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L-Arginine supplementation 0.5% of diet during the last 90 days of gestation and 14 days postpartum reduced uterine fluid accumulation in the broodmare

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ABSTRACT

L-Arginine is an essential amino acid in many species that has been shown to influence reproduction. However, in horses a dose of 1% L-arginine of total dietary intake impaired absorption of other amino acids, whereas a dose of 0.5% did not. The objectives of this experiment were to evaluate postpartum parameters on mares supplemented with 0.5% L-arginine through the last 90 d of gestation and 14 d postpartum. Sixteen light-horse mares were randomly divided in two groups: 8 mares supplemented with 0.5% L-arginine and 8 mares fed an isonitrogenous equivalent. Gestation length, days to uterine clearance and days to first ovulation were compared. Uterine body depth, diameter of uterine horns, and length of largest pocket of uterine fluid were recorded daily via transrectal ultrasound. Measurements of foal weight, height, and cannon bone circumference were recorded for 9 weeks. Arginine treatment had no effect on gestation length ($P = 0.58$). Supplemented mares cleared fluid quicker postpartum (6.8 ± 0.53 d; $P = 0.026$) compared to control (9.0 ± 0.38 d). Mares supplemented with L-arginine had smaller diameter of fluid present in the postpartum uterus ($P \leq 0.05$). Days to first postpartum ovulation were not affected by treatment nor any influence on uterine involution. Finally, treatment had no effect on any foal's measured parameters. L-Arginine supplementation fed at 0.5% of daily intake during the last 90 d of gestation and early postpartum in mares decreased uterine fluid accumulation, yet did not appear to have any effect on any other parameters measured.

1. Introduction

L-Arginine (L-Arg) is an essential amino acid in mammals, even when it can be endogenously synthesized, and requirements in most species exceeds its biosynthetic production (Castillo et al., 1994). L-Arg is also involved in diverse physiological functions as a mediator in endocrine and immune systems, and serves an essential role in the cardiovascular system by being a nitric oxide precursor. Furthermore, L-Arg is required for synthesis of proteins, urea, polyamines, creatine, and agmatine (Wu and Morris, 1998).

Numerous studies have evaluated the effects of arginine supplementation on animal models to include effects on hypertension (Sanders, 1996; Siani et al., 2000) preeclampsia (Kim et al., 2006), and growth hormone secretion (Fisker et al., 1999). In studying L-Arg and influences on reproduction, Mateo et al. (2007) reported L-Arg supplementation in pregnant gilts elicited an increase in litter survival with increased live litter birth weight. The author's

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hypothesized that supplemented gilts could have experienced enhanced placental angiogenesis and increased placental blood flow that promoted better circulation and improved environment for the fetuses. It has also been suggested that arginine is an insulin stimulator in human fetuses and newborns, therefore causing an anabolic response (King et al., 1971). L-Arg deficiencies have also been associated with fetal growth restrictions (Vosatka et al., 1998).

Mares supplemented with 1% of their diet with L-Arg had shorter gestation lengths, improved uterine arterial blood flow and decreased uterine fluid accumulation postpartum (Kelley et al., 2013, 2014a; Mortensen et al., 2011). However, recent evidence suggested that L-Arg supplemented at this amount reduced the absorption of other amino acids. Kelley et al. (2014b) reported, mares supplemented with 1% L-Arg had a decrease in plasma lysine, methionine, histadine, and proline. This was not observed in mares fed a single dose of 0.5% dose L-Arg.

The purpose of this experiment was to evaluate reproductive parameters of postpartum mares supplemented with 0.5% L-Arg of the total daily dietary intake through their last 90 d of gestation and first 14 d post parturition. Our hypothesis was mares supplemented with 0.5% L-Arg of the total for the 90 d leading up to their expected foaling date, would have a significantly shorter gestation length, greater reproductive performance postpartum and foals born from supplemented mares would report faster growth rates.

