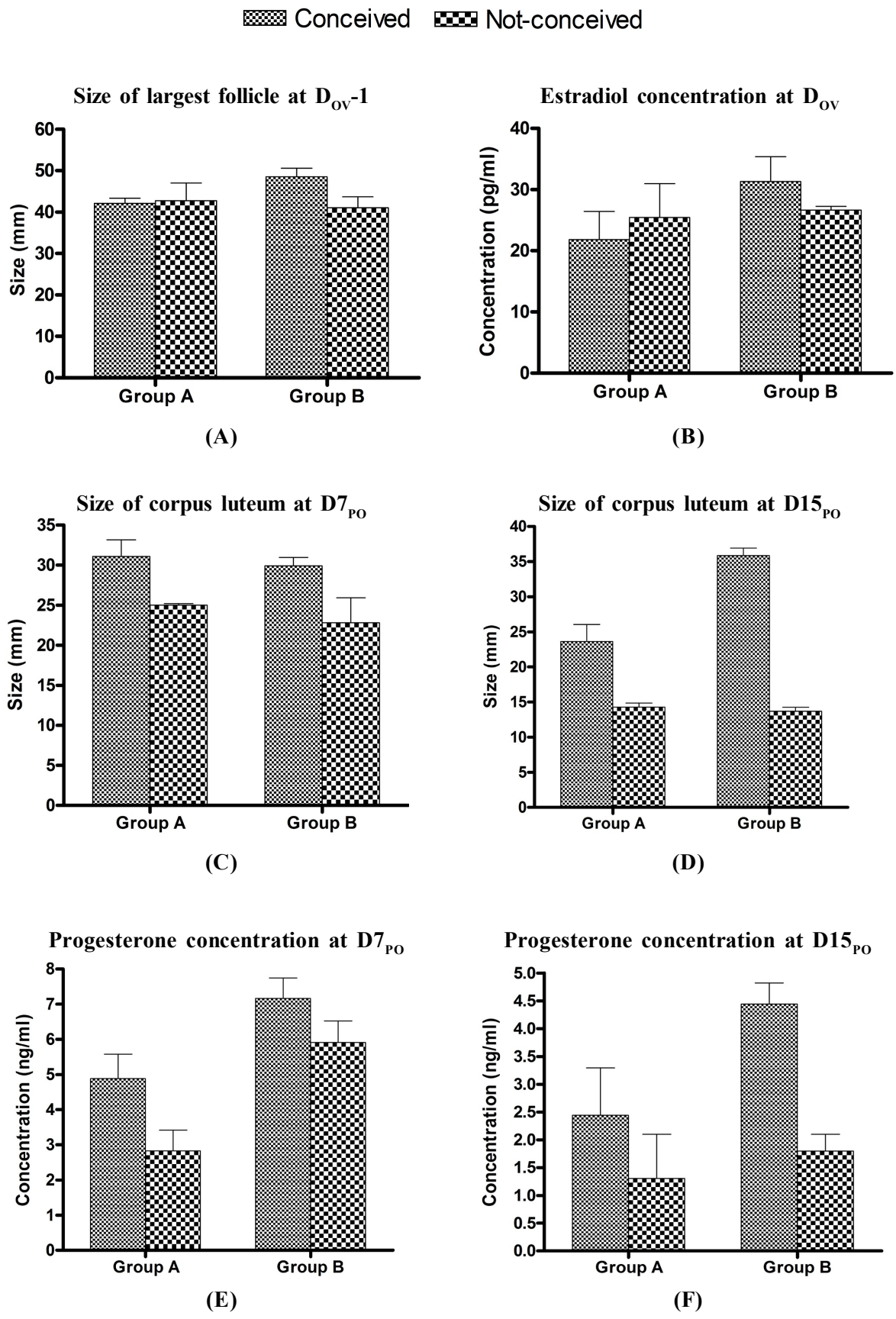


Fig. 12 : Bar diagrams depicting assocation between follicular size (A), plasma estradiol concentration (B), size of corpus leuteal (C and D) and plasma progesterone concentration (E and F) with outcome of AI

Note : Dov-1, indicates 1 day before ovulation

Table 5 : Biochemical parameters in Marwari mares fed either a routine (Gr. A) or fish oil supplemented diet (Gr. B)

Parameters	Group	Day of sampling						Effect
		D0	D3	D6	D_{ov}	$D7_{PO}$	$D15_{PO}$	
Plasma cholesterol concentration (mg/dl)	A	98.99±9.95	96.30±7.67	109.10±10.43	102.22±8.87	95.64±8.03	98.63±11.14	G= P>0.01
	B	103.84±2.52^{b}	106.63±7.90^{b}	103.37±5.12^{b}	96.83±2.77^{ab}	94.09±5.27^{ab}	88.0±4.04^{a}	D= P>0.05
								GXD= P>0.05
Plasma triglyceride concentration (mg/dl)	A	54.53±4.71^{ab}	60.83±3.76^{B,b}	52.82±3.72^{B,ab}	56.26±2.17^{B,ab}	47.02±3.23^{a}	51.05±3.44^{ab}	G= P<0.001
	B	43.87±3.45^{b}	46.62±4.24^{A,b}	32.34±3.65^{A,a}	47.83±3.23^{A,b}	38.88±2.70^{ab}	42.75±3.20^{b}	D= P<0.01
								GXD= P>0.05
Plasma non-esterified free fatty acid concentration (µg/dl)	A	365.51±17.22^{ab}	349.68±64.72^{B,ab}	383.19±18.49^{B,b}	329.70±9.58^{A,a}	351.74±10.77^{B,ab}	339.45±6.35^{a}	G= P>0.05
	B	335.74±7.78^{a}	318.20±7.79^{A,a}	339.74±9.78^{A,a}	378.84±18.72^{B,b}	323.90±7.85^{A,a}	349.29±12.85^{ab}	D= P>0.05
								GXD= P<0.01

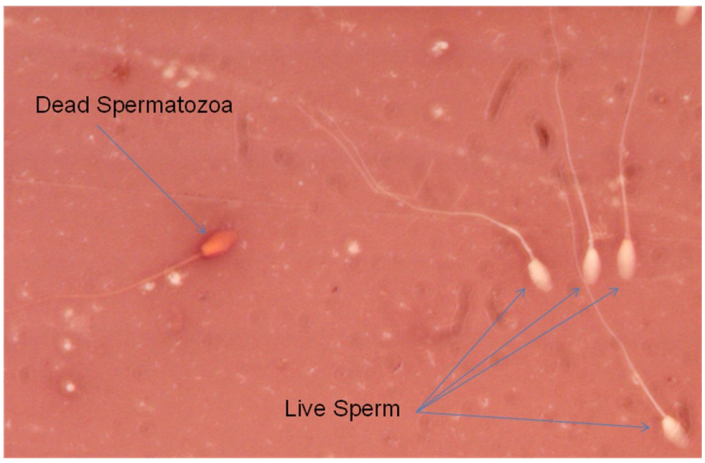
Uppercase (A, B) and lower case (a,b) superscripts denote significance (p<0.05) within column and within rows, respectively

4.2 Experiment II : To study the effect of dietary fish oil supplementation on semen quality in Marwari stallions

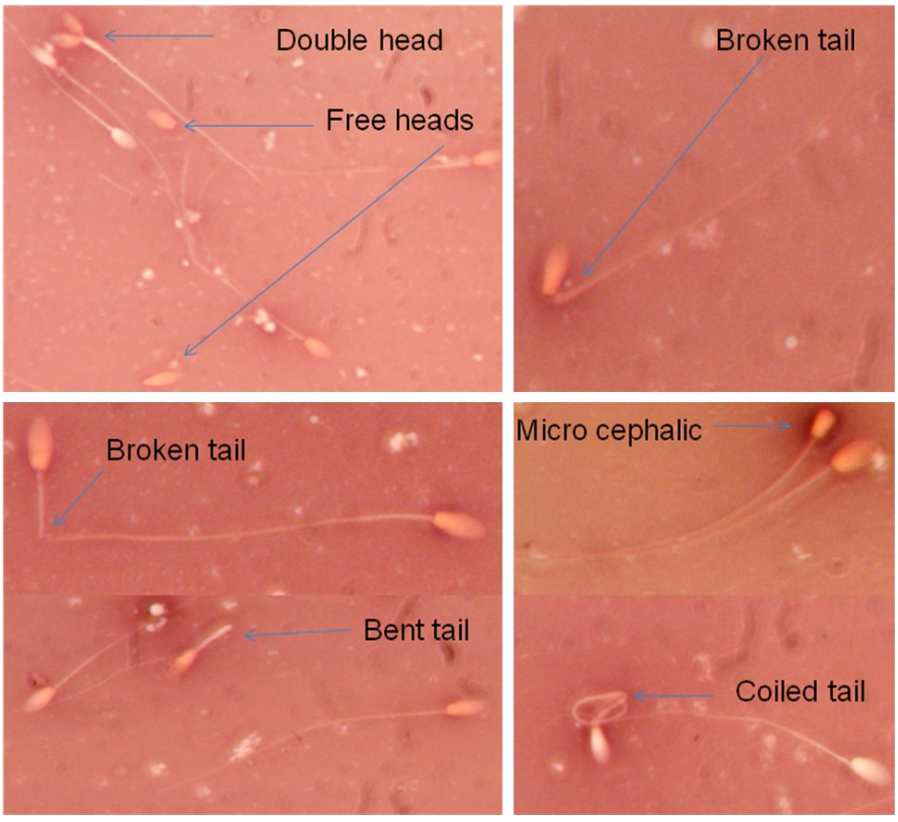
Six stallions were selected for experiment-II and ejaculates were collected before as well as after fish oil supplementation in diet for their evaluation both in fresh and after freezing. The seminal parameters observed in fresh semen included appearance, consistency, volume (total, gel in semen and gel free semen), pH, mass and progressive motility, sperm concentration, live and abnormal sperm percent. In frozen semen samples, sperm PTM, live and abnormal sperm as well as HOST reacted sperm percent were recorded.

