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Residual effect after salmon oil supplementation on semen quality and serum levels of testosterone in dogs

Marcelo Carvalho Santos[1] | Chiara Milani[2] | Paolo Zucchini[2] | Celia Raquel Quirino[1] | Stefano Romagnoli[2] | Isabel Candia Nunes da Cunha[1]

[1]Laboratory of Animal Reproduction and Genetic Improvement, North Fluminense State University (UENF), Campos dos Goytacazes, Brazil

[2]Department of Animal Medicine, Production and Health, University of Padua, Legnaro, Italy

Correspondence
Isabel Candia Nunes da Cunha, Laboratory of Animal Reproduction and Genetic Improvement, North Fluminense State University (UENF), Campos dos Goytacazes, Brazil.
Email: cunhaicn@uenf.br

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Abstract

The objective of present study was to evaluate the effects of oral supplementation of salmon oil on seminal parameters and testosterone serum levels in dogs, following also the residual effects for 60 days after treatment. Nine healthy male dogs with proven fertility, weighing between 10 and 36 kg, ageing from 2 to 11 years, of different breeds, fed diets supplemented with salmon oil at the manufacturer's recommended dosage. The parameters measured were sperm volume, motility, vigour, normal morphology and concentration, live/dead ratio, membrane viability by means of HOST test and serum testosterone levels. Evaluations occurred at baseline (D0), after 90 days of supplementation (D90) and at the end of the experiment, 60 days after supplementation cessation (D150). Results (mean ± *SD*) obtained at time D0, D90 and D150 were as follows: motility of 76.66% ± 13.7, 92.77 ± 4.41 and 93.0 ± 7.90 (p = .001); normal spermatozoa of 69.11% ± 24.90, 90.00% ± 5.15 and 80.66 ± 16.04 (p = .05); live/dead (%) from 64.44 ± 22.86 to 85.33 ± 8.41 (p = .001); and spermatozoa (%) with integral membrane in the membrane integrity (HOST) test ranging from 76.44 ± 20.74 to 91.22 ± 4.68 (p = .05). Serum levels of testosterone (ng/ml) increased from 5.50 ± 1.13 to 8.84 ± 1.13 at D90 (p = .003) and decreased after 2 months (D150) to 5.13 ± 1.13. In conclusion, a 90-day supplementation with salmon oil had a positive influence on semen quality and serum testosterone levels. The supplementation of omegas 3 and 6 at the ratio of 10:1 for 90 days determined an increase in concentration and motility of the sperm, and these effects were maintained for 60 days, with the only exception of testosterone levels.

KEYWORDS
omega-3, oxidative stress, polyunsaturated fatty acids, spermatozoa

1 | INTRODUCTION

The composition of fatty acids in sperm membranes is very important for their adequate function. The sperm cell membrane plays a critical role in the main fertilization events, such as capacitation, acrosome reaction and sperm–oocyte fusion (Flesch & Gadella, 2000). The quantity of PUFAs, particularly docosahexaenoic acid, in the sperm cell membrane diminishes as the spermatozoon matures (Ollero, Powers, & Alvarez, 2000). The DHA represents 20% of the content of fatty acids in mature spermatozoa, compared with only 4% in immature germinal cells (Lenzi et al., 2000). Futher, DHA has six double bonds, contributing to a peculiar folding of its molecule, which confers fluidity and flexibility that is highly characteristic of sperm cell membranes. The ingestion of these fatty acids and the foods containing them has been associated with improved semen quality in men (Afeiche et al., 2014; González-Ravina et al., 2018).

Diets supplemented with fish oil, which is rich in EPA and DHA, increase the concentrations of testicular DHA in rodents (Sebokova, Garg, Wierzbicki, Thomson, & Clandinin, 1990) and of DHA in the sperm cell membrane of humans (Safarinejad, 2010). Other researchers have demonstrated that levels of omega-3 (principally EPA) in the seminal fluid of dogs increase after fish oil supplementation (Risso, Pellegrino, Relling, & Corrada, 2015).

The composition of the fatty acids in the sperm cell membrane is due to highly specialized local metabolism. The Sertoli cells express D^6-desaturase and D^5-desaturase, which are responsible for the processes of elongation and desaturation of PUFAs, and their products are used in spermatogenesis (Saether, Tran, Rootwelt, Christophersen, & Haugen, 2003).