2. Materials and methods

2.1. Experimental design

The study was conducted from November 2012 to April 2013 at the Institute of Food and Agricultural Sciences (IFAS) Equine Science Center in Ocala, FL. The experiment was approved by the Animal Care and Use Committee at the University of Florida. A total of 16 light-horse mares and of their foals were used for this experiment and housed as previously described by Mortensen et al. (2011). Mares were blocked by parity, randomly assigned into either an L-Arg or control group and were weighed weekly for dietary estimation as described by Kelley et al. (2014b). The basal diet consisted of ad libitum access to Coastal Bermuda grass hay and 0.5–1% of their bodyweight of a grain-mix concentrate (minimum guarantees: 16% crude protein, 3.5% crude fat, 0.9% Ca, 0.55% P; Gest-O-Lac Ocala's Breeder's feed and Supply, Ocala, FL) during gestation. Amino acid analyses of diets used in this study are similar to those described in Kelley et al. (2014b). Briefly, estimated arginine content of forage was 0.32 g per 100 g and concentrate was 0.85 g per 100 g. After parturition, the offered grain increased to 1.25% of their body weight from the same concentrate. The treated group was supplemented with L-Arg (Ajinomoto AminoScience LLC, Raleigh, NC, USA) at 0.5% of the total estimated daily intake. The control group was fed an isonitrogenous equivalent urea supplement. Mares were fed individually and the supplement was mixed with the morning feed. Supplementation started 90 d before the expected foaling date and continued for 14 d postpartum.

2.2. Mare reproductive performance

Transrectal ultrasonographic measurements began within 24 h of foaling as previously described by Mortensen et al. (2011). Briefly, a digital color Doppler ultrasound (Micromaxx®, Sonosite, Bothell, WA) with a 10–5 MHz broadband and 52 mm linear probe was used and the same technician performed all the exams. Uterine body depth, diameter of the gravid and non-gravid horns, diameter (height × width) of the largest pocket of uterine fluid was recorded to estimate uterine involution. Spectral-Doppler blood flow measurements for uterine arteries were obtained operating an algorithm package of the Micromaxx ultrasound. Uterine arteries were classified as gravid or non-gravid according to the diameter of the ipsilateral uterine horn on the first post-partum measurement. The arterial spectrum obtained with Doppler, with two uniform cardiac cycles, was used to generate the resistance index (RI) [(peak systolic velocity (PSV) − end diastolic velocity (EDV))/PSV] (Ginther, 2007). Blood flow data was performed in triplicate and the average was considered for statistical analysis. Retrospectively identified dominant follicle diameters and presence of a corpus luteum was recorded to identify first postpartum ovulation. All exams were digitally recorded (Sony DVDIRECT®, San Diego, CA, USA) for future analyses.

2.3. Foal measurements

Within 24 h of foaling, all 16 foals were weighed using an electronic livestock scale and height and cannon bone circumference was recorded using a tape measure. Height was measured from the ground to the top of the foal's withers on a uniform concrete surface. Cannon bone size was calculated by measuring the circumference of the medial front right large metacarpal bone. Individual measurements of the foals continued weekly for the 9 weeks following parturition.

2.4. Statistical analysis

Variables were analyzed for normal distribution using Shapiro–Wilk test; in case of normal distribution, the Barlett test was used to verify the homogeneity of variances. If any of the assumptions were not assured, data was transformed using a Box–Cox transformation using the Lmsupport package on R, and subsequently assumptions were verified again. These statistical analyses were performed using R version 3.0.1 (R Foundation). Measurements collected at a single time point, such as gestation length, days to ovulation and days to uterine clearance were compared by one-way ANOVA. For all comparisons a P-value ≤ 0.05 was considered significant and a P-value < 0.1 and > 0.05 was considered a trend. Graphed values are given as mean ± SEM.