4.2.1 Effect of fish oil supplementation on semen quality

Mean values of various seminal parameters of stallions before and after fish oil supplementation is presented in Table 6. Appearance (milky white to creamy) and the consistency (variably thin) of ejaculates were not different following fish oil supplementation compared to their ejaculates collected before supplementation. Mean volume of ejaculate in total, gel in semen and gel free semen did not differ significantly ($P>0.05$) before and after fish oil supplementation and ranged from 10 to 140 ml vs. 15 to 125 ml, 0 to 95 ml vs. 0 to 55 ml and 10 to 100 ml vs. 15 to 100 ml, respectively. Similarly, there was no significant effect of fish oil supplementation on total or progressive sperm motility, seminal pH, sperm concentration, live sperm and abnormal sperm per cent in fresh semen. The range of values before and after fish oil supplementation in diet was same for sperm mass motility (50 to 95%), progressive sperm motility (40 to 90%) and seminal pH (7 to 8.5) whereas, the ranges respective to sperm concentration, live sperm and abnormal sperm per cent in fresh semen were 91.35 to 327.6 x 10^6 vs. 85.06 to 405.3 x 10^6, 38 to 78% vs. 35 to 80% and 6 to 23% vs. 5 to 27%, respectively. In frozen semen, sperm PTM and HOST reacted sperm per cent before and after fish oil supplementation was not significant ($P>0.05$), however, the difference tended to approach significance ($P=0.063$) with respect to per cent abnormal sperm which is lower in fish oil supplemented stallions as compared to non-supplemented (12.63±0.96% vs. 14.23±0.69%). It ranged from 30 to 60% for sperm PTM, 8 to 25% abnormal sperm and 18.5 to 53.5% HOST reacted sperm in frozen semen samples before fish oil supplementation and the corresponding values after fish oil supplementation ranged from 30 to 70%, 6 to 31% and 21 to 59.5%, respectively.



(A)



(B)

Plate 6 : Representative images depicting sperm viability (A) and sperm abnormalities (B)

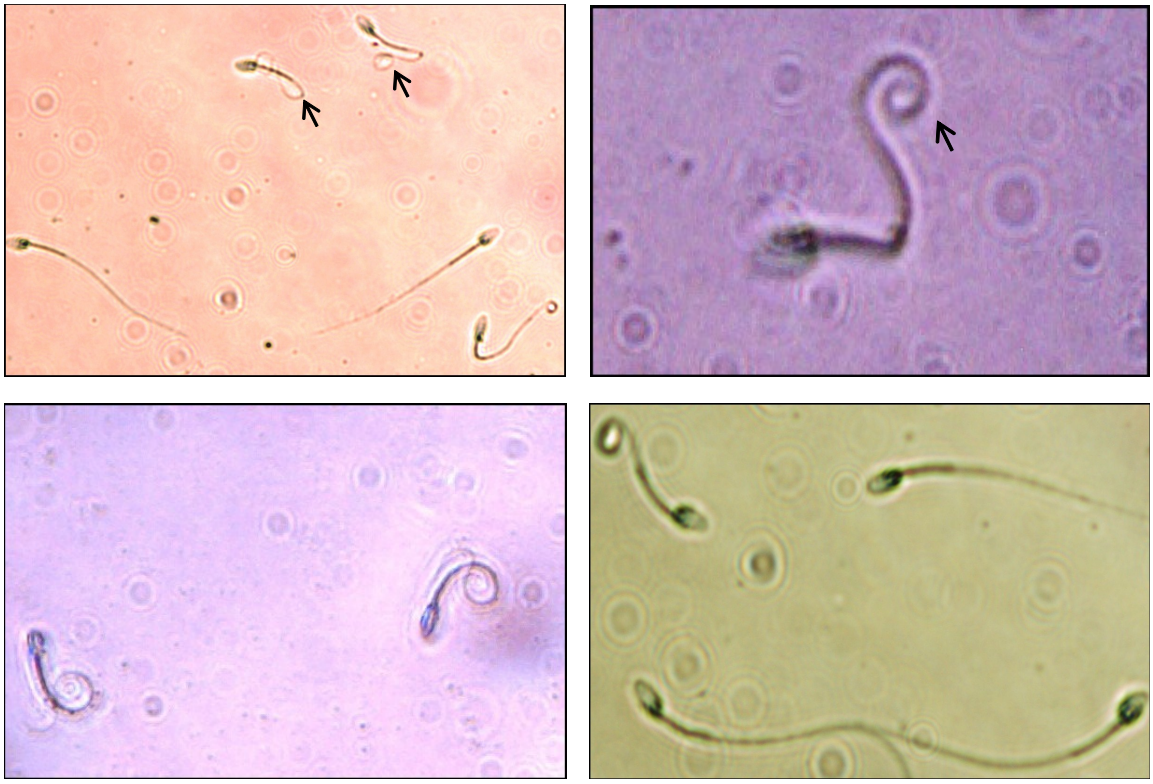
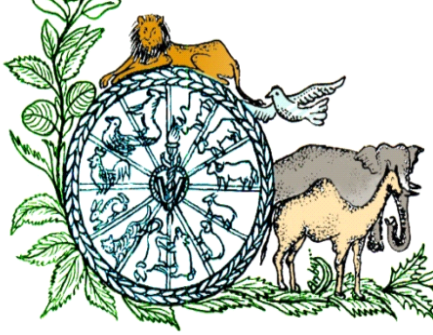


Plate 7 : Representative images depicting HOST reactive spermatozoa (arrow)

Table 6 : Seminal parameters before and after fish oil supplementation in Marwari stallions

Group	Number of total ejaculates	Total vol. (ml)	Gel vol. (ml)	Gel free semen vol. (ml)	pH	Mass motility (%)	Progressive motility (%)	Sperm conc. (10^6)	Live sperm in fresh semen (%)	Abnormal sperm in fresh semen (%)	PTM (%)	Live sperm in frozen semen	Abnormal sperm in frozen semen	HOST reacted sperm (%)
Before Supplementation	36	60.75 ± 6.17	20.64 ± 3.24	40.11 ± 3.85	7.74 ± 0.06	79.58 ± 2.26	72.50 ± 2.67	182.0 ± 11.89	79.97 ± 1.40	11.42 ± 0.68	38.83 ± 1.85	51.70 ± 2.96	14.23 ± 0.69	35.22 ± 1.86
After Supplementation	36	58.92 ± 5.38	18.05 ± 2.70	40.86 ± 3.74	7.64 ± 0.06	76.39 ± 2.63	70.14 ± 2.85	188.24 ± 12.94	78.97 ± 1.96	10.39 ± 0.86	39.63 ± 2.08	51.70 ± 2.06	12.63 ± 0.96	34.44 ± 1.78
Sig.		**0.886**	**0.699**	**0.864**	**0.242**	**0.309**	**0.536**	**0.772**	**0.552**	**0.126**	**0.775**	**0.574**	**0.063**	**0.765**

Discussion



5

DISCUSSION

This study was designed to investigate the effect of dietary fish oil, a rich source of n-3 PUFA on ovarian functions, embryonic development and quality of semen in Marwari breed of horses. In experiment I, influence of n-3 PUFA supplementation on follicular number and size, development of CL and embryonic vesicle including length and width of embryo proper as well as conception rate in mares was examined. Changes in plasma concentration of estradiol, progesterone and metabolites (cholesterol, triglyceride and NEFA) in experimental mares were estimated in relation to n-3 PUFA supplementation. The correlation between size of largest ovulatory follicle with plasma estradiol concentration and subsequently the CL size post-ovulation was examined. Further, correlation between progesterone concentration and CL size was also observed. In experiment II, various seminal parameters were compared in ejaculates from the same stallion before and after dietary fish oil supplementation.

5.1 Experiment I : To study the effect of fish oil supplementation on follicular, luteal and embryonic development in Marwari mares

5.1.1 Estrus duration and estrous cycle length

The finding that dietary fish oil supplement did not alter the estrous cycle length in mares is in accordance with earlier findings of Robinson *et al.* (2002) who also reported no effect on length of estrous cycle in cows supplemented with linolenic acid, an n-3 PUFA rich diet. Similarly, there was no effect found on estrous cycle length was revealed when whole raw soya beans or fish oil was added @2% to the diet in heifers (Childs *et al.,* 2008a). In contrary, supplementing n-3 PUFA in human diets shortens diestrus intervals (Juschem *et al.,* 2010). In cattle, increasing fat content in the diet was reported to shorten the interval to the first ovulation post partum (Lucy *et al.,* 1991). Usually, during the breeding season (spring and summer), estrous cycle length in mare is about 22 days with 5 to 7 days of estrus period (Aurich, 2011). Estrous cycle length is also affected by reproductive stage for example 21.2±1.8 days in lactating and 22.8±1.4

days in non-lactating mares as observed by Heidler *et al.* (2004). Even, between the individual mares, significant differences in estrous length have been reported (Aurich, 2011). Estrus duration and length of the estrous cycle in this experiment are within the range of 3 to 11 and 13 to 29 days, respectively as reported earlier by Arangasamy *et al*. (2008) in Marwari mares.

5.1.2 Follicular development

Consistent increase in size of pre ovulatory follicle from onset of estrus to an ovulatory size in this study is similar to earlier reports in cows fed diets rich in PUFAs (Bilby *et al.,* 2006a; Garnsworthy *et al.,* 2008; Ghasemzadeh-Nava *et al.,* 2011). Other studies in cattle (Lammoglia *et al.,* 1996; Robinson *et al.,* 2002; Staples and Thatcher, 2005; Ambrose *et al.,* 2006; Mendoza *et al.,* 2011) had also shown an increase in follicle size with fat supplemented diet. The diameter of the largest follicle was superior in cows (Lucy *et al*. 1991; 1993) and sheep (Herrera *et al.,* 2008; El-Shahat and Abo-El maaty, 2010) fed calcium salt of long chain fatty acids (Ca-LCFA) from palm oil or corn oil in diets. These evidence revealed that consumption of lipids accelerates the follicular growth.

