EFFECT OF DHA SUPPLEMENTATION DURING STALLION SEMEN CRYOPRESERVATION ON SPERM CHARACTERISTICS

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ABSTRACT

This study was conducted to evaluate the potential protective effects of adding Docosahexaenoic acid (DHA) in combination with α-tocopherol (vitamin E, VE) to semen extender, on freezeability of equine semen; in particular for ejaculates whose sperm do not tolerate the rigors of cooling and storage. In this experimental study two ejaculates from seven proven fertile stallions were used to investigate the effects of four different treatment, made up of four combination of DHA and VE. Semen was divided in four groups consisting of four levels of DHA (0, 1, 10, 20 ng/ml) and the same concentration of VE (0,2 mmol) in the semen extender. Motility, progressive linear motility (using optical microscope) and viability (using flow cytometry) of equine sperm, were investigated after 1 month of storage in a liquid nitrogen tank.

Our findings showed that freezing process has some detrimental effects on sperm quality, decreasing significantly most of measured variables of sperm as compared to fresh semen. Whereas the treatments did not improve sperm quality, not even in ejaculates which show more sensitivity to freezing procedures.

In conclusion, the addition of DHA in combination with vitamin E, at the studied levels, cannot be effectively introduced to conservation media in order to protect spermatozoa from freezing damages. It could be interesting to conduct further studies with higher concentration of DHA, in order to verify if it could have a protective effect on stallion semen during cryopreservation.
RIASSUNTO

Questo studio è stato condotto al fine di valutare i potenziali effetti protettivi dell’aggiunta di acido docosaesaenoico (DHA) in combinazione con tocoferolo (Vitamina E, VE) al diluitore del seme, sulla congelabilità del seme equino, con particolare attenzione agli eiaculati con scarsa tolleranza al raffreddamento e congelamento.

In questo studio sperimentale, sono stati usati due eiaculati da sette stalloni di provata fertilità, per valutare l’effetto di 4 diversi trattamenti consistenti in 4 diverse combinazioni di DHA e vitamina E. Il seme è stato diviso in quattro gruppi che consistevano in quattro livelli di DHA (0, 1, 10, 20 ng/ml) e la stessa concentrazione di vitamina E (0,2mmol) nel diluitore per il congelamento. Dopo un mese di stoccaggio in azoto liquido, sono state valutate motilità e motilità progressiva del seme con l’utilizzo di un microscopio ottico, mentre la vitalità è stata valutata con la citometria a flusso.

I risultati ottenuti mostrano che il processo di congelamento e stoccaggio ha avuto effetti negativi sulla qualità del seme, mentre i trattamenti non hanno migliorato la congelabilità del seme, nemmeno negli eiaculati più sensibili al congelamento.

In conclusione, l’aggiunta del DHA in combinazione con la vitamina E nel diluitore per il congelamento, ai livelli studiati, non può essere introdotta come mezzo di prevenzione efficace per i danni da congelamento-scongelamento sugli spermatozoi. Potrebbe essere interessante condurre ulteriori studi con concentrazioni maggiori di DHA, al fine di verificare se possa avere un effetto protettivo durante la crioconservazione del seme equino.
1. INTRODUCTION

Nowadays, for the most part, stallions are not selected as sires because of their intrinsic fertility. Conversely, stallions become commercial sires based on athletic performance record, pedigree, and conformation. As such, the equine breeding industry abounds with stallions whose level of fertility is less than optimal and veterinary intervention is sometimes required to maximize the fertility of many such stallions (Dickson, 2014). Cryopreservation of stallion sperm has critical importance for the equine breeding industry, because it allows for long-term storage and transportation. However, approximately one third of stallions yield unsatisfactory post-thaw sperm quality and therefore these stallions are not used in cryopreservation programmes. The hypothesis of this study was that the reason some stallions semen does not freeze well is due to a low level of DHA in the fatty acid profile of their semen and the addition of DHA in combination with vitamin E, to the semen extender, would enhance post thaw semen quality.

1.1 Artificial insemination

The increasing interest of the equine industry in respect of artificial insemination (AI) is leading to develop new and more efficient techniques to storage equine semen.

The principal benefits of AI are:

1. The removal of geographical restriction.
2. Reduction of disease transfer.
3. Increasing the number of mares that can be inseminated per ejaculate.
4. Improvement of native stock through semen importation.
5. Breeding of difficult mares or Stallions, those with physical problems.

Nowadays, the most important techniques used for AI in the equine breeding industry involve the use of cooled and frozen semen.
1.2 Cooled semen

In the last 30 years there has been a steady increase in the use of cooled transported semen in Equine breeding industry. This increase has been due mainly to the development of an effective passive cooling container and acceptance of the technology by numerous breed registries.

This demand for the use of cooled semen for all stallions, however, has revealed many disadvantages or problems. Some of these disadvantages are described below:

1. Not all stallions produce ejaculates suitable for cooling or storage;
2. Problems with scheduling of shipments of semen lead to poor timing of insemination for many mares;
3. The relatively short longevity of cooled semen (24–48 h) may require multiple shipments per cycle;
4. Stallions must be available and healthy at all times during the breeding season for semen collections upon mare owner requests;
5. Much of the semen is processed by owners, not professionals, which leads to a tremendous variability in quality of transported semen. (Loomis, 2001)

Some of this disadvantages, in particular the short longevity of the semen and the availability of the stallion, could be overcome by using of frozen semen.

1.3 Frozen semen

Transported frozen semen offers breeders additional benefits which are not available with cooled semen. Some of these advantages are:

1. Stallions need not be available for on-demand collections, allowing for involvement in performance events during the breeding season;
2. Illness, injury or death of a stallion does not prevent insemination of mares with his semen;
3. Scheduling of semen shipments is easier and can be shipped well in advance and maintained on the farm until the optimum time for insemination;
4. Frozen semen allows for international distribution;
5. Centralized processing of frozen semen by specialized laboratories may result in less variability in semen quality compared to cooled semen;
6. Less semen is wasted, because the whole semen collected from one ejaculate for freezing is usually processed and stored, resulting in an average of 10–12 insemination doses per ejaculate instead of one.

However, some important disadvantages impede the spread of frozen semen as a normal practice in equine breeding industry. Some of the impediments to the development of the frozen semen industry include:

1. Frozen semen results in lower fertility than with cooled semen for many stallions;
2. More technical expertise is required for processing frozen semen than cooled semen;
3. If frozen semen is marketed with a limited number of insemination doses available per cycle, mare management costs are higher than with cooled semen; more frequent examinations are required, since frozen semen has lower longevity in the mare, so closer synchrony between insemination and ovulation is needed to conserve doses (Loomis, 2001);
4. There are individual variations in the ability of sperm, from different stallions, to survive cryopreservation (Barbas and Mascarenhas, 2009).

Many studies tried to identify reasons of this decrease in semen characteristics of thawed stallion semen (Aurich et al., 1996; Blottner et al., 2001, Alvarenga et al., 2005; Vidament, 2005; Loomis and Graham, 2008)

Freezing and thawing causes major damage to the spermatozoa, particularly their membranes (plasma and organelle membranes). The most important factors causing cryoinjury are considered to be the toxicity caused by unequal distribution of cryoprotectants (such as glycerol), and the osmotic stress caused by dehydration of the extender and the cells during freezing and again at thawing. In addition, a number of other events occur during cooling: phase transitions in the plasmalemma, oxidative damage and premature ageing which contribute to sperm death or, if surviving, to
their shortened life-span. Furthermore, apoptosis-like phenomena have been identified as taking place in the spermatozoa of several species which explains not only cellular death, but also the different degrees of subtle cellular damage that most surviving spermatozoa disclose post-thaw (Macías García et al., 2009).

In addition to all these direct damages, some studies has shown that factors released from fragmented spermatozoa, are capable of inducing DNA fragmentation in accompanying intact spermatozoa (Pérez Crespo et al., 2008).

1.4 Anatomy and physiology of male reproductive system.

The male genital tract has as its primary product sperm. Hormones, such as testosterone, the epididymal fluid and seminal plasma contribute to the success of the production process. The testes have a huge production capacity in mammals (<1 to 25 billion sperm per day or 35000-200000 per second) (Dyce KM et al., 2006). The male reproductive system consists in scrotum, testes, spermatic cords, sperm ducts, accessory glands, urethra, penis, prepuce and muscles that contribute to erection and ejaculation.

The testicles, in addition to the gametogenetic function (spermatogenesis), exert an autocrine - paracrine - endocrine function by production of hormones such as testosterone (Roser, 2001).

The spermatic cord has an important thermoregulatory function, essential for spermatogenesis. Indeed, through one counter current heat exchange, the temperature of the blood is lowered to 4-6 °C before reaching the testis (Baron, 1994).

The sperm ducts extend from the testicles to the urogenital sinus. Its functions, modulated by testosterone and estrogen (Parleviet et al., 2006), consist of concentrate and make fertile spermatozoa (maturation) (Aman, 1993).

The ejaculate is composed by the sperm cells and the Seminal Plasma (SP). The latter is produced from the epididymis and accessory glands. The accessory glands in the stallion include ampullary glands, vesicular glands (gelatinous fraction of the semen), prostate (sperm-rich fraction) and the bulbourethral glands (pre-sperm fraction). Their activity is regulated from circulating testosterone (Chenier, 2000).
1.5 Biochemical composition of seminal plasma.

Seminal plasma (SP) is a mixture of fluid secreted from the testes, epididymis and accessory sex glands, and it is involved in a multitude of sperm functions and events preceding fertilization. Although data on the features of various biochemical components of SP have been published and progress has been made especially in the field of SP proteomics, the physiological role of SP is still not fully understood (Kareskoski and Katila, 2008).

Seminal Plasma is a nutrient media and protect the sperm. Some components of the SP are very important for the metabolism of spermatozoa, as well as for their survival and transport in the female reproductive tract.

The accessory glands, which are the seminal vesicles, prostate, and bulbo-urethral glands, contribute to the majority of the ejaculate volume while the secretion of the seminal vesicles makes up most of the SP.

It was believed that all the accessory glands could be anatomically and functionally homogeneous because of their very similar embryonic origin and their morphological structure. The discovery and identification of several substances in the secretion of accessory glands, such as citric acid, prostatic phosphatase, fructose and phosphorylcholine (Mann, 1964), has opened the way to study the diversity of function of the secretions of the various glands and their possible roles for spermatozoa.