Data collected at multiple time points was analyzed using the MIXED procedure in SAS (V.9.2, SAS Inst., Inc., Cary, NC). Fixed effects of treatment, day, and the interaction of treatment by day were assessed by the restricted maximum likelihood (REML) estimation method, with repeated measures over time (days). The mares

nested in treatment were considered a random effect, and a LSMEANS statement was used compare the treatment groups at each time point. The covariance structure selected for individual data analyses was determined by assessing the Bayesian information criterion fit statistic among the following covariance structures: ar(1), arh(1), csh.

3. Results

3.1. Mare reproductive performance

Supplementation with 0.5% L-Arg did not influence any parturition parameters measured and had no effect on gestation length. Supplemented mares had a mean gestation length of 338.8 ± 10.1 d, whereas isonitrogenous control mares had a mean gestation length of 336.1 ± 8.4 d (Table 1). Foals were born healthy with no noticeable impairment.

Table 1
Effect of arginine supplementation on reproductive parameters of postpartum mares.

	Arginine ($n = 8$)	Control ($n = 8$)	P-value
Gestation length	338.75 ± 3.59	336.13 ± 2.98	0.137
Days to uterine clearance	6.75[a] ± 0.53	9.00[b] ± 0.38	0.026
Days to ovulation	11.14 ± 0.63	11 ± 0.87	0.723

In the same row, values with different letter mean significant difference ($P < 0.05$). Values are mean ± SEM.

Mares supplemented with 0.5% L-Arg had a significant decrease ($P < 0.01$) in the time to uterine fluid clearance postpartum (Table 1). Postpartum days to uterine fluid clearance was observed within 6.8 ± 1.5 d in supplemented mares, compared to 9.0 ± 1.0 d in control mares. Additionally, supplemented mares had a significant decrease

