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Uterine Involution of Mares Supplemented with Dietary Algae-Derived Omega-3 Fatty Acids During the Peripartum Period

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ABSTRACT

Different approaches have been used to improve conception rates at foal heat. Omega-3 fatty acids family and derivatives have improved reproductive efficiency in ruminants, but literature lacks studies evaluating these components on equines. The objective of this study was to evaluate effects of mare dietary supplementation with microalgae rich in docosahexaenoic acid (DHA) during peripartum on follicular dynamics and uterine involution in early post-partum. Eighteen pregnant mares, no particular breed, 410 ± 39.5 kg body weight (BW), and 7.83 ± 2.01 yr old were used. Mares were randomly assigned to control (CONT) or supplementation with microalgae rich in DHA at 0.06 g/kg BW (ALG). Treatments were supplied from 90 d prior to expected foaling date until 7 d after first ovulation. Reproductive evaluations were performed during early post-partum until 7 d after first ovulation through rectal palpation and ultrasonography of the following parameters: uterine and endometrium diameters, intrauterine fluid (IUF), uterine echogenicity, uterus tone, and follicular dynamics. Endometrial cells samples were collected to assess mRNA expression of CRP, IL-1β and AKR1C4, using RT-qPCR. Data were analyzed by mixed procedure of SAS. ALG mares had smaller uterine horns diameters and greater uterine echogenicity during post-partum in comparison with CONT. No treatment effects were detected for other characteristics evaluated, but a day effect was observed for uterine and endometrium diameter, IUF, uterine echogenicity, and transcript abundance of endometrial AKR1C4. Supplementation with DHA during peripartum may benefit uterine involution process and odds of early conception, but more studies should be performed regarding fertility.

1. Introduction

Mares present a relatively long gestational length (around 11 mo) with the first estrus around 5–12 days after foaling, commonly referred to as the foal-heat. During the foal-heat, however, uterine involution process has not been completed [1]. Despite being a fertile heat, studies indicate that fertility and conception odds during the foal heat are relatively low [2]. In this scenario, the gestation success depends on appropriate ovulation, fertilization, and rapid uterine involution which involves endometrium regeneration, lack of inflammatory processes, and suitable response to ovary steroidal hormones [3–5].

Several factors can influence reproductive performance in mares. For instance, animals that require uterine treatments with lavages, antibiotic infusions, or oxytocin application after parturition, before or after mounting, and mares aging more than16 yr

Conflict of Interest: With the submission of this manuscript we would like to undertake that there is no conflict of interest.

Ethical statement: I am enclosing herewith a manuscript entitled "Uterine involution of mares supplemented with dietary algae-derived omega-3 fatty acids during the peripartum period" for publication in Journal of Equine Veterinary Science for possible evaluation. The corresponding author of this manuscript is Julia Rizzo de Medeiros Ferreira and contribution of the authors as mentioned below with their responsibility in the research. Julia Rizzo de Medeiros Ferreira, Saulo Baracat Villela, Camila Bianconi, Murillo Ormieres, Gabriela Dalmaso de Melo, Guilherme Pugliesi, Alexandre Augusto de Oliveira Gobesso.

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generally present poor reproductive performance [6]. Even with an appropriate body condition score (BCS), nutrient supply and metabolic status will impact fertility. Lactating mares exhibit lower conception rates compared to single mares, despite having a similar BCS, demonstrating the impact of appropriate nutrient supply in the post-partum period [7].

Supplementing mares with a diversity of nutrients can reduce the time for placental release, increase blood flow in uterus and ovaries, hasten the involution of uterine horns, and improve conception rates [8–11]. Among the nutrients capable of influencing reproduction, polyunsaturated fatty acids of omega-3 family or their derivatives have positively affected the reproductive efficiency when fed to beef cattle, dairy cattle, and sheep [12–20]. In ruminants, the dietary supplementation with relatively high levels of omega-3 fatty acids have shown anti-inflammatory effects through the modulation of eicosanoids synthesis, have increased the size of the ovulatory follicle and corpus luteum, and improved oocyte maturation and embryo survival during the first stages of gestation [21]. Recently, authors fed mares during the peri-conception period with algae rich in omega-3 fatty acids, especially docosahexaenoic acid (DHA), and reported a modulation for improved uterine environment and gene expression of embryo [22].

The available literature lacks studies evaluating dietary components, mainly derivatives from omega-3 fatty acids, on reproductive traits of mares after parturition. Thus, the objective of this study was to evaluate the effects of feeding a microalgae rich in DHA during the peripartum period on follicular dynamics and uterine involution of mares. The primary hypothesis of this study was that DHA supplementation during the peripartum period would benefit uterine involution, reducing the presence of uterine fluid, uterine size and the blood perfusion in uterine compartments. Secondly, we expected that DHA would modulate genes related with inflammatory processes, improving the uterine environment for embryo development.

2. Material and Methods

2.1. Local, Animals, And Management

This experiment was carried out on a stud farm (Vila Colonial Farm, Analândia, Brazil; 22° 07′ 35″ S latitude and 47° 39′ 47″ W longitude). Eighteen crossbreed recipient mares [410 ± 39.5 kg body weight (BW) and 7.8 ± 2.01 yr old] without known breed at the third gestation stage were used in this experiment. All mares were expecting Paint Horse foals with a similar proportion of fillies and colts. Mares were maintained on a pasture and supplemented with ration in a unit service with individual pens (90 cm width and 220 length). Animals were properly vaccinated, dewormed and sprayed against ectoparasites. Animals were handled in accordance with the Institutional committee for Ethics in research of the University of São Paulo (CEUA- FMVZ number: 7278221117).