In the SP have been identified amino acids and proteins, enzymes, organic ions, lipids and fatty acids, hormones, and sugars.

The function of SP in normal physiology is associated with ejaculation of sperm and their subsequent survival in the female reproductive tract. The role of SP on the maturation of spermatozoa has been widely studied with mixed results, and research in different species highlights the various roles, including:

• activation and increased sperm motility;

• it is considered a media to provide the optimal osmosis and nutriment;
• prevention of premature activation during transport of sperm and physiological stabilization of the plasma membrane with inhibitors of capacitation (Villemure et al, 2003);

• regulation of the transport of sperm and their Disposal (Troedsson et al, 2005);

• assistance in sperm-egg interactions (Souza et al, 2008);

• influence on fertility (Rozeboom et al, 2000).

It has also been reported negative effects of SP on the motility and viability of sperm after freezing-thawing (Garcia and Graham, 1987).

When the proportion of SP is reduced to about ≤ 5%, sperm are able to maintain more desirable motility compared to samples containing a higher proportion of SP (10–30%), during both cooled storage and cryopreservation. Sperm motility and DNA integrity are also superior in samples with complete removal of SP compared to samples with greater SP content. However, the effect of SP varies between individual stallions. The presence of some SP seems to be necessary for semen storage and fertility, but it is beneficial to remove most of the SP by centrifugation before storage, at least for those stallions whose ejaculates have poor tolerance to cooling and storage (Kareskoski and Katila, 2008).

1.6 Lipid profile in stallion semen.

Since the nineteenth century researchers have reported that lipids are a basic component of semen, contributing to the membrane structure of spermatozoa, the metabolism of the sperm cells and to their ability to capacitate and fertilize the female gamete (Mann and Lutwak-Mann, 1981). The fatty acid composition of the spermatozoa and seminal plasma from many animal species has been reported and a key finding was the presence of extremely high concentrations of polyunsaturated phospholipids (Kelso et al 1997b).

The lipid composition of semen is unique in its content of long chain polyunsaturated fatty acids (LC-PUFAs); they are essential components of all cell membranes and also give rise to many bioactive molecules, for example, eicosanoids. In most mammals,
sperm (similarly to the brain and retina) have a considerable amount of n-3 LC-PUFAs, mainly docosahexaenoic acid (DHA; C22:6, n-3). These compounds have an essential role in the development and function, regulation of cellular movement, lipid metabolism and fusion capacity of sperm. Differences among phospholipids in their PUFA composition may affect the flexibility and compressibility of cellular membranes. Furthermore, there is considerable evidence that the lipid composition of the sperm membrane is a major determinant of motility, cold sensitivity and overall viability (Samadian et al., 2010).

The increasing interest of the equine industry in respect of artificial insemination is leading to develop new and more efficient techniques for freezing equine semen. However, the large inter-individual variability in sperm quality, characteristic of the equine species, clouds this development (Macías García et al., 2009).

The physiological and biochemical reasons behind this variability remain still unexplained. In many species including horses, peroxidation of the lipids present in the plasma membrane [lipid peroxidation (LPO)] has been claimed to be a major factor causing differences in sperm quality, especially when spermatozoa are stored or processed for later use (Macías García et al., 2011). Unfortunately, many stallions produce semen of unacceptable motility after it undergoes the stresses of cooling and freezing, thus affecting storage capability (Brinsko et al., 2005).

This reduction in sperm quality seems due to cellular injury, which is associated with a disruption of membrane lipids, resulting damage to the plasma, mitochondrial and acrosomal membranes.

In the stallion, the majority of lipids composing this membrane are PUFA. These fatty acids are essential for the structure, function, and integrity of the plasma membrane because they maintain and modulate intracellular composition and protect the cell from extracellular and non-physiological influences. Thus, preservation of this membrane is critical for sustained cell viability (Grady et al., 2009).

Studies on lipid composition of mammalian spermatozoa (Parks and Lynch, 1992; Argov-Argaman, 2013), show some differences between domestic animal species. Most mammalian spermatozoa has a greater proportion of docosahexaenoic acid (DHA
22:6 n-3, an omega-3 fatty acid) than docosapentaenoic acid (DPA 22:5 n-6, an omega-6 fatty acid). Interestingly, stallion and boar spermatozoa, which are normally consider bad freezers, have a higher proportion of DPA. According to this, differences in the ability of sperm from various animals to resist cold shock appear to be related to their sperm membrane lipid composition. In particular, making a comparison (table 1) between the lipid composition of the sperm membrane in the bull and in the stallion, significant differences appear in the DHA percentage in the Fatty Acids profile.

**Table 1** Fatty acids profile, comparison of the percentage of the main fatty acids between stallion and bull.

<table>
<thead>
<tr>
<th>Fatty acids profile</th>
<th>Stallion (Macías García et al., 2011)</th>
<th>Bull (Argov-Argaman et al., 2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic 14:0</td>
<td>7.5 - 30.9</td>
<td>15.62 - 21.85</td>
</tr>
<tr>
<td>Palmitic 16:0</td>
<td>22.9 - 27.3</td>
<td>23.9 - 24.76</td>
</tr>
<tr>
<td>Arachidonic 20:4n-6</td>
<td>0.1 - 2.1</td>
<td>4.65 - 4.72</td>
</tr>
<tr>
<td>Eicosapentanoic acid (EPA) C20:5 n-3</td>
<td>0.04 - 0.03</td>
<td>0.15</td>
</tr>
<tr>
<td>Docosohexanoic (DHA) (C22:6 n-3)</td>
<td>0.6 – 2.8</td>
<td>24.35 – 31.91</td>
</tr>
</tbody>
</table>

The table shows the percentages of the principal fatty in the fatty acids profile of stallion and bull semen.

This difference raises the question as if this specific fatty acid composition might influence the freezability of the gamete. Lipid and fatty acid composition is associated with semen physiological characteristics, which is considered as important reproductive predictor. Increased concentrations of free cholesterol, free fatty acids, triacylglycerol, and cholesterol ester are associated with decreased sperm motility and fertility (Cerolini et al., 1997). Similarly, in chickens, reduced male fertility was associated with low lipid content in the seminal fluid and reduced content of polyunsaturated fatty acids (PUFAs) in the sperm membrane, most notably arachidonic acid and docosahexaenoic acid (DHA) (Kelso et al., 1997b).

Interestingly, in infertile human males has been reported significant difference about the DHA composition of the semen, in comparison with normal fertile male (table 2).

**Table 2**: Fatty acid profile of human spermatozoa (Safarinejad et al., 2010).

<table>
<thead>
<tr>
<th></th>
<th>Infertile men</th>
<th>Fertile men</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA</td>
<td>0.31 ± 0.11</td>
<td>0.62 ± 0.17</td>
<td>0.003</td>
</tr>
<tr>
<td>DHA</td>
<td>6.55 ± 1.17</td>
<td>9.58 ± 1.44</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 2 the table shows the percentages (mean % ± Standard Deviation) of Eicosapentanoic acid (EPA) and Docosohexanoic (DHA) in fatty acids profile of fertile and infertile human males. P values < 0.05 show that there are significant different level of the two fatty acids between fertile and infertile males.

All these studies lead to the conclusion that different percentages of DHA in the lipid profile are strongly related to the quality of the semen.

1.7 Lipid peroxidation

There are several low molecular weight antioxidants (enzymatic and nonenzymatic) present in seminal plasma to help protect sperm against oxidative damage by reactive oxygen species (ROS). All these antioxidants protect spermatozoa during their transit through both the male and female reproductive tracts (Bilodeau et al., 2000). The major antioxidants present in seminal plasma are glutathione peroxidase, superoxide dismutase and catalase (Kankofer et al., 2005). However, during preparation for cryopreservation, sperm are washed out of seminal plasma and resuspended in cryopreservation extender, which leaves them vulnerable to oxidative stress due to loss of antioxidant protection. The absence of seminal plasma, in combination with a membrane high in polyunsaturated fatty acids (PUFA), makes sperm particularly susceptible to lipid peroxidation and cell damage during cryopreservation. Two significant types of ROS free radicals generated under conditions of cellular stress are superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), both of which have been associated with cryopreservation and ensuing oxidative lipid and DNA damage (McCarthy and Meyers, 2011) which has significant genetic and developmental consequences. Indeed, the association of semen abnormalities such as oligozoospermia and asthenozoospermia with oxidative stress has been extensively documented in Humans (Filipcikova et al., 2013).

In frozen-thawed semen, there is a significant decline in motility, and a reduced viability of spermatozoa in the genital tract of the female, which are the major causes of low fertility after cervical insemination (Salamon and Maxwell, 2000). Furthermore, after cervical insemination, frozen–thawed spermatozoa were expelled faster than fresh spermatozoa from the female reproductive tract (Gillan and Maxwell, 1999). Despite many hypotheses, the exact molecular mechanisms responsible for decreased
sperm fertility during in vitro storage remain unclear. However, evidence is accumulating that the reduced fertility is related to the disruption and damage of the sperm membrane (White IG, 1993); in particular cryopreservation of sperm resulted in a 226% increase in oxidative membrane damage compared with their fresh counterparts (Martorana et al., 2014).

The particular susceptibility of the sperm plasma membrane of some individuals or species to peroxidative damage is attributed to their high content of highly polyunsaturated fatty acids (HUFAs) and, further, to the innate deficiency of spermatozoa regarding the availability of cytoplasmic protective enzymes, because they had lost most of them with the bulk cytoplasm loss during spermiogenesis (Lenci et al. 1996; Ladha 1998).

Highly polyunsaturated fatty acids have been detected in the sperm membrane of humans and other mammals (Whates et al. 2007). These unsaturated fatty acids give to the plasma membrane fluidity that spermatozoa needs to participate in the membrane fusion events associated with fertilization (Lenci et al. 1996; Flesch and Gadella 2000). However these molecules are also vulnerable to attacks by reactive oxygen species, free radicals that generate peroxyl (ROO•) and alkoxyl (RO•) radicals. These radicals become stable as molecules both subtracting hydrogen atoms from adjacent carbon atoms in a neighbouring lipid, and thus generating the correspondent acid or alcohol (Macías García et al., 2011). In this process, a ROS-mediated attack on unsaturated fatty acids in the plasma membrane generates peroxyl (ROO) and alkoxyl (RO) radicals that, in order to stabilize, abstract a hydrogen atom from an adjacent carbon, generating the corresponding acid (ROOH) or alcohol (ROH). The subtraction of a hydrogen atom from an adjacent lipid creates a carbon-centred radical that combines with molecular oxygen to create another lipid peroxide. In order to stabilize, the latter must abstract a hydrogen atom from a nearby lipid, creating yet another carbon radical. In this manner, a chain reaction is created that propagates the peroxidative damage throughout the plasma membrane (Bilesky et al., 1983; Halliwell and Gutteridge, 1999).