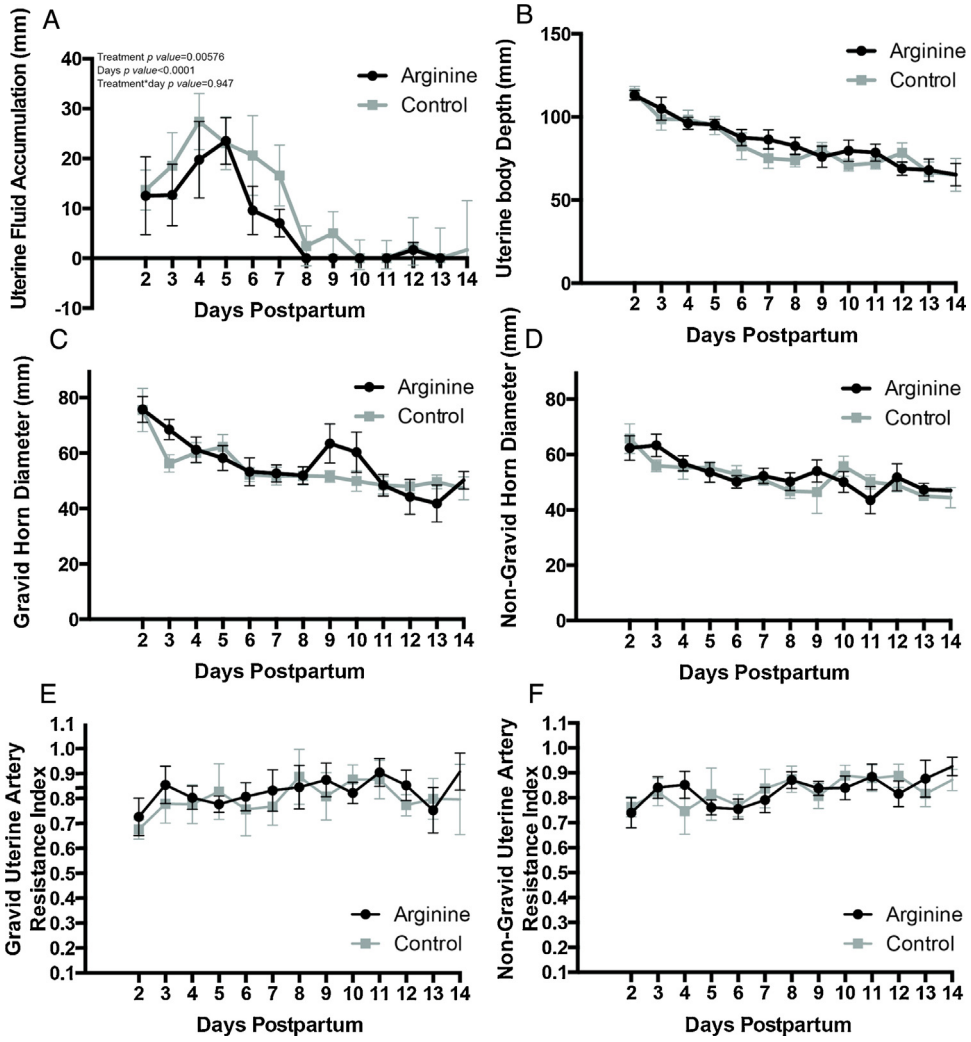

Fig. 1. Uterine involution in postpartum mares supplemented with L-arginine or an isonitrogenous control. (A) Mean (±SEM) maximal fluid accumulation (mm) in the postpartum uterus. (B) Depth (mm) of the uterine body. (C) Diameter (mm) of the formerly gravid uterine horn. (D) Diameter (mm) of the formerly non-gravid horn for 14 d postpartum. (E) Resistance index (RI) of the uterine artery ipsilateral to the gravid horn. (F) RI of the uterine artery contralateral to the non-gravid horn.

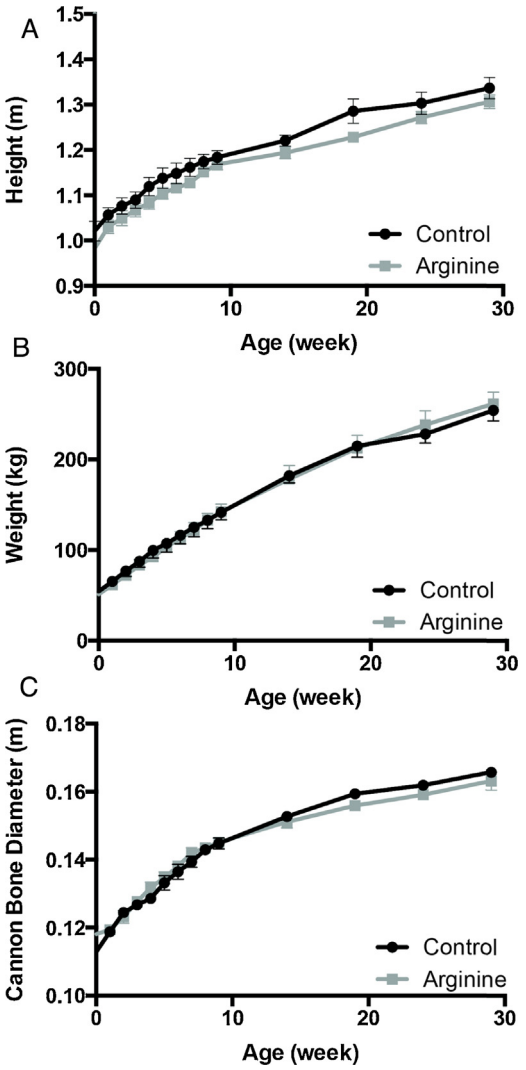

Fig. 2. Effect of maternal L-arginine supplementation on foal growth parameters. (A) Height, (B) Weight, (C) Cannon bone diameter. There was no difference ($P > 0.05$) between treatments.

($P < 0.01$) in size of fluid in the uterine lumen (Fig. 1). There was a significant reduction of fluid overtime but the interaction between treatment and time was not significant. Treatment had no effect on depth of uterine body or diameter of the former gravid or non-gravid uterine horns (Fig. 1). However, as expected all decreased over time ($P < 0.05$).