Oxidative stress has been identified as an important mediator in various masculine infertility aetiologies (Agarwal, Mulgund, Sharma, & Sabanegh, 2014). Oxidative stress occurs when the levels of reactive oxygen species (ROS) and other free radicals rise greatly, or the levels of antioxidants diminish substantially, so that the delicate balance between oxidants and antioxidants is upset (Agarwal, Hamada, & Esteves, 2012; Sharma & Agarwal, 1996). In other words, oxidative stress is a condition that reflects an imbalance between the ROS and a biological system's capacity to quickly detoxify (antioxidant defence) the reactive intermediates or to repair the resulting damages (Saalu, 2010; Zini & Al-Hathal, 2011).

Jones, Mann, and Sherins (1979) observed a correlation between rising content of lipid peroxide in human spermatozoa and severe loss of motility. Thus, the exposure of spermatozoa to ROS generated outside the cells induces a loss of motility that is directly correlated with the level of lipid peroxidation of the spermatozoa (Gomez, Irvine, & Aitken, 1998).

The quantity of DHA in the human sperm membrane has been associated with higher motility and concentration, as well as changes in morphology (González-Ravina et al., 2018; Gulaya et al., 2001; Safarinejad, Hosseini, Dadkhah, & Asgari, 2010; Tavilani et al., 2007). In other animal species, there are reports of improved acrosome and sperm membrane integrity in horses (Freitas et al., 2016); elevation of motility, viability and membrane integrity in roosters (Safari Asl, Shariatmadari, Sharafi, Karimi Torshizi, & Shahverdi, 2018); a significant increase in the semen volume and total number of spermatozoa in pigs and dogs (Murphy et al., 2017; da Rocha, Cunha, Ederli, Albernaz, & Quirino, 2009; Tufarelli et al., 2018); and improved sperm quality in water buffaloes (Tran et al., 2016) and sheep (Esmaeili, Shahverdi, Alizadeh, Alipour, & Chehrazi, 2012). However, the use of PUFAs as dietary supplements to increase the quality of semen or treat testicular degeneration in dogs has not yet been fully elucidated. All the studies in this respect have only observed the immediate results, with no measurement of the durability of the effect(s) after supplementation cessation.

Therefore, the objective of this study was to evaluate whether the ingestion of salmon oil would influence canine semen quality and serum testosterone levels 60 days after the end of treatment.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

Data were collected between September 2017 and March 2018. The study was approved by the ethics committee of Norte Fluminense State University (CEUA-UENF) under protocol number 393. Nine healthy male dogs were used, of different breeds, with live weight between 10 and 36 kg and ages ranging from 2 to 11 years, all with proven fertility. The dogs remained with their owners during the entire study. The animals were evaluated at three times: at the start of the experiment (D0), after 90 days (D90) and after 150 days (D150), the end of the study. The animals received salmon oil supplementation at the dose recommended by the manufacturer for 90 days (Grizzly Salmon Oil, Grizzly Pet Products, LLC), where each 3.5 ml of the product contained: omega-3—935 mg; omega-6—95 mg; DHA—355 mg; and EPA—320 mg.

2.2 | Collection of semen and evaluation of sperm

The semen was collected by manual stimulation. After collection, the volume of the sperm fraction (ml) was determined using a graduated tube. The sperm concentration (sptz $\times 10^6$/ml) was determined in a Bürker chamber (number of sptz counted × 100 × 1,000 × the dilution used) (WHO, 2010), followed by calculation of the total sperm count (sptz × 10^6ml × semen volume = total sptz). The vigour, a subjective measure, was defined according to the linearity and quality of sperm movement, assigned a score from 0 to 5, as described previously (CBRA, 2013). The motility (MOT), expressed as a percentage, was ascertained immediately after collection, by removing 10 µl of semen, which was deposited between a slide and coverslip and visualized by light microscopy with 400× magnification in 10 fields, on a plate heated to 37°C. A total of 200 spermatozoa were evaluated in a moist chamber with rose bengal staining (WHO, 2010) to determine the percentages of normal (normal sptz) and defective ones. The defective spermatozoa were classified as having head, midpiece or tail defects, with all values expressed in percentage. The number of detached heads was also evaluated (%). The live/dead ratio (%) was determined by the supravital test (eosin/nigrosin staining) (WHO, 2010). For the eosin–nigrosin staining, semen smears were prepared by gently mixing about 10 µl of eosin–nigrosin with a similar volume of semen on a microscope slide kept at 37°C. The mixture was spread gently on a clean glass slide, and each smear was air-dried (WHO, 2010). The integrity of the cytoplasmic membrane (%) of the spermatozoa was determined by the hypo-osmotic swelling test (HOST) (Drevius & Eriksson, 1966), where the spermatozoa are classified as swollen or HOS reactive (HOS +), meaning functionally intact membranes.

