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Oral L-arginine supplementation impacts several reproductive parameters during the postpartum period in mares

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ABSTRACT

L-Arginine is an amino acid which can alter pituitary function and increase blood flow to the reproductive tract. The objective was to determine the effect of supplementing 100 g of L-arginine on plasma arginine concentrations, follicular dynamics and ovarian and uterine artery blood flow during the estrus that occurs subsequent to foaling. In Experiment 1, mares were fed 100 g L-arginine for 1 day during the last 3 weeks of pregnancy and plasma samples taken for every hour for the first 4 h and every other hour until 12 h. L-Arginine supplementation elevated plasma arginine concentrations from 1 to 8 h post feeding; arginine peaked at 6 h (arginine: 515 ± 33 μmol/L; control: 80 ± 33 μmol/L). In Experiment 2, mares received either 100 g L-arginine or control diets beginning 21 d before the expected foaling date and continued for 30 d postpartum. The reproductive tract was evaluated by transrectal Doppler ultrasonography from Day 1 postpartum through Day 30. There were no differences in ovarian follicular dynamics, ovarian or uterine resistance indices between groups. Vascular perfusion of the F1 follicular wall was greater in L-arginine supplemented mares (37.3 ± 2.6%) than controls (25.4 ± 2.7%; $P < 0.05$). L-Arginine supplemented mares had a smaller uterine body and horns and accumulated less uterine fluid than controls ($P < 0.05$). The combination of reducing uterine fluid accumulation, while not altering follicular development, raises the possible use of L-arginine supplementation as a breeding management tool during the postpartum period to increase reproductive success.

1. Introduction

L-Arginine is one of ten essential amino acids in horses (National Research Council, 2007) and a biologically-active regulator of several physiological systems including the reproductive, cardiovascular, pulmonary, renal, and immune systems (Wu et al., 2009). L-Arginine can behave both as a receptor ligand (Joshi et al., 2007) and as a substrate for biosynthesis of nitric oxide (NO), polyamines, proline, glutamate, creatine, and agmatine (Wu and Morris, 1998). L-Arginine activates nitric oxide synthase (Joshi et al., 2007; Morrissey and Klahr, 1997) which catalyzes the conversion of L-arginine to NO and L-citrulline. Nitric oxide is a vasodilator that inhibits vasoconstrictor signals (Thiriet, 2008) and acts downstream of VEGF signaling to promote angiogenesis (Murohara et al., 1998). In gilts, oral supplementation of L-arginine starting on Day 30 of gestation increases litter size and birth weights (Mateo et al., 2007). Supplementing L-arginine (1% of diet) in pre- and postpartum mares increased uterine blood flow (Mortensen et al., 2011). Given that blood flow surrounding the dominant follicle is associated with increased pregnancy rates in mares (Silva et al., 2006); fertility may be improved in mares by feeding L-arginine.

In the present study, the impact of L-arginine supplementation on the reproductive characteristics of the

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postpartum mare are further examined. Mares are distinctive among domestic livestock in the ability to return to estrus and potentially conceive shortly after giving birth. This first postpartum estrous period has been coined 'foal heat', with mares having ovulations from 8 to 13 d after giving birth parturition (McCue and Hughes, 1990). Scant literature exists on ovarian follicular development and follicle selection during the first postpartum estrous cycle in mares. Shortly after parturition, the diameter of largest follicle ranges from 13 to 16 mm (Ginther et al., 1994) and reaches a diameter of 36.4 mm by Day 8 (Gündüz et al., 2008). The objectives of this study were to observe baseline follicular dynamics and ovarian blood flow during the first postpartum estrous cycle in mares and evaluate the influence of supplemental L-arginine on ovarian follicular dynamics, ovarian and uterine blood flow and uterine involution.