This has been suggested in earlier study (de Fries *et al.,* 1998) that dietary lipid may enhance follicular development via release of metabolic hormones that act on the central nervous system to stimulate GnRH secretion and also through metabolic hormones acting directly at ovarian level. In this context, both insulin and IGF-I are known to stimulate granulose cell proliferation (Webb *et al.,* 1992; Spicer and Echternkamp, 1995). Further, it has also been suggested that the fatty acids themselves and not the additional energy provided that had stimulated the ovarian function (Lucy, 2001). However, there are conflicting results from a number of studies in dairy cattle which indicated no change in follicle diameter (Burke *et al.,* 1996; Robinson *et al.,* 2002; Petit *et al.,* 2002, 2004; Bilby *et al.,* 2006a) when fed with diet supplemented with n-3 than n-6 PUFA.

There was no difference between groups in number of follicles under different class size (small, medium and large) or total number of follicles irrespective of class size counted on day of estrus onset in this study. This finding is in concurrence to Petit *et al*. (2002) who did not find the effect of n-3 PUFA feeding on the mean number of developing follicles. Contrary reports are also available indicating long-chain PUFAs induced

increase in number of total follicles (Robinson *et al.,* 2002; Ambrose *et al.,* 2006). Similarly an increase in number of 5 to 10 mm size follicles was observed in dairy cows that were fed a diet enriched with 5% n-3 PUFA derived from fish oil (Heravi-Moussavi *et al.,* 2007). Other researchers also observed an increase in the total number of follicles following fat supplementation in cattle (Lucy *et al.,* 1991a,b; Wehrman *et al.,* 1991; Thomas and Williams, 1996; Beam and Butler, 1997; Lammoglia, 1997).

5.1.3 Corpus luteum (CL) development

Increase in CL size with fish oil supplementation in this study is in close agreement with previous reports in cattle (Garcia-Bojalil, 1998; Petit *et al.,* 2002; Childs *et al.,* 2008b). Similarly, CL of greater diameter was observed in lactating dairy cows (Garcia-Bojalil, 1998b) were given a diet supplemented with CaLCFA. It has been indicated that larger size of CL could possibly be due to greater size of the ovulatory follicle (Lucy, 2001; Vasconcelos *et al.,* 2001). Antiluteolytic effects of EPA and DHA has been suggested for development larger corpora lutea in cows fed a diet high in omega-3 fatty acids (Petit *et al.,* 2002). Further, it was revealed that fish oil supplemented diet may lower plasma concentration of $PGF_{2á}$, thus inhibition of luteolysis and resulted in larger size of CL (Mattos *et al.,* 2004). The possible mechanism of PUFA's mediated inhibition of luteolysis was suggested from both *in vitro* (Mattos *et al.,* 2003) and *in vivo* (Mattos *et al.,* 2002; Mattos *et al.,* 2004) studies that PUFAs, in particular, n-3 PUFA can possibly alter the synthesis of $PGF_{2á}$ by partial replacement of arachidonic acid from phospholipids pool; through decreased arachidonic acid biosynthesis by inhibition of delta-6 and delta-5 desaturase enzymes that are necessary for conversion of linolenic acid (LA) to Arachidonic acid (ArA); by acting as a direct competitive inhibitor with arachidonic acid for PGHS (Prostaglandin H Synthase), and directly by decreasing gene expression of PGHS. Contrary to our results, some workers have not found such effect of lipid supplementation on size of CL in cows (Petit *et al.,* 2001; Petit *et al.,* 2004; Bilby *et al.,* 2006a; Ghasemzadeh-Nava *et al.,* 2011).

5.1.4 Embryonic development

In the present study, dietary fish oil increased the diameter of embryonic vesicle on day 15 post-insemination with greater length and width of embryo proper in mares on day 28 post-ovulation. Our findings are partially supported by previous observation

in the study of Thangavelu *et al*. (2007) that cows fed diet supplemented with unsaturated fatty acids had greater embryoic development. The possible reason of an alteration in uterine environment that may be more luteotrophic as a result of shift towards synthesis (Childs *et al.,* 2008c) and secretion of less potent PGE_3 than the $PGF_2\alpha$ (Calder, 2006). Recently, Nazir *et al.* (2013) also found a greater development of embryos with better survival rate during the embryonic period in Murrah buffaloes, fed diets supplemented with flaxseed. Beneficial role of n-3 PUFA for embryo survival has also been indicated due to reduced $PGF_{2á}$ secretion as evidenced by studies both *in vitro* (Mattos *et al.,* 2003; Caldari-Torres *et al.,* 2006) and *in vivo* (Cerri *et al.,* 2004; Petit and Twagiramungu, 2006; Thangavelu *et al.,* 2007; Childs *et al.,* 2008b).

Further other studies also indicated an improvement in embryo quality following n-3 PUFA supplementation (Petit *et al.,* 2002; Juschem *et al.,* 2004). Hammiche *et al.* (2011) also reported improved embryo morphology with n-3 PUFA supplements in women. Similarly, Cerri *et al*. (2009) also revealed that dairy cows supplemented with Ca-LCFA from palm oil between days 25 pre-partum and 80 days postpartum yielded a greater proportion of excellent and good quality embryos, in addition to higher number of blastomeres. A lower rate of late embryo loss was reported for dairy cows fed a source of linoleic acid compared with supplementation of saturated fatty acid (Petit *et al.,* 2002). Cerri *et al.* (2004) reported that fertilization rate and the proportion of high quality embryos were higher for super ovulated Holstein Friesian cows fed Ca-LCFA and long chain trans- fatty acids (LTFA) than those fed palm oil. Moreover, less number of embryonic mortality was also observed in dairy cattle fed whole flaxseed (Petit and Berthiaume, 2007).

On the contrary, in an experiment Childs *et al*. (2008b) fed cows with a ration enriched with *n*-3 PUFAs and did not observe any effect on the number of normal or good quality embryos. Similarly, Bilby *et al*. (2006a) did not find an effect of fatty acids in the ration of dairy cows on the quality of embryos after maturation and *in vitro* fertilization. Even the *in vivo* and *in vitro* studies have shown a better embryo development with rations supplemented with lipids, however, results are not consistent and it is important to ascertain in particular, which fatty acids are the most beneficial for embryo survival (Santos *et al.,* 2008).

5.1.5 Plasma estradiol and progesterone concentrations

A higher level of plasma estradiol concentration in fish oil supplemented mares at the onset and day 3rd of estrus is consistent with the findings of Robinson *et al.* (2002) who also observed higher concentrations of estradiol during follicular phase of the estrous cycle in cows supplemented with linolenic acid. The review of literature reveals variable effect of fat supplementation on estradiol concentration in cows viz. increased (Lammoglia *et al.,* 1997; Robinson *et al.,* 2002) and decreased level (Hightshoe *et al.,* 1991) or no change (Beam and Butler, 1997; Sartori *et al.,* 2004; Childs *et al.,* 2008b).

The plasma progesterone concentration in fish oil supplemented mares was inconsistent i.e. increased levels at day of ovulation and 15th day post-ovulation, whereas the levels did not change at day of estrus and 7 day post ovulation. Higher progesterone at day 15 post-ovulation could be due to ovulation of larger follicle as studied in cows supplemented n-3 PUFA that had larger corpora lutea with increased steroidogenic capacity as reported earlier by Mattos *et al.* (2000). Further, it was reported that the cows fed diet high in n-3 PUFAs produced significantly higher quantities of progesterone from luteal cells (Robinson *et al.,* 1998). Moreover, increase in concentration of progesterone could be due to reduced rate of its clearance as evidenced in cows supplemented with fat supplement of CaLCFA which had elevated progesterone even the corpora lutea were removed by ovariectomy (Hawkins *et al.,* 1995). It is also possible that fatty acids present in the fish oil reduced the sensitivity of the corpus luteum to $PGF_{2\alpha}$ that resulted in consistent release of progesterone from the CL as reported in cow (Mattos *et al.,* 2000). However, in contrast, Hinckley *et al.* (1996) observed that incubation of dispersed bovine luteal cells with PUFAs such as EPA and DHA decreased the secretion of progesterone. Thus, the literature related to effect of lipid supplementation on progesterone concentration is inconsistent with reports of increased (Hawkins *et al.,* 1995; Burke *et al.,* 1996; Stronge *et al.,* 2005), decreased (Robinson *et al.,* 2002) or no change in levels (Mattos *et al.,* 2002; Wamsley *et al.,* 2005; Childs *et al.,* 2008b).

5.1.6 Correlation between follicle size at D_{ov}-1 with plasma estradiol and CL size with plasma progesterone

In this study, we could not find correlation between largest ovulatory follicle size with size of CL at days 7th and 15th post ovulation. This finding is contrary to

previous studies in dairy cattle (Lopes *et al.,* 2007; Pfeifer *et al.,* 2009) and buffalo (Pandey *et al.,* 2010) who observed that a large POF generates a larger CL. However, our results are similar to the observations of Lynch *et al*. (2010) who could not find any correlation between POF size and CL diameter.

In the current study, no correlation was observed between the size of CL and circulating plasma progesterone concentration on concurrent days 7 and 15 post-ovulation is in agreement with earlier reports in heifers (Childs *et al.,* 2008a). However, Robinson *et al*. (2005) and Mann (2009) in cows and Pandey *et al.* (2010) in buffaloes observed a positive correlation between CL size and plasma progesterone on day 5, but this relationship was found in significant on day 8th and 12th during luteal phase.