2.2. Experimental Design and Diets

The experiment structure was a completely randomized design with repeated measures (Fig. 1). At the beginning of experiment, body condition score was evaluated using the 1-9 scale proposed by Henneke *et al.* [23]. Mares with 5.4 ± 0.61 BCS were randomly assigned to 2 experimental groups: 1) CONT: control, no microalgae supply; and 2) ALG: microalgae rich in DHA (All-G Rich, Alltech, Nicholasville, KY) supplementation at 0.06 g/kg BW. The microalgae product was fed only in the morning along with concentrate. According to Moran *et al.* [24], every 100 g of the microalgae product contains 66.9 g ether extract (EE), 12 g crude protein (CP), 3.66 g palmitic acid, 16.12 g DHA, 3.2 g ash, and 2.2 g moisture.

Diets were formulated according to NRC [25] to meet nutrient requirements of pregnant and lactating mares. The basal diet consisted of an ad libitum access to a pasture of Cynodon spp. cv. Tifton 85 (35.1% DM, 73.1% NDF, 38.4% ADF, 9.77% CP, 1.62% EE, 5.91 ash, 0.43% Ca, and 0.8% P) and the amounts of concentrate offered were based on the average body weight of animals. The commercial concentrate fed to mares (B150, Royal Horse, Campinas Brazil) contained 88.7% DM, 29.6% NDF, 16.5% ADF, 19.4% CP, 4.88% EE, 11.6% ash, 2.05% Ca, and 1.05% P. During the last third of gestation, 1 kg/d (as-fed) of the commercial concentrate was offered to animals twice daily in equal amounts at 8:00 and 15:00 h. After parturition, the amount of concentrate offered was increased to 2 kg/d/animal. Mares were fed experimental diets from 90 d prior to the expected foaling date until 7 days after the first ovulation [22]. At the end of supplementation, the body condition score was evaluated as described earlier.

2.3. Transrectal Palpation and Ultrasonography

Uterus and ovary transrectal palpation and ultrasonography evaluations were performed by a technician as described by Lemes *et al.* [26]. An ultrasound equipped with a multifrequency linear transducer (3.5-10 MHz; MyLabDelta, Esaote, Italy) in B mode and Doppler was used during the experiment. Uterine and ovarian evaluations were performed on days 3, 7, 11, and 15 post-partum and on d 4 and 7 after ovulation. Follicular dynamics were evaluated daily after detecting a 35 mm-dominant follicle until ovulation (disappearance of dominant follicle). The size of pre-ovulatory follicle was considered as the diameter of follicle measured on the day before of ovulation.

For uterine evaluation, the following traits were considered in both uterine horns: uterine horn diameter (endometrium + mesometrium), endometrium diameter, the presence and amount of intrauterine fluid (IUF), uterine echogenicity, and uterine tone. Endometrium and mesometrium blood perfusion [scores 1–4,26] were assessed only in the pregnant horn using the Doppler mode of ultrasound (6.3 MHz frequency, 61% gain, 720 Hz PRF, 4 WF, M/2 PRC, and 3 PRS). The uterine horn and endometrium diameters (mm) in pregnant and non-pregnant horns were individually evaluated and calculated by the mean between height and width of the median portion of uterine horn observed in the B-mode ultrasonography exam. Measurement of IUF considered height and width of the site where the maximal fluid accumulation was detected in the uterine lumen. Uterine echogenicity was evaluated in a 1-3 scale whereas 1 is hypoechoic and 3 is hyperechoic. Uterine tone was evaluated by digital palpation and scored on a scale of 1 to 4 (maximal or turgid to minimal or flaccid, respectively; Lemes *et al.* [26]).

2.4. Endometrial Cells Sampling

Epithelial cells from the endometrium were collected using brushes for uterine cytology (cytobrush) on d 7 and d 11 post-partum, and on d 4 after ovulation as described by Cardoso *et al.* [27] and adapted for equine. Together with the epithelial cells, leukocytes can be also present in the uterine lumen due to post-partum inflammation process; however, cytobrush samples were proven to be reliable for endometrial gene expression determination [28] as the proportion of polymorphonuclear leukocytes represent 3.66% of the cells recovered at the first week post-partum [29]. A disposable plastic tool equipped with cytological brush (Provar, São Paulo, Brazil) was inserted in the uterus through the cervix and rotated on the uterine body wall to collect endometrial cells. After sampling, the cytological brush was placed into a cryotube containing 1 mL of TRIzol (Life Technologies, California, USA)

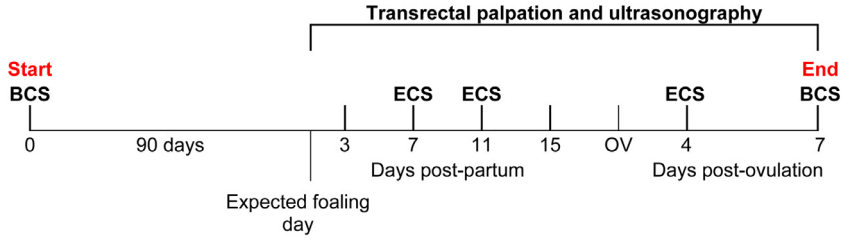

Fig. 1. Timeline of sampling collection. Start: first day of experimental diets. BCS: initial body condition score. ECS: endometrial cells sampling. OV: ovulation. End: last day of experimental diets.