Such peroxidation damage would disrupt the fusogenity of the sperm membrane and its ability to support key membrane-bound enzymes such as ATPases (Storey 1997).
Moreover, alterations in the fluidity of the sperm membranes could alter the activation of signal transduction pathways critical for sperm function (Macías García et al., 2011).

Spermatozoa have the ability to produce reactive oxygen species (ROS) which have physiological functions in signaling events controlling sperm capacitation, acrosome reaction and sperm-oocyte fusion as long as they are produced in a controlled manner (Aitken and Baker, 2004). While ROS are a normal by-product of cellular respiration, when they react with electrons in the lipids of sperm membranes lipid radicals are formed. These lipid radicals then react with oxygen molecules to create lipid peroxo radicals that in turn react with other membrane lipids to form lipid peroxides causing damage to cells (Aitken et al, 2006).

An imbalance in the production or degradation of ROS may have serious adverse effects on sperm function, i.e. sperm motility declines prior than detectable changes in membrane integrity. Oxidative stress appears as a consequence of the extreme ROS production and results in a decrease of intracellular ATP levels which initiates lipid peroxidation in the sperm plasma membrane (Deichsel et al., 2008).

In conclusion: Oxidative stress is defined as the imbalance between biochemical processes leading to production of reactive oxygen species and those responsible for the removal of ROS. It is known that excess of ROS impairs sperm cell function and plays a negative role on male fertility.

As said oxidative stress induced by the freezing-thawing process affects not only the fluidity of the sperm plasma membrane but also the integrity of DNA in the sperm nucleus. Try to avoid this deleterious mechanism with additions of treatments to the semen has been an objective of many studies. Omega-3 Fatty Acids (FA) are considered potentially important antioxidants and a considerable number of these studies suggest that antioxidants can prevent the oxidation of various macromolecules such as DNA, proteins, and lipids. Since omega-3 FAs are widely available and have an excellent safety profile, they have been used as nutraceuticals that improve semen quality (Safarinejad et al., 2010).
1.8 Docosahex-enoic acid (DHA)

Analysis of FA from head and tail of monkey sperm showed that DHA composed 1.1 and 19.6% of total FA of head and tail, respectively. This result is in accordance with that 99% of sperm DHA is present in the tail. This difference between lipid composition of head and tail may be necessary for specific functions of sperm because fats play a major role in integrity, fluidity, stability and permeability of plasma membrane. Therefore, high proportion of DHA in sperm tail may be necessary because they increase sperm motility via increasing membrane fluidity in sperm tail and thus improving sperm tail flexibility required for motility (Connor et al., 1998). In addition, improved fluidity and flexibility increases tolerance ability to freezing and prevents sperm cell membrane from being disintegrated caused by ice crystal formation during freezing process (Maldjian et al., 2005).

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 22:5 and 22:6 fatty acids. Sperm of bulls have higher levels of 22:6 fatty acids, whereas sperm from stallions and boars have higher levels of 22:5 fatty acids (Parks and Lynch, 1992). The major 22:6 PUFA in semen is DHA and the major 22:5 PUFA is DPA with the majority residing in sperm. Levels of DHA in both the seminal plasma and sperm of men with low (<50%) sperm motility were found to be significantly lower compared to men whose semen had normal sperm motility (Conquer et al., 1999). Increasing the ratio of DHA to DPA in semen has been shown to increase fertilizing capacity and semen quality (Blesbois et al., 1997; Penny et al., 2000). Conversely, reducing the ratio of DHA to DPA was accompanied by reductions in fertilizing capacity (Penny et al., 2000). DHA may contribute to the membrane fluidity that is necessary for the motility of sperm tails (Bwanga et al., 1991). Membranes with a high content of DHA in their phospholipids are, therefore, distinguishable by high levels of flexibility, compressibility, deformability, and elasticity. Hence, it can be suggested that DHA may have an
important role in the physiological and molecular mechanisms controlling the spermatozoa membrane fluidity (Gholami et al., 2010).

If DHA is essential for optimal fertility in stallion spermatozoa, as it is in other species, then it is possible that the addition of DHA to the Extender may be a means to increase after thawed semen quality.

1.9 Dietary polyunsaturated fatty acids

In previous studies, it has been tried to study the effect of dietary supplementation of DHA and other PUFAs on the quality of the semen. There are controversial results.

In example in the case of boar semen, Paulenz et al (1999) found that supplementing boar diets with cod liver oil did not improve the freezability of semen. However Penny et al. (2000) demonstrate that, when a formulation of DHA and antioxidants were added to boar rations, significant increases in semen quality and fertility were observed compared to boars fed a control diet.

Blesbois et al. (2004) demonstrated a significant improvement of reproductive performance in male turkey, following DHA supplementation in feed.

A study of Gholami et al., 2010, conducted on bull semen show that post-thawed sperm motility parameters did not significantly differ between the control and a fish oil enriched diet.

Whereas, in the ram, the addition of fish oil, rich in n-3poly-unsaturated fatty acids, on the diet, improved sperm characteristics after thawing (Esmaeili et al., 2014).

Regarding Stallion semen (Brinsko et al., 2005) has been showed that feeding a DHA rich diet, motion characteristics of frozen–thawed semen were improved even if less than optimal. Percentage of live, acrosome-intact sperm in frozen–thawed samples did not differ when stallions were fed the control diet and when they were fed the nutriceutic.

In contrast Grady et al. (2009) reported that adding fish oil to the diet failed to improve the quality of cryopreserved Stallion sperm.
Taken together, these studies have revealed inconsistent effects of PUFAs on sperm quality when they are used in the diet.

On the other hand, dietary supplementation experiments needs long time to see the effect on the semen. Thus, this study was designed to investigate the potential protective effects of DHA, added to extender, on stallion semen quality in response to freezing procedures.

1.10 Omega3 in the extender

As said, there are many experiments which investigate the effect of the dietary supplementation with PUFAs on semen parameters, in different species. Conversely, there are not many experiments trying to study the effect of the addition of n3 Fatty acids directly in freezing extenders.

In some of them, it seems to be a positive correlation between the post-thaw semen parameters and the addition of omega3. In the study of Nasiri et al. 2011, the results suggest that adding DHA accompanied with an antioxidant to an extender could improve cryosurvival of bull sperm via altering membrane lipid composition. The effect of the interaction of n-3 FA and vitamin E on freezing process was significant on sperm motility, progressive linear motility and viability.

The results of Towhidi and Parks (2012), on Brown Swiss bull semen, reinforced the idea that supplementing bull semen extender with n-3 FA and α-tocopherol improve post-thawed in vitro characteristics of bull sperm.

In addition to these, Ansari et al. (2012) showed that the adding n-3 FA accompanied by vitamin E to the extender increased post-thaw sperm quality in goat.

The improved characteristics of frozen-thawed sperm may be due to effective incorporation of DHA into the cell membrane before freezing and that has protective effects on sperm membrane.

The reason why n-3 FA increased motility and viability could be that they increased PUFA proportion in the sperm head and tail membrane and improved the fluidity that is necessary for sperm motility.
Even if an other recent study on bull semen (Abavisani et al, 2012) seem to be in contrast with this theory (they concluded that supplementation of semen extenders with omega 3 PUFAs did not significantly improve sperm resistance to cooling, especially to cryopreservation), it is interesting to investigate the effect of the addition of DHA in the extender, on stallion semen. There are apparently no reports regarding it.

1.11 Vitamin E (VE)

As explained, sperm cells contain very high proportions of polyunsaturated fatty acids, and the proportion of sperm PUFAs has been directly related to semen quality in different species.

Mammalian spermatozoa are sensitive to lipid peroxidation, due to the phospholipid content of sperm membranes with their high PUFA sidechains. Seminal plasma provided some protection against peroxidation via its constituent antioxidants. However, dilution of semen reduces antioxidant availability for sperm. On the other hand, supplementing semen extender with PUFAs during cryopreservation increases ROS production (Towhidi and Parks 2012), in particular Omega-3 fatty acids as docosahexaenoic acid (DHA) are susceptible to peroxidation due to its six double bonds (Filipcikova et al, 2013).

This high level of PUFAs increases the susceptibility of the cells to free radical induced peroxidative damages, considered a significant cause of male infertility.

Spermatozoa are protected against excessive levels of ROS by both enzymatic (superoxide dismutase, catalase, glutathione transferase, and glutathione peroxidase) and non-enzymatic antioxidants (ascorbate, reduced glutathione, urate, vitamin E and b-carotene) in seminal plasma, involving mechanisms to deal with free radicals which are constantly produced in cells and cause in turn lipid peroxidation. Vitamin E is one of the major natural lipid soluble antioxidants present in cell membranes, and plays a crucial role in breaking the chain reaction of peroxidation (Deichsel et al., 2008; Zaniboni et al., 2004).
Dietary supplementation with vitamin E, vitamin C, selenium and other antioxidative substances is claimed to be associated with improved antioxidant defense mechanism and prevention of free radical-associated damages in testes and epididymides. Recently an improvement in semen quality (semen motility, longevity, morphology as well as total sperm count) after dietary intake of antioxidants alone or in combination with polyunsaturated fatty acids has been reported in a variety of species. It has therefore become popular to add antioxidants to the diet of breeding animals of various species (Deichsel et al., 2008).

Apart of its effects after dietary supplementation, VE is also used as antioxidant in addition on PUFAs supplementation during semen cryopreservation. One key component of all diluents used during cooling and freezing is lipids; VE can break the covalent links that reactive oxygen species have formed between fatty acid side chains in membrane lipids. This result indicates that VE plays an important role in reducing membrane damage caused by excessive ROS production during cryopreservation (Nasiri et al., 2011).

