Theriogenology 81 (2014) 752–757

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Influence of L-arginine supplementation on reproductive blood flow and embryo recovery rates in mares

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ARTICLE INFO

Article history:
Received 17 April 2013
Received in revised form 6 December 2013
Accepted 8 December 2013

Keywords:
Mare
Reproduction
L-Arginine
Doppler
Ultrasonography

ABSTRACT

Supplementation with L-arginine can increase uterine arterial blood flow and vascular perfusion of the preovulatory follicle in mares. Increased vascular perfusion of the preovulatory follicle has been correlated with successful pregnancy in mares. The objective of this study was to determine if supplemental L-arginine would increase ovarian arterial blood flow, vascular perfusion of the preovulatory follicle, and embryo recovery rates in mares. Mares were blocked by age and breed and assigned at random within block to L-arginine supplementation or control groups. Mares were fed L-arginine beginning 17 days before and through the duration of the study. Transrectal Doppler ultrasonography was used to measure ovarian arterial blood flow and vascular perfusion of the preovulatory follicle daily when it reached 35 mm and subsequent CL on Days 2, 4, and 6. Mares, on achieving a follicle of 35 mm or more were bred via artificial insemination and an embryo collection was attempted 7 days after ovulation. Treatment did not affect interovulatory interval (arginine-treated, 18.1 ± 2.6 days; control, 20.7 ± 2.3 days) or embryo recovery rate (arginine-treated, 54%; control, 48%). Mares treated with L-arginine had a larger follicle for the 10 days preceding ovulation than control mares (30.4 ± 1.2 and 26.3 ± 1.3 mm, respectively; $P < 0.05$) and vascular perfusion of the dominant follicle tended ($P = 0.10$) to be greater for the 4 days before ovulation. No differences were observed between groups in diameter or vascular perfusion of the CL. Resistance indices, normalized to ovulation, were not significantly different between groups during the follicular or luteal phase. Oral L-arginine supplementation increased the size and tended to increase perfusion of the follicle 1, but had no effect on luteal perfusion or embryo recovery rates in mares.

1. Introduction

Proper vascularization plays an important role in the selection, growth and maturation of follicles [1], and the increased blood flow to the dominant follicle is associated with increased pregnancy rates in mares [2]. One way to improve blood flow to the reproductive tract is through administration of L-arginine [3]. This amino acid acts as a substrate for biosynthesis of nitric oxide (NO), polyamines, proline, glutamate, creatine, and agmatine [4]. Nitric oxide is a vasodilator that inhibits vasoconstrictor inputs [5], acts downstream of vascular endothelial growth factor signaling to promote angiogenesis [6], and is thought to modulate preovulatory ovarian blood flow [7,8]. Supplementation of women, who have a history of poor response to ovarian hyperstimulation, with L-arginine, increased ovarian blood flow, oocytes retrieved, and embryos transferred [9]. In mares, oral supplementation with L-arginine increased uterine arterial blood flow and hastened the involution period in postpartum mares [10]. The objective

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http://dx.doi.org/10.1016/j.theriogenology.2013.12.012

of this experiment was to determine if oral supplementation with L-arginine would increase ovarian blood flow and embryo recovery in mares.

2. Materials and methods

2.1. Animals

Animal use was approved by the University of Florida Institute of Food and Agricultural Sciences Animal Research Committee. Mares were blocked into a control or treatment group based on age and breed. The control consisted of five Thoroughbreds (mean age: 14.8 ± 1.4 years) and three American Quarter Horse (mean age, 6.7 ± 4.7 years) mares aged 2 to 17 years (control mean age, 11.8 ± 2.3 years). The treatment group contained three Thoroughbred (mean age, 14.3 ± 1.7 years) and four American Quarter Horse (mean age, 9.5 ± 3.4 years) mares aged 2 to 19 years (treatment mean age, 11.6 ± 2.1 years). Mares had apparently normal reproductive activity as determined using ultrasound examination of the cervix, uterus, and ovaries. All mares had uterine biopsies obtained before the study and were examined by a Diplomate of the American College of Theriogenology using the Kenney system [11]. All mares had either a biopsy score of II or IIA and there was no difference between groups in biopsy score as determined by chi-square test.

