In vitro addition of DHA and IGF-I increases the progressive motility of cryopreserved stallion semen

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Abstract

High seminal quality is important to achieve acceptable pregnancy rate following artificial insemination with cryopreserved semen in the equine industry. 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 Irish Sport Horse stallions were collected, the gel fraction was removed and semen was diluted in a 1:1 ratio using extender, centrifuged (1.000 g) for 10 minutes 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 seconds, 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 minutes, total motility (TM), rapid progressive motility (PM), viability and acrosome integrity were assessed. After 60 and 120 minutes, 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.

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, as cryopreservation is a technique that preserves semen to -196 °C, it 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 suitable for the production of cryopreserved semen (LOOMIS; GRAHAM, 2007). Cryoinjury of spermatozoa is mainly represented by membrane damage because of physical,

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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; LYNCH, 1992). Docosahexaenoic acid is an omega-3 PUFA which has been shown to improve the integrity of the spermatozoa cell membrane (SCHMID-LAUSIGK; AURICH, 2014) during temperature changes. PUFA, especially DHA, is correlated with human spermatozoa motility and viability after freezing/thawing (MARTÍNEZ-SOTO et al., 2013). Moreover, it is known 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 improve 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 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), Macpherson et al. (2002) demonstrated 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 to 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.

Material and methods

Experimental design

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 from Irish Sport Horse stallions (n=3) of proven fertility (17 years old) were collected between February and March 2016 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.

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 (□-Tocopherol, Arklow, Ireland, Sigma) at 0.02 mM to prevent posterior lipid peroxidation (NASIRI; TOWHIDI; 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 minutes 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 minutes using flow cytometry.

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, therefore these results are not presented herein.

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). Stain 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). Stain 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 period. 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. Stain 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 °C in the dark for the 22 incubation periods. Stain AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. Stain SYTO 16 was then added to the sample at a final concentration of 100 nM and incubated in the dark at 32 °C for 15 min. Following that, 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 minutes 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.

Statistical analysis

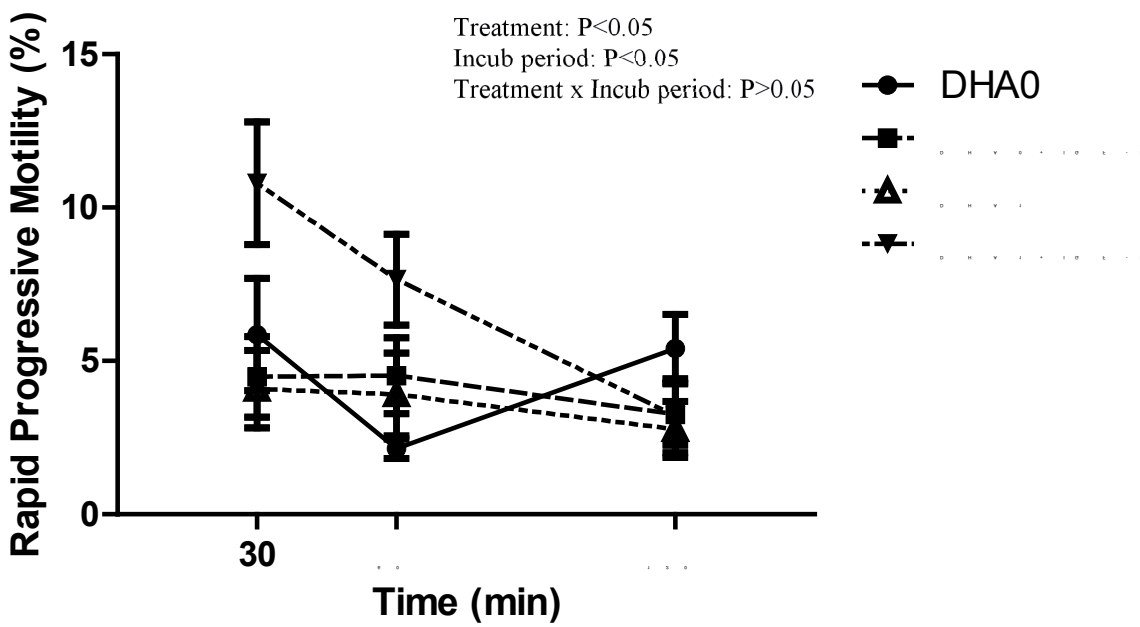
Data were examined for normality of distribution, tested for homogeneity of variance and analysed using an Analysis of Variance (ANOVA; flow cytometric analysis) or repeated measures ANOVA (CASA analysis) in the Statistical Package for the Social Sciences (SPSS; version 22.0, IBM, Armonk, USA). Post 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.).

