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ANIMAL SCIENCE

Fatty acid profiles of the plasma and follicular fluid mares fed a combination of linseed and salmon oil

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Abstract: This study evaluated the presence of polyunsaturated fatty acids in circulating blood and in the ovarian follicular fluid of mares, after supplementation of the diet with linseed oil. Six Mangalarga Marchador mares, weighing 397.00±31.89 kg, were kept on native pasture, and assigned to the current study. In a switch over design, mares were randomly allocated to receive 150 ml of vegetable oil daily, containing polyunsaturated fatty acids n3 (62.23 g ALA, 20.34 g LA, 2.27 g EPA, 2.32 g DHA), (n=3) or no supplementation (n=3) in two replicates. Blood and follicular fluid samples were taken on the first day (D0) and every 30 days until the end of the supplementation period (D60). After 60 days of supplementation, mares were switched across the treatments. Plasma concentrations of linolenic acid in total fatty acids were higher (P=0.006) in the supplemented compared to the control group (1.89±0.13 vs. 1.49±0.13%). There were positive correlations between plasma linoleic acid and follicular fluid arachidonic acid (P=0.0106; r^2=0.13) and between plasma alpha linolenic acid and follicular fluid EPA (P=0.0004; r^2=0.2544). Data indicated a low to moderate relationship between the dietary linseed-based oil supplementation studied and circulating and follicular fluid polyunsaturated fatty acids contents in mares.

Key words: DHA, equine, ovarian follicle, PUFA.

INTRODUCTION

A good nutritional balance must be applied in order to maintain adequate reproduction efficiency, especially for mares during the reproductive season (Soncin et al. 2009).

If nutrition is inadequate, often times, poor follicular development is found in mares which, as a consequence, do not ovulate normally or have deficient corpora lutea, leading, in turn, to early embryonic losses or underdeveloped fetuses (Shepherd et al. 2012). Studies have shown that diets rich in starch may result in decreased in the sensibility to insulin (insulin resistance), resulting in disruption of the hypothalmic-hypofhyseal ovariam axis compromising mare fertility (Jacobs et al. 2015).

In this sense, some studies have been carried out with supplementation of polyunsaturated fatty acids (PUFA) of breeding mares (Dinnetz et al. 2013, Jacobs et al. 2018), in order to improve reproduction.

Among PUFAs, the most important representatives are the fatty acids known as omega 3 (or n3) and omega 6 (or n6) (Vaz et al. 2014). Omegas 3 and 6 are found in large amounts in vegetable oils, most seeds and cereal grains, corn, soybean, sunflower and linseed oils (Martin et al. 2006). Equines respond quickly to dietary EPA supplementation, with a significant

increase in blood concentration from as early as three days of supplementation (King et al. 2008).

The main metabolic functions of these compounds are related to the maintenance of endothelial cell integrity, prevention of atherosclerosis and cardiovascular changes; stimulation of insulin release; inhibition of vasoconstriction and platelet aggregation; participation in placental development, fetal growth and neuronal development, participation in immunomodulatory functions, release of cytokines and synthesis of eicosanoids (NRC 2007, King et al. 2008). The addition of LNA, an Omega 3 fatty acid also assists in the triggering of estrous cyclicity in prepubertal animals (Moreira et al. 2016). In cattle, the addition of linolenic acid (LNA), omega 3 (n-3) in the diet promotes a direct decrease in the secretion of PGF2α, due to the inhibition of the action of prostaglandin H synthase or, indirectly, by means of the products of this desaturation, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can improve the rate of pregnancy in the initial phase of gestation (Ambrose & Kastelic 2003, Gregory et al. 2009).

In general, the role of PUFA metabolism in reproductive physiology is centered on the availability of substrates for estradiol synthesis in the follicles which is essential for normal follicular growth and ovulation (Wathes et al. 2007).

Considering the previous information, it is relevant to verify whether LNA, DHA, and EPA increase in the follicular fluid and plasma when mares are supplemented with linseed oil. The objective was to determine the concentrations of polyunsaturated fatty acids in the plasma and in the follicular fluid in mares supplemented with a linseed- based vegetable oil.