The study was conducted between May and September (Latitude 18′ 22″N; Longitude 10′ 3″W). The first estrus cycle was induced with an injection of PGF2α (Lutalyse; Pfizer, New York, NY, USA) on Day 7 after ovulation, as were subsequent estrous cycles. Data were collected from up to six cycles; however if uterine bacterial culture was positive, mares were treated and not bred. Mares were housed on pasture and supplemented with 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) and fed individually in paddocks once daily. All mares had free access to water and trace mineral salt. Treatment mares were fed 100 g of L-arginine (Ajinomoto, Raleigh, NC, USA) once daily in the morning beginning from 17 days before the start of the study and remained on supplementation once daily until the completion of study. Ovulation was determined using transrectal ultrasonography.

2.2. Doppler ultrasonography

Ovaries and ovarian arteries were examined via transrectal ultrasonography using Doppler ultrasonography with a 5 to 10 MHz broadband linear array (Micromaxx; Sonosite, Bothell, WA, USA) every other day during estrus until the largest follicle reached a diameter of 35 mm and then daily until ovulation. Profiles of the eight largest follicles (designated F1 through F8, respectively) were categorized using a nontracking method (without regard to day-to-day follicle identity). A retrospective analysis identified the dominant ovulatory follicle (F1) and the largest subordinate follicle (F2). Corpus luteum measurements were taken on Days 2, 4, and 6 postovulation. Diameters (average of height and width) of the largest follicle and CL were measured and recorded. Vascular perfusion to the follicle and CL was determined with color power Doppler ultrasonography. Resistance index (RI) was measured for both ovarian and uterine arteries as described by Smith, et al. [12]. All examinations were recorded digitally for future analysis. Images from the digital video disc recordings were used to determine the percent perfusion of the retrospectively identified dominant follicle and CL as previously described by Kelley, et al. [13] and Smith, et al. [12].

2.3. Embryo collections

Mares were bred with semen from one of two fertile stallions. Semen was collected using artificial vagina and evaluated for total progressive motility and sperm concentration. The concentration was estimated using an Equine Densimeter (Animal Reproduction Systems, Chino, CA, USA). Each mare received a total dose of 500×10^6 fresh, motile spermatozoa extended to a volume of 15 mL using E-Z Mixin original formula (Animal Reproduction Systems). Mares were bred every other day until ovulation via artificial insemination, beginning from the time they displayed estrus to a teaser stallion and had a follicle with a diameter of 35 mm or greater. All ovulations were spontaneous (not induced). Nonsurgical embryo collection was performed on Day 7 after ovulation. The uterus was infused four times with a 500-mL flush medium (Biolife Advantage; Agtech, Manhattan, KS, USA) utilizing a silicone 34 French catheter (Reproduction Resources, Walworth, WI, USA) and the effluent was collected through Y-junction tubing (Reproduction Resources) into a 75-μm filter (Reproduction Resources). Mares were administered PGF2α (Lutalyse; Pfizer), 10 mg intramuscularly, at the end of the flushing procedure. The contents of the filter were rinsed into collection dishes, which were examined using a stereomicroscope at magnification ×20. Embryos were evaluated based on a one (excellent) through four (nonviable) scale as previously described [14].

2.4. Statistical analysis

Mares were divided by age into young (<16 years; arginine, n = 4; control, n = 4) and old (≥16 years; arginine, n = 3; control, n = 4) for analysis to examine the effect of age. Follicular and RI data (during the follicular phase) were normalized to ovulation (Day 0). Corpus luteum and RI data (during the luteal phase) were normalized to ovulation beginning an estrous cycle (Day 0). Continuous data were analyzed using the SAS MIXED procedure (version 9.2; SAS Institute Inc., Cary, NC, USA). A random statement was used to account for variability of animals within treatment and cycle by animals within treatment. A repeated statement for day was used, with the subject being cycle by animals within treatment, using compound symmetry as the model best fitting the covariance matrix. A Tukey's multiple range test was used to detect differences among groups within days when there was a significant interaction between group and day. The embryo recovery rates were compared using a Chi-square test. Data are presented as least square means ± standard error of the mean. A significant

difference was denoted by $P < 0.05$, whereas a tendency was denoted by $0.05 \leq P \leq 0.10$.