Table 1
Forward (F) and reverse (R) primers sequences of target and reference genes analyzed using qPCR.

Gene	Forward primer	Reverse primer
CRP	GCAGCCGGTGCAAGATAGA	TTCCAAATCCCAGGCCATC
IL1β	TCCAGCCAACCTTCATTGC	ACAGCTCATTCTCGTCACTGTAGTAAG
AKR1C4	TCCTGTCCTGGGATTTGGAACCTT	ATCGGCAATCTTGCTTCGAATGGC
PPIA	GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT
18S	AACGACACTCTGGCATGCTAACTA	CGCCACTTGTCCCTCTAAGAA

and maintained on liquid nitrogen for transportation. Uterine samples were stored at -80°C until analysis.

2.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted from endometrial cells according to the manufacturer's recommendations. In brief, each sample containing 1 mL of TRIzol was homogenized on a vortex mixer for 5 min, the cytological brush was removed from the cryotube, and the cryotube was incubated for 5 min at room temperature before adding 266 mL of chloroform. Afterwards, samples were mixed on a vortex for 15 s, incubated for 5 min at room temperature, centrifuged at 12,000 x g for 15 min at 4 °C, and the supernatant transferred to another 2 mL polystyrene PCR tube. PCR tubes rested for 10 min at room temperature and frozen overnight at -80 °C. Isopropanol alcohol (333 mL) was added to PCR tubes and centrifuged (15,000 x g for 8 min at 4 °C). The supernatant was carefully removed, and samples were washed twice with 600 mL of 75% ethanol and centrifuged (15,000 x g for 5 min at 4 °C). The cell pellet rested at room temperature for 5 min, then 12 mL of nuclease-free water was added at 55°C (samples were homogenized 2 to 3 times during this step) for 15 min. The concentration and purity (A260/A280) of total RNA extracts were measured using a spectrophotometer (NanoVue, GE Healthcare, Chicago, USA). Therefore, the isolated RNA (1µg) from all mares was subjected to reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. The cDNA of each sample was stored at -20 °C until qPCR analysis.

2.6. Quantitative Polymerase Chain Reaction

The real-time qPCR analyses were performed in triplicate, using the same qPCR settings as described previously [30], and were carried out using a Step One Plus apparatus (Life Technologies). Several genes related to inflammation were selected (CRP, IL1β, AKR1C4, PTGES, PTGS2, TNFα, IL4, IFNΥ, IL22, and TLR4). The primers were selected and tested as previously described [31–35], but only CRP, IL1β, and AKR1C4 had their primers amplified and validated for equine cytobrush samples and used in the present study (Table 1). The genes GAPDH, PPIA, and 18S were tested as reference genes, and those with less variation, selected by the NormFinder software, were used (PPIA and 18S). The reactions were performed in triplicate on a 96-well plate. The gross fluorescence data were extracted from the Step One Plus apparatus and analyzed using the LinReg PCR software for baseline correction, determination of qPCR efficiency, and cycle quantification values per sample, as described by Ruijter *et al.* [36]. The log-linear portion of the amplification curve used for the analysis performed with the LinReg PCR software contained four points with the highest R^2 value. The expression of the target genes evaluated by qPCR was normalized with the two reference genes by the comparative Ct method [37]. The results were expressed as relative arbitrary units.

2.7. Statistical Analyses

Data were analyzed using the Mixed procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) according to the following model: $Y_{ijk} = \mu + T_i + \omega_{i:j} + D_k + T \times D_{ik} + e_{ijk}$, with $\omega_{i:j}$ » N (0, s^2_ω) and e_{ijk} » MVN (0, s^2_e); where Y_{ijk} is the observed value of the dependent variable, µ is the overall mean, T_i is the fixed effect of treatment (i = 1 and 2), $\omega_{i:j}$ is the error associated with treatment (random effect of animal within treatment; j = 1 to 18), D_k is the fixed effect of day (k = 1 to 4 for evaluations performed during the post-partum period and k = 1 and 2 for evaluations performed after ovulation), $T \times D_{ik}$ is the fixed interaction effect between treatment and sampling date, e_{ijk} is the experimental error, N stands for normal distribution, s^2_ω is the variance associated to the effect of animal, MVN indicates a multivariate analysis with normal distribution, and s^2_e is the residual variance. The autoregressive covariate matrix (AR1) was used for all variables assessed in this study. Interactions between treatment and sampling day were submitted to SLICE option when significant and treatment differences were evaluated through Fisher's test (LSD).

For evaluations of body condition score and interval from foaling to first ovulation, the following model was used: $Y_{ij} = \mu + T_i + e_{ij}$, with $e_{i:j} \approx N(0, s^2_e)$; where Y_{ij} is the observed value of the dependent variable, µ is the overall mean, T_i is the fixed effect of treatment (i = 1 and 2), e_{ij} is the experimental error, N stands for normal distribution, MVN indicates a multivariate analysis with normal distribution, and s^2_e is the residual variance. Significance level was set at $P < 0.05$ and tendencies were considered when $0.05 < P \leq 0.10$.

