DAIANE MOREIRA SILVA

EFEITO DA ADIÇÃO *IN VITRO* DE ÁCIDO DOCOSAHEXAENÓICO (DHA) E FATOR DE CRESCIMENTO SEMELHANTE À INSULINA TIPO I (IGF-I) NA QUALIDADE DO SÊMEN DE GARANHÕES

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutora.

Prof. Dr. José Camisão de Souza

Orientador

Prof. Dr. Sean Fair

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EFFECT OF THE *IN VITRO* ADDITION OF DOCOSAHEXAENOIC ACID (DHA) AND INSULIN-LIKE GROWTH FACTOR-I (IGF-I) ON THE QUALITY OF STALLION SEMEN

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para a obtenção do título de Doutora.

APROVADA em 15 de dezembro de 2016

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RESUMO

A qualidade do sêmen após os processos de resfriamento, congelamento e descongelamento decresce, diminuindo a taxa de gestação após a inseminação artificial. Ácido docosahexaenóico (DHA) e fator de crescimento semelhante à insulina tipo I (IGF-I) podem eliminar essa perda de qualidade ocasionada pela baixa temperatura. O objetivo deste estudo foi analisar o efeito da adição *in vitro* de DHA e IGF-I na qualidade do sêmen resfriado e criopreservado de garanhões. No Experimento 1, sêmen de 10 garanhões *Irish Sport Horse* foi coletado (3 ejaculados/garanhão) em 2014 na Irlanda. O sêmen foi transportado para o Laboratório de Reprodução Animal (*University of Limerick*), diluído a 100 x 10^6 de espermatozoides/mL, suplementado com 0,02 mM de vitamina E (VE) e 0, 1, 10 e 20 ng de DHA/mL e congelado. O sêmen foi descongelado e a motilidade total (MT), motilidade progressiva rápida (MP), integridade acrossômica, fluidez de membrana e morfologia foram analisadas. No Experimento 2, sêmen de 3 garanhões foi coletado (3 ejaculados/garanhão) em 2015 e congelado como no Experimento 1, mas VE e DHA foram adicionados após o descongelamento. MT e MP foram analisadas aos 30, 60 e 120 minutos e viabilidade, integridade acrossômica e fluidez de membrana aos 30 minutos após a adição de DHA. No Experimento 3, sêmen de 5 garanhões foi coletado (1-3 ejaculados por garanhão) durante a estação de monta de 2015 e diluído a 20 x 10^6 de espermatozoides/mL. Posteriormente, DHA e VE foram adicionados e o sêmen foi estocado a 4ºC. Depois de 1, 24, 48 e 72 horas, MT, MP, viabilidade, fluidez de membrana e peroxidação lipídica foram analisadas. No Experimento 4, sêmen de 3 garanhões (3 ejaculados por garanhão) foi coletado entre fevereiro e março de 2016. Os ejaculados foram processados como no Experimento 1 e suplementados com VE. Após o descongelamento, o sêmen foi diluído a 25 x 10^6 de espermatozoides/mL e dividido em 4 tratamentos, assim nomeados: DHA0 (0 ng de DHA/mL; controle), DHA0 + IGF-I (controle + 100 ng de IGF-I/mL), DHA1 (1 ng de DHA/mL) e DHA1 + IGF-I (1 ng de DHA/mL + 100 ng de IGF-I/mL). A adição de 20 ng/mL de DHA ao sêmen resfriado (Experimento 3) aumentou a MT comparada ao controle sem VE (52,9 ± 7,99% X 25,7 ± 5,23%; $P<0,05$); a adição de qualquer concentração de DHA melhorou a MP comparada aos controles (20,8 ± 5,86% X 4,3 ± 1,48%; $P<0.001$) e a adição de 10 e 20 ng/mL de DHA aumentou a fluidez de membrana comparada aos demais tratamentos (28,7 ± 4,35% e 29,4 ± 5,18%, respectivamente X 19,0 ± 3,85%; $P<0,001$). No entanto, a adição de DHA não afetou o sêmen congelado (Experimento 1 e 2). A adição simultânea de DHA e IGF-I ao sêmen de garanhões após o descongelamento (Experimento 4) melhorou a MP (7,2 ± 1,56% X 4,1 ± 1,15%; $P<0,05$) mas não afetou a MT, a viabilidade e a integridade acrossômica.

Palavras-chave: Espermatozoides. PUFA. Hormônio proteico.

ABSTRACT

Semen quality after cooling, freezing and thawing processes decreases, which consequently impairs the pregnancy rate following artificial insemination. Compound as docosahexaenoic acid (DHA) and insulin-like growth factor-I (IGF-I) can eliminate this loss of quality caused by low temperature. The aim of this study was to assess the effect of the *in vitro* addition of DHA and IGF-I on the quality of cooled and cryopreserved stallion semen. In Experiment 1, semen from 10 Irish Sport Horse stallions was collected (3 ejaculates per stallion) during 2014 in Ireland. Semen was transported to Laboratory of Animal Reproduction (School of Natural Sciences, Faculty of Science and Engineering, University of Limerick), diluted to 100 x 10^6 spermatozoa/mL, supplemented with 0.02 mM of vitamin E (VE) and 0, 1, 10 or 20 ng of DHA/mL and frozen. Semen was thawed and total motility (TM), rapid progressive motility (PM), acrosome integrity, membrane fluidity and morphology were assessed. In Experiment 2, semen from 3 stallions was collected (3 ejaculates per stallion) during 2015 season breeding and frozen as in Experiment 1, but VE and DHA were added after thawing. TM and PM were assessed at 30, 60 and 120 min and viability, acrosome integrity and membrane fluidity at 30 min after addition of DHA. In Experiment 3, semen from 5 stallions was collected (1-3 ejaculates per stallion) during 2015 season breeding and diluted to 20 x 10^6 spermatozoa/mL. Posteriorly DHA and VE were added and semen was stored at 4ºC. After 1, 24, 48 and 72 h, TM, PM, viability, membrane fluidity and lipid peroxidation were assessed. In Experiment 4, semen from 3 stallions (3 ejaculate per stallion) was collected between February and March 2016. Ejaculates were processed as per Experiment 1 and supplemented with VE. After freezing and thawing processes, semen was diluted to 25 x 10^6 spermatozoa/mL and split in 4 treatments, namely: DHA0 (0 ng of DHA/mL; control), DHA0 + IGF-I (control + 100 ng of IGF-I/mL), DHA1 (1 ng of DHA/mL) and DHA1 + IGF-I (1 ng of DHA/mL + 100 ng of IGF-I/mL). Addition of 20 ng/mL of DHA to cooled semen (Experiment 3) increased the TM compared to the control without VE (52.9 ± 7.99% X 25.7 ± 5.23%; $P<0.05$); addition of any concentration of DHA improved the PM compared to the controls (20.8 ± 5.86% X 4.3 ± 1.48%; $P <0.001$) and addition of 10 and 20 ng/mL of DHA increased the membrane fluidity compared to other treatments (28.7 ± 4.35% and 29.4 ± 5.18%, respectively X 19.0 ± 3.85%; $P<0.001$). However, addition of DHA did not affect frozen semen (Experiment 1 and 2). The simultaneous addition of DHA and IGF-I to stallion semen after thawing (Experiment 4) improved PM (7.2 ± 1.56% X 4.1 ± 1.15%; $P<0.05$), but did not affect the TM, viability and acrosome integrity.

Keywords: Spermatozoa. PUFA. Protein Hormone.

SUMÁRIO

FIRST PART

1 INTRODUCTION

Although equine breeding dates from several centuries ago, it remains strongly essential to this date. In the past, horses were mainly used as transportation in agriculture and wars, however modern activities were introduced in the life of these animals over time. Nowadays, horses are used in various sports and in a myriad of activities in connection to mankind.

In addition to equestrian sports, horses have been used in hippotherapy, as companion animals and even for meat consumption by humans. Thus, there is an ever crescent need to study and further develop the artificial reproduction techniques aiming to optimize quality of reproducers and their gametes, by increasing the number of superior genetic descendants in a shorter time, exchanging genetic materials between long distances, controlling diseases and improving general reproductive efficiency.

Stallion semen quality can be manipulated for the purpose of achieving improvements in seminal parameters such as spermatic motility, morphology, membrane integrity and fluidity, consequently expanding the use of cooled and frozen semen in artificial insemination (AI). There are several techniques that can be used in order to increase the quality of seminal parameters, including the addition of substances to semen extenders. These substances can act as an energy source for the spermatozoon, protection against thermal and osmotic shock, as well as participating directly in the spermatozoa metabolism.

Docosahexaenoic acid (DHA) and insulin-like growth hormone factor-I (IGF-I) are some examples of substances that can be added to semen extenders in an attempt to improve semen quality. DHA is a polyunsaturated fatty acid (PUFA) that can persist in the plasma membranes, promoting higher resistance and fluidity during cooling, freezing and thawing processes. IGF-I is a hormone that can act as an antioxidant and a promoter of energy source uptake by spermatozoa.

Thus, the objective of this study was to investigate the effect of the *in vitro* addition of DHA to cooled and cryopreserved stallion semen, besides to verify the effect of the *in vitro* addition of DHA in combination with IGF-I on the quality of frozen-thawed stallion semen.

2 LITERATURE REVIEW

2.1 Equine breeding industry

The world equine population is 58.9 million heads distributed in all continents with 55.2% of the population located in America, 24.4% in Asia, 10.3% in Africa, 9.4% in Europe and 0.7% in Oceania; the five largest equine producers are The United States, Mexico, China, Brazil and Argentina with 10.2, 6.3, 6.0, 5.4 and 3.6 million heads, respectively (FOOD AND AGRICULTURE ORGANIZATION OF UNITED NATIONS - FAO, 2014).

Horses have co-existed with humans throughout history and have served to a variety of practical purposes, including transport, draft animals in agriculture and in wars (HALL; RAUNE, 1993). Nowadays, horses have been used in various other activities, including hippotherapy (MORAES et al., 2016) and because of their power, agility, gracefulness and speed, they are mostly used for companion and in sports (ALDERSON, 2008). Equestrian sports have become more popular over the last decades both at amateur and professional levels (ROVERE et al., 2016).

Currently, the structure of equine breeding industry is more internationalized and the use of AI is becoming more frequent. This biotechnology has become a major reproduction tool, allowing breeders all over the world to benefit from the best stallions of most breeds, especially in relation to sport breeds (AURICH; AURICH, 2006).

2.2 Artificial insemination in the equine breeding industry

AI has emerged as a biotechnology that allows the use of semen of superior genetics with better efficiency, encompassing the insemination of a higher number of females per ejaculate. AI is used to deposit normal and live spermatozoa in the uterus at the appropriate time (SAMPER, 2000) and it has been widely used after its introduction in the equine breeding industry (HAADEM et al., 2015).