Blood flow measurements of the uterine arteries did not differ significantly between treatment or control mares (Fig. 1). Uterine arterial RI increased significantly over time to the gravid horn ($P < 0.05$) but this was not observed in the former non-gravid uterine horn.

3.2. Foal measurements

Supplementation with 0.5% L-Arg over the last 90 d of gestation had no significant difference on any of the foal parameters measured (weight, height, cannon bone circumference; Fig. 2). All significantly increased ($P < 0.05$) over time, but no interaction between treatment and time was evident.

4. Discussion

L-Arg supplemented mares had faster uterine fluid clearance than non-supplemented mares. Unlike our previous studies, mares did not have shorter gestation lengths or improved uterine arterial blood flow. Finally, resulting foals showed no difference in growth rates.

Our study demonstrated that mares supplemented with 0.5% L-Arg during the last 90 d of gestation and 2 weeks following parturition had less uterine fluid that cleared faster compared to isonitrogenous control mares. These data are consistent with our earlier studies demonstrating similar uterine clearance in mares supplemented with 1% L-Arg (Kelley et al., 2014b; Mortensen et al., 2011). This is important as fluid accumulation in postpartum mares has been shown to negatively affect pregnancy rates (Malschitzky et al., 2002). Therefore, data from this study may indicate that a nutraceutical intervention of 0.5% L-Arg fed once daily may be effective in reducing fluid accumulation following parturition.

Our data does differ with regards to vascular perfusion of the dominant follicle and involution rates in postpartum mares. In our previous studies, mares fed a higher dose of 1% L-Arg once daily were reported to have greater vascular perfusion of the dominant follicle (Kelley et al., 2013) and hastened uterine involution rates (Kelley et al., 2013; Mortensen et al., 2011). Greater vascular perfusion of the dominant follicle in mares has been correlated with greater pregnancy rate (Silva et al., 2006) and embryo recovery (Smith et al., 2012). Additionally, delayed uterine involution has been shown to correlate with lower pregnancy rates in the first postpartum estrous (Ball, 1993; Macpherson and Blanchard, 2005). Both vascular perfusion of the dominant follicle and involution rate in mares could be viewed as important predictors for breeding success in the first postpartum estrous cycle.

The differences in the present study compared to our past results could be due to the amount supplemented; 0.5% L-Arg in this study compared to 1% L-Arg in our previous work. When examining amino acid profiles in non-pregnant mares, plasma levels of arginine remained elevated past the 5 h observation period with 1% L-Arg which peaked at 278 ± 25 mmol/L (Kelley et al., 2014a). The duration of arginine elevation in the plasma for 1% L-Arg supplemented mares was not reported. However, when mares were fed 0.5% L-Arg, plasma levels remained significantly elevated for 8 h during the longer 12 h sampling period, but only peaked at 167 ± 19 mmol/L. The differences in the amount of arginine in the plasma could account for the differences observed in our postpartum mares in the present study. Clearly further work is warranted to either establish 1% L-Arg of the total daily diet for a longer duration during gestation is safe for both the pregnant mare and resulting neonate, or if supplementing 0.5% L-Arg twice daily in the diet is efficacious in improving reproductive health in the postpartum mare.

We propose that L-Arg can exert two major physiological pathways on the postpartum uterus: by acting as a