Table 2
Endometrium diameter, uterine diameter (endometrium + mesometrium), blood perfusion, presence of fluid, uterine echogenicity, and uterine tone of mares fed microalgae rich in docosahexaenoic acid during the peripartum period.

Item	Treatment[a]		SEM	P-value[b]		
	CONT	ALG		Treat	Day	Treat × Day
Endometrial diameter (mm)						
Pregnant horn						
Post-partum	31.1	30.2	0.72	0.550	<0.001	0.788
Post-ovulation	25.5	26.4	0.69	0.538	0.039	0.718
Non-pregnant horn						
Post-partum	28.1	27.9	0.64	0.850	<0.001	0.905
Post-ovulation	23.2	24.5	0.56	0.269	0.179	0.496
Uterine horn diameter (endometrium + mesometrium) (mm)						
Pregnant horn						
Post-partum	34.6	31.4	0.74	0.047	<0.001	0.220
Post-ovulation	27.6	28.6	0.67	0.463	0.016	0.028
Non-pregnant horn						
Post-partum	32.3	29.8	0.71	0.099	<0.001	0.536
Post-ovulation	27.8	26.7	0.58	0.349	0.037	0.964
Endometrium perfusion (1-4)						
Post-partum	2.19	2.33	0.133	0.935	0.510	0.757
Post-ovulation	2.17	2.22	0.132	0.713	0.930	0.141
Mesometrium perfusion (1-4)						
Post-partum	2.17	2.63	0.17	0.374	0.216	0.909
Post-ovulation	2.28	2.33	0.161	0.820	0.400	0.763
Intra-uterine fluid (mm)	2.25	1.33	0.523	0.542	0.010	0.891
Echogenicity (1-3)						
Post-partum	2.25	2.69	0.069	0.009	0.047	0.293
Post-ovulation	2.67	2.78	0.074	0.461	0.502	0.502
Uterine tone (1-4)						
Post-partum	2.11	2.31	0.157	0.344	0.174	0.174
Post-ovulation	2.56	2.56	0.167	1.00	0.115	1.00

[a] Treatments: Control (CONT) or dietary supplementation with algae-derived omega-3 fatty acids (ALG; All-G Rich, Alltech, Nicholasville, KY) at 0.06 g/kg BW per day.
[b] *P*-values for treatment effect (CONT vs ALG), day effect (evaluations on days 3, 7, 11, 15 post-partum and days 4 and 7 after the first ovulation), and interaction effect between treatment and day.

3. Results

3.1. Body Condition Score

Feeding ALG had no effect ($P > 0.10$) on body condition score at the end of supplemental period (5.78 ± 0.15 and 5.89 ± 0.15 for CONT and ALG, respectively).

3.2. Size of Uterine Horns

Treatments had no effect ($P > 0.10$) on diameter of endometrium, pregnant horn, and non-pregnant horn, regardless of the evaluation day (Table 2). A day effect ($P < 0.05$) was detected for endometrial diameter in both uterine horns during the post-partum and post-ovulation periods, as indicated by the progressive reduction in diameter until 15 days post-partum and between days 4 and 7 post-ovulation (Fig. 2A and 2B). Regarding the uterine diameter (endometrium + mesometrium), treatment and day effects were observed post-partum and day and treatment by day interaction effects after the first ovulation in the pregnant horn (Fig. 2C). The day effect revealed a progressive reduction from day 3 until day 15 post-partum and between days 4 and 7 post-ovulation. During the first 15 days post-partum, ALG group exhibited a smaller uterine diameter ($P < 0.05$) relative to CONT group, however, the uterine diameter in ALG group was higher on day 7 post-ovulation. The diameter of non-pregnant horn of mares fed ALG tended to be smaller ($P = 0.10$) in the postpartum period. A progressive reduction in the non-pregnant horn diameter was observed throughout the days after foaling and ovulation (Fig. 2D).

3.3. Uterine Blood Perfusion Score

Blood perfusion of the endometrium and mesometrium in the pregnant horn were not influenced by treatment nor by the period of evaluation (post-partum or post-ovulation; Table 2).

3.4. Presence of Intra-Uterine Fluid

The supplementation with ALG did not influence ($P > 0.10$) the presence of IUF (Fig. 3A), but the accumulation of IUF started to decrease after 7 days post-partum and no IUF was detected on day 15.

3.5. Follicle Dynamics

The foaling to the first ovulation interval was similar ($P > 0.10$) between treatments (13 ± 0.87 days and 12 ± 0.87 days for CONT and ALG, respectively). No treatment effect nor interaction effect between treatment and day were observed ($P > 0.10$) on the diameter of dominant follicle across treatments (35.5 ± 1.67 and 37.0 ± 1.67 mm for CONT and ALG, respectively). As expected, the diameter of dominant follicle progressively increased between days 3 and 11 after foaling (Fig. 3B).