2. Materials and methods

2.1. Animals and experimental design

Studies were approved by the Institute of Food and Agricultural Sciences Animal Care (IFAS) and Use Committee at the University of Florida and were conducted at the IFAS Equine Science Center (Latitude 29°18′12″N; longitude 82°10′3″W). In Experiment 1, six multiparous Quarter horse mares were randomly assign to either a control or L-arginine group ($n = 3$) during the last 3 weeks of pregnancy to evaluate plasma availability of arginine in response to a meal supplemented with L-arginine. Both groups were fed 2.4 kg of a commercial mixed concentrate ration formulated for gestating and lactating mares (minimum guarantees: 16% crude protein, 3.5% crude fat, 0.9% Ca, 0.55% P; Ocala Breeder's Feed and Supply, Ocala, FL,USA). Mares were fed individually in stalls. L-Arginine supplemented mares received 100 g of L-arginine (Ajinomoto AminoScience LLC, Raleigh, NC, USA) that was mixed into the grain ration. Mares in both groups were fed Coastal Bermuda grass (3.5 kg) hay 4 and 8 h after receiving grain. Control mares were not fed an isonitrogenous (diets equal in nitrogen) vehicle to ensure that the comparison would be between L-arginine and a standard pregnant mare diet. Blood samples were obtained via jugular catheters into heparinized tubes, prior to feeding (0 h) and at 1, 2, 3, 4, 6, 8, 10 and 12 h post feeding. Samples were immediately centrifuged at 11,000 *g* for 15 min and plasma was stored at -80 C until analysis.

In Experiment 2, 16 mares were blocked by age (range 5–19 yr), breed [Thoroughbred ($n = 8$) and Quarter Horse ($n = 8$)], and expected foaling date and assigned randomly to receive L-arginine or no supplementation ($n = 8$/group). The mean (±SEM) age of mares was 11.5 ± 1.7 yr for L-arginine and 11.4 ± 1.3 yr for controls. The basal diet consisted of ad libitum Coastal Bermuda grass hay and 3.8 ± 0.3 kg of a commercial mixed concentrate ration formulated for gestating and lactating mares (minimum guarantees: 16% CP, 3.5% crude fat, 0.9% Ca, 0.55% P; Ocala Breeder's Feed and Supply, Ocala, FL, USA). Mares were fed individually in stalls at 0700 and 1500 h. L-Arginine-supplemented mares received 100 g of L-arginine (Ajinomoto AminoScience LLC, Raleigh, NC, USA) that was mixed into the grain ration immediately before each morning feeding. Treatments began 21 d before expected foaling dates and continued for 30 d postpartum.

2.2. Amino Acid Analysis

Plasma samples from Experiment 1 were deproteinized using 35% (w/v) sulfosalicylic acid. The acid soluble fraction was separated by centrifugation (4 °C, 11,000 *g* for 20 min). The supernatant was filtered (0.2 μm, Fisher Scientific, Pittsburgh, PA, USA) and then mixed 1:1 with 0.02 N HCl (Boucher et al., 1997). Plasma samples were then analyzed for amino acid composition using an amino acid analyzer (L-8900, Hitachi-High Technologies, Pleasanton, CA) as previously described by Ma et al. (2010).

2.3. Ultrasonongraphy

In Experiment 2, ovaries and ovarian arteries were examined via trans-rectal ultrasonography daily from the day after foaling until the first postpartum ovulation. The uterus and uterine arteries were examined daily from foaling until 30 d post-foaling. Ultrasonographic exams (900–1100 h) were performed using a Micromaxx Sonosite digital color Dopplar ultrasonic equipment with a 5–10 MHz broadband 52 mm linear array (Bothell, WA, USA). Exams were recorded (Sony DVDIRECT®, San Diego, CA, USA) and subsequent videos were reviewed for analysis. The length of the first estrus subsequent to foaling was defined as the number of days from foaling to the first post partum ovulation.

Follicles were grouped by diameter (6 to 10, 11 to 15, 16 to 20 and >20 mm) each day without regard to day-to-day identity as previously described by Kelley et al. (2011). Data were normalized to the 10 d preceding ovulation with the day of ovulation considered Day 0. Following ovulation, retrospective analysis determined the largest (ovulatory) follicle (F1), largest subordinate follicle (F2) and the second largest subordinate follicle (F3). The diameter of the F1 and F2 at the time of follicular deviation was ascertained as described elsewhere (Kelley et al., 2011). Deviation was defined as occurring on the day (exam) prior to the examination with the greatest change in differences in diameter between the two largest follicles.

For uterine measurements the screen depth was increased to visualize the entire uterine section and an outer cross sectional measurement for the uterine body was taken at the point of maximum height using electronic calipers. The diameter of each uterine horn was calculated by averaging the height and width measurements and was taken at approximately the midpoint of each horn. Maximum intrauterine fluid accumulation was measured within the uterine body via ultrasonography using electronic calipers.