It was hypothesized that dietary 18:3n3 (omega 3) supplementation increases PUFA in the plasma and ovarian follicular fluid in mares, as a result of elongation and desaturation in the organism. A secondary hypothesis is that the concentrations of PUFAs in the plasma are correlated to those of the follicular fluid.

MATERIALS AND METHODS

This study is in accordance with the guidelines established by the Ethical Committee for the use of Animals in Research of the Federal University of Lavras (Protocol n° 005/11).

Animals, diets and experimental design

Six Mangalarga Marchador mares of 7.95±6.4 years old, weighing 397.00±31.89 kg and body scores ranging from 3.0 to 6.5 (1- emaciated to 9- excessively obese) (Henneke et al. 1983). Mares were kept during the dry season on native pastures in a continuous grazing system, and ad libitum access to water and mineral mix. All animals were previously dewormed and evaluated clinically and declared sound by an experienced veterinarian. Additionally, 2 Kg of a concentrate (18% crude protein), were fed twice daily (7 a.m. and 3 p.m.). The concentrate included corn grain, soybean meal and wheat bran, containing an estimated n6:n3 ratio of 1.03:1.00 (Table I).

Mares were randomly allocated to one of two treatments: Treatment A (n=6): unsupplemented regular diet; Treatment B (n=6 animals) = Regular diet plus 150 mL of PUFA n3 oil. The oil was offered on top of the concentrate, divided into two 75mL servings; one in the morning and the other in the afternoon feeding.

The linseed-based vegetable oil used contained poly-and mono-unsaturated fatty acids added of Canadian salmon oil. The fatty acid composition of the oil was determined by gas chromatography: 62.23 g of linolenic acid, 20.34 g of linoleic acid, 2.27 g of EPA and 2.32 g of

Table I. Estimation of PUFA concentration in the experimental diet*, on dry matter (DM) basis.

	Composition of feed[1]			Quantity consumed (g/d)			
Source	**EE%**	**Ômega 6 (%)**	**Ômega 3 (%)**	**Intake[2] (Kg/DM/day)**	**Intake n6**	**Intake n3**	**Relation n6/n3**
Corn grain	4.20	49	0.5	1.10	22.63	0.23	98/1
Soybean meal	1.10	53.0	8.0	0.43	2.51	0.38	66/1
Wheat bran	4.3	0	0	0.18	3.07	0	0
Native pasture	2.00	19.48	40.55	6.2	24.15	50.28	1/2.08
Oil UFLA[3]	--	--	--	20.34	62.23	1/3.06	20.34
Total	11.6	121.48	49.05	7.91	72.7	113.09	1/1.55

***Hypothetical estimate of preparation of grains in concentrated (18% CP: 61% corn grain, 24% soybean meal, 10% wheat bran. [1]Source: NRC (2007). [2]2% BW of 400 kg, concentrate 1.8 Kg, forage DM 6.2 Kg. [3]Analized values by gas chromatographic.**

DHA, considering that the density of the linseed oil was 0.9285 (Clark & Tschentke 1929). The anti-nutritional factors were deactivated and the supplement enriched with vitamin E.

Before the beginning of each trial, mares went through a fourteen-day adaptation period, during which the oil amount was increased daily until the total intake proposed was reached (150 mL of the oil daily). In a crossover design, animals were switched across the treatments in two replicates. In the first replicate, three mares were randomly allocated to the PUFA group or Control group for sixty days. Mares were reversed across the treatments after a sixty-day interval, in order to remove the possible PUFA residual effects and for adaptation.

Follicular fluid aspiration and blood sampling

Before the beginning of the experiment, mares received an intra-muscular (IM) injection of 5 mg of dinoprost tromethamine (Lutalyse®). Mares were submitted to four follicular aspirations (largest follicles detected) at 30-days intervals, as described below:

Period 1: follicular aspirations 30 days before the initiation of the treatments; Period 2: follicular aspirations 30 days after initiation of the treatments; Period 3: follicular aspirations 60 days after initiation of the treatments.