3.6. Uterine Echogenicity and Tone

The uterine echogenicity was greater ($P < 0.05$) in mares fed ALG compared to those fed CONT during the first 15 days post-partum (Fig. 4A). In addition, a day effect indicated an increase in uterine echogenicity between days 3 and 7 post-partum. After ovulation, effects of treatment, day nor interaction between treatment and day ($P > 0.10$) were observed on the uterine echogenic-

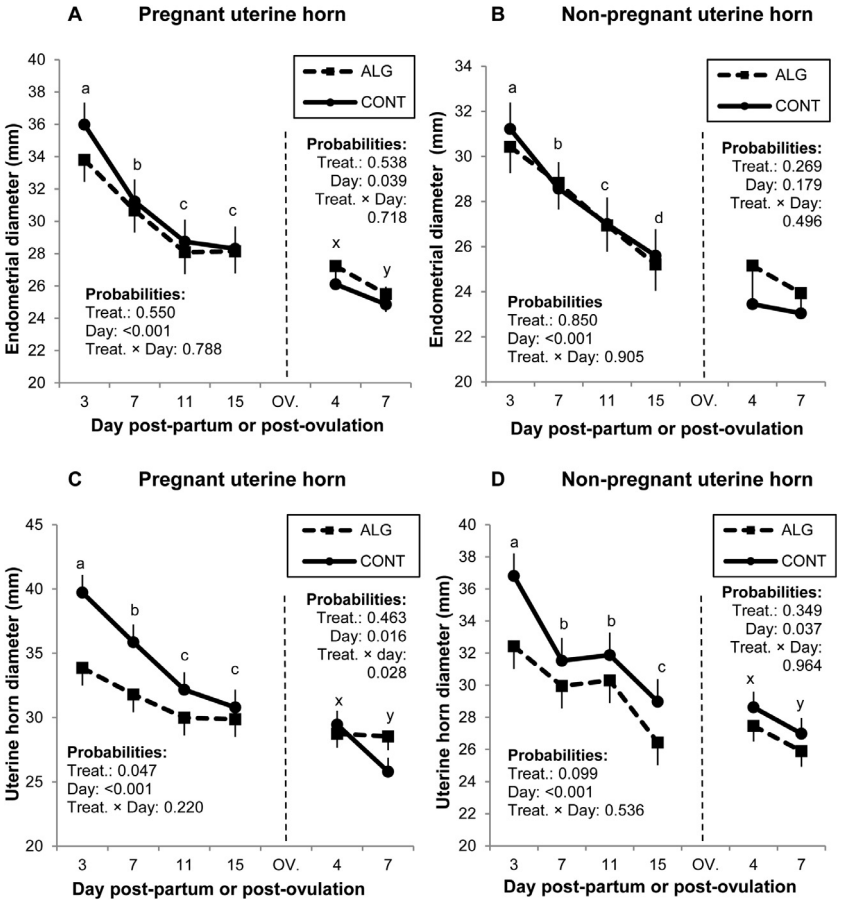

Fig. 2. Diameter of endometrium and uterine horns of post-partum mares supplemented (ALG) or not (CONT) with algae-derived omega-3 fatty acids (0.06 g/kg BW per day; All-G Rich, Alltech, Nicholasville, KY) from 90 d of the expected foaling date until 7 days after the first ovulation. (A) Endometrium diameter of pregnant uterine horn. (B) Endometrium diameter of non-pregnant uterine horn. (C) Uterine horn diameter of pregnant uterine horn. (D) Uterine horn diameter of non-pregnant horn. First ovulation date (OV.). Different letters mean statistical differences according to the evaluation day using the Fisher's test (LSD; $P < 0.05$).

ity (Table 2). Similarly, the uterine tone was similar between treatment groups and across day points during both the post-partum and post-ovulation periods (Fig. 4B).

3.7. Endometrial Gene Expression

Transcript abundance of IL1β and CRP did not differ between CONT and ALG treatment and across day points ($P > 0.10$; Fig. 5A and 5B). Although no treatment or treatment by day interaction effects were observed, there was a decrease ($P < 0.05$) in transcript abundance of AKR1C4 between days 7 and 11 post-partum (Fig. 5C). No differences in transcript abundance for any of the genes measures were observed between treatment groups on day 4 after the first ovulation.

4. Discussion

The current study demonstrated that dietary supplementation with microalgae rich in DHA resulted in a lower horn diameter such that the involution process required less effort to return to normal size prior to cycling and ovulation. The uterine involution process begins with a drastic decrease in diameter of uterine horns during the first wk after parturition. Similar to results from other studies, the diameter of both uterine horns decreased throughout the first day's post-partum until d 15 [26,38]. In the current study, we also observed a decrease in IUF, whereas mares under both treatments did not exhibit accumulation of IUF at 15 d post-partum. Lemes *et al.* [26] also observed the presence of IUF in early post-partum mares, where the fluid accumulation started to decrease 4 d post-partum. Other authors have reported a total uterine clearance after 9 d of parturition [4,38].