AI is an optimal way of disseminating superior male genetics to a large number of females (AKHTER et al., 2016). It also facilitates the exchanging of genetics between different regions, avoiding sanitary risks usually involved in natural breeding (AISEN et al., 2005). Although fresh semen can be used in AI, the improvements that the transport of cooled semen provides to mare owners are more significant as they are able to choose the most popular stallions which may otherwise be unavailable geographically (HAADEM et al., 2015). Fresh semen has a short lifespan thus currently cooling semen is the most popular method for storage of semen in equine industry. This technique is used to reduce the metabolic rate of spermatozoa, resulting in decreased reactive oxygen species (ROS) and

higher storage ability of semen for transportation over long distance compared to fresh semen. However cooled semen has been associated with increased permeability of the spermatozoa membrane and lipid peroxidation (DAVIES MOREL, 2008). Cooled semen presents other logistical limitations, such as the reduced storage capacity and consequent difficulties in transportation through long distances compared to cryopreserved semen (LOOMIS; GRAHAM, 2008). For frozen semen the main disadvantages are the high cost and the greater number of spermatozoa in the inseminating dose, which is necessary to compensate the poor spermatozoa survival (SAMPER; PLOUGH, 2010). In addition, differences in handling, cooling, freezing, shipping procedures and semen quality in individual ejaculates from the same stallion make fertility data hard to compare between AI centers (HAADEM et al., 2015). In the other hand, cryopreservation of semen offers many advantages to the animal industry, particularly in conjunction with genetic evaluation and selection programs (CHAUDHARI et al., 2015). Moreover, frozen semen can be used in mares even when the stallion is at performance events, ill, recovering from an injury or even after its death. Other advantages are long-term preservation of spermatozoa, distribution of semen to breeding farms worldwide, as well as, reduced potential for the transmission of contagious diseases (MILLER, 2008).

Higher pregnancy rates have been achieved using fresh-semen AI than with natural mating (LANGLOIS; BLOUIN, 2004), but fertility rate from AI with cooled semen is lower compared to fresh semen (JASKO et al., 1992; LANGLOIS; BLOUIN, 2004). Jasko et al. (1992) reported first cycle pregnancy rates of 76 and 65% for fresh and cooled semen, respectively. However, according to Candeias et al. (2012), the commercial interest in the AI technique using stored semen is notorious and, for this reason, investigative studies on the conservation of stallion semen have been increased. Also, the benefits of frozen semen have led breeders to look at it as a practical and efficient method for transporting or storing stallion semen for longer periods of time (LOOMIS; GRAHAM, 2008).

Uterine body insemination is one of the most popular AI methods, wherein either fresh or cooled semen is delivered by a standard AI catheter (RISCO et al., 2009). The standard inseminanting doses of 500 to 1000 million spermatozoa have been used for years for fresh and cooled semen, respectively. One-half to one-fifth of the fresh semen dose is suggested to achieve acceptable levels of fertility with frozen stallion semen, and it is widely accepted that fertility of frozen-thawed stallion semen is lower than that of fresh and cooled semen. The rectally guided deep horn insemination is a technique that places the catheter (a 65-cm-long flexible pipette) firstly in the uterine body, the hand is removed from the vagina and

introduced in the previously evacuated rectum, then the tip of the pipette is identified and advanced by rectal manipulation and gentle pressured towards of the horn ipsilateral to the ovary that has the preovulatory follicle, where the inseminating dose is placed (SAMPER; PLOUG, 2010). There are higher spermatozoa access to the oviduct and consequently superior fertilization rate when semen is deposited at the tip of the uterine horn as opposed to the uterine body (VARNER, 2016). Another AI-method is by hysteroscopy, wherein a videoendoscope is required. These two AI methods are more complex and expensive than the conventional technique, but a much lower spermatic concentration is needed (1 to 25 million spermatozoa) (SAMPER; PLOUG, 2010).

Besides techniques, the success of AI is related to the capacity of the diluent in providing protection of the various cell compartments during the cooling, freezing and thawing processes (CELEGHINI et al., 2008) regardless of animal species. Furthermore, extenders must offer benefits such as pH and osmolarity control, energy substrate to the spermatozoa and seminal plasma dilution, thus maintaining the fecundation capacity after AI (MARTINS et al., 2016). Therefore, the composition of stallion semen diluents may affect the outcome of spermatozoa preservation and consequently fertilization rates, which is currently one of the major concerns of researchers (CANDEIAS et al., 2012).

2.3 Quality of stallion semen

Gamete viability may influence conception rates (XAVIER et al., 2010). Despite the improvement in semen manipulation techniques in laboratories and in the AI protocols, a thorough knowledge of gamete quality is important. A stallion should only be considered for breeding if its fresh semen exhibits excellent spermatic parameters and also good resistance to the cooling, freezing and thawing processes (FAGUNDES et al., 2011). Many studies have been conducted aiming at improving the cooling (GOULART et al., 2004; MEIRELLES et al., 1998; NUNES; ZÚCCARI; SILVA, 2006) and freezing techniques (FURST et al., 2005; MURPHY et al., 2014; SILVA et al., 2009; TERRACIANO et al., 2008) of stallion semen, which results in a longer conservation of fecundation power and in a more rational use of the stallions in AI programs.

The conventional approach to evaluate stallion semen dates back several decades and consists in the assessment of spermatozoa concentration, seminal volume, sperm cell morphological characteristics, motility patterns and membrane resistance before and after storage (CELEGHINI et al., 2010). The percentage of morphologically normal spermatozoa is positively correlated with motility. Moreover morphological inspection provides additional

information about the characteristics of individual spermatozoa; semen can have good motility but high incidence of morphologic abnormalities, although stallions may have many spermatozoa abnormalities and still be fertile (VARNER, 2008). Therefore, the different spermatic characteristics must be analysed and interpreted together.

The stallion ejaculate volume varies widely depending on age, season, breed, semen collection system, prior sexual stimulation, among other factors, but in general the seminal volume is approximately 36 mL (GOTTSCHALK et al., 2016). Total motility (TM) of fresh semen for most stallions is between 70 to 90% (PONTHIER et al., 2014; WRENCH et al., 2010), progressive linear motility (PLM) is around 52% and the average concentration is 270 million spermatozoa per mL (TORRE et al., 2016). Darenius (1998) suggested that stallions to be used in AI program with cooled semen should have: weekly seminal production of 30-35 x 10^9 spermatozoa, 70% of morphologically normal cells, TM higher than 50% and, after 12 hours of cooling, a reduction of the initial TM of not more than 30%, considering the final TM at the end of this period of at least 40%. For frozen semen, Vidament et al. (1997) considered stallions that produce post-thaw TM of at least 35% to be acceptable for use in AI programs.

2.4 Addition of substances to stallion semen

Substances can be added to equine semen in an attempt to improve spermatic characteristics and preservation time (Table 1), thus guaranteeing better results with AI. Several studies have reported benefits in the quality of cooled stallion semen after addition of substances into the extender. Milk, caseinate (MARTINS et al., 2016), coenzyme Q10, vitamin E (NOGUEIRA et al., 2015), superoxide dismutase (COCCHIA et al., 2011) and cholesterol-loaded-cyclodextrin (HARTWIG et al., 2014) are some examples.

Lipid peroxidation is an important characteristic of the stress caused by semen cryopreservation (WATSON; DUNCAN, 1988). It occurs in the cellular membrane with consequent generation of free radicals and it may drastically damage the metabolic activity of spermatozoa, resulting in the loss of motility and spermatic function (AITKEN, 1994). Thus several current reports on cryopreserved stallion semen involve attempts to avoid reactive oxygen species (ROS) production and lipid peroxidation. For example, Franco et al. (2016) added vitamin E to stallion freezing extender and found a protective effect against oxidative damages. Another way to reduce oxidative stress is by adding butylated hydroxyanisole or butylated hydroxytoluene into the extender during the freeze-thaw process (SEIFI-JAMADI

et al., 2016) or even by adding a combination of several cryoprotetants such as glycol, dimethyl sulphoxide, methyl formamide and dimethylformamide (WU et al., 2015).

Glycerol alone (SCHERZER et al., 2009) or in combination with dimethylformamide can also be used as a cryoprotectant for stallion semen (ÁLVAREZ et al., 2014). Other substances such as dimethylacetamide (SANTIANI et al., 2016), cholestanol-loaded-cyclodextrine (MORAES et al., 2015), cholesterol-loaded-cyclodextrine (MURPHY et al., 2014), cholesterol (OLIVEIRA et al., 2010) and caseinate (LAGARES et al., 2012) have been added to freezing extender and induced improvements in stallion semen quality after thawing process.

Table 1. Substances that can be added to stallion semen and their benefits (Continue)

Substance	Kind of semen	Action/Benefits	Reference
Milk	Cooled	There are antioxidants in the milk such as lactoferrin and casein, besides its action as energy source (lactose) for the spermatozoa	Martins et al. (2016)
Caseinate	Cooled/Frozen	Casein bound to a calcium or to a sodium molecule which increases casein solubility	Lagares et al. (2012) and Martins et al. (2016)
Coenzima Q10	Cooled	Also called vitamin ubiquinone, it participates in the process of adenosine triphosphate (ATP) production	Nogueira et al. (2015)
Vitamin E	Cooled/Frozen	Antioxidant by protection of the spermatozoa membrane against lipid peroxidation	Franco et al. (2016) and Nogueira et al. (2015)
Superoxide dismutase	Cooled	Antioxidant enzyme	Cocchia et al. (2011)
Cholesterol	Frozen	It improves the fluidity and stability of the spermatozoa membranes	Oliveira et al. (2010)
Cholesterol-loaded-cyclodextrin	Cooled/Frozen	Cyclodextrin is an amphipathic molecule that helps the cholesterol to move into water	Hartwig et al. (2014) and Murphy et al. (2014)

Table 1. Substances that can be added to stallion semen and their benefits (Conclusion)

Substance	Kind of semen	Action/Benefits	Reference
Cholestanol-loaded-cyclodextrine	Frozen	Cholestanol is a molecule derived from cholesterol	Moraes et al. (2015)
Butylated hydroxyanisole	Frozen	Antioxidant by neutralization of ROS	Seifi-Jamadi et al. (2016)
Butylated hydroxytoluene	Frozen	Antioxidant by neutralization of ROS	Seifi-Jamadi et al. (2016)
Glycol	Frozen	Cryoprotectant	Scherzer et al. (2009)
Dimethyl sulphoxide	Frozen	Cryoprotectant	Wu et al. (2015)
Methyl formamide	Frozen	Cryoprotectant	Wu et al. (2015)
Dimethylformamide	Frozen	Cryoprotectant	Wu et al. (2015)
Dimethylacetamide	Frozen	Cryoprotectant	Santiani et al. (2016)

2.5 Lipid structure of the spermatozoa membrane

The spermatozoa plasmatic membrane of mammals is characterized by a high proportion of polyunsaturated phospholipids that give it special physical characteristics and compartmentalization of many of its component proteins and lipids into discrete domains on the head and tail (JONES, 1998). This membrane is physiologically asymmetric, with phosphatidylcholine and sphingomyelin located in the external leaflet of the lipid bilayer, while phosphatidylserine and phosphatidylethanolamine are located in the internal leaflet. This asymmetry probably represents different domains that may be related to specific functions (LÓPES et al., 1987). Polyunsaturated phospholipids are in a lamellar arrangement that organizes the fatty acid chain into a hydrophobic barrier, preventing the entry of water or other molecules (DARIN-BENNET et al., 1977). These phospholipids confer fluid consistency to the plasmatic membrane (Wolfe et al., 1998). The cholesterol/phospholipids ratio is also responsible for membrane fluidity (DARIN-BENNET et al., 1977). High cholesterol membrane regions are less fluid than portions with a higher proportion of phospholipids (Amann; Pickett, 1987).