Results and Discussion

Results

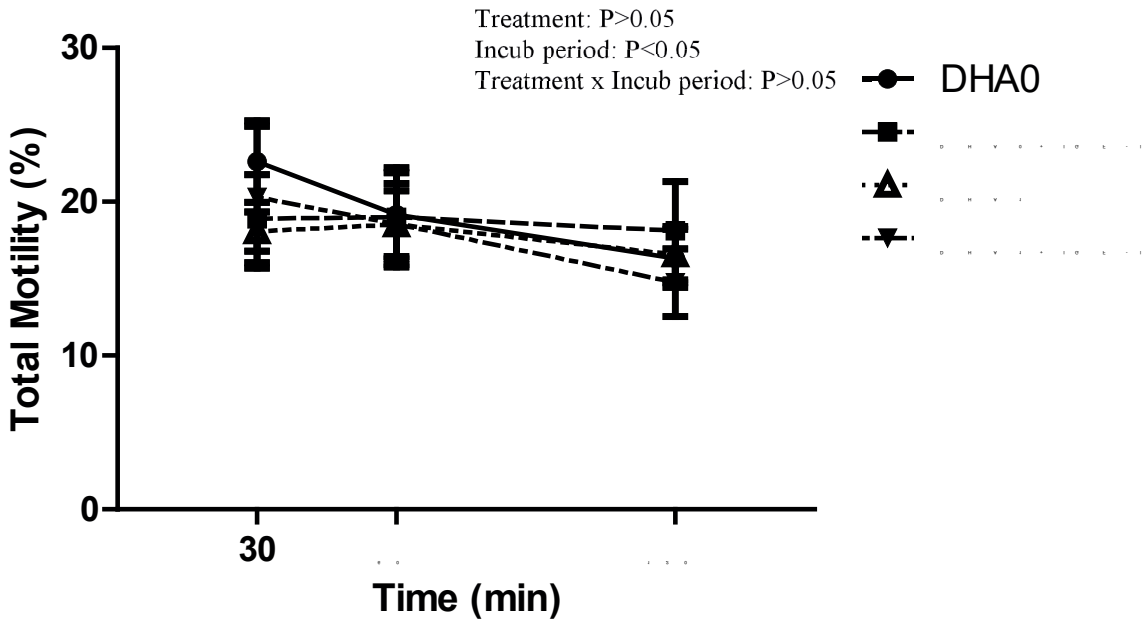
There was an effect of treatment on PM which was represented by the DHA1 + IGF-I treatment having higher PM than all the other treatments ($P<0.05$; FIGURE 1), however there was no effect of treatment on TM ($P>0.05$; FIGURE 2). There was an effect of incubation period on TM and PM ($P<0.05$), as both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

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.



Source: Elaborated by the authors (2018).

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.



Source: Elaborated by the authors (2018).

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%.

Discussion

Higher PM after adding DHA1 + IGF-I was observed. It is likely that 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), PM 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; PARKS, 2012; KAKA et al., 2015) also have reported improvements in TM. Progressive linear motility has been improved when DHA is added prior to freezing bull (TOWHIDI; 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 been shown to maintain the TM longer (CHAMPION et al., 2002). Selvaraju et al. (2009) demonstrated the *in vitro* addition of the same concentration of IGF-I to post-thawed buffalo semen increased both TM and PLM. 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; 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 et al. (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. It should be noted that the percent of acrosome intact spermatozoa in the current study was high across all treatmets and therefore there was limited scope for any further increases.

Conclusion

The present *in vitro* study demonstrated 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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Adição *in vitro* de DHA e IGF-I aumenta a motilidade progressiva do sêmen criopreservado de garanhões

Resumo

Alta qualidade seminal é importante para alcançar taxa de gestação aceitável por meio da inseminação artificial na equinocultura. Ácido docosahexaenoico (DHA) é um ácido graxo poliinsaturado também conhecido como ômega-3, que melhora a integridade da membrana espermática durante as mudanças de temperatura. Hormônio do crescimento semelhante à insulina I (IGF-I) é um hormônio proteico que auxilia principalmente a glicose a entrar no espermatozoide, além de ser um antioxidante. O objetivo deste estudo foi analisar o efeito da adição *in vitro* de DHA combinado com IGF-I no sêmen

congelado-descongelado de garanhões. Três ejaculados de cada um de três garanhões da raça *Irish Sport Horse* foram coletados, a fração gel foi removida e o sêmen foi diluído na proporção 1:1 usando diluidor, em seguida, o sêmen foi centrifugado (1000 g) por 10 minutos a 32°C e ressuspenso a 100 x 10^6 espermatozoides/mL em diluidor de congelamento. O sêmen foi resfriado a 4°C, envasado em palhetas de 0,5 mL, congelado e estocado em nitrogênio líquido a -196°C. As palhetas foram descongeladas a 37°C por 30 segundos, o sêmen foi diluído a 25 x 10^6 espermatozoides/mL e foi dividido em quatro tratamentos, nos quais se adicionou 0 ou 1 ng de DHA/mL e 0 ou 100 ng de IGF-I /mL: DHA0, DHA0 + IGF-I, DHA1 e DHA1 + IGF-I. O sêmen foi incubado a 32°C e, depois de 30 minutos, a motilidade total (TM), motilidade progressiva rápida (PM), viabilidade e integrade acrossômica foram avaliadas. Depois de 60 e 120 minutos, TM e PM foram analisadas novamente. A PM após o descongelamento foi superior ($P<0,05$) quando DHA1 + IGF-I foi adicionado, mas não houve efeito da adição de DHA e IGF-I na TM, viabilidade ou integridade acrossômica ($P > 0,05$).
Keywords: Equinos. Espermatozoides. Ômega-3. Antioxidante. Hormônios.

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