Despite changes observed in size and presence of fluid in uterine horns, no day or treatment effects were detected for blood perfusion scores of the endometrium and mesometrium. Lemes *et al.* [26], however, observed a progressive increase of endometrium vascularization between days 1 to 4 post-partum and an increase in mesometrium vascularization on the first 2 days post-partum followed by a decrease in vascularization until day 10. Further, they reported an increase of blood perfusion in the endometrium during the first five days after the first ovulation and a decrease of blood perfusion in the mesometrium between days 4 and 10 after ovulation. The increase in uterine blood perfusion observed by Lemes *et al.* [26] might be due to an inflammatory response that generally occurs after mounting and is controlled until 96 h after insemination [39]. In addition, the mares used in the previous study had moderately fleshy to fat body condition scores (6 to 8 in an 1-9 scale) [26]. A more acute inflammatory response is observed in overweight [40] animals which might have affected the blood perfusion of mares in Lemes *et al.* [26]. The lack of changes in uterine blood perfusion during post-partum or after ovulation in the current study may be associated with the fact that mares were not inseminated, their body condition score was moderate, or due to hormonal oscillations during the peripartum period.

In the current study, we observed a tendency for increased uterine echogenicity in mares of ALG group, without impacting the uterine tonus. These results disagree with reported by Fleury *et*

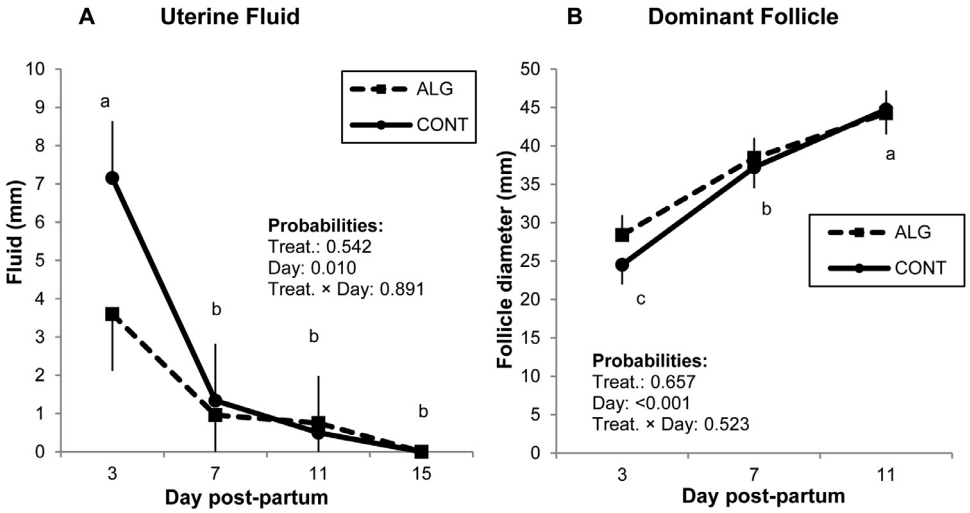

Fig. 3. Uterine fluid and dominant follicle diameter during early post-partum in mares supplemented (ALG) or not (CONT) with algae-derived omega-3 fatty acids (0.06 g/kg BW per day; All-G Rich, Alltech, Nicholasville, KY) from 90 d of the expected foaling date until 7 days after the first ovulation. Different letters mean statistical differences according to the evaluation day using the Fisher's test (LSD; $P < 0.05$).

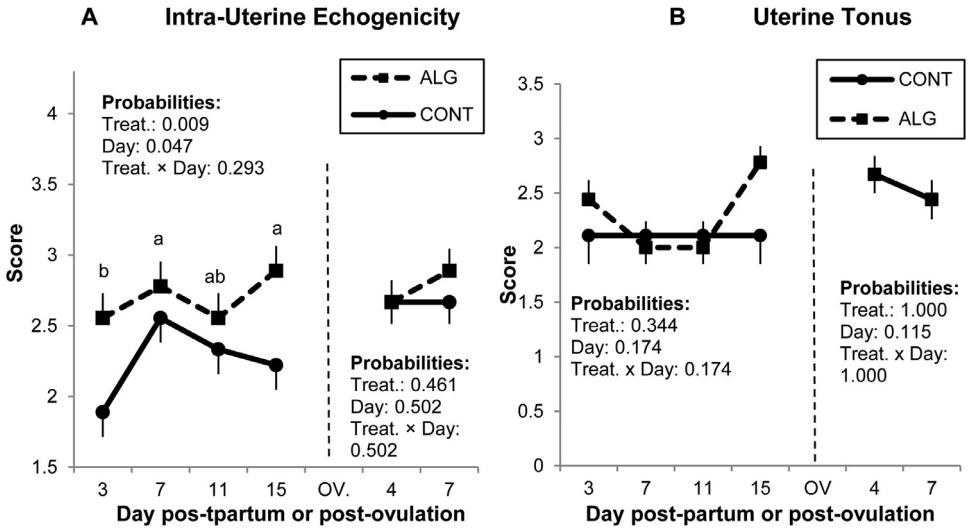

Fig. 4. Uterine echogenicity and tonus during early post-partum in mares supplemented (ALG) or not (CONT) with algae-derived omega-3 fatty acids (0.06 g/kg BW per day; All-G Rich, Alltech, Nicholasville, KY) from 90 d of the expected foaling date until 7 days after the first ovulation. First ovulation date (OV.). Different letters mean statistical differences according to the evaluation day using the Fisher's test (LSD; $P < 0.05$).