5.1.7 Effect of fish oil supplementation on biochemical parameters

5.1.7.1 Cholesterol concentration

There was no effect of fish oil supplementation on plasma cholesterol concentration in our study. This observation is in line with the results of Byers and Schelling (1988) in cows fed diet supplemented with fish oil. Similarly, Robinson *et al.* (2002) found that cholesterol concentration remained unchanged during the experimental period when cows were fed isoenergic control ration or a diet supplemented with commercially available supplement linpreme high in linolenic acid. Further, fish meal supplementation had no significant effect on cholesterol concentration in cows (Lammoglia *et al.,* 1997; Petit *et al.,* 2002) or buffaloes (Malik *et al.,* 2013). However, there are reports which indicate that serum cholesterol concentration increased during the supplementation period in horses receiving the vegetable oil or corn oil (Siciliano and Wood, 1993; Orme *et al.,* 1997) but not in horses receiving the fish oil (O'Connor *et al.,* 2007). Our finding is in contrast to previous observation which indicated an increase in total cholesterol concentration following fat (Grummer and Carrol, 1991; Hawkins *et al.,* 1995) fish meal (Thomas and William, 1996) or fish oil (Childs *et al.,* 2008b) supplementation in cattle. Increase in concentrations of total cholesterol by n-3 PUFA supplementation was suggested to have an effect on cholesterol synthesis (Childs *et al.,* 2008b). Furthermore, it has been opied that the increase in cholesterol might result at the expense of low-density lipoprotein (LDL) as fish oil supplementation is thought to reduce the activity of cholesterol ester transfer protein which is responsible for transfer of cholesterol esters from HDL to LDL (Nestel, 2000).

5.1.7.2 Triglyceride concentration

Triglyceride concentration was lower in mares given fish oil supplemented diet at different days of estrous cycle in the present study. Consistent with this finding, the studies in rats (Surette *et al.,* 1992; Fickova *et al.,* 1998) and humans (Saynor and Gillott, 1992; Christensen *et al.,* 1999) have demonstrated a decrease in circulating plasma triglycerides associated with n-3 fatty acid supplementation. Further, in horses fed fish oil had lower serum triglycerides compared to horses receiving corn oil at day 63rd of feeding supplement (O'Connor *et al.,* 2007). The exact mechanism responsible for this decrease has not yet been elucidated; however, several researchers have shown that n-3 fatty acids down regulate the enzymes associated with triglyceride synthesis (Marsh *et al.,* 1987; Surette *et al.,* 1992). Others have also reported decreased triglyceride concentrations when horses were fed soybean oil supplemented diets (Orme *et al.,* 1997; Geelen *et al.,* 1999). This decrease was attributed to increased lipoprotein lipase activity and a possible increase in fatty acid oxidation. In horses, very low density lipoprotein (VLDL) particles are the main transporters of triglycerides and contain 57% triglycerides and 15% phospholipids compared with low-density lipoprotein (LDL) particles, which contain 5.5% triglycerides and 22% phospholipids (Watson *et al.,* 1993). Increasing the activity of lipoprotein lipase might increase the clearance of the triglyceride-rich VLDL particles from the bloodstream, resulting in decreased circulating triglycerides. In contrast, others did not find any significant change in triglyceride concentration in cows fed fat in diet (Highshoe *et al.,* 1991; Lammoglia *et al.,* 1996) or buffaloes fed fish meal in diet (Malik *et al*., 2013). On the other hand, increased triglyceride concentrations have been shown in animals fed high lipid diets (Wehrman *et al.,* 1991) or fat infused abomasally (Oldick *et al.,* 1997). The differences in triglyceride concentration might be attributed to variability in metabolic status of selected mares as suggested by others (Wehrman *et al.,* 1991).

5.1.7.3 Non esterified free fatty acids (NEFA) concentration

The decreased Plasma NEFA concentration in fish oil supplemented mares is in concurrence with results of Childs *et al.* (2008b) who reported that increasing fish oil intake decreased the NEFA concentration in cattle. It was suggested that lower levels of NEFA resulted either from greater insulin-induced suppression of lipolysis during n-3 PUFA supplementation and due to an increase in insulin sensitivity that might have

occured due to incorporation of EPA and DHA into membrane phospholipids, resulting in a reduced NEFA release (Lovegrove *et al.,* 1997).

In contrast, cows receiving a high lipid diet during the puerperium exhibited a two to three fold lower concentration of serum NEFA than did cows receiving a normal lipid diet (Spoon *et al.,* 1990). Further, marginal increase of NEFA concentration was reported with consumption of whole sunflower seeds in dairy heifers (Park *et al.,* 1983), differ with either LA or ALA based supplements in lactating dairy cows (Robinson *et al.,* 2002) or heifers either fed dietary whole raw soya beans or fish oil (Childs *et al.,* 2008a). The NEFA profiles in equine blood have been suggested to vary according to the fatty acid composition in their diet (Orme *et al.,* 1994). The disagreement on systemic concentrations of NEFA and differences have been attributed to variances in the metabolic status of the animals employed in the different studies (Childs *et al.,* 2008a).

5.1.8 Effect of fish oil supplementation on conception

In this experiment, a higher conception rate (83.33% vs. 31.81%) in mares of fish oil supplemented group with relatively less number of AI per conception (1.00±0.32 vs. 2.00±0.75) than the control is well supported by earlier reports of cow fed fish meal supplemented in diets (Staples *et al.,* 1998). Similarly, Funston (2004) and Hess *et al.* (2008) observed a higher conception rate in cows supplemented with lipid. A number of other trials suggested that feeding fishmeal to cattle improves the conception rates (Bruckental *et al.,* 1989; Armstrong *et al.,* 1990; Carroll *et al.,* 1994; Burke *et al.,* 1997). Further, de Fries *et al*. (1998) reported that Brahman cows consuming 5.2% lipids in the ration showed a trend towards an increase in pregnancy rate than those cows which consumed only 3.7% lipids in the ration. Moreover, Ferguson *et al*. (1990) observed 2.2 times increase in pregnancy rate in lactating cows consuming 0.5 kg lipid per day. In another study, pregnancy rate at first service was 16% higher in grazing cows supplemented with fat than in cows which did not receive fat in the ration (Bader *et al.,* 2000). Similarly, pregnancy or embryo survival rate after first postpartum AI tended to be higher for dairy cows fed LTFA than those supplemented with palm oil (Juschem *et al.,* 2004).

The ability of n-3 PUFA supplements to delay luteolysis has been proposed for improving fertility by allowing the conceptus longer time to develop before the onset of

luteolysis (Mattos *et al.,* 2004). Diets high in n-3 PUFA may reduce $PGF_{2\alpha}$ synthesis which may prevent regression of the CL, allowing continued secretion of progesterone that may improve embryo survival (Inskeep, 2004, Naddafy *et al.,* 2005). Improvement in fertility of mares in this study might be associated with n-3 PUFA induced inhibition of $PGF_{2\alpha}$ secretion and/or decreased sensitivity of the CL to $PGF_{2\alpha}$ that resulted in reduced degree of embryonic loss in early pregnancy as indicated by Mattos *et al*. (2000) in ruminants. In present study, higher concepation rate was found to be associated with higher progesterone concentration on day 15 post-ovulation without significant change in cholesterol concentration. The higher levels of progesterone in treatment group seem to have resulted from formation of larger CL as reported in buffaloes (Pandey *et al.,* 2010) and/or also from reduced rate of progesterone clearance as opined by Hawkins *et al.* (1995).

5.1.9 Effect of largest follicle size, estradiol concentration, size of corpus luteum and progesterone concentration on outcomes of AI

In this study, there was no association of largest follicle size and estradiol concentration at day of ovulation with mares either conceived or not conceived, however, an association between CL size and progesterone concentration was observed on day 7 and 15 post-ovulation in PUFA non supplemented mares. In contrary to our finding, follicle size was reported to had influence on conception as evidenced in heifers on routine diet that ovulated follicles >10.7 mm in diameter with decreased pregnancy rates compared with heifers that ovulated follicles ≥12.8 mm (Perry *et al.,* 2007).

The serum concentration of estradiol and behavioral estrus influence the pregnancy rate which appears to be mediated through ovulatory follicle size, and management practices that optimize ovulatory follicle size that may improve fertility in heifers (Perry *et al.,* 2007). A delay in progesterone increase in post ovulatory period or low luteal phase after mating has been associated with poor embryonic development and fertility (Mann *et al.,* 1995; Larson *et al.,* 1997; Hommeida *et al.,* 2004). Increased concentration of plasma progesterone has been associated with improved conception rates in lactating ruminants (Staples *et al.,* 1998). The increased levels of progesterone may be achieved with additional cholesterol for its synthesis (Staples *et al.,* 1998) or by reduced rate of its clearance from the blood (Hawkins *et al.,* 1995; Sangritavong *et al.,* 2002). Other workers, however, found no changes in luteal progesterone secretion

following diets containing flaxseed and sunflower seed (Ambrose *et al.,* 2006) or, Menhaden fish meal (Mattos *et al.,* 2002) and fish oil (Bilby *et al.,* 2006b).

5.2 Experiment II : To study the effect of dietary fish oil supplementation on semen quality of Marwari stallions

5.2.1 Color and consistency

No difference was observed either in color or consistency of fresh semen following fish oil supplementation. Milky white to creamy color with variably thin consistency of ejaculate was similar to earlier usual observations in Marwari (Pal *et al.,* 2009) and Kathiawari horses (Ravi *et al.,* 2013) fed their routine diet. Ejaculate color is important and normally it is opaque white color and any deviation from this may indicate contaminants or abnormalities (Jasko, 1992). Common contaminants are urine (yellow color) and blood (red color) that may damage spermatozoa, if left in contact with these cells (Jasko, 1992). In general, creamy appearance of semen was observed very frequently irrespective of horses (Pal *et al.,* 2009) or jack (Gupta *et al.,* 2003; Roy *et al.,* 2003; Legha and Pal, 2012). Consistency of the semen was reported thick to thin in Poitou (Gupta *et al.* 2003) and Indian donkeys (Legha *et al.,* 2013). However, consistency may range from watery to creamy depending upon spermatozoa concentration in the semen sample and the creamy appearance of an ejaculate is an indication of more number of sperm, while a watery ejaculate often had fewer sperm cells (Kuklin, 1993).