nitric oxide precursor that can act as a vasodilator, or by stimulating the mammalian target of rapamycin (mTOR) pathway, or both. First, nitric oxide inhibits vasoconstrictor signals (Thiriet, 2008), promotes angiogenesis by acting downstream of VEGF signaling (Murohara et al., 1998) and can accomplish dual effects on myometrial motility by decreasing uterine contractions in the gravid uterus (Sladek et al., 1999). Furthermore, nitric oxide can potentially increase motility via prostaglandin synthesis (Franchi et al., 1994). Studies in rats have revealed that intrauterine infection promotes nitric oxide synthesis is important in the host response on restricting the infection (Dong et al., 1998; Fang et al., 1999). Second, mTOR is a protein kinase that participates in extra- and intra-cellular signaling and is associated with key regulatory processes in the cell. In environmental conditions where nutrients are sufficient, mTOR signaling is active, enabling protein synthesis, cell growth and proliferation. In contrast, when reserves are low, mTOR activity decreases and limits energy expenditure by reducing protein synthesis (Laplante and Sabatini, 2009). Therefore, amino acids represent a signal that positively regulates mTOR. For example, porcine conceptus trophectoderm (pTr2) cells cultured in arginine rich environment stimulated the mTOR-signaling pathway (Kong et al., 2012). Recent studies propose novel mechanisms by which mTOR can modulate innate, as well as adaptive immune responses. It has been documented postpartum uterine clearance is initiated by polymorphonucleated neutrophils (PMNs), and investigations have linked mTOR mediation with posttranscriptional gene regulation in human PMNs (Yost et al., 2004). However, more in depth studies are required to determine if any of these theories explain the improved uterine clearance in mares supplemented with arginine.

Many studies in other species have demonstrated L-Arg supplementation during gestation led to healthier and heavier offspring at birth (Lassala et al., 2011; Mateo et al., 2007; Vosatka et al., 1998; Wu et al., 2010). However, results from this study showed no differences in neonatal size at birth or growth rates for the 9 weeks following parturition. Compared to the report of Mateo et al. (2007) that showed heavier piglet size after L-Arg supplementation during pregnancy, in this study L-Arg was only fed for the last 90 d of a mean 337.4 d gestation length in mares. This was in comparison to an average 84 d supplementation period over a mean 114 d gestation length in gilts of the Mateo et al. (2007) study. The differences in the two studies could indicate that any potential benefit to offspring size at birth L-Arg supplementation is needed for either a longer duration during pregnancy or at a higher dose.

Supplementing pregnant animals with any nutraceutical should be attempted with caution. However in the case of L-Arg, the pharmacokinetic study by Wu et al. (2007) demonstrated long-term supplementation of L-Arg (<2.5% of dry matter intake) in the diet was safe for pregnant pigs, sheep and rats. These data are consistent with other findings in non-pregnant pigs (Edmonds et al., 1987; Hagemeier et al., 1983). Wu et al. (2007) also stated that available evidence does suggest that L-Arg in excess of 2.5% dry matter intake can result in adverse effects on the animal.

In our study, pregnant broodmares fed L-Arg at 0.5% of the total diet appeared to be a safe dose fed during the last 90 d of gestation; all supplemented mares delivered healthy foals. In our earlier studies, mares fed 1% L-Arg of the total diet during the 21 d of gestation also went on to deliver healthy foals (Kelley et al., 2014b; Mortensen et al., 2011). We chose not to supplement pregnant mares with 1% L-Arg for a longer duration during pregnancy because as previously mentioned, when examining amino acid profiles in non-pregnant mares our data appeared to show 1% L-Arg supplementation impaired absorption of other important amino acids (Kelley et al., 2014b). How this might affect neonatal outcomes in horses is currently unknown. However, Brown et al. (2011) stated that perinatal outcomes for mothers supplemented with large amount of protein are worse than non-supplemented mothers. The study of Edmonds et al. (1987) showed excess amino acids in the diets of pigs, to include 4% L-Arg, suppressed maternal weight gain and dietary intake during pregnancy.

In conclusion, L-Arg fed at 0.5% of the total daily dietary intake demonstrated to help improve uterine fluid clearance following parturition. However, L-Arg supplemented at 0.5% daily did not influence any other reproductive parameters in postpartum mares and may not represent the most effective dose. In addition, L-Arg supplemented at 0.5% over the final 90 d of gestation did not affect neonatal size at birth or subsequent foal growth. However, this experiment does demonstrate nutritional interventions can aid in postpartum clearance of uterine fluid in horses.

Conflict of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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