Spectral Doppler measurements of both ovarian arteries and uterine arteries were evaluated as described by Ginther (2007) and calculated by an algorithm package in the Micromaxx® ultrasonography. Retrospective analysis identified the ovarian arteries as either ipsilateral or contralateral to the ovulatory follicle. The sample cursor

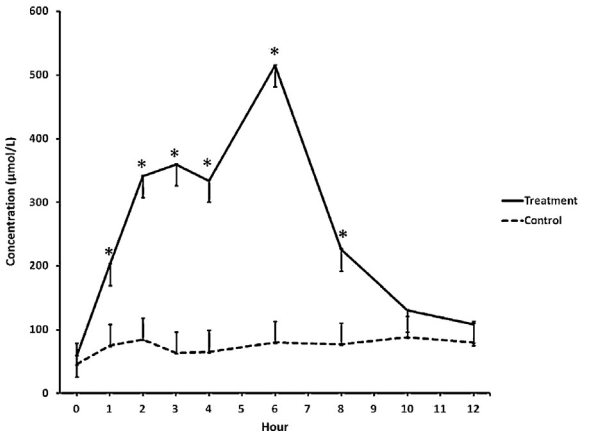

Fig. 1. Least-squares mean (±SEM) plasma arginine concentrations for 12 h following the consumption of 100 g L-arginine. An asterisk (*) denotes a difference ($P < 0.05$) between groups.

gate was set at 5 mm at a magnification depth of 7.7 cm. Resistance index (RI) [(peak systolic velocity (PSV) – end diastolic velocity (EDV))/PSV] was recorded without concern for the Doppler angle. The RI is a measure of spectral waveform variation and used to estimate downstream impedance of the vasculature. Generally, a decrease in RI indicates an increase in blood flow. The setting for the range of flow-velocity was adjusted to visualize the spectral graph, and Doppler spectrum with at least two uniform cardiac cycles generated, one of which was used for measurements. This was repeated with the mean of the two RI measurements taken were used for statistical analysis.

Follicular perfusion was defined as the blood-flow area surrounding the wall of the follicle using color Doppler ultrasonography (Ginther and Utt, 2004). Follicular perfusion was evaluated for the retrospectively identified dominant follicle (F1) using the color power Doppler (CPD) mode, which is more sensitive to low or weak blood flow than the color Doppler mode. Measurements for CPD perfusion were taken from DVD recordings using a radial grid for the 5 d preceding ovulation. Briefly, a radial grid was constructed by dividing a circle into 32 even slices, each slice covering 11.25° of the circle. The center of the radial grid was aligned to the center of the F1 and the slices containing perfusion, indicated by the color, were counted. The numbers of slices containing perfusion were then used to determine the percent [Percent perfusion = (slices with perfusion/total number of slices) × 100] of the F1 that contained perfusion.

2.4. Statistical analysis

Data were analyzed using the SAS MIXED procedure with a random statement to account for variability of mares within group and a repeated measures statement with the "cs" classification (SAS version 9.2: SAS Institute, Cary, NC, USA). Dietary treatment, day, and day x dietary treatment were included in the model as fixed effects. Data are represented as least squared means ± SEM. A probability