5.2.2 Semen volume

Total volume of ejaculate including gel in semen as well as gel free semen volume was not affected by n-3 PUFA supplementation. This finding is in agreement with the previous results in chicken (Cerolini *et al.,* 2006), turkey (Zaniboni *et al.,* 2006), goat (Dolatpanah *et al.,* 2008), buffalo (Adeel *et al.,* 2009), rabbit (Gliozzi *et al.,* 2009) and pig (Yeste *et al.,* 2011). Similarly, total semen volume did not differ in stallions (Brinsko *et al.* 2005) and Holstein bulls (Gholami *et al.,* 2010) fed DHA enriched nutriceutical or diet supplemented with fish oil in ram (Fair *et al.,* 2014). However, Strzezek *et al*. (2000) indicated that unsaturated fatty acid supplementation had positive effect on semen volume in boars. An average stallion generally produced 100 ml semen of which 20 to 40 ml was gel and 60 to 80 ml useful gel free semen (Dowsett and Pattie, 1982; Pickett *et al*., 1988; Rickets, 1993). Further, Ghei *et al*. (1994) reported a total

seminal volume varying between 60 to 120 ml and gel free seminal fraction from 50-100 ml in Indian stallions. The mean of total ejaculate volume as well as gel and gel free fraction in the present study was within the range and similar to earlier reports varying between 20 to 70 ml in stallions (Rodriguez-Martinez, 1996). Wide range in semen volume could be due to individual variations in semen production (Picket *et al*., 1976; Picket and Shiner, 1994) and the difference in teasing time (Ionata *et al*., 1991) which is largely due to an increase in the gel free fraction. Total ejaculate volume is found to increase through accessory gland secretion by excessive sexual stimulation of the stallion prior to collection (Pickett *et al.,* 1987). Some stallions rarely produce gel, whereas, some may produce extremely large quantities, particularly in the first ejaculate but the presence or absence of gel appears to have no effect on fertility (Pickett *et al.,* 1989).

5.2.3 Seminal fluid pH

There was no difference in mean value of seminal pH between the PUFA supplemented and non supplemented horses. No literature could be retrieved for effect of PUFA on seminal pH though a normal pH value 7.2±0.02 in Marwari stallions and 7.17±0.02 in Poitou donkeys was observed with normal diet (Pal *et al.,* 2009). Heckenbichler *et al.* (2011) reported a wider range of seminal pH i.e. from 6.0 to 7.5 in stallion semen. The measurement of pH is influenced primarily by the variation in secretions from the accessory sex glands. The pH of the accessory sex glands secretion is alkaline and it tended to more alkaline with increase in ejaculation frequencies. A high pH also suggests an ejaculatory failure apart from urine or infection in the ejaculate. Measurement of pH could be beneficial in determining if the first ejaculate was complete (McKinnon, 1996). Stallions under heavy exercise or workload tended to have lower pH than without workload (Hartlova *et al.,* 2013). Exposure to lubricant like KY jelly reported to cause marked decrease in seminal pH (Limone *et al.,* 2002). A lower pH than the reference range (6.0 to 7.5) may adversily affect spermatozoa quality (Moce and Graham, 2008) as acidity may alter motility and viability (VanDuijin and Hendriske, 1968). Seminal pH was also reported to have correlation with live: dead sperm ratio and to progressive sperm motility (Dogan *et al.,* 2009).

5.2.4 Sperm motility

The finding that total and progressive sperm motility did not improve following dietary n-3 PUFA supplementation is consistent with earlier reports in stallion (Brinsko *et al.,* 2005, Harris *et al.,* 2005), boar (Paulenz *et al.,* 1999; Strzezek *et al.,* 2004, Yeste *et al.,* 2011), broiler chickens (Surai *et al.,* 2004) and turkey (Zaniboni *et al.,* 2006) which indicated no evidence of a positive effect of dietary long chain fatty acids supplementation on fresh semen motility. Similarly, the motility of spermatozoa in the fresh semen did not differ in sunflower enriched diet given to buffaloes (Adeel *et al.,* 2009) or with fish supplementation in ram (Fair *et al.,* 2014). However, Gholami *et al.* (2010) found higher total and progressive sperm motility assessed by CASA in Holstein bulls fed DHA enriched nutriceuticals. Not able to observe any difference in sperm motility might be due to human errors in addition to the fact that the percentage of motile spermatozoa was subjectively assessed to the nearest 5% by analyzing various fields of view. Therefore, differences less than 5% of accuracy could not be well demonstrated in the present study.

Our results do not support the findings of other researchers in human (Conquer *et al.,* 2000), boar (Rooke *et al.,* 2001), goat (Dolatpanah *et al.,* 2008), and sheep (Towhidi *et al.,* 2008) who reported a significant correlation between dietary DHA supplementation and the number of motile spermatozoa. The values for both total and progressive sperm motility in our study are in compliance to values (79.76 and 73.33%, respectively) observed in Marwari stallions (Pal *et al*. 2009) and comparable to values (82.0±1.51 and 77.0±1.51, respectively) in Kathiawari horses (Ravi *et al.,* 2013). Evaluation of sperm cell motility is considered important as an indicator of fertilizing capacity (Varner, 2008). The percentage of motile spermatozoa and in particular those showing progressive motility, is a good indicator of the number of viable spermatozoa. The correlation between motility and morphologically normal spermatozoa is reported to be 0.63 (Long *et al*., 1983). Semen samples within excess of 40-50% progressively motile spermatozoa are considered to be appropriate for use in artificial insemination programme in equines (Pickett *et al*., 1988; Fayrer-Hosken and Caudle, 1989: Davis-Morel, 1990; Colenbrander *et al*., 1992; Rickets, 1993; Braun *et al*., 1993).

5.2.5 Sperm concentration

Mean sperm concentration in present study also remained similar in stallions with or without supplementation of dietary n-3 PUFA. These results are supported by earlier reports in chicken (Cerolini *et al.,* 2006), turkey (Zaniboni *et al.,* 2006), buffalo (Adeel *et al.,* 2009), rabbit (Gliozzi *et al.,* 2009), Holstein bulls (Gholami *et al.,* 2010) and pig (Yeste *et al.,* 2011). In contrary, Strzezek *et al.* (2000) indicated positive effect of unsaturated fatty acid supplementation on the sperm number in boar. Higher sperm concentration and total sperm production in boar (Rooke *et al.,* 2001) and stallion (Brinsko *et al.,* 2005, Harris *et al.,* 2005) are well documented with feeding of a source of omega-3 fatty acids. Mean sperm concentration in ejaculates of stallions being fed the nutriceuticals was 1.8 times higher than when stallions were fed the control diet (Brinsko *et al.,* 2005). Further, supplementation with fish oil in ram increased the sperm concentration (Fair *et al.,* 2014). Mean sperm concentration in horses of this study was comparable to its concentration (173.75±8.86 x 10^6 ml^{-1} and 192.0±9.3x 10^6 ml^{-1}) in Marwari stallions (Pal *et al*., 2009) and Kathiawari stallions (Ravi *et al.,* 2013), respectively. The values for sperm concentration in horse semen samples were reported to vary widely, ranging from 100 to 300×10^6 ml^{-1} (Pickett *et al*., 1988; Rickets, 1993). All values within the range may be considered acceptable and appropriate for use in artificial insemination programme. Calculating the sperm concentration together with knowledge of semen volume is important for determining the total sperm production and to decide the number of insemination doses (Sieme, 2009).

5.2.6 Live sperm count

Live sperm count both in fresh and frozen semen samples did not differ before and after supplementation of n-3 PUFA in the current study. This observation is supported by results of Brinsko *et al.* (2005) in stallions who did not observe any difference in percentage of live sperm in frozen–thawed and in fresh semen samples of pig (Yeste *et al.,* 2011) when they were fed the nutriceutical high in DHA. Further, Grady *et al.* (2009) also failed to observe any significant effect with n-3 PUFA on the percentage of live spermatozoa in fresh, cooled and frozen/thawed semen in stallions. On the other hand, increased number of viable sperm cells in fresh semen was reported with DHA

supplementation in bulls (Gholami *et al.,* 2010), boar (Rooke *et al.,* 2001) and Turkey (Zaniboni *et al.,* 2006).

5.2.7 Sperm morphology

In this study, there was no effect of n-3 PUFA supplementation on count of abnormal sperm percent in fresh semen; however, it tended to be lower after dietary supplementation of n-3 PUFA. Our results are in line with study of Grady *et al.* (2009) who also reported no change in morphologically normal spermatozoa percent in fresh, cooled and frozen/thawed semen of horses supplemented with n-3 PUFA. Similarly, Strzezek *et al.* (2004) also indicated that unsaturated fatty acid supplementation did not affect sperm percent with abnormal morphology. Moreover, fish oil supplementation with excess vitamin E also failed to improve percent sperm with normal morphology in ram (Dolatpanah *et al.,* 2008).

In contrary, dietary fat supplementation was found to increase the percentage of sperm with normal morphologies in human (Zalata *et al.,* 1998) and boar (Strzezek *et al.,* 2004; Yeste *et al.,* 2011). Further, Rooke *et al.* (2001) has also reported that diet supplemented with tuna fish oil with excess antioxidants in boar increased the population of spermatozoa with normal morphology. Similarly, DHA enriched supplement in stallions resulted in reduced percentage of abnormal spermatozoa especially the acrosome and midpiece abnormalities (Elhordoy *et al.,* 2008). It is well established that fertility get affected if the number of sperms with morphological defects are excessive (Rouge, 2014). Sperm morphology has also been extensively used to assess semen quality. Percent of morphologically normal spermatozoa is considered to be indicator of fertility as reported in bull (Wood *et al.,* 1986; Gravaance and Devis, 1995) and stallion (Jasko *et al.,* 1990; Gravaance and Devis, 1995).

5.2.8 Sperm post thaw motility (PTM)

The post thaw sperm motility was not affected by supplementation with n-3 PUFA in our experiment. This is consistent with previous reports in pigs (Paulenz *et al.,* 1999; Maldjian *et al.,* 2005; Castellano *et al.,* 2010), rabbits (Gliozzi *et al.,* 2009), and stallions (Grady *et al.,* 2009) who also did not find any improvement of stored

semen quality when the diet was supplemented with n-3 PUFA. There were no significant difference in the percentage of total or progressive motility of cooled (24 h or 48 h) or frozen–thawed spermatozoa with dietary supplementation of n-3 fatty acids and vitamin E in stallion (Gee *et al.,* 2010). The post-thawed sperm motility data obtained by subjective assessment and with CASA did not differ either fed or not the DHA enriched nutriceutical in Holstein bull (Gholami *et al.,* 2010).