The plasmatic membrane is more fragile than the acrossomal and mitochondrial membranes, thus the preservation of the plasmatic membrane is the critical point for the success of the cooling and cryopreservation (Valcárcel et al., 1997). At body temperature, the plasmatic membrane is in the liquid state and the lamellar arrangement allows the phospholipids to move freely along the bilayer (Amann; Pickett, 1987).

Cooling of semen causes transitions in the lipids, favoring the reduction of membrane fluidity and preventing the functionality of proteins which is necessary for the integrity and metabolism maintenance. Thus, these changes may lead to the loss of stability of the lipid

bilayer, structural deformations of cellular organelles and posteriorly, it can lead to cell death (HOLT, 2000; OEHNINGER; DURU; SRISOMBUT, 2000).

During cryopreservation, the plasmatic membrane undergoes modifications to adapt to changes in temperature, such as the translocation movement of phospholipid, with consequent phosphatidylserine externalization (LADHA, 1998). Mechanisms involved in the lipid exchange between the internal and external leaflets of the plasmatic membrane, such as tyrosine phosphorylation and cholesterol efflux, are also observed in cryopreserved spermatozoa (Silva; Gadella, 2006). Changes in the spermatozoon and its plasmatic membrane during the reduction of temperature generate lesions and decrease the fertilizing capacity of this gamete (SILVA; GUERRA, 2011).

2.6 Production of reactive oxygen species in spermatozoa

Spermatozoa undergo oxidative stress due to their high levels of PUFAs and low antioxidant response (GADELLA et al., 2001). It has been deemed necessary for low levels of ROS to be present within the reproduction process for spermatozoa capacitation and acrosome reaction (VASCONCELOS FRANCO et al., 2013). The superoxide anions produced during ROS generation induce hyper-motility of the spermatozoa and are involved in the binding to the zona pellucida (GADELLA et al., 2001). However excess ROS production in semen leads to sperm dysfunction and decreased fertilization capacity (MAIA; BICUDO, 2009).

Spermatozoa secrete ROS mainly due to the movement of the flagellum (VASCONCELOS FRANCO et al., 2013). ROS appear to be produced by sperm-specific NADPH oxidase which is present in the plasma of the spermatozoon head or the mid-piece (PENA et al., 2011). The ROS most commonly generated by spermatozoa are: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH*) (AITKEN, 1995). O_2^- is a free radical formed from molecular oxygen by the addition of an electron, it is generated spontaneously, especially in the mitochondrial membrane, through the respiratory chain and also by flavoenzymes, lipoxygenases and cycloxigenases. It is a small reactive radical and does not have the ability to penetrate the lipid membranes, acting only in the compartment where it is produced. H_2O_2 has a long life span and is able to cross biological membranes; it is produced from O_2^- and participates of the production of OH* in a reaction catalyzed by metal ions (Fe^{++} or Cu^+) called Fenton reaction. OH* is considered the most reactive free radical in biological systems and reacts rapidly with biomolecules and can trigger peroxidation of lipids in cell membranes (NORDBERG; ARNÉR, 2001).

As discovered during the examination of sperm, ROS generation shows increased levels in cryodamage, nonviable and morphologically abnormal stallion spermatozoa (BALL;

VO; BAUMBER, 2001). Vasconcelos Franco et al. (2013) also believe that the abnormal acrosome reactions can occur due to the oxidative stress produced in stallion semen, besides that ROS cause decreased spermatic motility and increased lipid peroxidation which consequently decreases the fluidity of the membrane and further reduces the potential fertilizing ability of the spermatozoa. In addition Baumber et al. (2000) demonstrated increased H_2O_2 and decreased sperm motility after induction of ROS production in stallion semen at room temperature.

2.7 Importance of docosahexaenoic acid to semen

PUFAs contain more than one double bond in their structure. The position of the first bond within the molecular structure distinguishes between the different types of fatty acids such as omega-3 (n-3), omega-6 (n-6) and omega-9 (n-9). PUFAs are involved in the gene expression of many cells types and are highly influential on changes within cell membranes. They are also involved in the regulation of cell signalling and membrane protein-mediated responses (YESTE et al., 2011).

The plasma membranes of most mammal spermatozoa appear to have higher DHA levels than those of docosapentanoic acid (DPA), however in stallions and boars the opposite is true (GADELLA et al., 2001; GRADY et al., 2009). Stallion spermatozoa have a high PUFA content and predominant PUFAs are variable between stallion but in general are arachidonic (18%), DPA (17%), DHA (8%) and oleic (5%) (CHOW; WHITE; PICKET, 1986). The spermatozoa membrane has high n-6 and low n-3 contents and DHA is the most important and predominant n-3 in spermatozoa. According to Gholami et al. (2010), these PUFAs are essential for spermatozoa's motility, viability and membrane integrity, but spermatozoa undergo oxidative stress due to their high levels of PUFA and low antioxidants response (GADELLA et al., 2001). Lipid peroxidation of these PUFAs occurs due to the effect of ROS and spermatozoa secrete ROS mainly because of the movement of the flagellum (FRANCO et al., 2013). Thereafter, ROS generation increases the cryodamaged, nonviable and morphologically abnormal spermatozoa (BALL, 2008). These events, in turn, cause decreased motility and increased lipid peroxidation, which reduce membrane fluidity and the potential fertilizing ability of spermatozoa.

Recent studies have reported positive effects in semen quality after dietary DHA supplementation in buffaloes (TRAN et al., 2016), bulls (GHOLAMI et al., 2010; MOALLEM et al., 2015), rams (ALIZADEH et al., 2014; SAMADIAN et al., 2010), rabbits (GLIOZZI et al., 2009; MOURVAKI et al., 2010) and men (SAFARINEJAD, 2011). Probably there are a direct mechanism that transfers PUFA from the diet to the testes and

spermatozoa (ESMAEILI et al., 2014). In addition an indirect effect of n-3 by stimulating gene expression of the elongase and desaturase enzymes with the availability of the required components can occur, which creates a rise in the n-3 levels (such as DHA) in the spermatozoa (ESMAEILI et al., 2012). However, according to other researchers, dietary DHA has failed to improve the quality of fresh (CASTELLANO et al., 2010b) and cryopreserved boar semen (CASTELLANO et al., 2010a) and the general reproductive capacity of spermatozoa in turkeys (ZANIBONI; RIZZI; CEROLINI, 2006). In stallions, feeding a DHA-enriched nutraceutical resulted in higher TM and PLM of cooled and frozen-thawed semen (BRINSKO et al., 2005).

Many authors have shown that the *in vitro* addition of DHA improves cryosurvival of spermatozoa by altering membrane lipid composition through incorporation of DHA between the phospholipids in the plasmatic membrane in bull semen (KAKA et al., 2015; NASIRI; TOWHIDI; ZEINOALDINI, 2012; TOWHIDI; PARKS, 2012), also it can increase acrosome integrity (CHANAPIWAT; KAEOKET; TUMMARUK, 2009), plasmatic membrane integrity and PLM in post-thaw boar semen (KAEOKET et al., 2010). On the other hand, Kiernan, Fahey and Fair (2013) concluded that the addition of exogenous DHA to liquid bull semen had detrimental effects on PLM and viability. No studies were found in the literature elucidating the effects of the *in vitro* addition of DHA to stallion semen.

2.8 Importance of insulin-like growth factor-I to semen

IGF-I is a polypeptide growth factor that has similar structural homology with proinsulin and it is responsible for activities in the metabolism, proliferation, growth and cellular differentiation in many cells in the body. This growth factor is a single chain molecule having a molecular weight of 7,649 daltons and exercises its actions by receptors IGF-IR interactions (JONES; CLEMMONS, 1995). IGF-I is also called somatomedin C. Growth hormone is the main promoter of IGF-I in the postnatal life and its concentration increases during puberty (MARTINELLI JUNIOR; AGUIAR-OLIVEIRA, 2005). IGF-I is produced mainly in the liver (BARTKE, 2000), but it is also expressed in several other tissues, including muscular (KAMANGA-SOLLO et al., 2004), adipose (SOUZA et al., 2010), osseous (MAINARDES et al., 2007), testicular (ROSER, 2001) and ovarian (ARMSTRONG; BENOIT, 1996). The secretion of IGF-I occurs according to its production and there is not a storage organ. Proper growth of tissues or organs is dependent on both the circulating IGF-I, particularly of hepatic origin, and on the IGF-I produced in other tissues.

This concept reinforces the existence of endocrine, paracrine and autocrine actions for IGF-I (YAKAR et al., 2002).

In the testicle, IGF-I is secreted by Sertoli and Leydig cells under the control of the follicle-stimulating hormone and the luteinizing hormone, respectively (LEJEUNE et al., 1996) and receptors for IGF-I can be found in spermatogonia, spermatocytes, spermatids and spermatozoa (HENRICKS et al., 1998; VANNELLI et al., 1988). The highest amount of IGF-I in seminal plasma is bound to proteins IGFBPs until the IGF-I is fixed to its receptor located on the membrane of spermatic cells. Posteriorly, the tyrosine kinase enzyme is phosphorylated resulting in a sign to the cell nucleus which consequently performs its biological function, as the stimulation of spermatic motility, for example (GUPTA, 2005). The action of IGF-I is similar to that of insulin, promoting glucose and amino acids uptake by cells (YOUNIS et al., 1998). Thus, the greater the amount of IGF-I in seminal plasma probably the higher is the energy production by spermatozoa and spermatic motility.

Studies have reported the importance of IGF-I to the general semen quality for human (LEE et al., 2016; NADERI et al., 2015) and mitochondrial potencial membrane of canine spermatozoa (SHIN et al., 2014). It was also reported that IGF-I also stimulates spermatic motility in bull semen (HENRICKS et al., 1998). Moreover, addition of IGF-I to liquid boar semen has promoted higher spermatic motility (MENDEZ et al., 2013) and increased glutathione peroxidase activity, indicating the antioxidant capacity of IGF-I (SILVA et al., 2011). Besides that, addition of IGF-I has increased *in vitro* fertilizing ability in cooled semen (MAKAREVICH et al., 2014) and spermatic motility and structural integrity of the plasmatic membrane in cryopreserved ram semen (PADILHA et al., 2012). Also, it was suggested that the addition of IGF-I prevents deterioration of spermatic functional parameters and fertility (SELVARAJU et al., 2010) and reduces lipid peroxidation levels (SELVARAJU et al., 2009) in frozen-thawed buffalo semen.