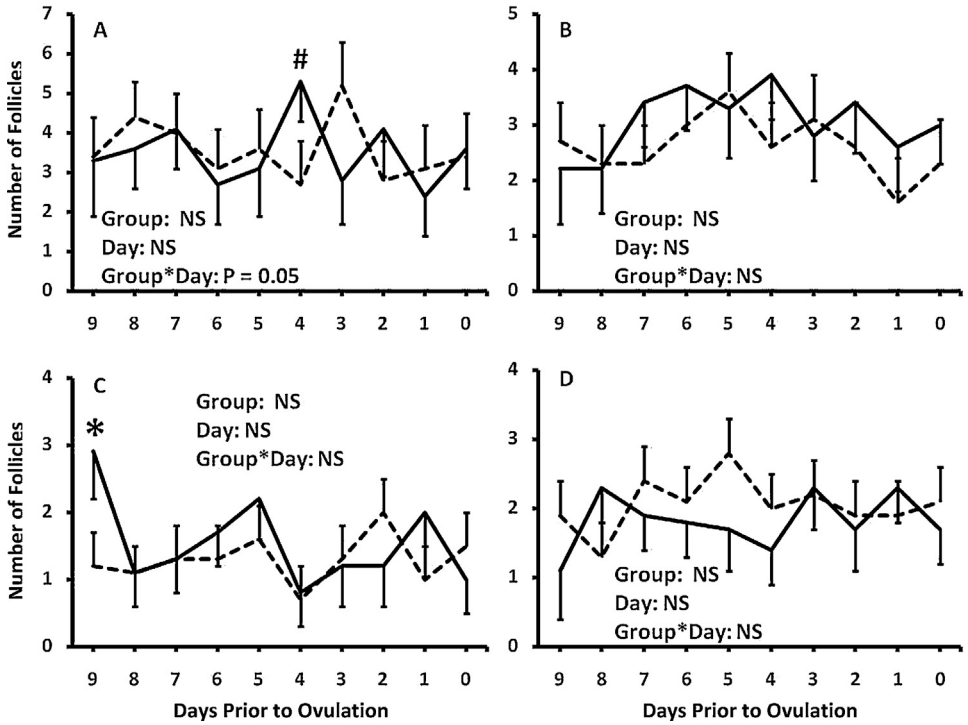

Fig. 2. Least-squares mean (±SEM) number of follicles for the 10 d preceding ovulation for L-arginine treated (solid line) and control (dotted line) mares. Panel A: diameter between 6 and 10 mm. Panel B: diameter between 11 and 15 mm. Panel C: diameter between 16 and 20 mm. Panel D: diameter greater than 20 mm. An asterisk (*) denotes a significant difference ($P < 0.05$) between groups, while a pound sign (#) indicates a trend toward significance ($P > 0.05$ and ≤ 0.10).

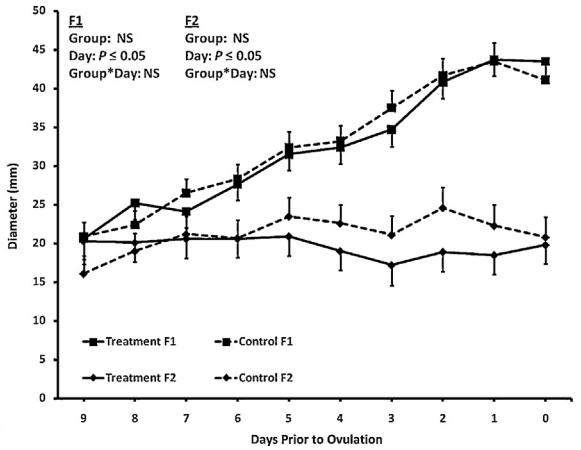

Fig. 3. Least-squares mean (±SEM) diameter of the dominant (F1) and largest subordinate (F2) follicles for L-arginine treated and control mares. There were no differences between treatments.

of $P < 0.05$ was considered significant and a probability between $P > 0.05$ and ≤ 0.10 indicated a trend toward significance.

3. Results

3.1. Plasma L-arginine concentrations

In Experiment 1, there were no significant differences in plasma arginine concentrations at 0 h between groups. Beginning 1 h post feeding and lasting until 8 h post feeding L-arginine supplemented mares had greater ($P < 0.05$) plasma arginine concentrations than control mares (Fig. 1). Plasma arginine concentrations were not statistically different between groups at 10 and 12 h post feeding.

3.2. Ovarian follicular dynamics, blood flow and follicular perfusion

All mares in Experiment 2 foaled without complications. Gestational length of L-arginine-supplemented mares was shorter than control mares and no differences were found between groups in placental weight. Mean length of the first estrous cycle after foaling for L-arginine supplemented and control mares was 12.6 ± 1.9 d and 16.5 ± 1.8 d ($P = 0.15$), respectively. All mares had an ovulation from a single follicle. No differences were observed between groups in the mean number of follicles within categories of 6–10, 11–15, 16–20, or > 20 mm (see Fig. 2A–D). Similarly, there was no difference in diameter of F1 or F2 (Fig. 3). The mean time from deviation to ovulation in L-arginine-supplemented and control mares was 8.0 ± 0.6 and 6.6 ± 0.6 d ($P = 0.10$), respectively. L-arginine-supplemented mares had a mean F1 and F2 diameter at deviation of 22.9 ± 1.2 mm and 20.5 ± 1.4 mm, respectively, which was not significantly different than controls (23.0 ± 1.2 mm and 20.9 ± 1.4 mm, respectively).