In contrast, Kaeoket *et al*. (2010) reported that supplementation of the semen extender with DHA by adding fish oil was effective for freezing boar semen and resulted in higher post-thaw sperm motility. In buffalo bulls, diets containing long-chain PUFA (sunflower oil) improved post-thaw sperm quality (Adeel *et al.,* 2009). In another studies, feeding a DHA-enriched nutriceutical improved the motility of stallion spermatozoa after 48 h of cryopreservation (Brinsko *et al.* 2005; Elhordoy *et al.,* 2008) and in frozen-thawed semen (Harris *et al.,* 2005). Mean value of PTM percent was within the range (30 to 45) as reported in Kathiawari horses (Ravi *et al.,* 2013). A wide variation in post thaw motility from 6.25 to 47.5% was reported in Marwari stallions (Pal *et al.,* 2011). The commercially used stallion frozen semen with atleast 30% of progressive sperm motility was considered acceptable for insemination (Samper, 1995).

5.2.9 Hypo osmotic swelling test (HOST)

No difference in HOS positive sperm number with n-3 PUFA supplementation is consistent with the reports of Gholami *et al.* (2010) who also not observed any change in post-thawed HOS positive sperm supplemented with DHA enriched nutriceutical; however, it improved HOS-positive proportion of spermatozoa in fresh semen of bulls. In contrast to our findings, feeding PUFA to pigs has been shown to increase the proportion of membrane intact spermatozoa (Strzezek *et al.,* 2004). Similarly, it has also been reported that dietary n-3 PUFA was transferred into chicken sperm membrane by fish oil feeding (Cerolini *et al.,* 2006). Kaeoket *et al.* (2010) reported that supplementation of the semen extender with DHA by adding fish oil was effective for freezing boar semen as it resulted in higher post-thaw plasma membrane integrity. Moreover, Adeel *et al.* (2009) in buffaloes also observed higher number of HOS positive spermatozoa in frozen-thawed semen fed with sunflower-enriched diets

but did not find such difference in the fresh semen. In a study, Selvaraju *et al*. (2012) examined the effect of different sources of dietary energy and fed rams either with maize or sunflower oil diet (linoleic acid) and found that PUFA enrichment influenced sperm quality by stabilizing membrane integrity. Our observation of HOS reacted sperm both before and after n-3 PUFA supplementation is comparable to value 30.2±10.1 in horses (Neild *et al.*, 1999).

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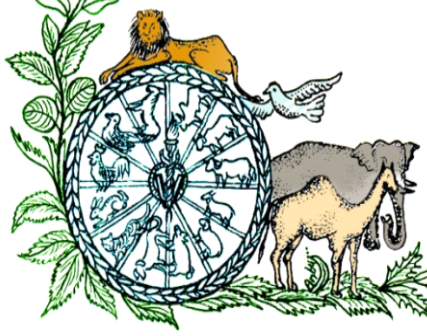
Summary and Conclusions



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SUMMARY & CONCLUSIONS

The present study was designed with three objectives (i) to study the effect of dietary n-3 PUFA supplementation on follicular, luteal and embryonic development in Marwari mares, (ii) to correlate the size of pre ovulatory follicle and corpus luteum with plasma estradiol and progesterone concentration and (iii) to study the effect of dietary n-3 PUFA supplementation on semen quality of Marwari stallion.

Experiment I : To study the effect of dietary fish oil supplementation rich in n-3 PUFA on follicular, luteal and embryonic development in Marwari mares

A total of twenty mares, either offered a routine diet (n=10; group A) or given fish oil @0.25 ml/kg BW to supplement n-3 PUFA in routine diet (n=10; group B) daily for a period of 70 days, respectively. The number of follicles was counted on day of estrus onset (D0) as well as diameter of the largest follicle was measured using trans-rectal ultrasonography on day 0 (D0), 2 (D2), 4 (D4), 5 (D5) day of estrus and one day before ovulation (D_{ov-1}) in experimental mares. Size of corpus luteum was measured subsequently on 7 and 15 day post-ovulation ($D7_{PO}$ and $D15_{PO}$, respectively). Blood samples were collected on D0, 3 (D3), 6 (D6) day of estrus, on day of ovulation (D_{ov}), $D7_{PO}$ and $D15_{PO}$. The plasma levels of estradiol, progesterone, cholesterol, triglyceride and non esterified fatty acids (NEFA) were estimated. Mares in both groups were inseminated with frozen semen at estrus in their second and subsequent cycle after start of feeding respective diets. Pregnancy diagnosis was performed on $D15_{PO}$ and if found pregnant, the diameter of embryonic vesicle was measured on 15, 22, 28, 35 and 42 day post ovulation. Length and width of developing embryo proper was also measured on 28, 35 and 42 day post ovulation. Conception rate and number of AI per conception was calculated in experimental mares.

There was no significant effect of fish oil supplementation on length of estrous cycle (25.87±0.58 vs. 24.93±0.67 days), however, estrus duration decreased significantly ($P<0.05$) in mares of Gr. B than A (7.45±0.2 vs. 8.26±0.20 days).

The diameter of largest follicle on D0 (26.27± 0.32 vs. 24.08± 0.87 mm; $P<0.05$) and subsequently on D2 (30.14±0.54 vs 27.08±0.70 mm; $P<0.01$), D4 (33.92±0.57 vs. 31.08±0.77 mm; $P<0.01$), D5 (40.00±0.40 vs. 37.44±0.60 mm; $P<0.01$), D_{ov}-1 (48.03±1.43 vs. 42.42±1.12 mm; $P<0.01$) was significantly higher in mares of Gr. B than A. However, number of small (6-10mm; 1.70±0.61 vs. 0.70±0.43), medium (10.1-25mm; 2.20±0.47 vs. 2.20±0.20) and large (>25 mm; 0.30±0.19 vs 0.50±0.21) size follicles or follicles in total (4.20±0.51 vs. 3.40±0.45) irrespective of class size on D0 did not differ significantly between the groups.

The diameter of CL was significantly ($P<0.01$) larger on $D7_{PO}$ (31.01±0.8 vs. 26.76±0.8 mm) and $D15_{PO}$ (35.48±0.86 vs. 23.73±1.0 mm) in fish oil supplemented mares. Further, there was significant ($P<0.01$) increase in CL size from $D7_{PO}$ to $D15_{PO}$ (31.01±0.8 vs. 35.48±0.86 mm) in group B, however, it did not differ significantly (26.76±0.8 vs. 23.73±1.0 mm; $P>0.05$) in group A. The effect of fish oil supplementation on CL size as well as interaction between group x day of CL size was significant ($P<0.001$), though, effect of days was not significant ($P>0.05$).

Dietary fish oil supplementation significantly ($P<0.01$) increased the diameter of embryonic vesicle on 15 day post-ovulation (21.41±0.71vs. 15.27±1.25 mm), however, it was not significant on 22 (27.20±1.32 vs. 26.99±1.29 mm), 28 (31.67±0.8 vs. 30.33±0.54 mm) 35 (44.04±0.8 vs. 43.31±1.41 mm) and 42 (57.77±1.4 vs. 55.90±1.8 mm) day post ovulation in group B and A, respectively. Embryonic vesicle diameter increased significantly ($P<0.01$) during the experimental period across the day 15, 22, 28, 35 and 42 post ovulation in mares of both the groups except no significant ($P>0.05$) from day 22 to 28 post ovulation in group A. Length and width of embryo proper was also significantly ($P<0.05$) higher on day 28 post-ovulation in group B (15.77±0.88 and 7.76±0.29 mm) than group A (12.93±1.08 and 6.03±0.34 mm). In both groups, increase in length of embryo proper was significant ($P<0.01$) across the days 28, 35 and 42 post-ovulation (12.93±1.08, 20.89±1.56, 30.11±1.68 mm and 15.77±0.88, 22.40±1.16, 35.15±2.10 mm, respectively). Similarly, there was significant ($P<0.01$) increase in width of

embryo proper in both Gr. A and B (6.03±0.34, 10.87±1.04, 16.40±0.92 mm and 7.76±0.29, 11.46±0.33, 18.45±1.16 mm, respectively). Effect of days post ovulation on diameter of embryonic vesicle as well as length and width of embryo proper was significant ($P<0.001$). However, the effect of treatment group and the two-way interaction (group X day) failed to achieve significance for all three observations ($P>0.05$).

Mean plasma estradiol concentration was significantly higher on D0 (27.63±3.42 vs. 17.76±2.38 pg/ml; $P<0.05$) and D3 (27.87±3.42 vs. 15.68±2.92 pg/ml; $P<0.01$) in fish oil supplemented mares than control. Plasma estradiol did not differ significantly on $D7_{PO}$ (15.81±0.75 vs. 19.85±3.25 pg/ml) and tended to approach significance ($P=0.059$) with higher value on D_{ov} (31.30±4.08 vs. 22.92±3.5 pg/ml), only in fish oil supplemented group. Plasma estradiol concentration did not vary significantly across the days of sampling D0, D3, D_{ov} and $D7_{PO}$ (17.76±2.38, 15.38±2.92, 22.92±3.50 and 19.85±3.25 pg/ml, respectively) in group A, whereas, in mares of Gr. B, the concentration was significantly ($P<0.05$) lower at $D7_{PO}$ (15.81±0.75 pg/ml) as compared to D0, D3 and D_{ov} (27.63±3.42, 27.87±3.42 and 31.30±4.08 pg/ml, respectively). There was significant effect of treatment group ($P<0.01$), day of sampling ($P<0.05$) as well as the group X day interaction ($P<0.05$) on plasma estradiol concentration.