On the other hand, using egg yolks enriched in n-3 FA without any antioxidant in diluent fail to improve the quality of sperm following cryopreservation, so for seeing the beneficial effects of n-3 FA, inclusion of an antioxidant in semen extender is unavoidable (Maldjian et al., 2005).

1.12 Flow cytometry

In recent years, flow cytometry has entered the andrology laboratory and has been extensively used to evaluate several different characteristics of sperm such as sperm viability.

Established microscopic procedures for evaluating populations of sperm cells are hindered by involved preparation and time-consuming analysis; consequently, sample size is small (Graham et al., 1990). The advantage of flow cytometry is that many thousands of cells can be analysed in a few seconds, giving a statistically more precise evaluation via a reproducible technique. Flow cytometric methods have been developed also for counting of sperm based on DNA staining.
In a flow cytometry analysis, labelled spermatozoa are driven within a laminar flow, passing one-by-one through a cell where they are illuminated by one or more lasers ('interrogation point'). The scattered or emitted light is filtered by mirrors and filters, reaching several photodetectors, where the signals are amplified. Finally, the information is digitalized and presented in different fluorescent intensity units to the researcher. Data is saved in standard FCS files (Flow Cytometry Standard). Thus, the information from each detector for each spermatozoon can be recovered and analysed afterwards. Typical sperm analyses usually render a few populations after manual classification (Martínez - Pastor et al., 2010).

Two of the most used stain, in flow cytometry are Syto 16 and Propidium Iodide. The nucleic acid stain Syto 16 has been demonstrated to be able to distinguish apoptotic from non-apoptotic cells in several apoptosis models. The apoptosis is a common cell-death pathway, which is initiated by various different stimuli. Sakkas et al. (1999) showed that apoptosis is a major mechanism in regulating spermatogenesis in the human and that there are significant differences in molecular markers of apoptosis between males with normal and males with abnormal sperm parameters. Accurate detection of apoptotic cells is important for the determination of cell viability. The recognition of early apoptotic events would markedly improve reliability and convenience of apoptosis assays. Apoptotic cells can be identified because they show a decreased (low) Syto 16 fluorescence, probably due to changes in their DNA structure whereas viable cells can be identified because they show an intact (high) Syto 16 fluorescence (Perticari et al, 2007).

Propidium Iodide (PI) cannot pass through an intact plasma membrane, but passes into and stains the nuclei of degenerated spermatozoa (Garner et al., 1986): entering cells with a broken plasmalemma and emitting red fluorescence when binding to nucleic acids. Graham et al (1990) proved that assays using PI and assays using eosin/nigrosin stains for intact plasma membranes produce nearly equivalent results, indicating that PI is an accurate supravital stain for sperm analyzed by flow cytometry. The correlation for the two assays was not perfect, probably due to differences in the size of the populations assayed (200 cells for eosin/nigrosin and 10000 cells for flow cytometry). Garner et al. (1986) showed a significant negative correlation with the
population of cells stained with PI and the percentage of motile sperm, which is reasonable since dead sperm are not motile.

1.13 Aim of the thesis

The current study was designed to investigate the potential protective effects of DHA, added to extender, on stallion semen quality in response to freezing procedures. If DHA is essential for optimal fertility in stallion spermatozoa, as it is in other species, then it is possible that the addition of DHA to the Extender may be a method to increase after-thawed semen quality, in particular for ejaculates with low tolerance to the rigor of freezing process.
2. MATERIALS AND METHODS

2.1 Semen collection

Seven mature stallion (between 7 and 28 years): Three Connemara Pony, Two Irish Sport Horse, a Selle Francais and a Duch Warmblood were used in this study. The semen was collected throughout the breeding season at Philip Mc Manus Clinic in Galway (Ireland) Ejaculates were collected using a Colorado style artificial vagina, lubricated and pre-warmed to 45–50°C after exposure of the stallion to a stimulus mare until erection and readiness to mount, followed by mounting of a dummy.

Figure 1: Colorado style artificial vagina utilized for the collection of stallion semen.
Figure 2: collection of the semen from a stallion with the use of a dummy and an artificial vagina.

The collected ejaculate was immediately transported to the laboratory for evaluation. Immediately after collection, the gel fraction of the ejaculate was removed and semen was filtered through sterile gauze and kept in a water bath at 34 °C until examination.

Sperm total motility was evaluated using a 7 µl drop of the raw semen split in a pre-warmed slide and covered with a pre-warmed cover-slide on a phase-contrast microscope at 400x. The value of gross motility was estimated within a scale 1 to 5 considering 1 as 0% motile spermatozoa and 5 as 100% motile spermatozoa.

Sperm concentration was assessed by Neubauer Haemocytometer (Marienfeld, Germany) after dilution (1:100 v/v). One drop of this dilution was placed into the chamber and the number of spermatozoa was evaluated under the phase contrast microscope 400× magnification. Sperm concentration was expressed as spermatozoa × 10^6 sperm/ml.
After the evaluation of the total volume using a graduated cylinder, the semen was diluted 1:1 with EQUIPRO extender (EquiPRO extender, minitube, Germany, which is composed of a blend of glucose, sucrose, non-fat dry milk and antioxidants with antibiotic) split in 15ml tube and centrifuged at 581 g (Regen Centrilab 80-2, Zengji Instruments, figure 3) for ten minutes at 32°C to remove the majority of the seminal plasma because of its detrimental effect on sperms over an extended period of time.

**Figure 3**: centrifuge (Regen Centrilab 80-2, Zengji Instruments) utilized in the experiment, with four 15ml tubes properly balanced inside it.

Ninety percent of the supernatant was aspirated and discharged, the pellet with the spermatozoa was gently resuspended and put in a common tube where was mixed with the other pellets got from the other tubes of the same ejaculate and the total volume obtained, named Volume After Centrifugation was assessed with a graduated cylinder. Post-centrifuge concentration was evaluated using a drop taken from this tube.

Semen was split in four 15 ml tubes and depending on the post-centrifuge concentration, the sample was diluted with a calculated volume of Gent extender (Equine Extender for semen freezing, 45 ml, Minitüb, Germany; contains: egg yolk,
glycerol and antibiotics) in order to obtain a volume of 10 ml and a final concentration 100 millions spermatozoa per ml in each of the four aliquots.

An aliquot served as the control and was frozen with only semen extender and vitamin E added. The other three aliquots served as test for the different concentrations of DHA.

Fifty µl of a stock of vitamin E, previously diluted 1:6 with ethanol to make it water-soluble, was added to each aliquot in order to obtain a final 0.02 m-molar concentration of vitamin E.

Subsequently DHA was added in order to obtain 4 different concentrations according to the 4 treatments: treatment 1: DHA 0 ng/ml; treatment 2: DHA 1ng/ml; treatment 3 DHA 10 ng/ml; treatment 4 DHA 20ng/ml.

The DHA had been previously prepared in different dilutions with ethanol in order to obtain the different final concentrations after the addition of 50µl of each dilution in the respective aliquot.

As said, the solutions of DHA and vitamin E to add to the extender, had been previously prepared, put in 500 µl eppendorf tubes and stored at -18°C; 30 min before the utilization, they had been thawed at room temperature and wrapped with aluminum foil to keep them safe from UV radiations.

The four tubes, containing the samples with the four different treatments, were kept in a water-bath at 32°C until they were stored in the straws.

2.2 Samples storage

The samples were then packaged into 0.5 mL straws (Minitüb, Germany), using a vacuum pump to fill the straws. The filled straws were closed and frozen to −110°C over 20 min (5.7°C/min) in a programmable freezer (IceCube 14 S-B: Automatic Freezer, Minitüb, Germany), followed by immersion and storage in liquid nitrogen until use.

In detail, the half-ml straws contained a "wick and powder" sealant at one end, which upon coming into contact with moisture (the semen) became solidified, and seals the
top end (the "open" end of the straw was immersed into the semen and suction was applied, with the help of the vacuum pump, sucking the semen into the straw until the sealant at the top end prevented further suction by becoming solid).

The bottom end of the straw was then sealed by using polyvinyl alcohol powder which was then dipped into water, and solidified.

All the straws carried a variety of information written on the outside: The stallions identification number, the number of the ejaculate, and the number of the treatment.

To make the straws easier to recognize and to have a double check on the process, different coloured straws were used for the different treatments and were labelled with different colours depending on the ejaculate.

After filling, the straws were placed horizontally on a rack and all the racks with the straws were wrapped with tissue paper and incubate in a fridge for 30-40 minutes to let the temperature decrease slowly and reach 5°C.

**Figure 4:** The freezing machine (IceCube 14 S-B: Automatic Freezer, Minitüb, Germany) utilized in the experiment to freeze the straws containing the stallion semen extended with the different concentration of DHA (0, 1, 10, 20ng/ml).
Figure 5: straws placed horizontally on a rack and put into the chamber of the control rate freezer (IceCube 14 S-B: Automatic Freezer, Minitüb, Germany) utilized in the experiment to freeze the straws containing the stallion semen extended with the different concentration of DHA (0, 1, 10, 20ng/ml).

Just before put the racks in the freezing machine, a straw from the control treatment was used to check the Progressive Linear Motility (PLM) of the semen before the freezing process, in order to use this data in comparison with the post thawing PLM (see below).

Once the straws had been frozen they were moved into a polystyrene-box filled with liquid nitrogen waiting to be split in the goblets and stored in a liquid nitrogen tank. A map of the positions occupied in the tank was updated every time straws of different ejaculates were added to it.

2.3 Total Motility and Progressive Linear Motility (PLM)

After a month of storage in the tank, the straws were thawed for the assessment of motility and viability. Four straws of the same ejaculate at once, one for each treatment, were taken from the tank and thawed in a beaker containing water at 37°C; after 30 sec the straws were opened and the content spilled into 4 labelled Eppendorf tube (one for each straw) and kept at 32 °C on a heater (Block Heater Model SBH130D, Stuart).

Sperm total motility and Progressive sperm motility were subjectively evaluated by visual estimation by the same person throughout the study.
Sperm Total Motility was evaluated with a phase-contrast microscope (magnification x 400) using an aliquot of 7µl of raw sample on a pre-warmed slide; the percentage of Total Motile spermatozoa was estimated using a 0 to 10 scale (0 corresponding to 0% motile spermatozoa and 10 corresponding 100% motile spermatozoa).