There was no effect of treatment effect on RI for either the ovarian artery ipsilateral or contralateral to ovulation (Fig. 4A and B). The mean percent perfusion

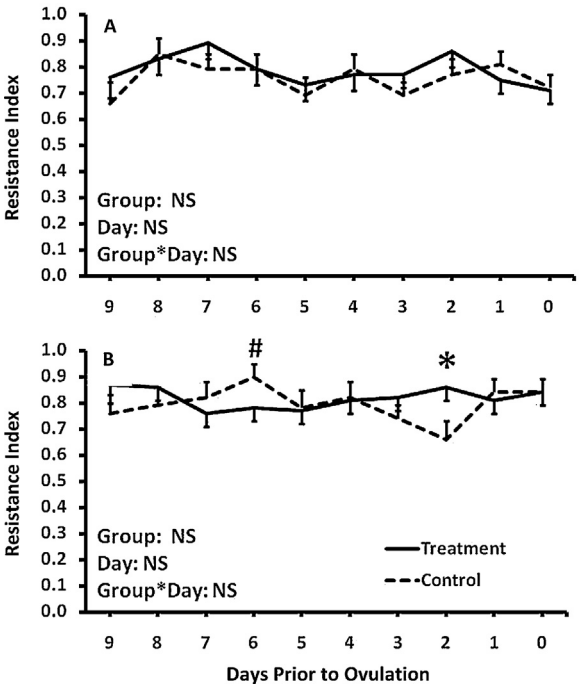

Fig. 4. Least-squares mean (±SEM) resistance index (RI) values in the ovarian arteries for the 10 d prior to ovulation. Panel A: RI of the ovarian artery ipsilateral to the ovary with the ovulatory follicle. Panel B: RI of the ovarian artery contralateral to the ovary with the ovualtory follicle. An asterisk (*) denotes a difference between groups ($P < 0.05$), while a pound sign (#) indicates a trend toward significance ($P > 0.05$ and ≤ 0.10).

for the F1 was greater in the L-arginine-supplemented group (37.3 ± 2.6%), than, control (25.4 ± 2.7%; $P < 0.05$). L-Arginine-supplemented mares had increased perfusion ($P < 0.05$) on Days -2 and -3 from ovulation and there was trend toward increased perfusion ($P = 0.07$) on the day of ovulation (Fig. 5).

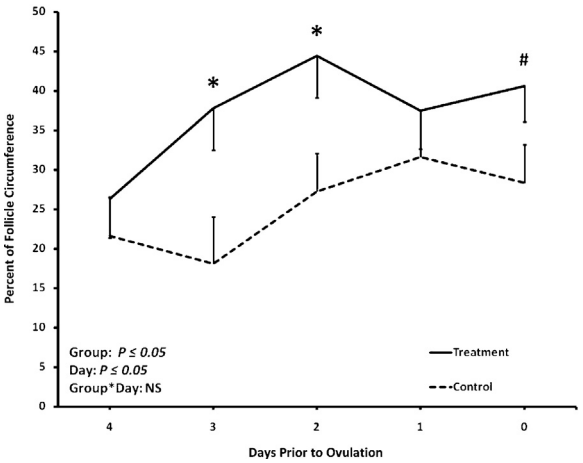

Fig. 5. Least-squares mean (±SEM) percent of vascular perfusion of the circumference of the ovulatory follicle as indicated by color power Doppler. An asterisk (*) denotes a difference between groups ($P < 0.05$), while a pound sign (#) indicates a trend toward significance ($P > 0.05$ and ≤ 0.10).