Plasma progesterone concentration was significantly ($P<0.01$) higher in mares of Gr. B than A on D6 (0.51±0.1 vs. 0.11±0.01 ng/ml) and $D15_{PO}$ (4.50±0.4 vs. 2.04±0.7 ng/ml), whereas its concentration between groups did not differ on D0 (0.94±0.65 vs. 0.42±0.19 ng/ml) and $D7_{PO}$ (4.79±0.99 vs. 4.57±0.58 ng/ml), respectively. The level of progesterone was significantly ($P<0.05$) higher during luteal phase at $D7_{PO}$ (4.57±0.58 and 4.79±0.99 ng/ml) and $D15_{PO}$ (2.04±0.7 ng/ml and 4.50±0.4 ng/ml) compared to follicular phase on D0 (0.42±0.19 and 0.94±0.65 ng/ml) and D6 (0.11±0.01 and 0.51±0.1 ng/ml) in group A and B, respectively.

The correlation of estradiol concentration on day of ovulation (D_{ov}) and largest ovulatory follicle size one day before ovulation (D_{ov-1}) was highly significant ($r=0.958$; $P<0.001$) in group B, whereas in group A, the correlation was moderate and approached to significance ($r=0.531$; $P=0.057$). Further, the correlation of progesterone concentration with CL size on $D15_{PO}$ was significant ($r=574$; $P<0.05$)

and tended to approach significance on $D7_{PO}$ (r=0.706, P=0.058) in mares of group B, whereas no significant correlation was observed in group A. There was no correlation (P>0.05) between follicle size at D_{ov}-1 and CL size ($D7_{PO}$ and $D15_{PO}$) in both the groups.

Conception rate was significantly (P<0.01) higher in mares of Gr. B (83.33%) than that of group A (31.81%), with lesser number of AI per conception 1.00±0.32 vs. 2.00±0.75, respectively. Retrospective investigation for association of the largest follicle on D_{ov-1}, estradiol concentration on $D_{ov,}$ CL size and progesterone concentration on $D7_{PO}$ and $D15_{PO}$ a revealed a greater CL size on $D7_{PO}$ (31.09±2.05 vs. 24.99±0.21 mm) and $D15_{PO}$ (23.61±2.43 vs. 14.27±0.59 mm) as well as progesterone concentration on $D7_{PO}$ (4.88±0.69 vs. 2.83±0.59 ng/ml) in conceived than that of non conceived mares (P<0.05, P=0.05; P<0.01).

The mean plasma cholesterol concentration did not differ significantly (P>0.05) between the groups at different days of sampling. Further, cholesterol concentration was also not significantly not different (P>0.05) at D0, D3, D6, $D_{ov,,}$ $D7_{PO}$ and $D15_{PO}$ (98.99±9.95, 96.30±7.67, 109.10±10.43, 102.22±8.87, 95.64±8.03 and 98.63±11.14 mg/dl, respectively) in mares of Gr. A. However, its concentration was significantly (P<0.05) higher on D0, D3 and D6 (103.84±2.52, 106.63±7.90 and 103.37±5.12 mg/dl, respectively) as compared to $D15_{PO}$ (88.0±4.04 mg/dl) in mares of Gr. B. The effect of group and day as well as group-by-day interaction on plasma cholesterol concentration was non-significant (P>0.05).

Plasma triglyceride concentration was significantly (P<0.05) lower in mares of group B at D3 (46.62±4.24 vs. 60.83±3.76 mg/dl), D6 (32.34±3.65 vs. 52.82±3.72 mg/dl) and D_{ov} (47.43±3.23 vs. 56.26±2.17 mg/dl), as compared to group A, whereas no significant (P>0.05) difference was observed on D0 (43.87±3.45 vs. 54.53±4.71 mg/dl), $D7_{PO}$ (38.88±2.70 vs. 47.02±3.23 mg/dl) and $D15_{PO}$ (42.75±3.20 vs. 51.05±3.44 mg/dl). Further, triglyceride concentration was significantly (P<0.05) lower at D6 (32.34±3.65 mg/dl) as compared to D0, D3, D_{ov} and $D15_{PO}$ (43.87±3.45, 46.62±4.24 and 42.75±3.20 mg/dl, respectively) but not different from value at $D7_{PO}$ (38.88±2.70) in group B. No difference in plasma triglyceride level except a significant (P<0.05) lower concentration on $D7_{PO}$ than D3 (47.02±3.23 vs. 60.83±3.76 mg/dl) was observed in group A. Effect of group

and day was significant (P<0.001 and P<0.01, respectively), however, the group-by-day interaction was non-significant (P>0.05).

Mean plasma NEFA concentration was also significantly (P<0.05) lower in mares of Gr. B on D3 (318.20±7.70 vs. 349.68±10.47 µg/dl), D6 (339.74±9.78 vs. 383.19±18.47 µg/dl) and $D7_{PO}$ (323.90±7.85 vs. 351.74±10.77 µg/dL) than group A, respectively. However, there was no difference at D0 (335.74±7.78 vs. 365.51±17.22 µg/dl), $D15_{PO}$ (349.29±12.85 vs. 339.45±6.35 µg/dl). NEFA concentration was significantly (P<0.05) higher at D6 (383.19±18.47 µg/dl) as compared to D_{ov} and $D15_{PO}$ (329.70±9.58 and 339.45±6.35 µg/dl) in mares of group A. On the other hand, NEFA concentration was significantly (P<0.05) higher at D_{ov} (378.84±18.72 µg/dl) as compared to D0, D3, D6 and $D7_{PO}$ (335.74±7.78, 318.20±7.79, 339.74±9.78 and 323.90±7.85 µg/dl, respectively) in mares of Gr. B. Effect of group and day was not significant, however, the group-by-day interaction was significant (P<0.01).

Experiment II : To study the effect of dietary fish oil supplementation rich in n-3 PUFA on semen quality of Marwari stallions

'Appearance of fresh ejaculate was milky white to creamy with variably thin consistency. Mean volume of total, gel in semen and gel free semen (60.75±6.17 vs. 58.92±5.38 ml, 20.64±3.24 vs. 18.05±2.70 and 40.11±3.85 vs. 40.86±3.74) did not differ significantly (P>0.05) before and after fish oil supplementation, respectively. Similarly, there was no significant effect of fish oil supplementation on mass sperm motility (79.58±2.26 vs. 76.39±2.63%), progressive sperm motility (72.50±2.67 vs. 70.14±2.85%), seminal pH (7.25±2.67 vs. 7.14±0.28), sperm concentration (182.0±11.89 vs. 188.24±12.94 $x10^6$), live sperm (79.97±1.40 vs. 78.97±1.96%) and abnormal sperm (11.42±0.68 vs. 10.39±0.86%) in fresh semen. In frozen semen samples, sperm PTM (38.83±1.85 vs. 39.63±2.08), live sperm (51.70±2.96 vs. 51.70±2.06) and HOST reacted sperm (35.22±1.86 vs. 34.44±1.78%) before and after fish oil supplementation was not significant (P>0.05), however, the difference tended to approach significance (P=0.063) with respect to abnormal sperm which is lower in fish oil supplemented stallions as compared to non-supplemented (12.63±0.96% vs. 14.23±0.69%).

From this study it can be condluded that fish oil supplementation @0.25 ml/kg BW :

1. Improves the follicular luteal and embryonic development as well as fertility rate in mares, when given 10 weeks.
2. Increases the size of pre-ovulatory follicle and corpus luteum with increased estradiol and progesterone secretory capacity, respectively.
3. Did not alter the seminal parameters under investigation when diet in breeding stallion was supplemented for 14 weeks.

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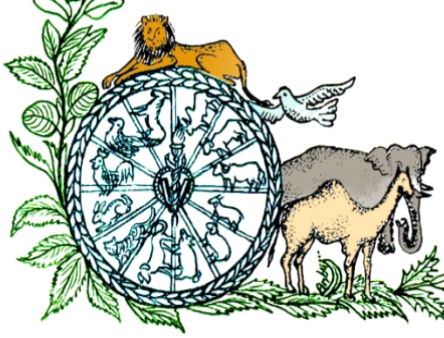
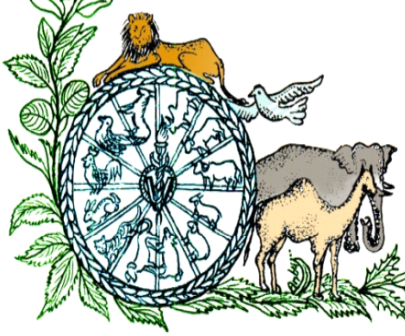
Mini Abstract



7 MINI ABSTRACT

The present study was conducted to investigate the effect of dietary fish oil, a rich source of n-3 PUFA on development of follicle (number and size), corpus luteum (CL) and conceptus as well as changes in plasma estradiol, progesterone, metabolites (cholesterol, triglyceride, NEFA) and conception rate in mares [experiment I; n=10 each in group A (routine diet) and B (fish oil supplemented in diet @0.25 ml/kg BW daily) for 70 days]. Blood sampling was done on day of estrus (D0), 3, 6 of estrus, day of ovulation (D_{ov}) and 7, 15 day post ovulation (PO). In stallions, influence of n-3 PUFA [(experiment II; 6 stallions x 6 ejaculates from each stallion before and after fish oil supplementation @0.25 ml/kg BW daily) for 14 weeks] was examined on various seminal parameters. There was no significant ($P>0.05$) effect of dietary fish oil supplementation on total number of follicles in different class size (small, medium, large) or irrespective of class size counted on D0, whereas diameter of follicle was significantly greater on D0 ($P<0.05$) and day 2, 4, 5 of estrus ($P<0.01$); and one day before ovulation (D_{ov-1}; $P<0.01$). There was significant ($P<0.01$) increase in CL diameter both on 7 and 15 day PO with fish oil supplementation. Plasma estradiol was significantly higher on D0 ($P<0.05$), day 3 of estrus ($P<0.01$) and tended to rise on D_{ov} ($P=0.05$). Plasma estradiol concentration on D_{ov} had significant ($P<0.001$) positive correlation with ovulatory follicle diameter on D_{ov-1} in mares of Gr. B, whereas the positive correlation between two reached to significance ($P=0.05$) in group A. Progesterone concentration was significantly ($P<0.01$) higher on 15 day PO and had significant ($P<0.05$) positive correlation with concurrent CL size in mares of Gr. B. There was no correlation ($P>0.05$) between ovulatory follicle size one day before ovulation and the subsequent CL size on 7 and 15 day PO in either group A or B. There was significant increase in diameter of embryonic vesicle ($P<0.01$) on 15 day PO as well as length and width of embryo proper ($P<0.01$) on 28 day PO. Conception rate was significantly ($P<0.01$) higher with less number of AI per conception in mares of Gr. B. The concentration of cholesterol did not alter significantly ($P>0.05$), whereas significant ($P<0.05$) decrease in triglyceride and NEFA was inconsistent with respect to day of sampling in mares of Gr. B. The effect of fish oil supplementation was not evident on fresh (semen volume, color, consistency, pH, total and progressive sperm motility, sperm concentration, live and abnormal sperm percent) and frozen semen (post-thaw sperm motility, hypo osmotic swelling reacted sperm, live and abnormal sperm percent) parameters. From this study, it was concluded that dietary fish oil supplementation increased the size of follicle, CL, embryonic vesicle, length and width of embryo proper, plasma estradiol and progesterone, with higher conception rate and less number of AI per conception in mares. However, the fish oil supplementation did not improve fresh and frozen semen quality in stallion under this study which needs further investigation.