50µl of each sample were diluted 1:5 in 200 µl of media (Trixcell semen preservation media, IMV technologies) to make easier the assessment of the PLM. An aliquot (7 µl) of diluted semen was placed on a pre-warmed (37 °C) microscope slide, covered with a coverslip; 10 fields were examined by phase contrast microscope counting the number of progressive linear motile spermatozoa out of fifty cells. The count was repeated 2 times and the average of the two counts was used to calculate the percentage of PLM spermatozoa.

2.4 Viability

The viability was assessed with the use of a flow cytometer (Guava EasyCyte HT flow cytometer, Merck, Millipore).

**Figure 6:** Flow cytometer utilized to asses the viability of the post thawing semen.

The samples to tests for the flow cytometer were previously prepared adding in order: trixcell media, the thawed semen, the life stain and the dead stain in 1ml Eppendorf tubes and kept in the heater at 32°C until the transferring on a 96 wells plate for the reading.
Figure 7: 96 wells plate containing the samples of thawed semen (with trixcell media and the respective stains: Syto16 and PI), ready to be placed in the dedicate space in the flow cytometer, for the acquiring process.

The four straws of each ejaculate (one for each treatment) were read at once in the machine. Thus, for every reading 12 Eppendorf tubes were prepared: four of them worked as a control and the remaining eight were used to test the four treatments: two sample for each treatment in order to have a double check reading.

In the 1st Eppendorf tube just 500ul of pre-warmed trixcell media were put for the un-stain control; 495µl were put in the 2nd and the 3rd Eppendorf tube which were used to test the two stains (life stain and dead stain) separately.

The 4th sample served as a “dual control” to check the two stains working together and 490 µl of media were put in it. The remaining 8 Eppendorf-tube for the tests were prepared with 490 µl of media as well.

Once all the Eppendorf tube had been filled with the correct amount of media, 1.5 µl of thawed semen were added to all of them in order to obtain a 300,000 spz/ml final concentration.

The 1.5 µl, to add to the four controls and to the two samples testing the 1st treatment, were taken from the thawed semen of the control treatment (treatment 1). Other 1.5µl of thawed semen were taken from the 2nd treatment straw, and added to other 2 testing samples; the same procedure was replicate for the last two treatments (treatment 3 and 4).

Subsequently 5µl of a “ready to use solution” containing Syto16 life stain (Life technologies, S7578) were added to ten Eppendorf tubes (eight Eppendorf tubes of the
test, one for the dual control and one of the two Eppendorf tubes for the single stain control) to give a final 100 nano molar working concentration of syto 16 stain.

Finally 5µl of “ready to use solution” of Propidium Iodide (PI) (Life technologies P3566) were added to ten samples (eight for the tests and two for the controls: the dual control and the dead stain control) to give a final 15 micro molar working concentration of PI stain.

After an incubation at 32°C for 15 min, each sample were transferred in a respective well on the pre-warmed 96-well-plate and moved in the appropriate space in the flow cytometer.

The machine had been accurately cleaned and prepared before the reading process, and each position of the wells had been labelled (on the software) in order to identify the sample and get clear data table as result.

Once the 96-well-plate had been transferred into the machine, the control wells were used to adjust the settings before start the reading to get more accurate results. After the adjusting, the reading process started and 10.000 cells were analysed by the machine for 180 seconds for each well.

This staining procedure distinguished two sperm subpopulations: the live sperm coloured by syto16 stain (green fluorescence), and the dead one coloured by PI stain (red fluorescence).

With the use of the software (Guavasoft, Merck, Millipore) data were analysed in a computer and Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified (Fig 8).
2. Materials and Methods

**Figure 8:** Examples of flow cytometry analyses for assessing spermatozoa viability. Fluorescence dot plot and histograms show the distribution of PI Red and Syto16Green fluorescence in stallion sperm as determined by flow cytometry.

The population of sperm was identified based on forward/side scatter variables and discriminated from debris. The fluorescence dot plot on the left reports the sperm population positive for PI and Syto16, and unstained sperm which is negative for both probes. The histogram on the right, represents a Syto16 Green single colour control and displays the proportion of negative (unstained) and positive events for Syto16Green fluorescence in the detector.

2.5 Data analysis

The effect of the treatments was observed on the totality of the ejaculates.

A comparison between two classifications of the ejaculates was obtained dividing the ejaculates depending on the PLM after thawing of the control treatment (class 1 PLM: more than 20%; class 2 PLM: less than 20%) and the Delta PLM, obtained from the difference between the before-freezing and after-thawed values of PLM (class 1 delta PLM: more than 60%; class 2 delta PLM: less than 60% of difference).

The means of the After Thawed PLM and Viability in the four treatments were compared between the classes to study the effects of the treatments in the different groups.
A third classification, Final Concentration Times (FCT) classification, was created to study the different effects of the treatments depending on the number of times the semen was diluted with gent extender to obtain the final 100 millions spermatozoa/ml concentration in the final sample used to fill the straws.

FCT classification was based on two classes depending on FCT value: 1\textsuperscript{st} class consist of the group of ejaculates which had been concentrated less times (less than 25 times in respect of the initial concentration); 2\textsuperscript{nd} class consist in the group of ejaculates which had been concentrated more (more than 25 times in respect of the initial concentration) times.

The FCT parameter was obtained dividing the Concentration Post Centrifugation value by the Initial Rate Dilution value (IRD).

IRD was another parameter obtained multiplying the Concentration before Centrifugation value with the Volume After Centrifugation value and dividing the result of this count by the discarded volume. The latter consist of the difference between the initial volume of semen and the one was utilized after the centrifugation process and the discard of the supernatant.

The data obtained were analyzed the GLM procedure of the Software SigmaStat 2.03 through an ANOVA considering the classes of classification (PLM; Delta PLM; FCT and IRD) as independent variables and the evaluated parameters (collection volume (mL); total motility before freezing; PLM before freezing (%); concentration before centrifugation; volume after centrifugation (mL); discarded volume; IRD (initial rate dilution); concentration after centrifugation; final concentration times (FCT); treatment; Syto 16 count; PI count; viability % after thawing; PLM % after thawing; total motility% after thawing; Delta PLM) as dependent variables. The comparisons are tested with Tukey’s Test and considered with a P>0.05. Moreover a correlation study was carried out considering the Pearson coefficient.
3. RESULTS

The mean percentages of post-thawed sperm characteristics of different groups are shown in table 1, 2 and 3.

3.1 Total motility

The effect of DHA supplementation in semen freezing extender (the four treatments are shown in fig. 9) on sperm motility percentage was not significant. The percentage of motile spermatozoa, presented as mean and standard deviation was 22.38 ± 9.79, 23.46 ± 9.88, 20.46 ± 8.69 and 24.85 ± 12.96 for 0, 1, 10, 20ng/ml of DHA respectively. (P>0.05).

**Figure 9:** Variation of the post-thawed percentage of sperm Total Motility in the totality of stallion ejaculates studied, between the different treatments: corresponding different concentrations of Docosahexaenoic acid DHA (0; 1; 10; 20ng/ml respectively for treatment 1, 2, 3 and 4) added in the semen extender prior to cryopreservation.
3.2 Progressive Linear Motility

The effect of DHA supplementation in semen freezing extender (the four treatments are shown in fig. 2) on sperm Progressive Linear Motility percentage was not significant.

The PLM percentage of sperm, presented as mean and standard deviation, was 16.62 ± 8.39, 17.15 ± 5.81, 12.08 ± 7.05 and 16.61 ± 11.88 for 0, 1, 10, 20ng/ml of DHA respectively.

The differences in the mean values among the treatment groups were not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.120).

**Figure 10:** Variation of the post-thawed percentage of sperm Progressive Linear Motility in the totality of stallion ejaculates studied, between the different treatments: corresponding different concentrations of Docosahexaenoic acid DHA (0; 1; 10; 20ng/ml respectively for treatment 1, 2, 3 and 4) added in the semen extender prior to cryopreservation.
3.3 Viability

The effect of DHA supplementation in semen freezing extender (the four treatments are shown in fig. 11) on sperm viability was not significant.

The viability of sperm, presented as mean and standard deviation, was $18.82 \pm 10.36$, $18.42 \pm 10.37$, $17.52 \pm 9.20$ and $19.65 \pm 9.78$ for 0, 1, 10, 20ng/ml of DHA respectively.

The differences in the mean values among the treatment groups were not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($P = 0.841$).

**Figure 11**: Variation of the post thawed sperm viability in the totality of the stallion ejaculates studied, between the different treatments: corresponding different concentrations of Docosahexaenoic acid DHA (0; 1; 10; 20ng/ml respectively for treatment 1, 2, 3 and 4) in the semen extender prior to cryopreservation.

3.4 Progressive Linear Motility classification

One of the hypotheses of this study was that the addition of DHA in semen extender would have improve, in a more significant way, post-thaw semen characteristics of that ejaculates which are more sensitive to cold shock.
The totality of the ejaculates were split in two different groups depending on the value of post thawed PLM they showed in the control treatment. The mean of the values of PLM: 20%, was used as a threshold between the class1 ejaculates that showed a more satisfactory value of PLM (>20%) and the class2 ejaculates that showed worst PLM (<20%).

A comparison between Class1 and Class2 ejaculates was done to evaluate if the supplementation of DHA in the extender lead to different effects in the two groups. The results for the Total Motility are shown in fig. 12.

The percentages of motile spermatozoa of “PLM class1”, presented as mean and standard deviation were: 32.5 ± 4.63, 32.5 ± 4.63, 31.25 ± 2.31 and 36.25 ± 4.43 for treatment 1, 2, 3 and 4 respectively.

The percentages of motile spermatozoa of “PLM class2”, presented as mean and standard deviation were: 17.89 ± 7.90, 19.44 ± 8.89, 15.67 ± 5.44 and 19.78 ± 12.27 for treatment 1, 2, 3 and 4 respectively.

**Fig 12:** Variation of Total motility (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of progressive linear motility (PLM).
The percentages of progressive linear motile spermatozoa of “PLM class1”, presented as mean and standard deviation were: 27 ± 2, 20.5 ± 2.78, 17.25 ± 7.98 and 14.25 ± 10.80 for treatment 1, 2, 3 and 4 respectively.

The percentages of progressive linear motile spermatozoa of “PLM class2”, presented as mean and standard deviation were: 12 ± 5.34, 15.67 ± 6.23, 9.78 ± 5.35 and 13.22 ± 10.95 for treatment 1, 2, 3 and 4 respectively.