Stallion semen has both IGF-I and IGF-IR, suggesting that this hormone is involved in the development and the maintenance of spermatogenesis (YOON; BERGER; ROSER, 2011). Testes contribute to the highest amount of IGF-I in stallion seminal plasma (HESS; ROSER, 2001), however accessory sex glands act producing the final ejaculation fluid and this fluid contains IGF-I. Likewise, epididymal tissue possibly contributes to the presence of IGF-I in seminal plasma and the total concentration of IGF-I in stallion seminal plasma is around 10.3 ng/mL. An association of the concentration of IGF-I in the seminal plasma with spermatic motility and normal morphology was reported in stallions and IGF-I may also be related to the fecundation capacity of the spermatozoa, since it would be involved with the

continuity of the motility after the ejaculation (MACPHERSON et al., 2009). In addition, it was reported that motility in stallion semen was maintained longer after treatment with 100 ng/mL of recombinant human IGF-I at room temperature (CHAMPION et al., 2002).

3 CONCLUSION

Due to the global crescent equine market, reproduction techniques such as AI should be enhanced, therefore the analysis of stallion semen quality has become increasingly important for the development of this biotechnology. The addition of certain substances to both cooled and cryopreserved stallion semen, including DHA and IGF-I may improve spermatic quality, especially with regard to the integrity of plasmatic membranes and motility characteristics.

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SECOND PART – PAPERS

PAPER 1 - *IN VITRO* ADDITION OF DOCOSAHEXAENOIC ACID IMPROVES THE QUALITY OF COOLED BUT NOT FROZEN-THAWED STALLION SEMEN

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In vitro addition of docosahexaenoic acid improves the quality of cooled but not frozen–thawed stallion semen

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ABSTRACT

The aim of the present study was to assess the effect of the addition of docosahexaenoic acid (DHA) on the *in vitro* quality of cooled and frozen–thawed stallion semen. In Experiment 1, semen from 10 stallions was collected (three ejaculates per stallion). Semen was diluted to 100×10^6 spermatozoa mL^{-1} with 0.02 mM vitamin E (VE) and 0, 1, 10 or 20 ng mL^{-1} DHA and frozen. Semen was thawed and total motility (TM), rapid progressive motility (PM), acrosome integrity, membrane fluidity and morphology were assessed. In Experiment 2, semen from three stallions was collected (three ejaculates per stallion) and frozen as in Experiment 1, but VE and DHA were added after thawing. TM and PM were assessed at 30, 60 and 120 min and viability, acrosome integrity and membrane fluidity were evaluated at 30 min. In Experiment 3, semen from five stallions was collected (one to three ejaculates per stallion), diluted to 20×10^6 spermatozoa mL^{-1} and stored at 4°C. After 1, 24, 48 and 72 h, TM, PM, viability, membrane fluidity and lipid peroxidation were assessed. The addition of DHA had no effect on frozen semen (Experiments 1 and 2) but improved TM, PM and membrane fluidity in cooled stallion semen.

Keywords: equine, fertility, polyunsaturated fatty acid (PUFA), spermatozoa.

Introduction

Artificial insemination (AI) with cooled and frozen–thawed semen is widely used in the equine sport horse breeding industry (Aurich and Aurich 2006). To achieve acceptable pregnancy rates with cooled stallion semen, it must be inseminated within 24–36 h of semen collection (Lindahl *et al.* 2012), compared with 3 days with liquid bull semen (Murphy *et al.* 2015) and 5 days with boar semen (Johnson *et al.* 2000). The pregnancy rates achieved following the use of frozen–thawed stallion

semen are lower (~45%; Miller 2008) than those in cattle (~54%; Odhiambo *et al.* 2014), but are also highly dependent on the individual stallion (Haadem *et al.* 2015).

During temperature decreases, stallion spermatozoa undergo a membrane lipid phase change, in which membrane lipids transition from a liquid to a gel phase with peak phospholipid transition thought to occur in stallion spermatozoa at ~20°C (Parks and Lynch 1992). The cholesterol:phospholipid ratio is thought to affect sperm membrane fluidity (Klein *et al.* 1995), as well as the stability of the membrane during sperm cooling, freezing and subsequent thawing (Darin-Bennett and White 1977). Thus, intact lipid molecules are necessary for a good functional sperm membrane (Bustamante-Filho *et al.* 2014) that can survive the freeze–thaw process. In addition, there are differences in the lipid composition of the plasma membrane between different stallions, which may help explain the variations in sperm resistance to the cooling and freeze–thaw processes (Batellier *et al.* 2001). The phospholipid profile of the stallion sperm plasma membrane is similar to that of the boar. It contains high levels of docosapentaenoic acid (DPA), an omega-6 polyunsaturated fatty acid (PUFA), and docosahexaenoic acid (DHA), an omega-3 PUFA (Parks and Lynch 1992). Although it may be desirable to increase the omega-3 PUFA content of spermatozoa using dietary or *in vitro* supplementation so as to increase membrane fluidity, this strategy may also promote susceptibility of the spermatozoon to lipid peroxidation, resulting in membrane damage (Schimid-Lausigk and Aurich 2014). Thus, the beneficial and detrimental effects of PUFA enrichment are closely balanced in stallions (and other species) due to sensitivity to reactive oxygen species (ROS; Peña *et al.* 2011). The relationship between the lipid profile of the plasma membrane and sperm quality in stallions remains poorly understood. In contrast, this relationship has been studied in humans (Lewis 2007; Aitken *et al.* 2012), bulls (Rodrigues *et al.* 2015) and boars (Radomil *et al.* 2011; Barranco *et al.* 2015; Chung *et al.* 2015).

Several studies supplementing PUFAs in the diet have demonstrated a beneficial effect on semen quantity and quality in bulls (Gürler *et al.* 2015; Moallem *et al.* 2015), boars (Liu *et al.* 2015) and rams (Fair *et al.* 2014). Brinko *et al.* (2005) reported beneficial effects of dietary supplementation of stallions with a DHA nutraceutical on both frozen–thawed and cooled semen quality, with the most notable improvements in stallions whose semen did not tolerate cooling well. In an attempt to compensate for the damage caused to stallion spermatozoa during storage, researchers have studied the effects of the addition of substances to semen during processing. Several studies have shown that the *in vitro* addition of fatty acids to semen improves the quality of cryopreserved (Büyükleblebici *et al.* 2014; Kaka *et al.* 2015*a*; Sampaio *et al.* 2015) and liquid-stored (Kiernan *et al.* 2013) bull semen, as well as cryopreserved boar semen (Chanapiwat *et al.* 2009), but there is no published study on the effect of the exogenous DHA addition to stallion semen.

Thus, the aim of the present study was to assess the effects of the *in vitro* addition of DHA to stallion semen before freezing, after thawing and before cooling on a range of *in vitro* sperm quality parameters.

Materials and methods

Experimental design

Animal ethics

All experiments were performed according to appropriate ethical and legal standards under the approval number 2014_11_11_ULAEC (University of Limerick, Ireland).

Experiment 1: effects of DHA addition to stallion semen before freezing

The aim of this experiment was to assess the effect of the addition of DHA (*cis*-4,7,10,13,16,19-docosahexaenoic acid; Sigma) and vitamin E (VE; α-tocopherol; Sigma) to semen before freezing. Semen from 10 Irish Sport Horse stallions of proven fertility, ranging between 13 and 28 years of age, was collected at a commercial stud in Ireland using an artificial vagina (three ejaculates from each stallion with a rest interval of at least 3 days between ejaculates) and all ejaculates were processed individually. Following collection, the gel fraction was removed, after which total motility (TM) was assessed subjectively using a phase contrast microscope (minimum TM of 70% was used; data not shown). The ejaculate was diluted 1 : 1 in INRA 96 extender (IMV Technologies) and centrifuged at 600*g* for 10 min at 32°C, after which the sperm concentration was assessed using a photometer (SDM6; Minitube) after the addition of 120 μL of the pellet to a cuvette containing 3 mL of 0.9% NaCl. The sperm concentration was validated using a haemocytometer and all samples were within 10% of the target concentration. The pellet was then diluted to a concentration of 100×10^6 spermatozoa mL^{-1} in Gent freezing extender (Minitube) in the presence of: (1) 0 ng mL^{-1} DHA + 0.02 mM VE (control; $T0_{VE}$); (2) 1 ng mL^{-1} DHA + 0.02 mM VE ($T1_{VE}$); (3) 10 ng mL^{-1} DHA + 0.02 mM VE ($T10_{VE}$); or (4) 20 ng mL^{-1} DHA + 0.02 mM VE ($T20_{VE}$). Because there was no similar published study adding DHA to stallion semen, the literature in other species was assessed as a starting point. Thus, the concentrations of DHA and VE were adapted from the study of Nasiri *et al.* (2012), in which 0.02 mM VE and 0–10 ng mL^{-1} DHA were added to frozen–thawed bull semen, with a positive effect demonstrated on sperm characteristics following the addition of 10 ng mL^{-1} DHA. The diluted semen was cooled slowly to 4°C over 60 min and then packaged into 0.5-mL straws (Minitube) and sealed using polyvinyl alcohol (PVA) powder (Minitube). Straws were frozen to –110°C (13.9°C min^{-1}) in a programmable freezer (IceCube 14S; Minitube), after which they were plunged into liquid nitrogen at –196°C. The sperm concentration within straws was confirmed using a haemocytometer and was ±10% of the target concentration.

One straw of each treatment was thawed at 37°C for 30 s (10 stallions with three ejaculates per stallion = 30 ejaculates) and maintained at 32°C in a heated block until TM, rapid progressive motility (PM) and kinematic parameters were analysed using computer-assisted sperm analysis (CASA). A

further two straws from each treatment were thawed (five stallions with three ejaculates per stallion = 15 ejaculates) and assessed for acrosome integrity and membrane fluidity using flow cytometry. Another straw from each treatment was thawed (five stallions with three ejaculates per stallion = 15 ejaculates) and assessed for morphology. Different straws for motility, morphology and flow cytometry measurements were assessed due to logistical constraints because there was limited time to analyse all parameters simultaneously. In addition, sperm viability and motility decrease over time and having a time delay between thawing and analysis would have led to inaccurate results.