al. [41] and Lemes *et al.* [26] where there is an increase in echotexture, which is aligned with a decrease in echogenicity. Low echogenicity is related to high levels of circulating estradiol that causes the emergence of edema and heat behavior [42]. Thus, the greater average score of echogenicity during the post-partum period suggests that mares under ALG treatment might have presented lower circulating estradiol concentrations. Although circulating estradiol concentrations were not determined in the herein study, a reduction in estradiol concentration is consistent with that found in women supplemented with DHA during in vitro fertilization programs, who produce fewer follicles but produce embryos with better morphology [43]. In addition, beef heifers supplemented with omega-3 fatty acids had lower transcript abundance of estrogen receptor (ESR1) in the endometrium compared to counterparts [44], suggesting a lower influence of circulating estradiol on animals supplemented with omega-3 fatty acids. Alternatively, the observed changes on uterine echogenicity could be also associated to uterine inflammation causing edema and consequently reduced echogenicity. Therefore, mares from CONT group could have a more inflamed uterus post-partum leading to less echogenicity. In addition, the pregnant and non-pregnant uterine horns diameters in CONT group were larger than ALG group, despite of there were no problems during foaling or foal gender differences between groups. Greater pregnant uterine horn diameter is related to delayed uterine involution, as well as intra-uterine fluid accumulation and endometrial edema. All these responses are associated with mild degree of systemic inflammation combined to oxidative stress [45]. So, in this case, edema could be related with inflammatory response due to involution process rather than endocrinological effects, suggesting that supplementation with DHA could decrease post-partum endometrial inflammation.

After ovulation, the echogenicity score did not differ between mares receiving or not the ALG but a progesterone-induced increase in uterine echogenicity is expected in mares after ovulation [26] and that are prepared to receive embryos [41]. Yet, Lemes *et al.* [26] observed decreased uterine tonus between days 0 and 3 relative to ovulation. We did not observe treatment effects on development and diameter of dominant follicle, contrasting with re-

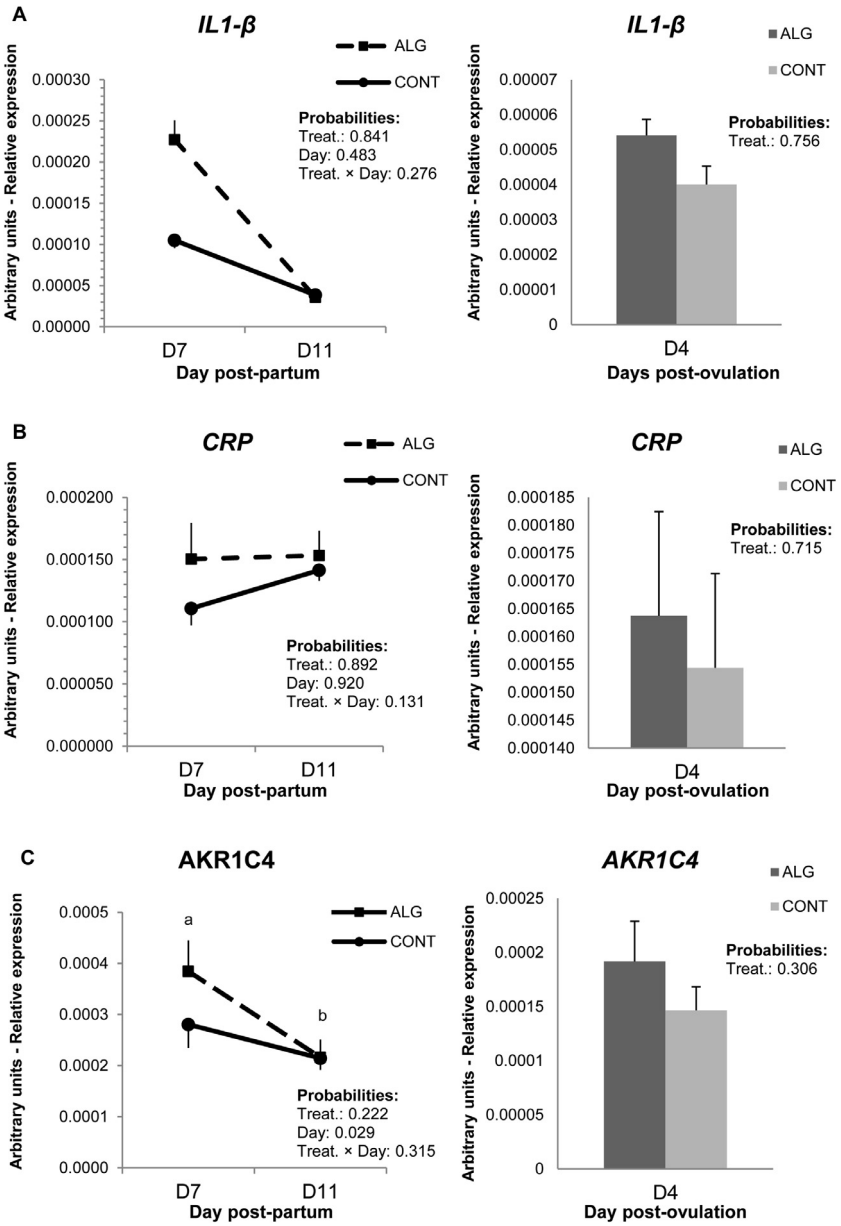

Fig. 5. Transcript abundance in endometrium of mares with (ALG) or without (CONT) dietary supplementation (ALG) of algae-derived omega-3 fatty acids (All-G Rich, Alltech, Nicholasville, KY) from 90 d relative to the expected foaling day until day 4 after the first ovulation. The following transcripts: IL1-β (A), CRP (B), and AKRC1C4 (C) were evaluated on days 7 and 11 post-partum and on d 4 after the first ovulation. Different letters mean statistical differences according to the evaluation day using the Fisher's test (LSD; $P < 0.05$).

ported by Dirandeh *et al.* [46] in dairy cattle. No treatment differences in the interval between parturition and first ovulation were detected in this study.