लघु सारांश



8 लघु सारांश

वर्तमान अध्ययन में मछली के खाद्य तेल, औमेगा–3 वसा अम्ल के एक प्रचुर स्त्रोत का मादा अश्व की अंडाशय पुतिका (संख्या एवं आकार), पीत–पिण्ड तथा भ्रूण पुटिका के विकास एवं प्लाज्मा एस्ट्राडाइओल, प्रोजेस्टेरॉन हारमोन, मेटाबोलाइट्स (कॉलेस्ट्राल, ट्राइग्लिसराइड, नेफा) और उनकी गर्भाधान दर पर प्रभाव का विवेचन किया गया। [प्रयोग–I: समूह 'अ' (सामान्य आहार) तथा 'ब' (मछली का तेल पूरक आहार 0.25 मि.ली. प्रति कि.ग्रा. शरीर भार प्रतिदिन) प्रत्येक में 10 मादा अश्व 70 दिनों के लिए] रक्त के नमूने का चयन मद के शुरू होने के दिन, मद के 3, 6 और डिम्बक्षरण के दिन तथा डिम्बक्षरण के 7 और 15 दिन पश्चात किया गया। नर अश्व में औमेगा–3 फेटी एसिड का प्रभाव (प्रयोग II: 6 नर अश्व X 6 वीर्य) नमूने, प्रत्येक नर अश्व से मछली तेल का पूरक आहार @0.25 मि.ली. प्रति कि.ग्रा. शरीर भार प्रतिदिन देने के पहले तथा बाद में 14 सप्ताह तक वीर्य के विभिन्न मापदंडों का परीक्षण से पता लगाया गया। मछली के तेल के पूरक आहार का अंडाशय की पुटिका के विभिन्न वर्ग आकार (छोटे, मध्यम तथा बड़े) की संख्या और वर्ग से निरपेक्ष कुल संख्या पर कोई सार्थक प्रभाव नहीं देखा गया। जबकि, अंडाशय पुटिका का व्यास मद के शुरू, और 2, 4, 5 दिन में एवं डिम्वक्षरण के एक दिन पहले महत्वपूर्ण रूप से अधिक पाया गया। मछली के तेल पूरक आहार से डिम्बक्षरण के 7 एवं 15 दिन पश्चात पीत–पिण्ड के व्यास में सार्थक रूप से वृद्धि देखी गई। प्लाज्मा एस्ट्राडाइओल मद के शुरू के और तीसरे दिन महत्वपूर्ण रूप से अधिक था एवं डिम्बक्षरण के दिन में वृद्धि की प्रवृति देखी गई। समूह 'ब' में की मादा अश्व में प्लाज्मा एस्ट्राडाइओल की मात्रा का डिम्बक्षरण पुटिका के साथ महत्वपूर्ण साकारात्मक सह सम्बन्ध पाया गया। जबकि समूह 'अ' में दोनों में साकारात्मक सह–संबंध महत्व तक पहुंचा। समूह 'ब' की मादा अश्व में डिम्बक्षरण के 15 दिन पश्चात् प्रोजेस्ट्रान की मात्रा महत्वपूर्ण रूप से अधिक थी तथा इसका समवर्ती पीत–पिण्ड के आकार से महत्वपूर्ण साकारात्मक सह–सम्बन्ध था। समूह 'अ' अथवा 'ब' में डिम्बक्षरण के एक दिन पहलेडिम्बक्षरण पुटिका के आकार तथा डिम्बक्षरण के 7वें एवं 15वें दिन के पित पिंड के आकार में कोई सह–संबंध नहीं था। डिम्बक्षरण के 15वें दिन भ्रूण पुटिका के व्यास साथ ही भ्रूण विशिष्ट की लंबाई तथा चौड़ाई डिम्बक्षरण के 28वें दिन महत्वपूर्ण रूप से अधिक थी। समूह 'ब' की मादा अश्व में प्रति गर्भ कम कृत्रिम गर्भाधान के साथ गर्भाधान की दर महत्वपूर्ण रूप से अधिक थी। समूह 'ब' की मादा अश्व में कोलेस्ट्राल की मात्रा पर कोई प्रभाव नहीं देखा गया जबकि ट्राइग्लिसराइड एवं नेफा में कमी रक्त के नमूने के दिनों की तुलना में असंगता थी। मछली के तेल के पूरक आहार से ताजा (वीर्य परिमाण, रंग, घनापन, पी.एच. कुल एवं प्रगतिशील शुक्राणु गतिशीलता, जीवित एवं आसामान्य शुक्राणु प्रतिशत) एवं हिमीकृत वीर्य (पिघलाने के पश्चात शुक्राणु गतिशीलता), कम परासरणी सूजन प्रतिकृत शुक्राणु, जीवित एवं असामान्य शुक्राणु प्रतिशत के मापदण्डों में कोई सार्थक प्रभाव स्पष्ट नहीं हुआ। इस अध्ययन से यह निष्कर्ष निकला कि मछली के तेल पूरक आहार से मादा अश्व में अंडाशय की पुटिका का आकार, पीत–पिण्ड, भ्रूण पुटिका, भ्रूण विशिष्ट की लम्बाई एवं चौड़ाई, प्लाज्मा एस्ट्राडाइओल एवं प्रोजेस्ट्रान हारमोन में वृद्धि हुई, साथ ही गर्भाधान दर में सार्थक रूप से वृद्धि ज्ञात हुई। तथापि मछली के तेल पूरक आहार से बीजाश्व के ताजा एवं जड़ीकृत वीर्य की गुणवता में कोई सुधार नहीं पाया गया, जिसके आगे अन्वेषण की आवश्यकता है।

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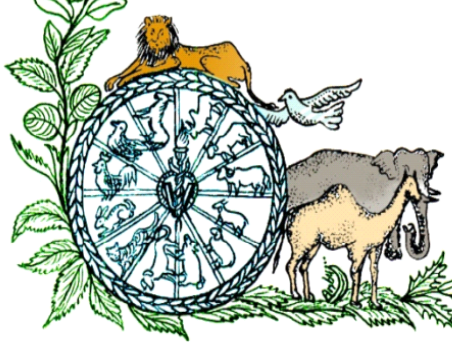

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Appendix

APPENDIX

AVESTIA

Laboratory Analysis Report

Product Name: Omega 3 Fish Oil Refined Dt of Mfg: 05/02/2013

Batch No.: RFO0133D13 Dt. Of Expiry: 04/02/2014

PARAMETER	STANDARD	RESULT
Free Fatty Acid	NMT 1.5%	0.73%
Iodine Value	145 – 170 **gI2**/100 g	163.23 gI2/100g
Acid Value	NMT 3mg of KOH/kg	2.15 mg of KOH/kg
Moisture	NMT 0.5 %	0.11%
Peroxide Value	NMT 10 meq/kg	6.93 meq/kg
Colour	NMT 30 units	26.4 Units
EPA	NLT 14%	15.28%
DHA	NLT 10%	10.41%
Total Plate Count	NMT 5 lac	1.3 lac
E. Coli	<20/gm	Absent
Shigella	Absent/25 gm	Absent
Salmonella	Absent/25 gm	Absent

Storage: It may be stored on room temperature and avoid direct sunlight.

Expiry: Expiry can be increased by addition of antioxidants at specific intervals.

Analysed By

QC Manager

AVESTIA PHARMA MUMBAI 400 101

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Curriculum Vitae

Author was born on January 5th, 1983 in Hazaribag district of Jharkhand (India). He passed his Secondary School Examination in the year 1997 from Dr. B.N. Singh High School and Higher Secondary School Examination in 1999 from St. Columba's College, Hazaribag. He obtained his B.V.Sc. & A.H. degree from Birsa Agriculture University, Ranchi (Jharkhand) in May, 2006 and M.V.Sc. degree (Veterinary Gynaecology and obstetrics) from G. B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand) in August, 2008. He joined the Ph. D program (Animal Reproduction) in September, 2008 at Indian Veterinary Research Institute, Izatnagar. He joined Indian Council of Agricultural Research as Agriculture Research Scientist on December 15th, 2009 and presently posted to Equine Production sub campus, National Research Centre on Equines at Bikaner. He worked under following research projects:

1. Cryopreservation of equid semen using amides.
2. Cryopreservation of embryos for conservation of Marwari horses.
3. Development of Intra-vaginal device for estrus control in mares.
4. Endocrine, biochemical and gene expression profiling of reproductive states in Marwari mares.

He published 13 research papers, 1 technical bulletin, 1 review, 1 popular article, 1 book chapter and 17 lectures in training compendium. He is a life member of Indian Society for the study of Animal Reproduction.

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