Experiment 2: effects of DHA addition to stallion semen after thawing

The aim of this experiment was to assess the effect of the addition of DHA to semen after thawing. Semen from three Irish Sport Horse stallions of proven fertility, ranging between 7 and 17 years of age, was collected at a commercial stud in Ireland using an artificial vagina (three ejaculates from each stallion), processed and frozen as per Experiment 1, but without the addition of DHA or VE. Two straws were thawed per ejaculate at 37°C for 30 s and the semen was diluted to a final concentration of 25×10^6 spermatozoa mL^{-1} in INRA 96 containing the following: (1) 0 ng mL^{-1} DHA (control; T0); (2) control + 0.02 mM VE ($T0_{VE}$); (3) 1 ng mL^{-1} DHA + 0.02 mM VE ($T1_{VE}$); (4) 10 ng mL^{-1} DHA + 0.02 mM VE ($T10_{VE}$); and (5) 20 ng mL^{-1} DHA + 0.02 mM VE ($T20_{VE}$). All treatments were maintained at 32°C until analyses were completed (three stallions with three ejaculates per stallion = nine ejaculates). TM, PM and kinematic parameters were assessed at 30, 60 and 120 min after the addition of DHA using CASA (as per Experiment 1), whereas viability, acrosome integrity and membrane fluidity were assessed at 30 min after the addition of DHA using flow cytometry.

Experiment 3: effects of DHA addition to stallion semen before cooling

The aim of this experiment was to assess the effects of the addition of DHA to semen before cooling. Semen from five Irish Sport Horse stallions of proven fertility, ranging between 7 and 20 years of age, were collected, diluted and centrifuged as per Experiment 2. The pellet was resuspended to 20×10^6 spermatozoa mL^{-1} in INRA 96 using the same treatments as per Experiment 2. Semen was maintained at 15°C for 2 h, packaged in 0.5-mL straws, sealed using PVA powder and, following a gradual temperature reduction, stored at 4°C. Liquid stallion semen is normally stored or transported in large syringes, but the use of straws in the present study kept consistency across experiments and allowed 1 unit (straw) to be sampled without compromising the remainder of the sample. In addition, the sperm concentration was in line with that used in syringes and the straw is a similar anaerobic environment to the syringe. After 1 h (Day 0), one straw from each treatment group was warmed to 32°C, after which motility (TM and PM) and kinematic parameters were assessed (five stallions with one to three ejaculates per stallion = 12 ejaculates) using CASA (as per Experiment 1). Another straw from each treatment was warmed to 32°C, after which viability, membrane fluidity and lipid

peroxidation were assessed (three stallions with three ejaculates per stallion = nine ejaculates) after 24 h (Day 1), 48 h (Day 2) and 72 h (Day 3) using flow cytometry.

Sperm functional assessments

CASA

Motility and kinematic parameters were analysed using negative phase contrast (×100) brightfield microscopy on an Olympus BX60 fitted with a CASA system (Spermatozoa Class Analyser (SCA); Microptic). SCA Evolution software (Microptic) preset to record stallion parameters was used. A drop (5 μL) of diluted semen was placed on a prewarmed chamber (37°C; Leja counting chambers; Microptic) and analysed for sperm motion and kinematic characteristics immediately after thawing. A minimum of five microscopic fields with at least 100 spermatozoa was analysed in each sample using a phase contrast microscope at ×100 fitted with a prewarmed (37°C) stage. Objects incorrectly identified as spermatozoa were edited out using the playback function. The CASA-derived kinematic and motility characteristics assessed were average path velocity (VAP; >10 μm s^{-1}), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), TM (VCL >10 μm s^{-1}) and PM (VCL >90 μm s^{-1} and STR >75%). None of the treatments in any of the three experiments significantly affected any of the kinematic parameters and so these results are not presented herein.

Morphology

Spermatozoa were fixed with 0.2% glutaraldehyde, after which a 10-μL aliquot of the solution was placed on a slide and covered with a coverslip. After placing a drop of immersion oil on the coverslip, the percentage of morphologically normal and abnormal spermatozoa was assessed using a phase contrast microscope at ×1000. At least 100 spermatozoa were assessed in each sample.

Assessment of acrosome integrity, membrane fluidity, viability and lipid peroxidation

Samples were diluted using phosphate-buffered saline (PBS) to a concentration of 6×10^6 spermatozoa mL^{-1} and analysed using a flow cytometer (Guava EasyCyte 6HT-2L; Merck Millipore) equipped with both a krypton (642 nm) and an argon (488 nm) laser. Appropriate single-colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in conjunction with the forward scatter (FSC) and side scatter (SSC) signals to discriminate spermatozoa from debris. Fluorescent events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample, 10000 gated events were captured.

Acrosome integrity was assessed using a method adapted from Murphy *et al.* (2015). Briefly, the fluorescent stain Alexa Fluor 647 PNA (AF647; lectin peanut agglutinin from *Arachis hypogaea*;

excitation and emission wavelengths 650 and 688 nm respectively; Life Technologies) was added to a final concentration of 6 μg mL^{-1} and samples were incubated at 32°C in the dark for 15 min. AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. In addition, 100 nM SYTO 16 (excitation and emission wavelengths 488 and 518 nm respectively; Life Technologies) was added to samples and they were incubated in the dark at 32°C for 15 min. SYTO 16 works by binding to nucleic acids. Following this incubation period, the fluorescent stain propidium iodide (PI; 15 μM; excitation and emission wavelengths 535 and 617 nm respectively; Life Technologies) was added to samples and they were incubated for a further 15 min at 32°C. Because PI cannot permeate live cells, it is used to detect dead cells. PI binds to DNA by intercalating between the bases with little or no sequence preference. After incubation, samples (200 μL) were transferred to a 96-well microplate and analysed. AF647, SYTO 16 and PI fluorescence was analysed using red2 detector (664/20 nm band-pass; BP; filter), green detector (525/30 nm BP filter) and yellow detector (583/26 nm BP filter) respectively; no compensation was needed. The percentage of viable spermatozoa with intact acrosomes was calculated as the percentage of AF647-negative cells of the PI-negative population as initially gated based on controls, FSC and SSC.

Membrane fluidity was assessed using a method adapted from Murphy *et al.* (2014). The apoptotic stain Yo-Pro-1 (excitation and emission wavelengths 491 and 509 nm respectively; Life Technologies) was added to a final concentration of 50 nM and samples were incubated at 32°C in the dark for 10 min. Yo-Pro-1 works by identifying apoptotic cells. After the incubation period, to assess membrane fluidity, the fluorescent probe merocyanine 540 (M540; excitation and emission wavelengths 555 and 576 nm respectively; Sigma) was added to a final concentration of 10 μM and samples were incubated in the dark for a further 15 min at 32°C. M540 binds to the surface of polarised membranes and fluoresces upon membrane depolarisation, thus indicating increased membrane fluidity. After incubation, samples (200 μL) were transferred to a 96-well microplate and analysed. Fluorescence of Yo-Pro-1 and M540 was read using the greendetector (525/30 nm BP filter). High membrane fluidity was defined as the percentage of viable cells (Yo-Pro-1 negative) positive for M540.

Viability was assessed using two fluorescent stains, namely SYTO 16 and PI, using a method adapted from Murphy *et al.* (2015). SYTO 16 was added to a final concentration of 100 nM and samples were incubated at 32°C in the dark for 15 min. Subsequently, 15 μM PI was added to the samples and they were incubated for a further 15 min at 32°C in the dark. After incubation, samples (200 μL) were transferred to a 96-well microplate (Corning) and analysed. SYTO 16 and PI were read with a green and yellow detector, respectively (525/30 and 583/26 nm BP filters respectively); no compensation was needed. The percentage of viable cells was determined as the percentage of cells positive for SYTO 16, but negative for PI.

Lipid peroxidation was assessed using two fluorescent stains, namely BODIPY C_{11} (excitation and emission wavelengths 581 and 591 nm respectively; Life Technologies) and PI. BODIPY C_{11} was

added to a final concentration of 2 nM and samples were incubated at 32°C in the dark for 15 min. BODIPY C_{11} works by detecting ROS in cells and membranes. After the incubation period, PI was added to a final concentration of 15 μM and samples were incubated for further 15 min. After incubation, samples (200 μL) were transferred to a 96-well microplate and analysed. Fluorescence of BODIPY C_{11} and PI was read with a yellow detector (583/26 nm BP filter). Lipid peroxidation was defined as the percentage of viable cells (PI negative) positive for BODIPY C_{11}.

Statistical analysis

Data were examined for normality of distribution, tested for homogeneity of variance and analysed using analysis of variance (ANOVA; Experiments 1 and 2) or repeated-measures ANOVA (Experiments 2 and 3) in SPSS version 22.0 (IBM). The final statistical model used included the main effects of treatment, incubation period, stallion and their interactions. Post hoc tests were conducted using the Tukey test and $P < 0.05$ was deemed to be statistically significant. All results are reported as the mean ± s.e.m.

Results

Experiment 1: effects of DHA addition to stallion semen before freezing

There was no effect of treatment on any of the *in vitro* parameters assessed ($P > 0.05$), with an overall post-thaw TM of 50.2 ± 4.0% and a PM of 9.1 ± 1.5%. The percentage of spermatozoa with intact acrosomes in the live population was 95.7 ± 0.7%, the percentage of spermatozoa with high membrane fluidity in the live population was 31.0 ± 3.5% and the percentage of spermatozoa with normal morphology was 74.6 ± 2.3%. There was an effect of stallion ($P < 0.001$) on all motility and flow cytometric parameters assessed, but there was no treatment × stallion interaction.

Experiment 2: effects of DHA addition to stallion semen after thawing

There was no effect of treatment, stallion or their interactions on TM or PM ($P > 0.05$). Over all time points assessed (30, 60 and 120 min after the addition of DHA) TM was 29.6 ± 5.1% and PM was 14.4 ± 4.5%. There was an effect of incubation period, with TM and PM decreasing over time ($P < 0.001$). There was an interaction effect between incubation period and stallion on both TM and PM ($P < 0.001$).

There was no effect of treatment, stallion or their interaction on viability ($P > 0.05$), and there was no effect of treatment or the treatment × stallion interaction on acrosome integrity and membrane fluidity ($P > 0.05$). Overall viability was 30.4 ± 4.2%, the percentage of spermatozoa with intact acrosomes in the live population was 97.6 ± 0.3% and the percentage of spermatozoa with high

membrane fluidity in the live population was 52.9 ± 4.9%. There was an effect of stallion ($P < 0.001$) on acrosome integrity and membrane fluidity.

Experiment 3: effects of DHA addition to stallion semen before cooling

There was an effect of treatment on TM, with the $T20_{VE}$ treatment having greater TM than the T0 treatment ($P < 0.05$; Fig. 1). TM declined with day of storage ($P < 0.001$), but this was not affected by treatment ($P > 0.05$). There was an effect of stallion on TM ($P < 0.001$), but no stallion × treatment interaction ($P > 0.05$). There was an effect of treatment on PM, with all the DHA treatments having greater PM than both the T0 and $T0_{VE}$ treatments ($P < 0.001$; Fig. 2). PM declined with day of storage ($P < 0.01$), but was not affected by treatment ($P > 0.05$). There was an effect of stallion on PM ($P < 0.01$), but no stallion × treatment interaction ($P > 0.05$).