A mild sedation and epidural anesthesia were given before follicular aspirations according to previously described procedures (Carnevale & Ginther 1993). A minimum of 0.5 mL of follicular fluid was obtained from the largest follicle detected on either ovary.

The follicular aspiration was guided by a 5.0 MHz microconvex transducer (UST974-5) with an Aloka SSD 500V unit connected to an aspiration guide, and using an 18G needle and aspiration line and 50 mL centrifuge tubes connected to a vacuum pump adjusted for 150 mmHg (Mozzaquatro 2008). Once the procedure was finished, mares received an antibiotic and anti-inflammatory combination IM injection (1 mL per 25 kg of bodyweight, Pencivet® Plus).

The follicular fluid was immediately stored in sterile plastic tubes at 4°C; later the fluid was centrifuged (1500 x g for ten minutes) and the supernatant collected and stored at -20°C until analysis (Gastal et al. 2010).

Blood samples (5 mL) were obtained by jugular venipuncture in a closed system

(Vaccutainer®) containing EDTA in the same day as the follicular aspirations. Blood samples were centrifuged (1500 x g for ten minutes). Plasma was separated and stored at -20°C (Soncin et al. 2009).

Lipid extraction and profile determination

Determination of the lipid profile was according to a previously described methodology (Folch et al. 1957). Once the original samples were prepared, 1 mL subsamples were used for the chromatography readings, which were done at the Animal Metabolism and Growth Laboratory of the University of São Paulo (USP) in Piracicaba-SP- Brazil. A 1µL aliquot of the esterified extract was injected into the chromatography unit and the identification of the fatty acids done by comparison between the retention times and the percentage of the fatty acids present, performed with the *Chromquest* 4.1 software (Thermo Electron). The standard from Supelco TM Component FAME Mix, cat 18919 was used. It was used a column of gas chromatography of 100m.

Statistical analyses

All data were analyzed by the procedures of the SAS® package (Statistical Analysis System® 1998). The fixed effects of treatment, time and interactions on follicular fluid and plasma lipid concentrations were submitted to the Mixed procedure using the lowest Akaike criterium value as the covariance structure in each case. The effect of animal within treatment was the error term. Means were compared by orthogonal contrasts. The GLM procedure was used to obtain the means and the standard errors.

Variables were checked for residual normality (Univariate) and submitted to the Bartlett test to evaluate variance homogeneity. Data which did not meet analysis of variance criteria were transformed (base-10 logarithm; inverse - 1/X; or squared-X**- 2). Variables that could not be adjusted to normality (non-parametric) after transformation were evaluated by the Wilcoxon test (NPAR1WAY) and submitted to the Genmod procedure considering the Poisson distribution and repeated options.

Statistical differences and tendencies were defined as probabilities of 5 and 15%. Serum fatty acid concentrations were regressed onto those of the follicular fluid by the Reg procedure.

RESULTS

Fatty acid profiles of follicular fluid

Omega 3 and 6 supplementation increased the concentration of linolenic acid in the plasma (P=0.006). Plasma LNA increased while mare were suplemmented between 30 and 60 days (P=0.0001), however no interaction was observed between these variables (Table II).

The concentrations of linoleic acid LN tended to be lower (P=0.10) in period 1 compared to those of period 3 (Table III). The concentration of LNA in period 2 was higher (P=0.05) compared to those of periods 1 and 3 (Table IV).

The concentrations of EPA in the follicular fluid tended (P=0.11) to be lower in period 1 compared to that of period 2, but were similar to that of period 3 (Table IV). A greater (P=0.06) concentration of DHA was found in the follicular fluid of controls mares compared to supplemented (Table IV).

The concentrations of the omega 3 fatty acid series were lower (EPA 0.07% e DHA 0.03%) than those of the omega 6 series (dihomo-gamalinolenic 0.16% and arachidonic acid 4.40%) in the circulation (Tables II and III). Higher omega 6 fatty acid concentrations in the follicular fluid were also evident (Table V).