3. Results

The mean interovulatory interval for mares supplemented with L-arginine was 18.1 ± 2.6 days and was not different than for control mares 20.7 ± 2.3 days (NS); however there was a tendency ($P = 0.08$) for a longer interovulatory interval in young (22.6 ± 2.6 days) when compared with older (16.3 ± 2.3 days) mares. There was no difference between stallions on embryo recovery rate, thus datum from both stallions were combined for further analysis. There was no significant difference in embryo recovery rates of arginine (13/24, 54%) and control mares (16/33, 48%); or between young (14/25, 56%) and old (15/32, 47%) mares. In addition, there was no difference between treatments or ages in the stage of embryonic development or embryo score (Table 1).

There was no effect of cycle on diameter of F1, F2, or CL or vascular perfusion of F1 or CL and therefore data are combined across cycles for additional analysis. Arginine-treated mares had a larger ($P < 0.05$) diameter F1 follicle (30.4 ± 1.2 mm) than control (26.2 ± 1.2 mm) mares during the 10 days preceding ovulation (Fig. 1A) and during this time, younger mares had a larger ($P < 0.05$) F1 (30.2 ± 1.2 mm) than older mares (F1: 26.3 ± 1.3 mm; Fig. 1A). No difference between groups was observed between F2 follicles (Fig. 1B); however, younger mares had a larger ($P < 0.05$) F2 (21.8 ± 1.1 mm) than older mares (18.5 ± 1.1 mm) during 10 days preceding ovulation.

There was a tendency ($P = 0.10$) for arginine-treated (45.1 ± 1.7%) mares to have greater perfusion to the F1 for the 4 days preceding ovulation compared with control

Table 1
Number of recovered embryos classified by grade [14] for each group (control or arginine) or based on mare age (young < 16 years; old ≥ 16 years).

Item	Group		Age	
	Control	Arginine	Young	Old
Uterine flushes performed	24	33	25	32
Embryos recovered	16 (54%)	13 (48%)	14 (56%)	15 (46%)
Embryos classified by grade				
1	10 (63%)	9 (69%)	11 (79%)	8 (53%)
2	4 (25%)	4 (31%)	2 (14%)	6 (40%)
3	1 (6%)	0 (0%)	1 (7%)	0 (0%)
4	1 (6%)	0 (0%)	0 (0%)	1 (7%)

There was no difference in embryo score between groups or ages.