There was no effect of treatment, day of storage or their interaction on viability ($P > 0.05$), with overall viability (on Days 0–3) of 55.2 ± 7.1%. There was an effect of stallion on viability ($P < 0.05$), but no stallion × treatment interaction ($P > 0.05$).

There was an effect of treatment on membrane fluidity ($P < 0.001$), with the $T10_{VE}$ (28.7 ± 4.4%) and $T20_{VE}$ (29.4 ± 5.2%) groups having a greater percentage of spermatozoa with high membrane fluidity in the live population than the other treatment groups (19.0 ± 3.9% over Days 0–3). There was an effect of day of storage, day × treatment interaction ($P < 0.001$), stallion and stallion × day interaction ($P < 0.001$) on membrane fluidity, but no treatment × stallion interaction ($P > 0.05$).

There was an effect of treatment on lipid peroxidation ($P < 0.05$), with the $T0_{VE}$ group (13.0 ± 4.4%) having lower lipid peroxidation than the $T10_{VE}$ group (21.3 ± 5.2%). Although there was an effect of day of storage ($P < 0.001$), there was no treatment × day interaction ($P > 0.05$). There was an effect of stallion ($P < 0.001$) on lipid peroxidation, but no stallion × treatment interaction ($P > 0.05$).

Discussion

The present study is the first published study to assess the effect of the *in vitro* addition of exogenous DHA to stallion spermatozoa; it demonstrates beneficial effects of DHA on cooled semen, but not when DHA is added before freezing or after thawing.

In contrast with findings in other species, the addition of DHA to stallion semen before freezing did not affect any of the parameters of sperm quality assessed. Increased post-thaw motility (Nasiri *et al.* 2012; Towhidi and Parks 2012; Kaka *et al.* 2015*b*), improved morphology, acrosome integrity and membrane integrity (Kaka *et al.* 2015*b*) have been reported after the *in vitro* addition of DHA to bull semen before freezing; this may be the result of DHA accumulation in the membrane of the spermatozoon and a consequent increase in resistance to degradation caused by ice crystal formation (Nasiri *et al.* 2012). Others have used a semen extender supplemented with DHA from fish oil (Kaeoket *et al.* 2010) or a combination of L-cysteine and DHA-enriched hen egg yolk (Chanapiwat

et al. 2009) before freezing boar spermatozoa and have reported improved post-thaw motility and acrosomal integrity. It may be that stallion sperm membranes are more sensitive to the freeze–thaw process than sperm membranes from other farm animal species and that the *in vitro* addition of DHA to semen was not enough to promote improvements in the semen quality in the present study. It is plausible that higher concentrations of DHA, or a more prolonged incubation period, may be required for stallion spermatozoa so as to avoid the disruption caused by ice crystal formation during the cryopreservation process.

The addition of DHA to stallion semen after thawing did not affect any parameters of sperm quality analysed. There is a dearth of published studies that have added DHA to semen after thawing. However, after dietary supplementation with a nutraceutical rich in DHA, improvements in freezability consequently resulting in increased motion characteristics have been reported for frozen–thawed stallion semen (Brinsko *et al.* 2005). It is not known why dietary supplementation of DHA seems to be more effective in improving frozen–thawed stallion semen quality than *in vitro* addition of DHA to frozen–thawed stallion semen, but it may be due to the duration of exposure of the spermatozoa to DHA or even the low concentrations of exogenous DHA.

The improvements observed in the present study in TM, PM and membrane fluidity when DHA was added to cooled semen are likely related to the incorporation of DHA into the sperm membrane, thus protecting the membrane against the damage caused by the temperature changes (Nasiri *et al.* 2012). Therefore, DHA seems to be important in preserving membrane functionality when the temperature change is not severe, from 37°C to 4°C, but the DHA concentrations used in the present study were not enough to protect the membrane when sub-zero temperatures were applied (as in Experiments 1 and 2). In contrast, there was no effect on TM in cooled stallion semen after dietary supplementation with a DHA-enriched nutraceutical (Brinsko *et al.* 2005); this suggests that the *in vitro* addition of DHA to cooled stallion semen is more efficient in improving TM than supplementation of DHA in the diet.

Although motility and kinematic parameters in stallion semen can be variable due to stallion-related effects such as breed and age, as well as frequency of semen collection (Gamboa *et al.* 2009), the main reason for low PM in all the experiments in the present study is the strict criteria used in defining PM. The SCA system (as used in the present study) only analysed the rapid population of spermatozoa (VCL >90 μm s^{-1}) for progressive motility (STR >75%). In the present study, the addition of DHA to cooled stallion semen yielded higher PM than the controls up to Day 3 of storage, which may be associated with the capacity of DHA to prevent the disruption of lipid membranes (Meryman 1966). Similarly, an improvement in PM was observed on both Days 1 and 2 of cooled semen storage when the stallions with <40% initial PM had their diets supplemented with DHA (Brinsko *et al.* 2005). Conversely, the *in vitro* addition of exogenous DHA to cooled bull semen had detrimental effects on PM and viability (Kiernan *et al.* 2013) and this may be due to DHA accelerating

the production of ROS. Therefore, the use of antioxidants such as VE along with PUFA supplementation to protect spermatozoa seems essential.

The addition of exogenous DHA to cooled stallion semen yielded a greater percentage of spermatozoa with high membrane fluidity and this may be explained by the several roles of PUFAs, for example their ability to confer upon the sperm plasma membrane the fluidity it needs to fertilise the oocyte (Wathes *et al.* 2007). The percentage of spermatozoa with high membrane fluidity decreased from Day 0 to Day 1, which may be due to stallion spermatozoa being susceptible to the drop in temperature due to the phase transition of lipids, which can damage the plasma membrane (Moran *et al.* 1992). After Day 1, spermatozoa with high membrane fluidity survived whereas spermatozoa with low membrane fluidity did not; therefore, it would appear that the percentage of spermatozoa with high membrane fluidity in the live population increased after Day 1.

Similarly to the present study, Kaka *et al.* (2015*b*) found that lipid peroxidation increased with increasing concentrations of DHA added. The presence of high levels of PUFA requires efficient antioxidant levels to protect spermatozoa against lipid peroxidation (Aitken and Baker 2004) and it seems that the 0.02 mM VE used in the present study was not sufficient to prevent lipid peroxidation.

In conclusion, the present *in vitro* study has demonstrated that the addition of DHA has a positive effect on the quality of cooled stallion semen in terms of increased TM, PM and percentage of spermatozoa with high membrane fluidity; however, there was no effect on frozen–thawed semen. A field fertility trial is required to establish whether the *in vitro* addition of DHA to cooled stallion semen can increase *in vivo* fertility.

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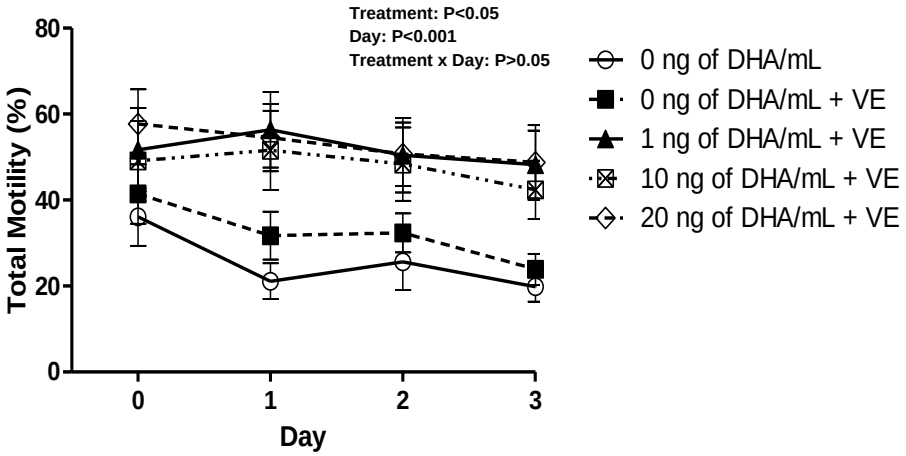

Figure 1. Experiment 3: effects of the addition of docosahexaenoic acid (DHA) in the presence of 0.02 mM vitamin E (VE) before cooling to 4°C on the total motility of stallion semen (n = 5 stallions). Data are the mean ± s.e.m.

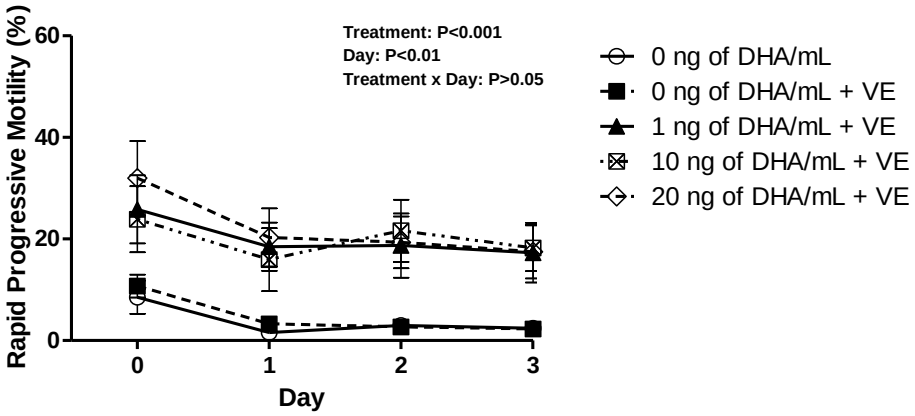

Figure 2. Experiment 3: effects of the addition of docosahexaenoic acid (DHA) in the presence of 0.02 mM vitamin E (VE) before cooling to 4°C on the rapid progressive motility (VCL > 90μm s^{-1} and STR > 75%) of stallion semen (n = 5 stallions). Data are the mean ± s.e.m.

PAPER 2 – *IN VITRO* ADDITION OF DOCOSAHEXAENOIC ACID IN COMBINATION WITH INSULIN-LIKE GROWTH FACTOR-I INCREASES THE PROGRESSIVE MOTILITY OF CRYOPRESERVED STALLION SEMEN

This paper will be submitted to Animal Reproduction (impact factor 0.780) and this is a preliminary version before the Journal's review.