2.3 | Evaluation of serum testosterone

To ascertain the serum level of testosterone (T), the dogs were submitted to the HCG stimulation test (Romagnoli et al., 2012), involving intramuscular application of 50 µg of gonadorelin GnRH (Fertagyl®), to avoid fluctuation of the level of the hormone in the blood during

TABLE 1 Mean and *SD* of fresh semen parameters of nine dogs submitted to dietary supplementation with salmon oil for 90 days (D0–D90) and after supplementation (D150)

Parameter	D0	D90	D150	*p*-value
Volume (1.0–6.0)	1.90 ± 0.85	3.27 ± 1.60	2.55 ± 1.71	.15
Motility (50.0–98.0)	76.66 ± 13.7[b]	92.77 ± 4.41[a]	93 ± 7.90[a]	.001
Vigour (2.5–5.0)	3.27 ± 0.44	3.67 ± 0.61	3.89 ± 0.82	.15
sptz/ml(×10^6) (80.0–1614.0)	283.44 ± 125.88	357.44 ± 291.90	455.33 ± 483.17	.56
sptz/ml(×10^6) × volume (80.0–2421.0)	585.11 ± 442.34	1,000.38 ± 616.40	1,020.16 ± 819.56	.29
Normal sptz (%) (43.0–97.0)	69.11 ± 24.90[b]	90.00 ± 5.15[a]	80.66 ± 16.04[ab]	.05
Head defect (%) (0.0–3.0)	0.33 ± 0.70 [b]	0.89 ± 0.93a[ab]	1.33 ± 0.70[a]	.04
Midpiece defect (%) (1.0–62.0)	19.22 ± 21.15	2.78 ± 2.73	10.33 ± 14.95	.08
Tail defect (%) (0.0–29.0)	7.44 ± 8.55	5.11 ± 3.82	6.78 ± 5.02	.71
Detached head (%) (0.0–5.0)	0.56 ± 0.88	1.22 ± 1.92	1.11 ± 1.54	.61
Live/dead (%) (20.0–99.0)	64.44 ± 22.86[b]	85.33 ± 8.41[a]	92.89 ± 5.35[a]	.001
HOST (%) (31.0–98.0)	76.44 ± 20.74[b]	91.22 ± 4.68[a]	87.67 ± 5.85[a]	.05
Testosterone (ng/ml) (1.58–13.37)	5.50 ± 1.13[b]	8.84 ± 1.13[a]	5.13 ± 1.13[b]	.003

Note: Different uppercase letters in the row indicate significant differences between the sample collection periods (D0, D90 and D150) ($p \le .05$).

the day. The blood samples were obtained by venipuncture of the cephalic vein on days D0, D90 and D150. The blood samples were centrifuged at 1,400 *g* for 5 min, and the serum was collected and stored in a freezer at −20 °C. The testosterone level was determined by chemiluminescence with an Immulite® 1000 immunoassay system. The concentrations of all samples were measured in one single procedure, at the end of evaluation period.

2.4 | Statistical analysis

Data were tested for normal distribution, and the descriptive statistics were computed using the PROC UNIVARIATE and PROC MEANS programs (SAS, 2016). Data were then submitted to ANOVA considering the fixed effect of the time period and the repeated effect of each animal (PROC MIXED, SAS, 2016). Finally, the pdiff test was performed to compare the means, with a significance level of $p < .05$.

3 | RESULTS

Results of the sperm analyses and serum levels of testosterone are reported in Table 1. There was an increase in motility on D90, the ending date of treatment, and a decline on D150. The percentage of live spermatozoa increased during the treatment period (until D90) and then remained at the same level until D150 ($p = .05$). This parameter also presented a smaller dispersion of the data (as indicated by the *SD*) on D90 in comparison with the other periods (D0 and D150), indicating homogeneity of the dogs at this moment (D90). There was an increase in the percentage of live spermatozoa ($p = .001$) on D90, with the level being maintained until the end of the experiment (D150). The same pattern was observed for the percentage of spermatozoa with intact membranes in the hypo-osmotic test, with increased values on D90 and maintenance until D150. With respect to the serum testosterone levels, these peaked on D90 ($p = .003$) and then declined afterwards (Figure 1). The other variables were not affected by the treatment with salmon oil.