The difference in the mean values among the different treatment was not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences within PLM classes. There is not a statistically significant difference (P = 0.058).

The results for the PLM are shown in fig. 13.

**Fig 13:** Variation of progressive linear motility (PLM) percentage (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of progressive linear motility.

*Class 1 (blue): ejaculates with more than 20% PLM after thawing in the control treatment (T1); Class 2(red): ejaculates with less than 20% PLM after thawing in the control treatment (T1).*

The percentages of viable spermatozoa of “PLM class1”, presented as mean and standard deviation were: 23.32 ± 10.45, 21.56 ± 9.73, 22.68 ± 8.41 and 24.74 ± 9.84 for treatment 1, 2, 3 and 4 respectively.
The percentages of viable spermatozoa of “PLM class2”, presented as mean and standard deviation were: 16.83 ± 9.95, 17.03 ± 10.60, 15.22 ± 8.78 and 17.39 ± 9.12 for treatment 1, 2, 3 and 4 respectively.

The difference in the mean values among the different treatments was not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences within PLM classes. There is not a statistically significant difference (P = 0.791).

The results for the viability are shown in fig. 14.

**Figure 14:** Variation of viability (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of progressive linear motility (PLM).

**Class 1 (blue):** ejaculates with more than 20% PLM after thawing in the control treatment (T1); **Class 2 (red):** ejaculates with less than 20% PLM after thawing in the control treatment (T1).

### 3.4 Delta Progressive Linear Motility classification

A second classification was done in order to distinguish the ejaculates more sensitive to cold shock from the less sensitive. This second classification was based on the “Delta PLM”: a parameter obtained doing the difference between the before-freezing and the after-thawing PLM of the control treatment (treatment 1). This parameter tell us how much the freezing process has influenced the PLM.
The totality of the ejaculates were split in two different groups depending on their value of Delta PLM:

Class Delta PLM 1, includes the ejaculates which have a Delta PLM < 60%; Class Delta PLM 2 includes the Ejaculates which have a Delta PLM > 60%. The value of 60% was chosen because it correspond to the mean of the delta PLM values. Class 2 ejaculates are considered less sensitive to cold shock than class 1 ejaculates.

The changing in the semen characteristics between the different treatments, within the two classes of Delta PLM are showing in Fig 15, 16 and 17.

Fig 15 show the trend of Total Motility within the two classes.

The percentages of motile spermatozoa of “Delta PLM class 1”, presented as mean and standard deviation were: 20 ± 7.34, 21.43 ± 9.08, 17.14 ± 4.69 and 22.29 ± 12.71 for treatment 1, 2, 3 and 4 respectively.

The percentages of motile spermatozoa of “Delta PLM class 2”, presented as mean and standard deviation were: 26.5 ± 13.38, 28.75 ± 9.54, 25.25 ± 12.08 and 28 ± 13.50 for treatment 1, 2, 3 and 4 respectively.

**Figure 15**: Variation of total motility (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of Delta progressive linear motility (Delta PLM).

*Class 1 (blue): ejaculates which decrease more than 60% in PLM after thawing in the control treatment (T1);*
3. Results

Class 2(red): ejaculates which decrease less than 60% in PLM after thawing in the control treatment (T1).

Fig 16 shows the trend of PLM within the two classes.

The percentages of progressive linear motile spermatozoa of “Delta PLM class1”, presented as mean and standard deviation were: 13.29 ± 5.04, 17.14 ± 6.29, 10.85 ± 4.91 and 16 ± 10.90 for treatment 1, 2, 3 and 4 respectively.

The percentages of progressive linear motile spermatozoa of “Delta PLM class2”, presented as mean and standard deviation were: 21.25 ± 10.83, 18 ± 4.54, 10 ± 5.71 and 15.5 ± 8.02 for treatment 1, 2, 3 and 4 respectively.

The difference in the mean values of PLM among the different treatments (0, 1, 10, 20ng/ml of DHA) is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences within Delta PLM classes. There is not a statistically significant difference (P = 0.432).

**Figure 16:** Variation of progressive linear motility (PLM) percentage (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of DeltaPLM.

Class 1 (blue): ejaculates which decrease more than 60% in PLM after thawing in the control treatment (T1);
Class 2(red): ejaculates which decrease less than 60% in PLM after thawing in the control treatment (T1).

Fig 17 show the trend of Viability within the two classes.
The percentages of viable spermatozoa of “Delta PLM class1”, presented as mean and standard deviation were: 18.56 ± 10.27, 18.11 ± 11.87, 16.15 ± 9.55 and 19.29 ± 9.32 for treatment 1, 2, 3 and 4 respectively.

The percentages of viable spermatozoa of “Delta PLM class2”, presented as mean and standard deviation were: 20.51 ± 13.43, 21.11 ± 10.10, 20.11 ± 10.82 and 22.51 ± 12.55 for treatment 1, 2, 3 and 4 respectively.

The difference in the mean values of PLM among the different treatments (0, 1, 10, 20ng/ml of DHA) is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences within Delta PLM classes. There is not a statistically significant difference (P = 0.940).

**Figure 17:** Variation of viability (mean) among the four different treatments supplemented to stallion semen extender (T1, T2, T3 and T4, respectively: 0; 1; 10; 20ng/ml DHA) within the two classes of Delta progressive linear motility (Delta PLM).

*Class 1 (blue): ejaculates which decrease more than 60% in PLM after thawing in the control treatment (T1);*  
*Class 2(red): ejaculates which decrease less than 60% in PLM after thawing in the control treatment (T1).*

### 3.5 Final Concentration Times (FCT) class

Some statistically relevant difference has been found out studying the variation of sperm characteristics between the two FCT classes (Fig 18).
The difference in the mean values of post thawing PLM among the different FCT classes is greater than would be expected by chance after allowing for effects of differences in the treatments. There is a statistically significant difference ($P = 0.008$).

**Fig 18**: Variation of the PLM values among the two Final Concentration Times (FCT) classes within the different treatments of DHA (0, 1, 10, 20 ng/ml respectively for T1, T2, T3 and T4) added to semen extender.

**FCTClass 1 (blue)**: ejaculates that had been concentrated less than 25 times prior to the freezing process.

**FCTClass 2 (red)**: ejaculates that had been concentrated more than 25 times prior to the freezing process.

3.6 Study of correlations.

Correlation indexes among all the data has been studied highlighting the more significant one ($P<0.05$); the results obtained has been reported in the following table.
### Table 3: Pearson correlation indexes

| Collection Volume (mL) | Total Motility before freezing (%) | PLM before freezing (%) | Concentration before Centrifugation (x 10^6)/mL | Volume after Centrifugation (mL) | discarded volume (mL) | IRD (initial rate dilution) | Concentration after Centrifugation (x 10^6)/mL | FCT (final concentration times) | Treatment | SYTO 16 | PI | VIABILITY% after thawing | PLM % after thawing | PLM CLASS | TOTAL MOTILITY % after thawing | DELTA PLM | DELTA PLM CLASS |
|-----------------------|------------------------------------|-------------------------|-----------------------------------------------|---------------------------------|-----------------------|-------------------------|-----------------------------------------------|---------------------------------|-----------|--------|-----------------|-------------------|-----------|------------------------|--------------|---------------|
|                       |                                    |                         |                                               |                                 |                       |                         |                                               |                                 |           |        |                 |                    |           |                        |              |               |
| Collection Volume (mL) | 0.513                              | 0.466                   | 0.991                                         | -0.383                          |                       |                         |                                               | 0.385                                          | 0.475      | -0.225 | 0.219           | -0.245            | 0.579     |                        |              |               |
| Total Motility before freezing (%) | 0.54                              | -0.289                  |                                               |                                 |                       |                         |                                               | 0.544                                          | 0.498      | 0.413  | 0.413           | 0.222            | 0.39     | -0.25                  |              |               |
| PLM before freezing (%) | -0.466                             | 0.483                   | 0.466                                         | -0.341                          |                       |                         |                                               | 0.553                                          | 0.542      | 0.338  | 0.421           | 0.421            | 0.505     | -0.497                  |              |               |
| Concentration before Centrifugation (x 10^6)/mL | -0.429                             | 0.313                   | 0.351                                         | 0.222                           |                       |                         |                                               | 0.221                                          | -0.241     |        |                |                    |           |                        |              |               |
| Volume after Centrifugation (mL) | 0.345                              | 0.459                   | 0.555                                         | -0.407                          |                       |                         |                                               | 0.314                                          | -0.228     | 0.349  | 0.349           | -0.342            | 0.265     |                        |              |               |
| discarded volume (mL) | -0.475                             | 0.261                   | 0.469                                         | 0.531                           |                       |                         |                                               | -0.266                                         | 0.247      | 0.573  | 0.573           | -0.258            | 0.388     |                        |              |               |
| IRD (initial rate dilution) | 0.599                              | -0.801                  | -0.556                                        | 0.225                           |                       |                         |                                               | -0.43                                           | 0.327      | -0.276 | -0.276         | 0.361             |           |                        |              |               |
| IRD CLASS | 0.466                              | -0.37                   |                                               |                                 |                       |                         |                                               | 0.289                                          | 0.378      |        |                |                    |           |                        |              |               |
| Concentration after Centrifugation (x 10^6)/mL | 0.5                                | 0.582                   |                                               |                                 |                       |                         |                                               | -0.504                                         | 0.667      |        |                |                    |           |                        |              |               |
| FCT (final concentration times) | 0.846                              | -0.222                  |                                               |                                 |                       |                         |                                               | 0.211                                          | 0.316      |        |                |                    |           |                        |              |               |
| Treatment |                                    | 0.094                   |                                               |                                 |                       |                         |                                               | 0.342                                          | -0.354     | 0.432  | -0.354         | 0.432             |           |                        |              |               |
| SYTO 16 |                                    | 0.949                   |                                               |                                 |                       |                         |                                               | 0.223                                          | -0.491     |        | 0.223          |                    |           |                        |              |               |
| PI |                                    | 0.094                   |                                               |                                 |                       |                         |                                               | 0.342                                          | -0.354     | 0.432  | -0.354         | 0.432             |           |                        |              |               |
| VIABILITY% after thawing | 0.238                              | 0.094                   |                                               |                                 |                       |                         |                                               | 0.223                                          | 0.328      |        |                |                    |           |                        |              |               |
| PLM % after thawing | -0.491                             | 0.094                   |                                               |                                 |                       |                         |                                               | 0.342                                          | -0.354     | 0.432  | -0.354         | 0.432             |           |                        |              |               |
| PLM CLASS | 0.238                              | 0.094                   |                                               |                                 |                       |                         |                                               | 0.223                                          | 0.328      |        |                |                    |           |                        |              |               |
| TOTAL MOTILITY % after thawing | -0.47                             | -0.429                  |                                               |                                 |                       |                         |                                               | 0.47                                           | -0.47      |        |                |                    |           |                        |              |               |