In vitro addition of docosahexaenoic acid in combination with insulin-like growth factor-I increases the progressive motility of cryopreserved stallion semen

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Article type: Biotechnology

Running title: Effects of DHA and IGF-I on thawed stallion semen

ABSTRACT

High seminal quality is important to achieve acceptable pregnancy rate following artificial insemination with cryopreserved semen. Docosahexaenoic acid (DHA) is an omega-3-polyunsaturated acid, which improves the integrity of the spermatozoa membrane during temperature changes. Insulin-like growth factor-I (IGF-I) is a protein hormone that helps mainly glucose to enter spermatozoa and it is an antioxidant. The aim of this study was to assess the effect of the *in vitro* addition of DHA in combination with IGF-I to frozen-thawed stallion semen. Three ejaculates from each of three stallions were collected, the gel fraction

was removed and semen was diluted in a 1:1 ratio using extender, centrifuged (1000 g) for 10 min at 32°C and ressuspended to 100 x 10^6 spermatozoa/mL in freezing extender. Semen was cooled to 4°C, packed into 0.5 mL straws, frozen and stored under liquid nitrogen at -196°C. Straws were thawed at 37°C for 30 sec, semen was diluted to 25 x 10^6 spermatozoa/mL and split in four treatments adding 0 or 1 ng of DHA /mL and 0 or 100 ng of IGF-I /mL: DHA0, DHA0 + IGF-I, DHA1 and DHA1 + IGF-I. Semen was incubated at 32°C and after 30 min, total motility (TM), rapid progressive motility (PM), viability and acrosome integrity were assessed. After 60 and 120 min, TM and PM were assessed again. Post-thawed PM was higher ($P<0.05$) when DHA1 + IGF-I was added but there was no effect of the addition of DHA and IGF-I to TM, viability or acrosome integrity ($P>0.05$).

Keywords: Equine, Spermatozoa, Omega-3, Antioxidant, Hormone

1 Introduction

The use of frozen-thawed stallion semen allows genetics to be transported across countries and to be stored for long periods (Wu et al., 2015). However, cryopreservation induces partially irreversible damage to spermatozoa that results in reduced fertility compared to fresh or cooled semen (Moraes et al., 2015) and approximately 50% of the stallions are not suitably for the production of cryopreserved semen (Loomis and Graham, 2007). Cryoinjury of spermatozoa is mainly represented by membrane damage because of physical, osmotic and oxidative stress due to extracellular ice formation during the phase transition from liquid to frozen (Pena et al., 2011).

One way to improve stallion post-thaw semen quality is through the addition of different cryoprotectants as glycerol, methyl formamide, (Wu et al., 2015), cholesterol (Blommaert et al., 2016) or cholestanol (Moraes et al., 2015) into extenders. Another way is

by adding substances such as growth hormone (Champion et al. 2002), caffeine or pentoxifylline (Milani et al., 2010; Barakat et al., 2015) after thawing in an attempt to improve mainly post-thawed motility.

Semen from all domestic species contains high levels of polyunsaturated fatty acids (PUFA), in particular, docosahexaenoic acid (DHA) and docosapentaenoic acid (Parks and Lynch, 1992). DHA is an omega-3 PUFA which has been shown to improve the integrity of the spermatozoa cell membrane (Schmid-Lausigk and Aurich, 2014) during temperature changes. PUFA, specially DHA, is correlated with human spermatozoa motility and viability after freezing/thawing (Martínez-Soto et al., 2013). Moreover, it is known that the addition of DHA to an extender causes significant improvements in the *in vitro* characteristics of bull (Nasiri et al., 2012) and boar (Chanapiwat et al., 2009; Kaeoket et al., 2010;) spermatozoa and supplementation of DHA in the diet improves stallion semen quality (Brinsko et al., 2005). Thus the addition of DHA to post-thawed stallion semen might increase the incorporation of DHA in the spermatozoa membrane and consequently be a chance to ensure high stallion semen quality after cryopreservation.

It is possible to improve the stallion semen quality by addition of insulin-like growth factor-I (IGF-I) to extenders (Champion et al., 2002). IGF-I is a protein hormone that helps glucose (Hernandez-Grazón et al., 2016) and other energy sources as fructose (Selvaraju et al., 2009), lactate and oxygen to enter into the cell (Travascio et al., 2014) resulting in improved mitochondrial membrane potential during hypothermic storage of semen (Shin et al., 2014). Also, there is a relevant function for IGF-I in β-oxidation and cholesterol synthesis and this growth factor has an important antioxidant activity (De Ita et al., 2015) by stimulation of glutathione peroxidase. In general, IGF-I has been shown to improve the quality of bovine (Henricks et al., 1998), bubaline (Selvaraju et al., 2016), ovine (Makarevich et al., 2012; Padilha et al., 2012), swine (Silva et al., 2011) and canine (Shin et al., 2014) spermatozoa. In

addition, post-thawed supplementation of IGF-I has improved semen quality in buffalo spermatozoa (Selvaraju et al., 2009; Selvaraju et al., 2010). Stallion seminal plasma has approximately 20 ng of IGF-I per mL (Lackey et al., 2002) and Macpherson et al. (2002) demonstrated that first-cycle pregnancy rate was greater when stallions had high concentration of IGF-I (10.2 ng of IGF-I per mL; pregnancy rate of 76%) compared with stallions with low concentration of IGF-I (0.7 ng of IGF-I per mL; pregnancy rate of 55%) in the seminal plasma.

However, there is no study in the published literature which has reported the effects of the DHA in combination with IGF-I in the stallion spermatozoa. Thus, the aim of this study was to assess the effect of the addition of DHA and IGF-I to frozen-thawed stallion semen on a range of *in vitro* spermatozoa quality parameters.

2 Material and methods

2.1 Experimental design

Animal ethics

The experiment was performed according to the appropriate ethical and legal standards under the approval number: 2014_11_11_ULAEC (University of Limerick, Ireland).

Semen collection and freezing

Semen from Irish Sport Horse stallions (n=3) of proven fertility (17 years of age), were collected between February and March at a commercial stud in Ireland using an artificial vagina. Three ejaculates were collected from each stallion with a rest interval of at least 3 days between ejaculates and all ejaculates were processed individually. Following semen

collection, the gel fraction was removed and the total motility (TM) assessed subjectively using a phase contrast microscope (minimum TM of 70% was used; results not presented). The ejaculate was diluted in a 1:1 ratio using INRA 96 extender (IMV Technologies, L'Aigle, France) and centrifuged at 1000 *g* for 10 min at 32°C following which the concentration of the spermatozoa in the pellet was assessed using a photometer (SDM6, Minitube, Tiefenbach, Germany). The pellet was diluted to 100 x 10^6 spermatozoa per mL in Gent freezing extender (Minitube). Semen was then cooled to 4°C over 2 h, packed into 0.5 mL straws (Minitube) and sealed using polyvinyl alcohol powder (Minitube). Straws were frozen to -110°C (13.9°C/min) in a programmable freezer (IceCube 14S, Minitube) following which they were plunged into liquid nitrogen at -196°C.

Thawing, treatments and analysis

Straws were thawed as described previously and semen was diluted to a final concentration of 25 x 10^6 spermatozoa per mL in INRA 96 supplemented with vitamin E (α-Tocopherol, Arklow, Ireland, Sigma) at 0.02 mM to prevent posterior lipid peroxidation (Nasiri, Towhidi and Zeinoaldini, 2012) of the DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid, Sigma, Arklow, Ireland, 25 mg) added. The sample was then split in four treatments, namely: (i) DHA0 (0 ng of DHA per mL; control), (ii) DHA0 + IGF-I (control + 100 ng of IGF-I per mL), (iii) DHA1 (1 ng of DHA per mL) and (iv) DHA1 + IGF-I (1 ng of DHA per mL + 100 ng of IGF-I per mL). The concentration of DHA was adapted from previous experiments (Silva et al., 2017) as the study demonstrated higher rapid progressive motility (PM) following the addition of 1 ng of DHA per mL to cooled stallion semen. The concentration of IGF-I (human recombinant IGF-I, Sigma, Arklow, Ireland, 100 μg) was adapted from Champion et al. (2002) as this study demonstrated greater longevity of stallion spermatozoa after the addition of 100 ng of IGF-I per mL to room temperature stallion semen.

In all treatments samples were maintained at 32°C in a heated-block until analysis were completed. Motility (TM and PM) and kinematic parameters were assessed at 30, 60 and 120 min following the addition of DHA and IGF-I using Computer Assisted Sperm Analysis Software (CASA; Sperm Class Analyser, SCA, Microptic, Barcelona, Spain). These time points were pooled for statistical analysis. Viability and acrosome integrity were assessed at 30 min using flow cytometry.

2.2 Assessment of total motility, rapid progressive motility and kinematic parameters

Motility and kinematic parameters were analysed using negative phase contrast (×100) brightfield microscopy on an Olympus BX60 fitted with a CASA system (Spermatozoa Class Analyser (SCA); Microptic). SCA Evolution software (Microptic) preset to record stallion parameters was used. A drop (5 μL) of diluted semen was placed on a prewarmed chamber (37°C; Leja counting chambers; Microptic) and analysed for sperm motion and kinematic characteristics immediately after thawing. A minimum of five microscopic fields with at least 100 spermatozoa was analysed in each sample using a phase contrast microscope at ×100 fitted with a prewarmed (37°C) stage. Objects incorrectly identified as spermatozoa were edited out using the playback function. The CASA-derived kinematic and motility characteristics assessed were average path velocity (VAP; >10 μm s^{-1}), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), TM (VCL >10 μm s^{-1}) and PM (VCL >90 μm s^{-1} and STR >75%). None of the treatments in any of the three experiments significantly affected any of the kinematic parameters and so these results are not presented herein.

2.3 Flow cytometric analysis

Viability and acrosome integrity were assessed using a method adapted from Murphy et al. (2015) for bull semen. Preliminary tests were conducted using 3 different concentrations and 22 different incubation periods (0 to 42 min every 2 min) for each stain to optimize the flow cytometric protocols specifically for stallion semen.

Three ejaculates were collected from one stallion with a rest interval of at least 3 days between ejaculates. Following the collection, the gel fraction was removed. Semen evaluation, dilution, freezing and thawing were conducted as described previously.

Samples were diluted using phosphate buffered saline (PBS) medium to a concentration of 6 x 10^6 spermatozoa per mL and were analysed using a flow cytometer (Guava EasyCyte 6HT-2L, Merck Millipore, Billerica, MA, USA), equipped with both a krypton (640 nm) and an argon (488 nm) laser. Appropriate single colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in conjunction with the forward scatter and side scatter signals to discriminate spermatozoa from debris. Fluorescent events were recorded using GuavaSoft (Version 2.7, Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample a minimum of 10,000 gated events were captured.

Viability was assessed using the fluorescent stains SYTO 16 (Ex/Em: 488/518 nm; Life Technologies, Carlsbad, USA) and propidium iodide (PI; Ex/Em: 535/617 nm; Life Technologies). SYTO 16 was added at three different concentrations (20, 100 and 200 nM) and incubated at 32°C in the dark for 22 different incubation periods (0 to 42 min, each 2 min). SYTO 16 works by binding to nucleic acids. Subsequently, PI was added at three different concentrations (3, 15 and 30 μM) and incubated for the 22 incubation periods. Since PI is not permeant to live cells it is used to detect dead cells, PI binds to DNA by intercalating

between the bases with little or no sequence preference. Post incubation, samples (200 µL) were transferred to a 96-well microplate (Corning Inc., Midland, USA) and analysed. SYTO 16 was read with the photodetector (525/30 nm band-pass filter) and PI was read with the photodetector (583/23 nm band-pass filter), no compensation was needed. The percentage of viable cells was expressed as the percentage of cells positive for SYTO 16, but negative for PI.