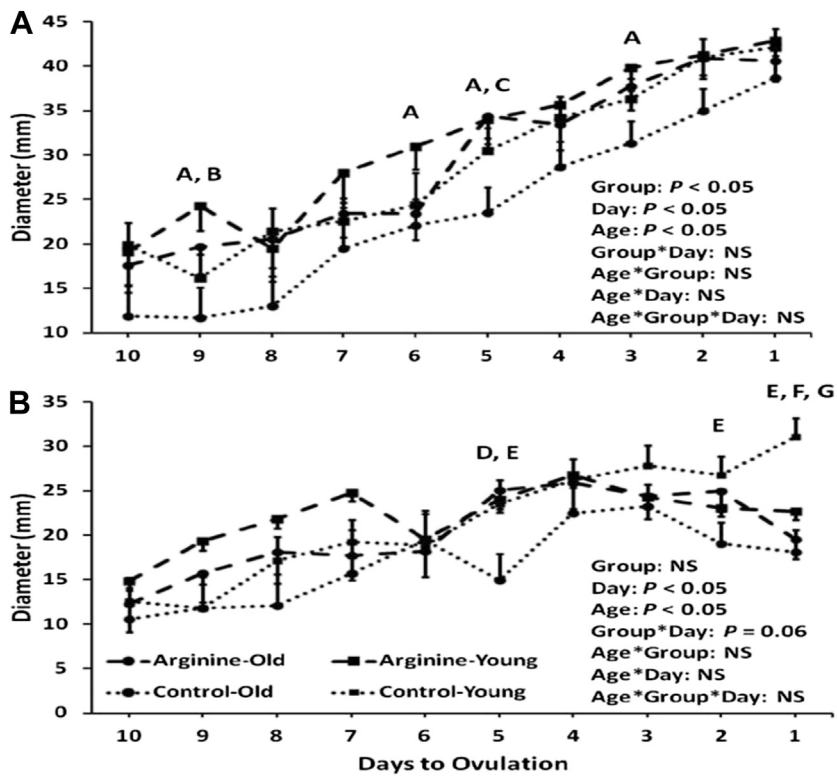

Fig. 1. Least squares mean ± standard error of the mean diameter (mm) of the (panel A) retrospectively identified dominant (F1) and (Panel B) the largest subordinate (F2) follicles for L-arginine–supplemented and control mares separated by age (young < 16 years; old ≥ 16 years). In panel A, an A denotes a significant difference ($P < 0.05$) between control-old and arginine-young mares; a B denotes a significant difference ($P < 0.05$) between control-young and arginine-young mares; and C denotes a significant difference ($P < 0.05$) between control-old and arginine-old mares on a given day. In panel B, a D denotes a significant difference ($P < 0.05$) between control-old and arginine-young mares; an E denotes a significant difference ($P < 0.05$) between control-old and control-young mares; an F denotes a significant difference ($P < 0.05$) between control-young and arginine-old mares; and a G denotes a significant difference ($P < 0.05$) between control-young and arginine-young mares on a given day.

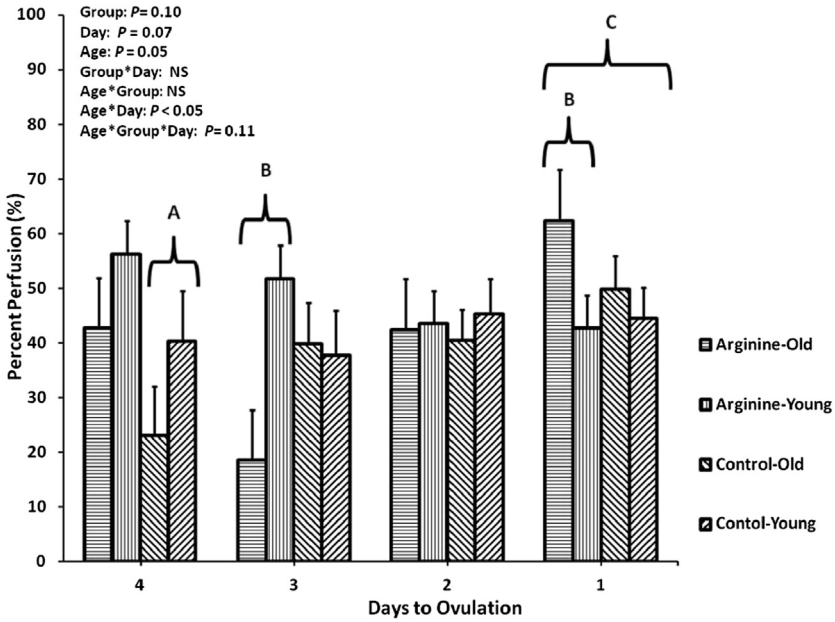

Fig. 2. Least squares mean ± standard error of the mean percent perfusion to the retrospectively identified dominant (F1) follicle for 4 days before ovulation. An A denotes a significant difference ($P < 0.05$) between control-old and control-young mares; a B denotes a significant difference ($P < 0.05$) between arginine-old and arginine-young mares; and a C denotes a significant difference ($P < 0.05$) between control-young and arginine-old mares on a given day.