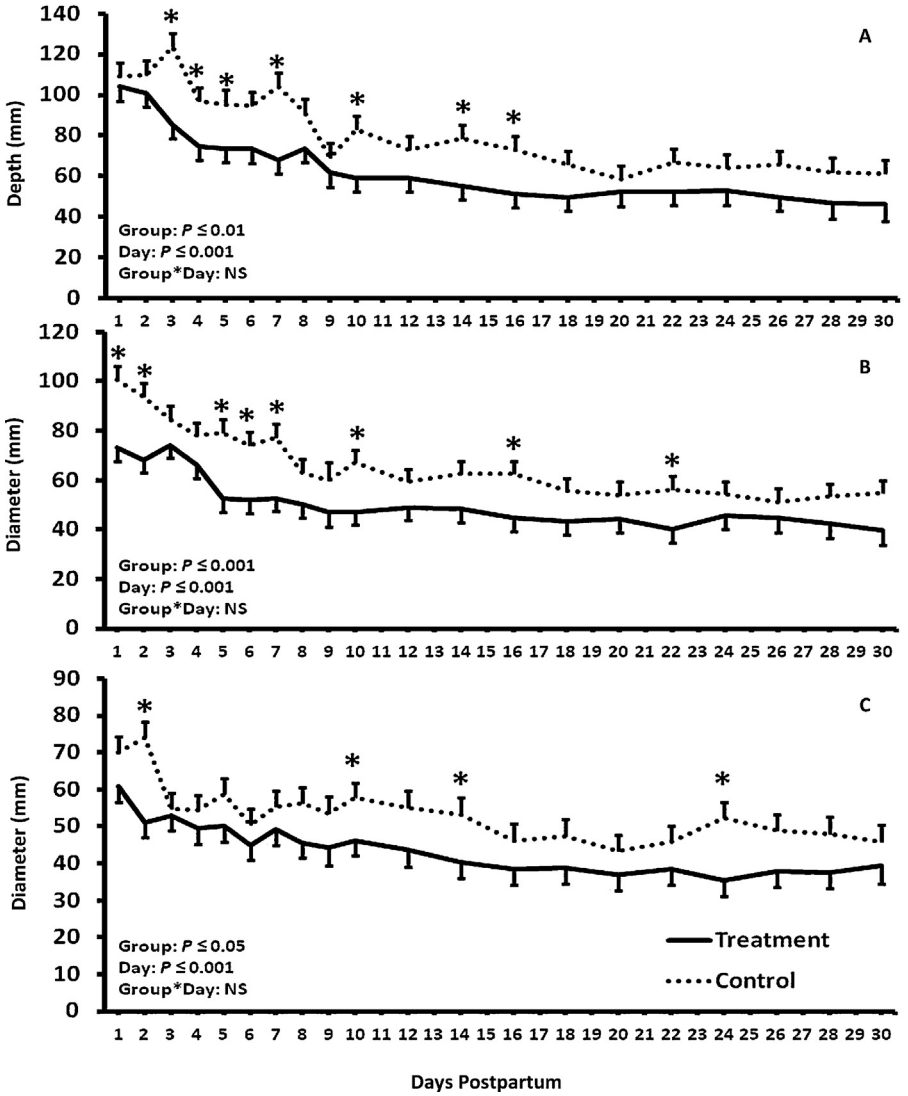

Fig. 6. Least-squares mean (±SEM) Panel A: depth of the uterine body, Panel B: diameter of the formerly gravid uterine horn and Panel C: diameter of the formerly non-gravid horn for 30 d postpartum. An asterisk (*) denotes a difference ($P < 0.05$) between groups.

3.3. Uterine involution and fluid clearance

L-Arginine-supplemented mares had a smaller mean diameter in the formerly gravid and non-gravid uterine horns as well as the uterine body ($P < 0.05$; Fig. 6). Diameter of the formerly gravid horn in L-arginine-supplemented mares was smaller on Days 1, 2, 5, 6, 7, 8, 10, 16 and 22 ($P < 0.05$) relative to controls. Diameter of the formerly non-gravid horn was smaller on Days 2, 10, 14, and 24 ($P < 0.05$) in L-arginine-supplemented mares. The depth of the uterine body was decreased in L-arginine-supplemented mares on Days 3, 4, 6, 7, 10, 14, and 16 ($P < 0.05$). Fluid accumulation was less than controls (Fig. 7), on Days 3 and 4 ($P < 0.05$). There was a trend ($P = 0.10$) toward less fluid on Day 4 postpartum. L-Arginine-supplemented mares had detectable fluid present for a mean of 3.4 ± 1.5 d after foaling compared to control mares, 7.1 ± 3.1d ($P < 0.05$).

3.4. Uterine blood flow

No difference within groups in RI was observed for the uterine artery of either the formerly gravid or formerly non-gravid side of the uterus ($P < 0.05$; Fig. 8A and B). RI increased in the uterine artery supplying the formerly gravid horn in L-arginine-supplemented mares from Day 1 to Days 12, 13, 15 and 29 and from Day 1 to Days 4, 8, 12, 15, 17, 18, 21 and 23 ($P < 0.05$) for uterine artery to the formerly non-gravid uterine horn,. In control mares RI increased from Day 1 to Day 3 through 30 ($P < 0.05$) in the formerly gravid horn and from Day 1 to Days 13, 15, 25 and 27 ($P < 0.05$) in the formerly non-gravid horn. There were no differences ($P > 0.05$) in the RI between the uterine artery on the formerly gravid side and the non-gravid side within groups or between groups for the 30 d following parturition.