Dietary supplementation with polyunsaturated fatty acids from omega-3 family (PUFA-3) has been shown to alter gene expression in the endometrium impacting its function and immunity [22,44,47]. In mares, feeding PUFA-3 has increased the expression of genes associated with prostaglandin signaling (PGE2 and PGF2α), pro-inflammatory signaling (SSA), anti-inflammatory signaling (IL-10), and nutrient transport [22]. Regarding the effects of dietary supplementation with microalgae rich in DHA on inflammatory and immune responses in the uterus, this study assessed the endometrial abundance of transcripts associated with immune and inflammatory responses (IL-1β and CRP) and a transcript for an enzyme involved in eicosanoids synthesis (AKR1C4). Eicosanoids, including prostaglandins and leukotrienes, are synthesized from arachidonic acid and regulate uterine immunity with pro- or anti-inflammatory stimulus. The synthesis of prostaglandins is stimulated by the cytokine IL-1β [48]. The PGF2α and leukotrienes in endometrium activate neutrophils and increase chemotaxis, migration, phagocytosis, and cytotoxic capacity mediated by cells independent of antibodies. These molecules are critical during the post-partum period in order to prevent infections [49]. In dairy cows, there is a positive relationship between the presence of B4 leukotriene and uterine involution [50]. After ovulation and conception, however, the synthesis of leukotrienes and PGF2α needs to be suppressed in endometrium to avoid luteolysis and allow embryo development [51].

In the current study, no differences in transcript abundance of IL-1β, CRP, and AKR1C4 across treatments were observed during the post-partum period. The IL-1β is a pro-inflammatory cytokine which usually increases when there is a disturbance in uterine en-

vironment such as the presence of pathogenic bacteria [52]. The uterus contamination during parturition is frequent and bacteria can be detected in the uterus of most mares at the foal heat [3]. Therefore, an increased inflammatory response is expected during first day's post-partum, and this response may occur during foal heat. However, in the present study, IL-1β abundance did not change between days 7 and 11 post-partum. The C reactive protein (CRP) is an acute phase protein which gradually increases in pregnant mares during the last 2 mo of gestation and slightly decreases after 7 d postpartum [53]. Therefore, the greater gene expression of CRP in the endometrium of mares during the post-partum period is physiological, likewise as the increase in cytokines, acute phase proteins when the uterine environment is contaminated [54].

The AKR1C4 gene encodes one of the major enzymes involved on PGF2α synthesis [55] besides having a critical role on progesterone metabolism in bovine [56] and androgen synthesis in the human fetus [57,58]. The expression of AKR1C4 in the uterus of mares is likely related with the uterine contractility required for a suitable uterine involution in an endocrine environment of low progesterone [48]. The decline in transcript abundance of AKR1C4 between days 7 and 11 observed in this study is consistent with a greater PGF2α requirement during the first days after parturition when compared to days around the first ovulation [48]. Thus, the decrease of pathogens in uterine environment and approach of ovulation, which occurred between days 12 and 13 post-partum, might have influenced the abundance of AKR1C4 on day 11. The decrease in AKR1C4 might also be associated to the beginning of foal heat, since a lower synthesis of prostaglandins was observed during heat in comparison with the early post-partum in dairy cows [49].

5. Conclusions

Dietary supplementation with microalgae rich in DHA during the peripartum period may benefit uterine involution as supplemented mares had lower diameter of the pregnant uterine horn and greater echogenicity of uterus, without differences in body condition score or growth of the dominant follicle during the postpartum period. On the other hand, other uterine characteristics such as blood perfusion, IUF absorption, uterine tone, and the endometrial transcript abundance of genes involved in the inflammatory response were not affected by dietary supplementation with microalgae rich in DHA. Finally, the use of polyunsaturated fatty acids from omega-3 family in diets of mares during the peripartum period may benefit uterine involution and early conception of mares; however, more studies evaluating pregnancy outcomes should be performed.

Author Contribution

Julia R. M. Ferreira: Data curation; Writing, review & editing;

Saulo Baracat Villela: Conceptualization; Project administration; Investigation;

Camila Bianconi: Investigation;

Murillo Ormieres: Investigation;

Gabriela Dalmaso de Melo: Investigation; Data curation;

Guilherme Pugliesi: Conceptualization; Formal analysis; Review & editing;

Alexandre A. O. Gobesso: Conceptualization; Funding acquisition; Review & editing.

With the submission of this manuscript I would like to undertake that:

1 All authors of this research paper have directly participated in the planning, execution, or analysis of this study;
2 All authors of this paper have read and approved the final version submitted;
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6 There are no directly related manuscripts, published or unpublished, by any authors of this paper;
7 My Institute's University of São Paulo representative is fully aware of this submission.

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