(40.2 ± 2.1%; Fig. 2) and a tendency ($P = 0.05$) for younger mares (45.3 ± 1.8%) to have increased perfusion of the F1 than older mares (39.9 ± 2.0%; Fig. 2). No differences were observed between groups or age, in diameter or vascular perfusion of the CL, and data from both ages were combined. The diameter of the CL for arginine-treated and control mares, respectively, on Day 2 was 29.1 ± 1.8 and 32.1 ± 1.7 mm (NS), Day 4 was 28.7 ± 1.7 and 31.7 ± 1.5 mm (NS), and Day 6 was 28.5 ± 1.8 and 31.8 ± 1.8 mm (NS). The perfusion of the CL for arginine-treated and control mares, respectively, on Day 2 was 37.1 ± 5.7% and 37.5 ± 6.2% (NS), Day 4 was 37.4 ± 5.2% and 37.3 ± 5.7% (NS), and Day 6 was 47.9 ± 5.6% and 44.8 ± 5.1% (NS). The mean CL perfusion was 47.4 ± 5.2% in arginine and 42.5 ± 4.0% in control mares (NS). There was a tendency ($P = 0.08$) for CL perfusion to increase from Days 4 to 6 postovulation.

As no difference in RI of the ovarian artery was found between the two age groups during the follicular or luteal phase, data were combined for analysis. No differences were observed in RI between groups during the follicular phase in the retrospectively identified ovulatory or non-ovulatory ovarian artery for the 10 days preceding ovulation. The mean RI of the ovarian artery ipsilateral to ovulation was 0.95 ± 0.05 for arginine-treated and 0.86 ± 0.05 for control mares. The mean RI of the ovarian artery contralateral to ovulation was 0.88 ± 0.02 for arginine-treated and 0.87 ± 0.01 for control mares. Resistance index was also normalized to ovulation and examined during the luteal phase (Days 2, 4, and 6 postovulation). There was no difference between RI in the ovarian artery ipsilateral to the CL. The RI of the ovarian artery ipsilateral to the CL for arginine-treated and control mares, respectively, on Day 2 was 0.98 ± 0.03 and 0.84 ± 0.03 ($P < 0.05$), Day 4 was 0.88 ± 0.03 and 0.89 ± 0.03 (NS), and Day 6 was 0.85 ± 0.03 and 0.86 ± 0.03 (NS), with mean values of 0.90 ± 0.02 and 0.87 ± 0.02, for arginine-treated and control mares. The RI of the ovarian artery contralateral to the CL for arginine-treated and control mares, respectively, on Day 2 was 0.88 ± 0.04 and 0.93 ± 0.04 (NS), Day 4 was 0.91 ± 0.04 and 0.87 ± 0.04 (NS), and Day 6 was 0.85 ± 0.04 and 0.89 ± 0.04 (NS), whereas mean RI values were 0.89 ± 0.03 and 0.90 ± 0.03 (NS) for arginine-treated and control mares.

4. Discussion

Oral supplementation of mares with L-arginine increased the mean dominant follicle diameter during the 10 days preceding ovulation and tended to increase perfusion of the dominant follicle for the 4 days before ovulation, but had no effect on embryo recovery rates, CL diameter, CL perfusion, or RI. Additionally, older mares (≥16 years) had a significantly smaller dominant and the largest subordinate follicle than younger mares for the 10 days preceding ovulation. There also was a tendency for older mares to demonstrate less perfusion to the dominant follicle for the 4 days before ovulation and these mares tended to have a shorter interovulatory interval. No differences were found by age with regards to CL diameter, perfusion, embryo recovery rates, or RI. Our findings demonstrate that L-arginine can increase growth of the dominant follicle possibly by increasing blood flow.

Recent work [31] has demonstrated that L-arginine supplementation during the first postpartum estrous cycle increased perfusion of the ovulatory follicle; however, no differences between the sizes of the ovulatory or the largest subordinate follicle were found. It remains unclear why ovulatory follicular diameter was increased in our study but was unaltered in the study by Kelley, et al. [31]. On

possibility is the increased metabolic demand of lactation during the postpartum period, which has been shown to reduce the prominence of dominant follicle in llamas [32]. Additionally, in postpartum cycling, lactating dairy cattle, folliculogenesis, and steroidogenesis are altered during the postpartum period [33]. Thus, it remains possible that differences between our study and Kelley, et al. [31] may be due in part to the effect of the postpartum period on follicular development and additional metabolic demands of lactation.