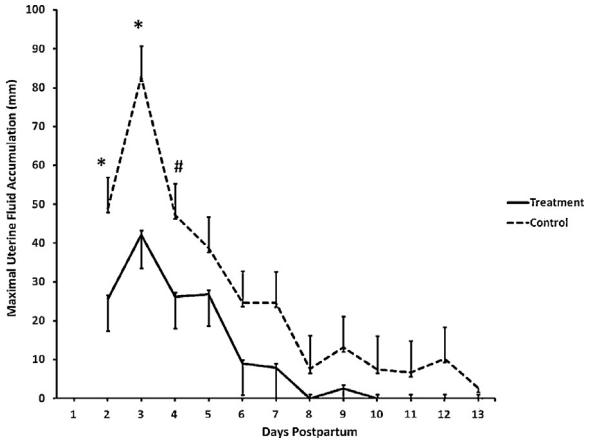

Fig. 7. Least-squares mean (± SEM) maximal fluid accumulation in the postpartum uterus. An asterisk denotes a difference ($P < 0.05$) while a pound (#) sign indicates a trend ($0.10 \geq P > 0.05$) between groups on a given day.

4. Discussion

Although the present study cannot separate the effect of additional nitrogen from L-arginine supplementation in the diet, it demonstrated that supplementation of mares with 100 g/d of L-arginine increased plasma arginine concentrations, increased perfusion of the ovulatory follicle, and reduced uterine diameter and uterine fluid accumulation postpartum. L-Arginine had no effect on the length of the first estrus after foaling, follicular development or RI for either the ovarian or uterine arteries. Results lead to the possibility that supplementation of mares that foal later in the breeding season with L-arginine may be beneficial to hastening uterine fluid clearance without altering follicular development or duration of the first estrus after foaling in an effort to retain an annual foaling interval.

Mares supplemented with L-arginine had a decrease in uterine size and fluid accumulation. L-Arginine can potentially affect many biological pathways that impact growth hormone, polyamine and nitric oxide synthesis. L-Arginine supplementation increases growth hormone production in humans (Merimee et al., 1969). In rats, growth hormone increased uterine proliferation and cell growth (Kennedy and Doktorcik, 1988; Gunin, 1997), while in sheep growth hormone increases the weight of the myometrium and endometrium (Jenkinson et al., 1999). L-Arginine is a precursor for both polyamines and nitric oxide (Wu and Morris, 1998). Polyamines can exert many actions but their role in reproduction remains unclear. Spermine, a polyamine, has been found to increase $PGF_{2\alpha}$ synthesis and inhibit PGE_2 production (Igarashi et al., 1981; Maruta et al., 1985). The role of other polyamines and their effect on prostaglandins is an area in need of further investigation. Nitric oxide is a smooth muscle relaxant (Thiriet, 2008) and has been found to be elevated in mares with a predisposition to breeding-induced endometritis (Alghamdi et al., 2005) although it was not established as to whether the increase in nitric oxide was a cause or result of breeding-induced endometritis. Mares susceptible to post-breeding endometritis have elevated concentrations of $PGF_2\alpha$ metabolite in peripheral