Table II. Effect of linseed-based vegetable oil with addition of salmon oil Canada supplementation on the plasma concentrations of polyunsaturated Omega 3 fatty acid (%) in Mangalarga Marchador mares.

		Period¶				Effect P*		
FA	Treat	1	2	3	Mean Treat	T	P	TxP
1†	Suppl.	1.26±0.21	1.84±0.24	2.57±0.24	1.89±0.13[a]	0.006	0.0001	0.66
	Control	1.03±0.19	1.74±0.24	1.67±0.24	1.48±0.13[b]			
	Mean	1.14±0.14[a]	1.79±0.17[b]	2.12±0.17[b]				
2‡	Suppl.	0.06±0.01	0.10±0.02	0.06±0.02	0.07±0.01	0.11	0.11	0.23
	Control	0.03±0.01	0.07±0.02	0.05±0.02	0.04±0.01			
	Mean	0.04±0.01[A]	0.08±0.01[B]	0.05±0.01[AB]				
3‡†	Suppl.	0.04±0.02	0.04±0.04	0.01±0.05	0.03±0.02	0.92	0.49	0.38
	Control	0.07±0.03	-0.02±0.06					
	Mean	0.06±0.01	0.01±0.03					

Distinct letter indicate differences (P<0.05) and tendencies (P<0.10). FA= Fatty Acid; 1†= C18:3 n3 (Linolenic Acid); 2‡ = C20:5 n3 (EPA); 3‡†= C22:6 n3 (DHA); ¶Period 1: follicular aspirations 30 days before the initiation of the treatments; Periods 2: follicular aspirations 30 days after the initiation of the treatment; Periods 3: follicular aspirations 60 days after initiation of the treatments. *Effect of P: T= Effect of treatment; P= Effect of period; TxP = interaction Treatment vs. Period.

Table III. Effect of linseed-based vegetable oil with addition of salmon oil Canada supplementation on the plasma concentrations of polyunsaturated Omega 6 fatty acid (%) in Mangalarga Marchador mares.

		Period¶				Effect P*		
FA	Treat	1	2	3	Mean Treat	T	P	TxP
1†	Suppl.	14.40±2.30	19.75±2.7	25.73±2.7	19.1±1.5	0.17	0.10	0.58
	Control	15.56±2.26	24.84±2.8	28.60±2.8	23.0±1.5			
	Mean	14.10±1.60[A]	22.30±1.9[AB]	27.17±1.9[B]				
2‡	Suppl.	0.16±0.03	0.13±0.03	0.20±0.03	0.16±0.02	0.21	0.87	0.15
	Control	0.11±0.02	0.13±0.03	0.09±0.03	0.11±0.02			
	Mean	0.13±0.02	0.13±0.02	0.14±0.02				
3‡†	Suppl.	4.26±0.70	3.76±0.80	5.13 ±0.80	4.40±0.44			
	Control	4.49±0.64	4.40±0.80	4.53 ±0.80	4.50±0.43			
	Mean	4.38±0.46	4.08±0.55	4.83 ±0.55				

Distinct letter indicate differences (P<0.05) and tendencies (P<0.10). FA= Fatty Acid; 1† = C18:2 n6 (Linoleic Acid); 2‡=C20:3n6 (dihomo-gama linolenic); 3‡†=C20:4n6 (Arachidonic Acid). ¶Period 1: follicular aspirations 30 days before the initiation of the treatments; Periods 2: follicular aspirations 30 days after the initiation of the treatment; Periods 3: follicular aspirations 60 days after initiation of the treatment. *Effect of P: T= Effect of treatment; P= Effect of period; TxP = interaction Treatment vs. Period.

Table IV. Effect of linseed-based vegetable with addition of salmon oil Canada supplementation on the follicular fluid concentrations of polyunsaturated Omega 3 fatty acid (%) in Mangalarga Marchador mares.