Table 3: the table reports the r values of all the significant (P<0.05) correlations among the data collected in the current study.
The following are the most noticeable correlations, with $P < 0.05$, between the studied parameters:

- Positive correlation of the Collection volume with the “PLM before freezing”.
- Positive correlation of the Collection volume with the viability after thawing.
- Negative correlation between the “PLM before freezing” and the concentration before centrifugation.
- Negative correlation between the “PLM before freezing” and the concentration after centrifugation.
- Negative correlation between the discarded volume and concentration after centrifugation.
- Negative correlation of collection volume with PLM after thawing.
- Positive correlation of collection volume with “delta PLM”.
- Positive correlation of discarded volume with “delta PLM”.
- Positive correlation between the volume after centrifugation and viability after thawing.
- Positive correlation of the total motility before freezing with the parameters of fertility post thawing (total motility, PLM, viability).
- Positive correlation of “PLM before freezing” with the parameters of fertility post thawing (total motility, PLM, viability).
A recapitulatory table (table 4) with the mean values of total motility, PLM and viability after thawing for each treatment and for each class, is reported below.

Table 4:

<table>
<thead>
<tr>
<th></th>
<th>TREATMENT</th>
<th>PLM class1</th>
<th>PLM class2</th>
<th>Delta class1</th>
<th>PLM class1</th>
<th>PLM class2</th>
<th>FCT class1</th>
<th>FCT class2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL</td>
<td>T1</td>
<td>32,50 ± 1,64</td>
<td>17,89 ± 1,86</td>
<td>20,00 ± 1,96</td>
<td>26,50 ± 4,73</td>
<td>25,83 ± 3,07</td>
<td>17,20 ± 2,61</td>
<td></td>
</tr>
<tr>
<td>MOTILITY</td>
<td>T2</td>
<td>32,50 ± 1,64</td>
<td>19,44 ± 2,10</td>
<td>21,43 ± 2,43</td>
<td>28,75 ± 3,37</td>
<td>27,50 ± 3,23</td>
<td>18,00 ± 2,26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>31,25 ± 0,82</td>
<td>15,67 ± 1,28</td>
<td>17,14 ± 1,25</td>
<td>25,25 ± 4,27</td>
<td>20,00 ± 2,46</td>
<td>18,20 ± 2,60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>36,25 ± 1,57</td>
<td>19,78 ± 2,89</td>
<td>22,29 ± 3,40</td>
<td>28,00 ± 4,77</td>
<td>22,67 ± 4,34</td>
<td>24,40 ± 3,94</td>
<td></td>
</tr>
<tr>
<td>PROGRESSIVE LINEAR MOTILITY</td>
<td>T1</td>
<td>27,00 ± 0,71</td>
<td>12,00 ± 1,26</td>
<td>13,29 ± 1,35</td>
<td>21,25 ± 3,83</td>
<td>18,83 ± 1,99</td>
<td>13,20 ± 2,40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>20,50 ± 0,98</td>
<td>15,67 ± 1,47</td>
<td>17,14 ± 1,68</td>
<td>18,00 ± 1,61</td>
<td>19,33 ± 1,81</td>
<td>14,20 ± 1,72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>17,25 ± 2,82</td>
<td>9,78 ± 1,26</td>
<td>10,86 ± 1,31</td>
<td>10,00 ± 2,02</td>
<td>13,50 ± 2,67</td>
<td>9,40 ± 1,41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>24,25 ± 3,82</td>
<td>13,22 ± 2,58</td>
<td>16,00 ± 2,91</td>
<td>15,50 ± 2,84</td>
<td>18,50 ± 3,81</td>
<td>13,00 ± 3,73</td>
<td></td>
</tr>
<tr>
<td>VIABILITY</td>
<td>T1</td>
<td>23,32 ± 3,69</td>
<td>16,83 ± 2,35</td>
<td>18,56 ± 2,74</td>
<td>20,51 ± 4,75</td>
<td>18,38 ± 2,93</td>
<td>18,27 ± 3,96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>21,57 ± 3,44</td>
<td>17,03 ± 2,50</td>
<td>18,11 ± 3,17</td>
<td>21,11 ± 3,57</td>
<td>18,11 ± 3,32</td>
<td>19,27 ± 3,61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>22,68 ± 2,97</td>
<td>15,22 ± 2,07</td>
<td>16,15 ± 2,55</td>
<td>20,12 ± 3,83</td>
<td>16,81 ± 2,64</td>
<td>17,78 ± 3,46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>24,74 ± 3,48</td>
<td>17,39 ± 2,15</td>
<td>19,29 ± 2,49</td>
<td>22,52 ± 4,44</td>
<td>21,28 ± 2,72</td>
<td>18,79 ± 3,85</td>
<td></td>
</tr>
</tbody>
</table>

The table reports the values of total motility, progressive linear motility (PLM), and viability of stallion ejaculates (expressed as mean percentage ± standard error) divided by the treatment and the class. Four treatments with different concentrations of Docosahexaenoic acid (DHA) were tested in the experiment (T1, T2, T3, T4, respectively for 0, 1, 10, 20ng/ml of DHA). The ejaculates studied were classified basing on three classifications, including two classes each classification: PLM class 1 (ejaculates with PLM after thawing >20%), PLM class 2 (ejaculates with PLM after thawing <20%); Delta PLM class 1 (ejaculates which decrease more than 60% after freezing), Delta PLM class 2 (ejaculates which decrease less than 60% after freezing); FCT class 1 (ejaculates concentrated less than 25 times during the processing), FCT class 2 (ejaculates concentrated more than 25 times during the processing).
5. DISCUSSION

4.1 Effects of docosahexaneoic acid in the totality of the ejaculates

As expected from previous studies cryopreservation, had a detrimental impact (compared to fresh semen) on sperm characteristics, motility parameters and viability. The results of this study show no remarkable protective effect of DHA for any criteria: total motility, PLM and viability.

The role of Ω-3 PUFA as docosahexaneoic acid (DHA) on sperm resistance to cooling and freezing procedures is controversial, and may be related to the type of long chain PUFA content (Castellano et al., 2010). Although both positive and negative actions are theoretically possible, the overall effects of PUFAs on fertility are not fully understood. With regard to male fertility, PUFAs are essential substances for male fertility as they give appropriate fluidity to the sperm plasma membrane (Wathes et al., 2007).

This study is the first attempt to administer DHA to stallion semen in vitro in order to evaluate its possible protective effects. These relate first of all to the possible exchanges of lipid components between the spermatozoa and two egg yolk-based diluents (normal and n-3 enriched yolks) during cryopreservation, and how this relates to the quality of the fresh semen.

Actually, the aim of the study was to determine whether supplementation of semen extender with various concentrations of DHA (1, 10, 20ng/ml) could enhance the thermal resistance of stallion semen during frozen storage procedures.

To be sure that the effects which were going to be observed, were due to the different levels of DHA, and not because of vitamin E concomitant supplementation (which was unavoidable because of the need of its antioxidant protection on the DHA), an equal concentration of vitamin E was guaranteed also in the control treatment, where no DHA had been added. Anyway, none statistically relevant differences has been noticed between the four treatments: neither in viability nor in motility parameters.
This result could be apparently in contrast with the effects expected according to other studies.

Brinsko et al. (2005) studied the effects of a DHA rich nutraceutical (omega 3) on fresh, cooled and frozen semen quality. Stallions were randomly assigned to one of two treatment groups (n=4 per group). Stallions were fed their normal diet (control) or their normal diet top-dressed with 250 g of a DHA-enriched nutraceutical. Feeding trials lasted for 14 weeks, after which a 14-week washout period was allowed and the treatment groups were reversed for another 14 weeks feeding trial. Feeding the nutraceutical resulted in a three-fold increase in semen DHA levels and 50% increase in the ratio of DHA to DPA in semen. Sperm motion characteristics in fresh semen were unaffected by treatment. After 24 h of cooled semen storage, total and progressive motility did not differ between treatment groups, but sperm from stallions fed the nutraceutical exhibited higher velocity and straighter projector (P<0.05). After 48 h of cooled storage, increases in the percentages of sperm exhibiting total motility (P=0.07), progressive motility (P=0.06) and rapid motility (P=0.04), were observed when stallions were being fed the nutraceutical.

Elhordoy et al. (2008) investigated the action of the polyunsaturated fatty acids (PUFAs), in particular decosahexanoic acid (DHA; 22:6 n-3, omega-3 fatty acid), on the quality of fresh, cooled and frozen stallion semen. Six stallions were randomly assigned to two treatment groups (n=3 per group) and were kept under the same management conditions. Stallions of one group (group A) were fed 30 g of DHA per day for 80 days, while the others (group B) did not receive any supplementation. The treatment groups were then reversed, with the group B fed with 30 g of DHA and group A serving as a control. Both groups were subjected to a washout period before the second stage of this switch-back study. This period was established to allow DHA levels of treated stallions to return to pre-treatment levels. Preliminary results show that treated stallions experienced an increase in total spermatozoa number per ejaculate (P<0.05), motility (P<0.05), and a reduced percentage of dead and abnormal spermatozoa (P<0.05), especially in the acrosome and mid-piece abnormalities (P<0.001), in comparison with the control treatment. Mean percentage of progressively motile
spermatozoa improved in some of the stallions after 48 h of cooled-semen storage and after semen cryopreservation.

Looking at these studies some consideration need to be done.

The first objection is that the two of these studies concerned with DHA dietary supplementation and not the adding of DHA to semen extender. That enhanced DHA content of the plasma membranes, induced by dietary supplementation (which has been widely demonstrated: Kelso et al., 1997; Paulenz et al., 1999; Castellano et al., 2010), could be probably more effective that the one it is possible to obtain by the addition during cryopreservation. It is reasonable to think that the longer duration of the dietary treatment can result in a more significant absorption of DHA in the membranes of the spermatozoa.