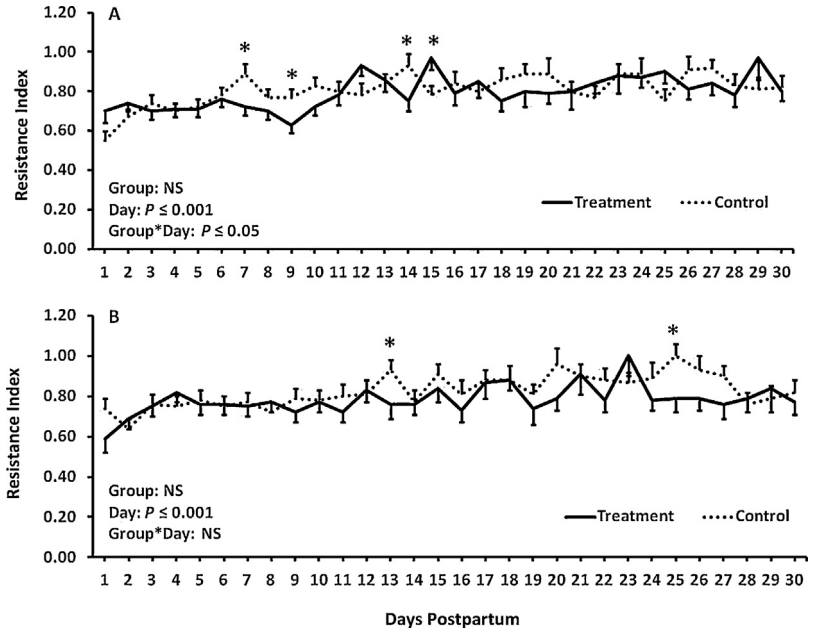

Fig. 8. Least squares mean (±SEM) resistance index of the uterine artery on the side of formerly gravid and non-gravid uterus. Panel A: Uterine artery on the side of the formerly gravid uterine horn. Treatment mares had a significant increase in RI between Day 1 and Days 12, 13, 15 and 29 in the uterine artery on the side of the formerly gravid horn while RI was increased in control mares between Day 1 and Day 3 through 30. Panel B: Uterine artery on the side of the formerly non-gravid uterine horn for 30 d postpartum. Treatment mares had in increase in RI between Day 1 and Days 4, 8, 12, 15, 17, 18, 21 and 23 ($P < 0.05$). Control mares had an increase in the uterine artery on the non-gravid side of the uterus between Day 1 and Days 13, 15, 25 and 27 ($P < 0.05$). An asterisk (*) denotes a difference ($P < 0.05$)between groups on a given day.

blood (Nikolakopoulos et al., 2000). Nitric oxide affects cyclooxygenase activity to increase prostaglandin synthesis and effect uterine contractility in rat uterine cultures (Franchi et al., 1994). Interestingly, nitric oxide decreased uterine contractions during times of increased myometrial motility, but increased myometrial contractions when there is very little motility. Using pre-term mice induced to undergo labor, Cella et al. (2010) found that treatment with low concentrations of a nitric oxide donor (SNAP) decreased prostaglandin production whereas a greater amount of SNAP increased prostaglandin production. This result raises the possibility that treatment with L-arginine may increase prostaglandin production in the uterus and impact uterine contractions. Although this study did not evaluate uterine motility, prostaglandin or local nitric oxide synthesis, it is possible that L-arginine supplementation increases nitric oxide concentrations in the uterus enough to increase prostaglandin production. Further research is needed to test this hypothesis.

Supplemental L-arginine increased perfusion of the ovulatory follicle. Silva et al. (2006) reported mares that became pregnant had greater vascular perfusion of the ovulatory follicle compared to those that did not become pregnant. Additionally, in the present study there was less perfusion (25–37%) during the immediate postpartum estrous cycle compared to previous reports in mares from non-periparturient cycles (approximately 50% in non-pregnant and 75% in pregnant mares; Silva et al. (2006) Although the present study did not evaluate fertility, further research is needed to determine whether increasing blood flow to the dominant follicle increases fertility.

There was no difference in RI between groups for any arterial measurements. The RI was selected as the blood flow measurement as it cancels out errors caused by not knowing the Doppler angle and is perceived to be more accurate than pulsatility index (Dickey, 1997). These data are interpreted to mean there was no difference in the amount of blood flow to the ovaries or uterus between groups. Interestingly, the amount of perfusion to the ovulatory follicle was increased in L-arginine supplemented mares, as evaluated by color power Doppler ultrasonography. This observation raises the possibility that L-arginine supplementation increased perfusion of the ovulatory follicle may be caused by a local angiogenic effect at the ovary rather than the vasodilatory actions of L-arginine.

There were no differences in the number of follicles, the diameter of F1 or F2, and deviation parameters between groups. Interpretation of the results of the present study is that L-arginine supplementation has no effect of follicular growth and development. In comparing the first estrus post-foaling to non-periparturient estrous cycles, mares from both groups during the first estrus post-foaling appear to have fewer follicles 6 to 10 and 11 to 15 mm than previously reported by Pierson and Ginther (1987) where non-periparturient ponies had between six and ten follicles, compared to 2 to 6, between 6 and -10 mm. Additionally, Pierson and Ginther (1987) reported four to seven follicles in the 11 to 15 mm range compared to the value of 2 to 4 obtained in the present study. Furthermore, in the present study the number of follicles that were 16 to 20 and >20 mm remained relatively steady throughout the first estrus post-foaling while Pierson and Ginther (1987) observed an increase in the number of these follicles over time, followed by a decrease. Deviation diameters of the F1 and F2 follicles during the first estrous cycle post-foaling were similar to previous reports in mares with a single dominant follicle. Jacob et al. (2009) reported diameters of 22.7 and 21.7 mm for F1 and F2 in mares having single ovulations.

5. Conclusion

Supplementation of mares with L-arginine increased vascular perfusion to the retrospectively identified dominant follicle without altering follicular growth and development or ovarian artery RI. Mares supplemented with L-arginine had a reduction in uterine size and fluid accumulation but no change in uterine artery RI. The combination of reducing uterine fluid accumulation, while not altering follicular development, raises the possibility of using L-arginine supplementation as a breeding management tool during the postpartum period to increase reproductive success.

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