4 | DISCUSSION

Various researchers have used different periods of supplementation with PUFAs, such as 60 days (stallions), 16 weeks (boars), 30 weeks (stallions) and 174 days (boars) (Freitas et al., 2016; Lin et al., 2016; Liu et al., 2017; Rodrigues et al., 2017). We believe our experiment is the first assessing the possible alterations at 60 days after the end of treatment. We observed that the effects of the treatment on the parameters motility, percentage of normal spermatozoa, live/dead percentage and membrane integrity (HOST) were maintained for 2 months after the end of the dietary supply of salmon oil.

The testosterone levels behaved differently, with the values declining after the end of the treatment (Figure 1). Risso et al. (2015) reported no increase in the serum testosterone levels of dogs fed with fish oil supplementation for 120 days. In contrast, in our study the testosterone levels increased, peaking on D90, before declining. That difference can perhaps be explained by the percentage of omega-3 present in the fish oil, which was 25.6% in the study of Risso et al. (2015) versus 29% in the product used in our experiment. We therefore believe that the higher dose used in our experiment influenced the higher serum testosterone levels.

Our hypothesis that the use of the salmon oil would influence the semen quality parameters was confirmed by the improved motility, increased percentage of live spermatozoa and elevation of the serum testosterone level. These results are in line with those described by other authors (González-Ravina et al., 2018; Nassan, Chavarro, & Tanrikut, 2018; Risso et al., 2015; Rocha et al., 2009; Santos, Souza, Quirino, Bastos, & Cunha, 2016).

The improving of sperm quality can be associated with changes in phospholipid profile of the spermatozoa due to ingestion of a substance rich in EPA and DHA (salmon oil) (González-Ravina et al., 2018). Although the transformation of spermatogonia into mature spermatozoa involves a large number of complex steps, the focus on some important changes can facilitate discussion of how nutritional supplementation affects this complex process. In the first place, spermatozoa lose most of their cytoplasm before leaving the testis, and the chromatin condensation of spermatozoa occurs after progressing from the head to the tail of the epididymis (Johnson, 1995). These processes include repeated oxidation reactions (Flesch & Gadella, 2000). Oxidative stress is a condition that reflects an imbalance between the systemic manifestation of ROS and the capacity of a biological system to promptly detoxify itself (antioxidant defence) (Agarwal, Mulgund, et al., 2014).

Many studies have demonstrated that low and controlled (physiological levels) concentrations of ROS play an important role in normal physiological processes such as capacitation, hyperactivation, acrosome reaction and sperm–oocyte fusion in order to ensure appropriate fertilization (Agarwal, Virk, Virk, Ong, & Plessis, 2014). However, an excessive increase in the free radicals in the spermatozoa can be harmful and cause subfertility (Agarwal, Virk, et al., 2014).

The level of DHA is higher in immature than in mature spermatozoa, evidencing the antioxidant role of docosahexaenoic acid, and thus minimizing the peroxidative damage (Ollero et al., 2000). Therefore, it is possible that DHA also can play a role in the regulation of spermatogenesis. If this is the case, interventions aimed at altering the DHA content in spermatozoa from immature germinative cells could affect sperm maturation. The increase in ROS production can affect the mitochondrial function of spermatozoa, and consequently their motility (Ollero et al., 2000; Tadros & Vij, 2019); a finding that has been repeatedly observed in independent studies (Aitken & Fisher, 1994; Aitken, 1999, 2004; Aitken & Clarkson, 1987; Alvarez, Touchstone, Blasco, & Storey, 1987; Sharma & Agarwal, 1996). We also observed this pattern, just after the beginning of the salmon oil ingestion there was an increase of sperm motility, as measured on D90, and this higher level was maintained until the end of the experiment (D150). These results are similar to those of Rocha et al. (2009), who reported positive effects (increased sperm motility and vigour and reduced level of sperm defects) using a diet supplemented with omega-3, 6 and 9 and enriched with vitamin E.

Other researchers have concluded that higher saturation of phospholipids in the sperm cell membrane reduces seminal quality (Esmaeili, Shahverdi, Moghadasian, & Alizadeh, 2015; Martínez-Soto, Landeras, & Gadea, 2013; Ramezani, Feltham, Suh, & Nutritional, 2018). On the other hand, the proportion of highly unsaturated fatty acids (PUFAs) was positively correlated with the proportion of spermatozoa with intact membranes (Tran et al., 2016). That pattern was also observed in our study, by the increase in the percentage of spermatozoa with intact membranes (live/dead ratio) on D90. The increase in the percentage of live spermatozoa after supplementation can also be based on the reduced damage to the DNA, since dysfunctional spermatozoa suffer from a debility in chromatin compaction, making them more vulnerable to attack from ROS, provoking fragmentation of the spermatic DNA followed by cell death (Agarwal, Mulgund, et al., 2014; Aitken, Iuliis, & Drevet, 2019). Another possible explanation for the better sperm quality values is the occurrence of an increase in the concentration of DHA in the epididymal fluid, mainly in the tail of the epididymis (Lenzi et al., 2000), where it is incorporated in the sperm membrane. In dogs, this incorporation process occurs in the final stages of maturation (Ramos Angrimani et al., 2017).