		Period¶				Effect P*		
FA	Treat	1	2	3	Mean Treat	T	P	TxP
1†	Suppl.	0.32±0.10	0.80±0.12	0.31±0.12	0.47 ±0.07	0.48	0.05	0.43
	Control	0.44±0.10	0.49±0.12	0.28 ±0.12	0.40 ±0.07			
	Mean	0.38±0.07^{a}	0.63±0.09^{b}	0.30 ±0.09^{a}				
2‡	Suppl.	0.08±0.03	0.08±0.04	0.07±0.04	0.08 ±0.02	0.11	0.08	0.74
	Control	0.06±0.04	0.26±0.04	0.19±0.04	0.16±0.02			
	Mean	0.07±0.02^{A}	0.17±0.03^{B}	0.13 ±0.03^{AB}				
3‡†	Suppl.	0.01±0.01	0.00±0.01	0.01±0.01	0.01±0.00^{A}	0.06	0.49	0.92
	Control	0.02±0.01	0.07±0.02	0.03±0.01	0.04±0.00^{B}			
	Mean	0.02±0.00	0.04±0.01	0.03±0.01				

Distinct letter indicate differences (P<0.05) and tendencies (P<0.10). FA= Fatty Acid; 1†= C18:3n3 (Linolenic Acid); 2‡ = C20:5 n3 (EPA); 3‡†= C22:6 n3 (DHA). ¶Period 1: follicular aspirations 30 days before the initiation of the treatments; Periods 2: follicular aspirations 30 days after the initiation of the treatment; Periods 3: follicular aspirations 60 days after initiation of the treatments. *Effect of P: T= Effect of treatment; P= Effect of period; TxP = interaction Treatment vs. Period.

Fatty acid profiles in blood plasma

There was a positive correlation (P=0.0140; r^2=0.2677) between plasma LNA and follicular fluid EPA concentrations. Similarly, there was a positive correlation (P=0.0238; r^2=0.2113) between plasma LN and arachidonic acid concentrations in the follicular fluid.

DISCUSSION

The amount of fatty acids present in the oils fed influences their plasma concentrations. It has been shown that EPA and DHA were only found in appreciable amounts in the plasma of horses supplemented with oils that contained these fatty acids, e.g., fish-based oils (Vineyard et al. 2009). Thus, the lower concentrations of EPA and DHA observed

In the plasma of the supplemented group (Table II) in comparison to an experiment (King et al. 2008), in which higher doses of 10, 20 e 40 g/day of EPA (17.81 g) and DHA (21.32 g) containing oil sources were used. In the current study low fatty acid concentrations were seen.

The relatively low concentrations of EPA and DHA in the plasma of mares supplemented with linseed oil (63.23g of LNA) indicate that the conversion rate of PUFA's is limited in equines, in the present experiment. Compatible results were into the tissues, rather than remain in circulating plasma.

In humans, the bioconversion rate from LNA to EPA is lower than 10% and to DHA is below 0.10% (Willians & Burdge 2006).

Vegetable oil rich in Omega 3 was offered to the animals, expecting its bioconversion into its derivatives (EPA and DHA). According to one previous report (Pawlosky et al. 2001), only 0.2% of the linolenic acid was used for the biosynthesis of EPA in human plasma. The best biosynthetic pathway for very long chain fatty acids is from EPA, so that supplementing with this fatty acid is a better alternative to obtain DHA and that the

Table V. Effect of linseed-based vegetable with addition of salmon oil Canada supplementation on the follicular fluid concentrations of polyunsaturated Omega 6 fatty acid (%) in Mangalarga Marchador mares.

		Period¶				Effect P*		
FA	Treat	1	2	3	Mean Treat	T	P	TxP
1†	Suppl.	5.75±3.33	12.87 4.09	14.21 ±4.09	10.9±2.23	0.74	0.31	0.75
	Control	7.55±3.34	9.01±4.08	6.27±4.08	7.61±2.22			
	Mean	6.65±2.36	10.94±2.90	10.24±2.90				
2‡	Suppl.	0.07±0.02	0.11 ±0.02	0.07 ±0.03	0.08±0.01	0.71	0.43	0.72
	Control	0.10±0.02	0.09±0.03	0.07±0.03	0.09±0.02			
	Mean	0.09±0.01	0.10±0.02	0.07±0.02				
3‡†	Suppl.	6.39±1.41	6.11±1.72	10.41±1.72	7.64±0.94	0.94	0.95	0.92
	Control	7.13±1.41	8.16±1.72	9.18±1.72	8.16±0.93			
	Mean	6.76±1.00	7.14±1.22	9.80±1.22				