Our study found older mares have reduced follicular blood flow for 4 days before ovulation and smaller dominant and the largest subordinate follicles for the 10 days before ovulation as compared with younger mares. These results are consistent with observations in women, in which increasing age was associated with a decrease in ovarian blood flow [15–18]. This is in contrast to findings of Altermatt, et al. [19], who reported increased blood flow to the ovulatory follicle in mares greater than 20 years of age compared with younger mares in the 24 hours before ovulation treated with FSH or LH. Additionally, Ginther, et al. [20] found no difference in follicular vascularity in young and old mares for the 4 days leading to ovulation. Our study also demonstrated that older mares have reduced dominant and the largest subordinate follicle diameters during the 10 days preceding ovulation. The reduction in preovulatory follicle diameter has been found in older mares, and a reduction in the number of follicles throughout the interovulatory interval [21]. In cattle, a reduction in follicle diameter has been associated with a smaller CL and reduced progesterone secretion and decreased fertility [22]. It is unclear, if the reduction in blood flow with age is a cause or result of the smaller follicular diameter in older mares.

Our study found a tendency for increased vascular perfusion of the F1 follicle with arginine treatment in younger mares. Silva, et al. [2] found that increased blood flow to the preovulatory follicle was positively correlated with pregnancy rates in mares with hCG-induced ovulations. Our study may lack enough statistical power to detect a difference in embryo recovery rate that may be associated with alterations in follicular blood flow. To detect a 10% difference with a power of 0.80 at $\alpha = 0.05$, we would need 411 animals per group. Additionally, Altermatt, et al. [19] found a positive correlation of follicular blood flow with the number of oocytes collected and blastocyst development in culture. Our study did not detect a difference in either embryo developmental stage or embryo grade between age groups or treatment groups and in embryo recovery rates between young and old mares. This is likely because of a lack of statistical power. Recovery rates in mares range from 50% in mares to 90% [23,24]. Embryo recovery rates in our study were low and this may be due in part to no rest cycles between flushes, use of subfertile mares and environment factors, such as heat and humidity [25–27].

Arginine supplementation in women that poorly respond to a follicular hyperstimuation protocol by either failing to achieve an adequate number of mature follicles and/or an adequate serum estradiol concentration after hyperstimulation, increased the number of oocytes retrieved and embryos transferred [9]. In contrast, women supplemented with arginine, who responded normally to a hyperstimulation protocol and then underwent an IVF program had decreased embryo quality and pregnancy rates [28]. Battaglia, et al. [28] hypothesized that a reduction in embryo quality was related to NO_2-/NO_3-concentrations in follicular fluid and adversely affect ATP production resulting in the formation of oxidizing molecules.

Resistance index was not different between groups in our study for arterial measurements during the luteal phase. This is in contrast to Takasaki, et al. [29], which found women treated with arginine starting after ovulation and through the luteal phase had a decreased RI compared with previous cycles. A decrease in RI is generally associated with improved blood flow through the examined vessel [30]. Based on our findings, arginine supplementation seemed to have little effect on luteal blood flow.

4.1. Conclusions

Supplementation of mares with arginine resulted in a larger dominant follicle in the 10 days preceding ovulation and tended to increase ovarian blood flow, but had no effect on embryo recovery rates. Additionally, older mares had a smaller dominant follicle and tended to have reduced blood flow but no difference in embryo recovery rates. It is well established that older mares are less fertile than younger mares, and the number of animals used in this study yielded a low statistical power and thus it was unlikely to detect differences in embryo recovery between groups or ages. This research raises the question, whether follicular blood flow plays a factor in mare fertility and if developing methods to alter follicular blood flow will enhance fertility in mares.

Acknowledgments

The authors would like to extend their appreciation to A.D. Ealy and P.J. Hansen for taking time to edit this manuscript.

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