Acrosome integrity was assessed using the fluorescent stain Alexa Fluor 647 PNA (AF647; lectin peanut agglutinin from *Arachis hypogaea;* Ex/Em: 650/688; Life Technologies) which was added at three different concentrations (1.2, 6 and 12 µg per mL) and was incubated at 32^{o}C in the dark for the 22 incubation periods. AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. SYTO 16 was then added to the sample at a final concentration of 100 nM and incubated in the dark at 32^{o}C for 15 min. Following this the fluorescent stain PI was added to the sample at a final concentration of 15 µM and incubated for further 15 min. Post incubation, samples (200 µL) were transferred to a 96-well microplate and analysed. Samples were analysed for AF647, SYTO 16 and PI via the photodetector 661/19, 525/30 and 583/23 nm band-pass filters, respectively, no compensation was needed. The percentage of viable spermatozoa with intact acrosomes was calculated as the percentage of AF647 negative cells of the PI negative population as initially gated based on controls, forward scatter and side scatter.

There was no effect of different concentrations of stains or incubation period in the capacity of reading intensity of stains in the post-thawed stallion semen, therefore based on these protocol optimizations, the concentrations of 100 nM, 15 µM and 6 µg per mL of SYTO16, PI and AF647, respectively and a 15 min incubation period for every stains were validated and posteriorly used to assess viability and acrosome integrity after addition of DHA and IGF-I to post-thawed stallion semen.

2.4 Statistical analysis

Data were examined for normality of distribution, tested for homogeneity of variance and analysed using an Analysis of Variance (ANOVA; flow cytometric analysis) or repeated measures ANOVA (CASA analysis) in the Statistical Package for the Social Sciences (SPSS; version 22.0, IBM, Armonk, USA). Post hoc tests were conducted using the Tukey test and $P<0.05$ was deemed to be statistically significant. All results are reported as the mean ± the standard error of the mean (s.e.m.).

3 Results

There was an effect of treatment on PM which was represented by the DHA1 + IGF-I treatment having higher PM than all the other treatments ($P<0.05$; Fig. 1) but there was no effect of treatment on TM ($P>0.05$; Fig. 2). There was an effect of incubation period on TM and PLM ($P<0.05$), whereas both decreased over time. There was no interaction between treatment and incubation period in both parameters ($P>0.05$).

There was no effect of treatment on viability and acrosome integrity with an overall post-thaw viability of 23.3 ± 4.99% and the percentage of spermatozoa with intact acrosomes in the live population of 98.3 ± 0.16%.

4 Discussion

Higher PM after adding DHA1 + IGF-I was observed. Probably IGF-I acts better in combination with DHA than alone due to the necessity of the incorporation of DHA between

the phospholipids in the spermatozoa membrane, keeping the resistance of the membrane under low temperature, then the IGF-I can connect to its receptor and consequently improve the uptake of energy sources and finally increase the PM. According to Morrell et al. (2014), progressive motility is highly correlated with stallion fertility and pregnancy rates. Supplementation of DHA in the diet improved the TM and progressive linear motility (PLM) in cooled (after 48 h) and frozen-thawed stallion semen (Brinsko et al. 2005). Several studies which have supplemented exogenous DHA before freezing to bull semen (Towhidi and Parks, 2012; Kaka et al., 2015) also have reported improvements in TM. PLM has been improved when DHA is added prior to freezing bull (Towhidi and Parks, 2012) and boar (Chanapiwat et al., 2009; Kaeoket et al., 2010;) semen and it has been hypothesised that this may be due to DHA aggregation in the flagellum of the spermatozoa (Nasiri et al., 2012). The *in vitro* addition of 100 ng per mL of IGF-I to stallion semen stored at room temperature has maintained the TM longer (Champion et al., 2002). Selvaraju et al. (2009) demonstrated that the *in vitro* addition of the same concentration of IGF-I to post-thawed buffalo semen increased both TM and PLM of the spermatozoa. Addition of IGF-I before freezing to ram semen also increased PLM (Padilha et al., 2012) while the addition of IGF-I to cooled ram (0 to 5°C for 48 h; Makarevich et al., 2014) and boar (15°C for 72 h; Mendez et al., 2013) semen increased TM. IGF-I improves spermatozoa motility by reducing oxidative stress (Selvaraju et al., 2016). Thus, the addition of IGF-I in combination with a PUFA is important to avoid increased lipid peroxidation, although Kiernan et al. (2013) found detrimental effects on PLM after the addition of 10 to 100 μM of IGF-I to liquid stored bull semen.

Supplementing DHA in the extender before freezing has been shown to increase viability in bull (Towhidi and Parks, 2012; Kaka et al., 2015) and boar (Kaeoket et al., 2010) semen which in turn may be related to increased membrane resistance to disintegration caused by ice crystal formation as a result of increased proportion of DHA in the plasma membrane

(Nasiri et al., 2012). The addition of 50 to 150 ng of IGF-I per mL in cooled (15°C for 72 h) boar semen increased viability (Silva et al., 2011), while, Kiernan, Fahey and Fair (2013) reported reduced viability after supplementing DHA to liquid stored bull semen. Although no improvement in the acrosome integrity was found in this study, the addition of IGF-I to post-thawed buffalo semen (Selvaraju et al., 2010) increased acrosome integrity probably due to the antioxidant capacity of the IGF-I. The *in vitro* addition of DHA prior to freezing of bull (Kaka et al., 2015), buffalo (Selvaraju et al., 2010), ram (Padilha et al., 2012) and boar (Chanapiwat et al., 2009; Kaeoket et al., 2010) semen improved acrosome integrity.

In conclusion, the present *in vitro* study demonstrated that the simultaneous addition of DHA and IGF-I to stallion semen after thawing increased PM but had no effect on TM, viability and acrosome integrity. More research should be conducted in order to test other concentrations of DHA and IGF-I to optimize stallion semen quality.

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Abbreviation list

AF647: Alexa fluor 647 PNA (lectin peanut agglutinin from *Arachis hypogaea*)

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

BCF: Beat cross frequency

CASA: Computer assisted sperm analysis

DHA: Docosahexaenoic acid

IGF-I: Insulin-like growth factor-I

LIN: Linearity

PBS: Phophate byffered saline

PI: Propidium iodide

PLM: Progressive linear motility

PM: Rapid progressive motility

PUFA: Polyunsaturated fatty acid

SCA: Sperm class analyser

SPSS: Statistical Package for the Social Sciences

STR: Straightness

TM: Total motility

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight line velocity

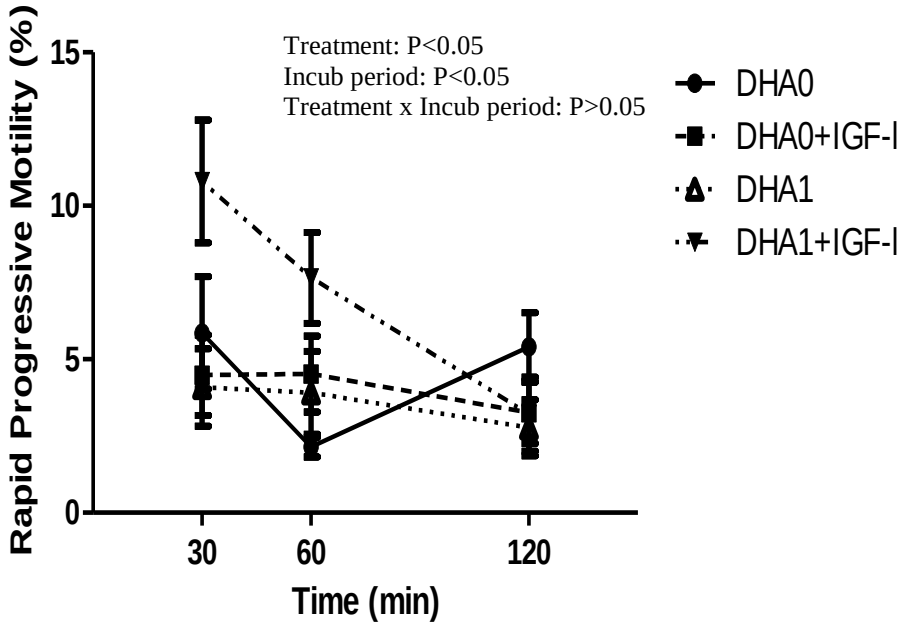

Figure 1. Effect of the *in vitro* addition of docosahexaenoic acid (DHA) and insulin-like growth factor-I (IGF-I) after thawing on rapid progressive motility of stallion spermatozoa (n=3). Vertical bars represent s.e.m.

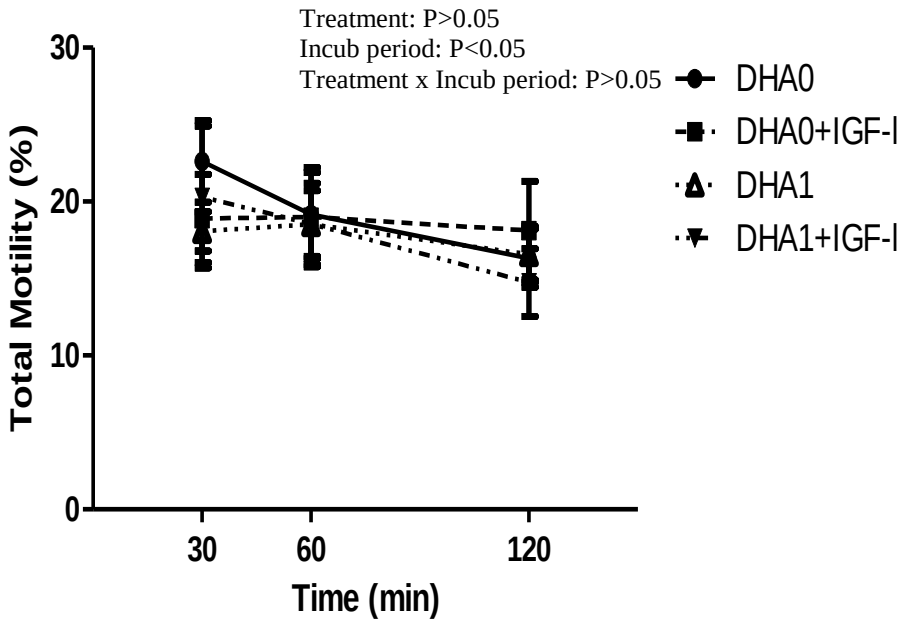

Figure 2. Effect of the *in vitro* addition of docosahexaenoic acid (DHA) and insulin-like growth factor-I (IGF-I) after thawing on total motility of stallion spermatozoa (n=3). Vertical bars represent s.e.m.