Distinct letter indicate differences (P<0.05) and tendencies (P<0.10). FA= Fatty Acid; 1† = C18:2 n6 (Linoleic Acid); 2‡ = C20:3n6 (dihomo-gama linolênic); 3‡†= C20:4n6 (Arachidonic Acid). ¶*Period 1: follicular aspirations 30 days before the initiation of the treatments; Periods 2: follicular aspirations 30 days after the initiation of the treatment; Periods 3: follicular aspirations 60 days after initiation of the treatments.* *Effect of P: T= Effect of treatment; P= Effect of period; TxP = interaction Treatment vs. Period.

utilization of linolenic acid may not be the best option (Pawlosky et al. 2001).

Therefore, it is concluded that the Omega 3 amount offered was minimally used for its main purpose (very long chain fatty acid synthesis), which was demonstrated by the low DHA concentrations in the plasma.

Alfa linolenic (LNA) acid may be metabolized by elongation and dessaturation to EPA, DPA and DHA. The eicosapentaenoic acid (DPA, 22:5n3) is formed by the elongation of the EPA chain and this conversion occurs in various steps. Firstly, the elongation of EPA to DPA (22:5n3) takes place, followed by the dessaturation of the tetracosapenthaenoic acid (24:5n3) to tetracosahexaenoic (24:6n3) and finally the oxidation to DHA (22:6 n3) in the peroxisome (Kaur et al. 2011).

The high LNA percentage (45.1%) offered as a supplement may explain the low DHA concentrations observed in the plasma of linseed oil- supplemented animals.

The highest concentration of DHA in the control group could be due to a residual effect from the first replicate. The interval for the exchange of groups into the treatments may have been insufficient to reduce the follicular fluid PUFA concentration (Table IV).

Linoleic acid uses Δ6dessaturase for its bioconversion into its metabolites, similarly to the α-linolenic acid. Thus, they compete for this enzyme, resulting in greater concentrations of omega 6 fatty acids. Moreover, the Δ6dessaturase have greater affinity for the omega-6 series fatty acids and this cause a restriction depending on the omega-6 diet content. Jacobs et al. (2018) supplemented pregnant mares with a marine source and observed greater DHA/EPA endometrial-tissue incorporation.

In the present study the highest concentrations of omega-6 fatty acids (dihomo-gamalinolenic 0.16% and arachidonic acid 4.40%) relative to omega-3 fatty acids (EPA and DHA 0.07% and 0.03%, respectively) in the

circulation (Tables II and III) suggests that a greater bioconversion of the series 6 into series 3 fatty acid may have occurred.

The positive correlations between plasma linolenic acid and follicular fluid EPA and between linoleic and arachidonic acid concentrations in the follicular fluid indicate the possible conversion of the former fatty acid in these two later very long chain derivatives. These correlations establish an important link between nutrition and reproductive function.

With longer supplementation periods, it would be expected that greater elongation rates and dessaturation of this fatty acid would occur, which could be benefic to equine reproduction.

There were no effects of treatment, period or interaction on the other PUFA plasma or follicular fluid concentrations analyzed (Tables II to V).

The dietary supplementation with linseed oil enriched with 63.23 g of α-linolenic acid, 20.34 g of linoleic acid, 2.27 g of EPA and 2.32 g of DHA influenced their concentrations in the plasma of supplemented mares however did not influence the n3 PUFA concentrations in the follicular fluid. This demonstrates that there is low conversion in the horse organism. Thus, it is necessary to provide nutritional sources with higher concentrations of EPA and DHA for a period exceeding 30 days in order to use PUFAs as a reproductive nutraceutical in mares.

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