DHA is positively correlated with ejaculate concentration and total sperm number; therefore, by supplementing the DHA level in the feed, its bioavailability could be increased and this could allow spermatogenesis to operate to its maximum extent, assuming that such activity is suppressed by a lack of essential fatty acids. Indeed, there may be a general lack of n-3 fatty acids in commercial feeds. An increased amount of DHA should increase membrane fluidity, and it can therefore be hypothesized that it would decrease the damage to the plasma membranes during freezing (Maldjian et al., 2005).

Another consideration concerned the fact that the parameters (velocity, projector and rapid motility) which show significant (P<0.05) improvement in the cited studies, were not considered in our study. Indeed, looking at the total motility or at PLM, they do not show a clear improvement in frozen thawed semen results.

4.2 Effect of docosahexaneoic acid on bad freezers ejaculates

According to some studies regarding DHA enriched diets (Brinsko et al., 2005; Elhordoy et al., 2008), it appears that feeding the nutraceutical may improve the motion characteristics of cool-stored stallion semen most beneficially for stallions of marginal fertility whose sperm do not tolerate the rigors of cooling and storage. Furthermore, it seems that the lack of DHA in semen is related with the decreasing quality of the
ejaculates: decreasing in DHA proportion of sperm phospholipids has been demonstrated to be accompanied by a decrease in the number of sperms and motility in aged bull ejaculations (Kelso et al, 1997a).

In other words, the supplementation may correct the lack of DHA, which seems to be responsible of the decreasing quality of the semen, which is linked with the less tolerance to cooling and freezing process.

Thus, one of the hypothesis of the current study was that: the supplementation of the extender with DHA, would have improve, in a more significant way, the semen characteristics of ejaculates which show worst resistance to freezing process than the ones which freeze well.

In order to verify this hypothesis ejaculates were divided in two classes depending of the PLM Post thawing. “PLM class 2” which had a lower PLM after thawing (less than 20%) was expect to show more differences between the treatments compared to “PLM class 1” which had a greater PLM after thawing (more than 20%).

The results obtained from both PLM and viability do not show any statistically different values among the treatments, between the two classes.

Trying to have a more accurate discrimination between the ejaculates more sensitive to cold shock and the less sensitive ones, the ejaculates had been classified with a second classification that highlight the decrease of PLM (Delta PLM) due to the freezing process, in the control treatment.

The two groups obtained were named “Delta PLM class 1” (which includes the ejaculates more sensitive to cold shock: Delta PLM>60%) and “Delta PLM class2” (which includes the ejaculates less sensitive to cold shock: Delta PLM<60%). A comparison within the two classes has been done to see if in one of them there was a significant difference in the sperm characteristics among the four treatments.

Neither this classification show any relevant difference between the treatments (0, 1, 10, 20 ng/ml of DHA), it seems that the addition of DHA during stallion semen cryopreservation has no useful effect, even in ejaculates which less tolerate freezing procedure.
The current results, are the first obtained for stallion semen, but other studies has been conducted about extender supplementation with omega-3 fatty acids in bull and goat semen.

Nasiri et al. 2011 investigated the effect of adding n-3 fatty acids (FA) and α-tocopherol (VE) to semen extender on freezing ability in Iranian Holstein bulls. Semen was divided into 12 groups including four levels of n-3 FA (0, 0.1, 1, 10 ng/ml) and three levels of VE (0, 0.1, 0.2 mmol) According to this study the treatment of 0.2 mmol VE and 10 ng/ml n-3 FA had the best post-thawed sperm characteristics (P < 0.01). Mean total motility and PLM percentages were significantly lower in 0 and 0.1 levels than that in 1 and 10 ng/ml levels of n-3 FA (P < 0.01). The viability, as well, was significantly lower in 0 and 0.1 ng/ml than those in 1 and 10 ng/ml n-3 FA.

The same treatments were tested in Goat semen (Ansari, 2012), for the total motility and the PLM; the treatment with 10 ng/ml of DHA had significantly highest value (P≤0.05). The motility and PLM percentage was significantly higher in 0.1 FA and 0.2 VE and 10 ng/ml FA, 0.2 mmol VE groups than other treatment groups.

Another study (Towhidi and Parks, 2012) showed similar results testing the combined effects of adding source of n-3 fatty acids and α-tocopherol to semen extender on freezability in Brown Swiss bull sperm. Semen was divided into 12 groups including 4 levels of n-3 FA (0, 1, 10, 100 ng/ml) and 3 levels of VE (0, 0.2, 0.4 mM); motility and viability were assessed. Mean percentage of total motility and mean percentage of PLM were significantly higher with 10 ng/ml n-3 FA than with other FA levels (P<0.05). The percentages were significantly higher in combined 10 ng/ml FA and 0.4 mM VE than for other treatment combinations. Furthermore for the different levels of FA, viability was significantly higher in 10 ng/ml than 0, 1 and 100 ng/ml n-3 FA. The treatment 10 ng/ml FA, 0.4 mM VE had the highest (P≤0.05) viability compared with other groups.

A more recent work (Abavisani et al., 2013) conducted to investigate possible protective effects of different levels of Ω-3 PUFA in extender on bovine chilled as well as frozen sperm, suggest that supplementation of semen extenders with Ω-3 PUFAs did not significantly improve sperm resistance to cooling, especially to
cryopreservation. In spite it reveals that the addition of Ω-3 PUFAs in bull extender is not a useful method for improvement of bull sperm, is difficult to make a comparison with the previous studies, in particular because the latter does not take into account of the supplementation of vitamin E, as extracellular antioxidant, which is able to reverse the negative effect of PUFA supplementation on mammalian spermatozoa.

Considering all these controversial results is difficult to establish whether the addition of PUFAs, and in particular of DHA, is a useful method to improve the freezability of semen or not. For sure, differences between bull and stallion semen composition should be consider as a possible cause of a probable different semen behaviour after the addition of DHA.

For this reason, investigations on porcine semen, whose lipid profile is considered closer to the equine one, could be more helpful to understand the effect of the addition of DHA in the extender.

In a study conducted on boar semen (Maldjian et al., 2005), two types of yolk have been considered as ingredients in diluents for cryopreservation: yolks with a standard fatty acid composition and yolks enriched in docosahexaenoic acid (DHA). The yolks enriched in n-3 fatty acids failed to improve the quality of sperm following cryopreservation, even if the proportion of DHA was significantly higher in spermatozoan phospholipids from thawed cells that had been in contact with n-3 yolks. On the other hand, Kaeoket et al. (2010) reported that supplementation of yolk freezing extender with DHA by adding fish oil was effective for freezing boar semen as it resulted in higher post-thaw plasma membrane integrity and progressive motility. Apparently, the reason of these opposite results might be a higher concentration of DHA used in the latter study. This explanation leads to another possibility, which would be interesting to investigate: as stallion and boar semen have a lower DHA content in their semen, might be that an higher concentration of DHA in the extender is necessary, to see the same effects seen in bull semen.

Anyway, to get clearer results it would be interesting to study the different characteristics of the semen of the three species before and after the addition of DHA and vitamin E. Subsequently, evaluate if there are beneficial effects, and in which
species. A unique method to process the samples and to assess their parameters would be recommend. The use of a CASA system would help to test more parameters and to get more objective and accurate results.

It is also recommendable to analyze more sample for the same treatment in the different ejaculates in order to have more significant data.

Finally, an important consideration need to be done: effects on the cryopreserved semen have to be evaluate after a month of storage, change in the sperm characteristics may occur in a longer time.

4.3 Effect of docosahexaneoic acid in Final Concentration Time classes

Class FCT 1 showed significant higher PLM values in the whole treatments, it suggest that depending on the final concentration time value we can aspect to have better o lower value of PLM. According to this result we can see that the initial dilution rate is inversely related with the PLM in the sample, meaning that the more a semen sample is originally diluted, the lower value of PLM it will have after thawing.

This result could be explained with the fact that an originally diluted semen, will probably have less concentrated enzyme and antioxidant in its seminal plasma, and this can lead to a loss of protection and a more susceptibility of the spermatozoa to lipid peroxidation and other damages during freezing-thawing process.

Anyway, none of the two classes reveals significant difference between the treatments, so the treatments had no relevant effects on the two classes.

4.4 Study of correlation

The study reveals some interesting correlations. Particularly, regarding the collection volume, it has shown to be positively related with the PLM before freezing, as well as it has shown a negative effect on the on the same parameter after thawing. All this reflects in a positive correlation between the collection volume and Delta PLM. These correlations suggest a positive effect of the SP on the fresh semen, probably due to its
several properties and components. In spite of this, as an increasing volume of seminal plasma can be read as a dilution of its antioxidants (which would be deleterious to overcome cooling process) it leads to decreasing values of PLM after thawing. However, this suspected loss of antioxidant protections does not seem to affect the viability after thawing, whose correlation with the collection volume is still positive.

PLM before freezing parameter, show another interesting correlation: it seems to be negative related with the concentration of the semen. This result could be due to the decrease in collection volume, and mainly in the volume of SP, normally associated with the increase of the concentration. Anyway, more accurate techniques for the study of PLM (as the use of a CASA system) could help to investigate the real significance of these correlations.
5. CONCLUSIONS

This study was conducted to investigate possible protective effects of different levels of DHA in extender on equine frozen semen. In contrast to the hypothesis, supplementation of semen extenders with DHA, in combination with vitamin E, did not significantly improve sperm resistance to cryopreservation, not even in ejaculates with lower toleration of freezing procedures. So, it reveals that the addition of DHA in stallion semen extender, at the studied concentrations (0, 1, 10, 20 ng/ml) is not a useful method for improvement of stallion sperm. Further investigations should be conducted with higher concentrations of DHA in order to convey its protective properties on sperm membranes. An accurate investigation of the biochemical composition of the SP of the ejaculates utilized, including the antioxidant components, could help to understand the influence of the dilution on the semen freezability. It is therefore recommendable to investigate the fatty acids profile and the level of peroxidation of the ejaculates, before and after the treatment, in order to evaluate the true effects of the addition of DHA on semen characteristics. Finally, a higher number of straws, for the same treatment, of each ejaculate should be analysed to get more significant results.
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