The testosterone produced by the Leydig cells in the testes is essential for the development and maintenance of spermatogenesis and other male characteristics. Fatty acid supplements can have energy effects on reproduction by increasing the hypophysis or gonadal function, or both (Wathes, Abayasekara, & Aitken, 2007). Several researchers have reported that unsaturated fatty acids improve steroidogenesis in vivo through the expression of the steroidogenic acute regulatory (StAR) protein (Hughes et al., 2011; Jefcoate, 2006; Wade, Kraak,

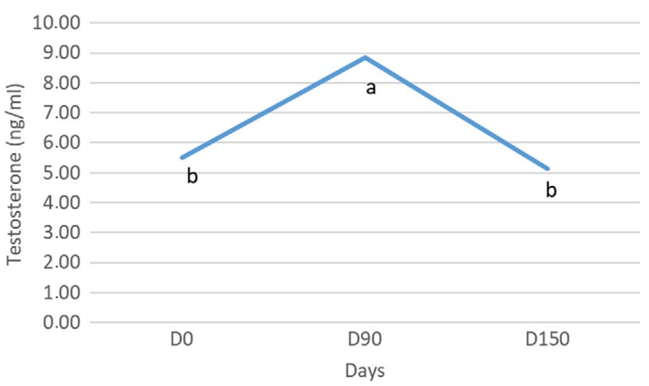

FIGURE 1 Effect of salmon oil supplementation (90 days) on the serum levels of testosterone in 9 male dogs during 150 days

Gerrits, & Ballantyne, 1994; Wang, Walsh, Reinhart, & Stocco, 2000), and alter the function of the transcription factors (Wathes et al., 2007). Sebokova et al. (1990) postulated that the ingestion of PUFAs alters the responsiveness of the Leydig cells, changing the activity of adenylyl cyclase and the synthesis of testosterone, suggesting that the change in the lipid composition of the testicular plasma membrane by dietary supplementation affects the accessibility of the LH/hCG receptors and that the LH bond is associated with the higher number of receptors. In our study, the serum testosterone levels increased after supplementation (D90). This finding indicates that ingestion of salmon oil possibly affected the phospholipid composition of the plasma membrane of the testicular cells, altering the expression and affinity of the gonadotropin receptors and influencing the testosterone synthesis rate.

From the results of this study, we can infer that the germinative cells and Sertoli cells have an active lipid metabolism that causes a rearrangement of the constitution of the fatty acids, in turn causing elongation and desaturation of those acids, which are essential during spermatogenesis and also possibly during the maturation of the spermatozoa. That metabolism is possibly responsible for the alteration of the phospholipid profile of the sperm membrane (with more DHA), reducing oxidative stress and favouring improved semen parameters.

5 | CONCLUSION

The dietary supplementation with salmon oil for 90 days positively influenced the semen quality and serum levels of testosterone of the dogs. These residual effects lasted for 2 months after the end of treatment, with the exception of the testosterone level, which initiate the declined after the end of the supply returning to initial values after 60 days of supplementation interruption.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

1. Marcelo Carvalho dos Santos. Doctoral thesis, (a) substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, (b) drafting and revising the article and (c) final approval of the version to be published.
2. Chiara Milani. (a) Substantial contributions acquisition of data, analysis and interpretation of data, (b) revising manuscript critically and (c) final approval of the version to be published.
3. Paolo Zucchini. (a) Substantial contributions acquisition of data.
4. Celia Raquel Quirino. (a) Substantial contributions analysis and interpretation of data.
5. Stefano Romagnoli. Doctoral thesis co-supervisor. (a) Substantial contributions to analysis and interpretation of data and (b) final approval of the version to be published.
6. Isabel Candia Nunes da Cunha. Doctoral thesis supervisor. (a) substantial contributions to conception and design, analysis and interpretation of data, (b) revising manuscript critically for important intellectual content and (c) final approval of the version to be published.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Isabel Candia Nunes Cunha https://orcid.org/0000-0002-6261-7431

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