BRIEF COMMUNICATION: Intracellular concentrations of free amino acids are reduced in skeletal muscle of late gestation twin compared to single fetuses

D. PACHECO[1], B.P. TRELOAR[1], P.R. KENYON[2], H.T. BLAIR[2] and S.A. McCOARD[1]*

[1]AgResearch Grasslands, Private Bag 11-008, Palmerston North 4442, New Zealand
[2] Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand
*Corresponding author: sue.mccoard@agresearch.co.nz

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INTRODUCTION

A cost-effective way to increase productivity measured as kg lamb weaned per ewe, from sheep farming is to increase lambing percentage. However, late fetal and early neonatal twin lambs have reduced body weight (McCoard *et al.,* 2000) and ~20% less skeletal muscle mass than singletons (McCoard *et al.,* 2001) with carryover effects on post-natal growth performance (Afolayan *et al.,* 2007; De Blasio *et al.,* 2007), and thus profits. Increased knowledge of the impact of nutrition and other environmental factors on the mechanisms regulating fetal growth, in particular skeletal muscle growth, is essential in order to develop new strategies to enhance meat production performance.

Intracellular free (non-protein bound) amino acids (FAA) serve as regulators of key metabolic and cell signalling pathways necessary for maintenance, growth, reproduction and immunity (Wu, 2009). For example, arginine has an important role in the regulation of nutrient metabolism (Tan *et al.,* 2008; Kim & Wu 2009). In pigs arginine-supplementation increases muscle protein synthesis (Yao *et al.,* 2008) and bodyweight gain (Kim & Wu 2004). However, the mechanisms of action of arginine on muscle growth are largely unknown. Furthermore, in ruminants the association between intracellular FAA profiles and phenotypes, such as muscle mass, has not been explored.

We hypothesised that the smaller muscle mass of twins compared to that of single late gestation fetuses would be associated with differing concentration of intracellular FAA in skeletal muscle. If that is the case, specific amino acids (AA) might be identified that are limiting fetal skeletal muscle growth.

MATERIALS AND METHODS

All procedures involving these animals were carried out in compliance with the guidelines of the Massey University Animal Ethics Committee.

Skeletal muscle tissue samples from single and twin fetal lambs at 140 days of gestation were sourced from a subset of animals used in a larger study. Details of the animals and experimental conditions have been published elsewhere (Firth *et al*., 2008; Kenyon *et al.,* 2009). Both single and twin-bearing ewes were fed on a maintenance pasture-only nutritional regimen from Day 21 post-insemination until 140 days of pregnancy and grazed such that total increase in maternal body weight during pregnancy approximately equalled the expected conceptus mass at term, that is the ewes maintained a conceptus-free weight.

At Day 140 of pregnancy, five twin bearing ewes and ten singleton-bearing ewes were euthanised. Fetal sex, weight and crown-rump length were recorded and the *M. semitendinosus* was excised, weighed and snap frozen in liquid nitrogen and stored at -80°C until analysis. Nine samples were obtained for each litter size group. Samples of approximately 120 mg of tissue were analysed for FAA concentrations using lithium-based high-performance liquid chromatography as described by Pacheco *et al.* (2010).

Differences between singletons and twins were defined by performing an analysis of variance, using the MIXED procedure in SAS, Version. 9.1 (SAS Institute, Cary, North Carolina, USA). Several models were tested, which included litter size (singleton vs. twins), sex of the fetus (male vs. female) and the interaction between main effects. Ewe live weight was included as a covariate. Results reported herein are those from the most parsimonious model, containing only litter size.

RESULTS

Ewe live weight did not differ between single and twin-bearing ewes. Twin fetuses had a smaller ($P < 0.001$) crown-rump length, body weight and *M. semitendinosus* weight than their single counterparts (Table 1).

For all AA, there were no significant ($P > 0.05$) interactions between litter size and sex of the fetus. For essential AA, the absolute intracellular concentrations (μmol/g tissue) of arginine, histidine, glutamine and valine were less ($P < 0.01$) in twins compared to singletons, while methionine was greater ($P = 0.01$) (Table 2). For non-essential AA, the concentrations of alanine, proline and tyrosine

were less in twins (P <0.05), while the concentration of glycine was greater (P <0.01) (Table 2). The relative concentrations (g/100 g total AA) followed similar patterns as those described for the absolute concentrations, as the total AA concentrations did not differ between single and twin fetal lambs.

DISCUSSION

Intracellular FAA concentrations are the result of complex interactions involving protein synthesis and degradation, FAA concentrations in extracellular fluids, and trans-membrane transport. Nevertheless, the concentrations of FAA themselves have been described as regulators of intracellular metabolic pathways (Haussinger *et al.*, 1996). This is the first report of differences in the concentrations of intracellular FAA in ovine skeletal muscle tissue of single and twin fetuses. The preliminary findings reported herein indicate that the reduced *M. semitendinosus* mass in the late gestation twin fetus is not associated with reduced overall AA availability. Rather, reduced muscle mass in twin lambs is associated with reduced intracellular concentration of specific AA namely, arginine, alanine, histidine, proline, tyrosine and valine.

Previous studies have reported that fetal growth restriction during late gestation in sheep, resulting from maternal nutrient restriction, is associated with reduced concentration of all FAA in fetal plasma and amniotic and allantoic fluids (Kwon *et al.,* 2003, 2004). Similar results have been reported for growth-restricted human fetuses (Cetin *et al.,* 1996). Therefore, while total concentrations of FAA in the plasma is likely to be important for overall fetal growth, the results of the present study suggest that skeletal muscle growth does not depend on total FAA levels. Rather, a decrease in the concentration of a few specific AA known to be involved in the regulation of muscle protein synthesis, notably arginine, glutamine, histidine and alanine, indicate that these AA may have a regulatory role in skeletal muscle growth in growth-restricted lambs. Interestingly, intracellular concentrations of leucine, a potential stimulator of muscle protein synthesis in pigs (Suryawan *et al.,* 2008) did not differ between single and twin lambs, suggesting that the effects of individual AA on skeletal muscle protein synthesis may be species-specific. The mechanisms involved have not been defined, but are likely to involve AA signaling pathways such as the nutrient-sensing mechanistic target of rapamycin (mTOR) pathway (Liao *et al.,* 2008).

TABLE 1: Mean ± standard error of mean for effect of litter size on crown-rump length, live weight and *M. semitendinosus* weight in single and twin fetal lambs at 140 days of gestation.

Measurement	Singles[1]	Twins[2]	P value
Ewe data			
Ewe live weight (kg)	69.9 ± 1.4	72.0 ± 1.5	0.312
Foetal data			
Crown-rump length (cm)	61.1 ± 0.6	54.8 ± 0.9	<0.001
Fetal weight (kg)	6.4 ± 0.2	4.8 ± 0.1	<0.001
M. semitendinosus weight (g)	11.4 ± 0.4	6.6 ± 0.5	<0.001
M. semitendinosus (% of foetal weight)	0.18 ± 0.01	0.14 ± 0.01	<0.001

[1]Ewe data n = 10, Fetal data n = 9.
[2]Ewe data n = 5, Fetal data n = 9.

TABLE 2: Mean ± standard error of mean of the concentration (µmol/g tissue) and the relative change (%) of intracellular free amino acids in the *semitendinosus* muscle from nine single and nine twin fetal lambs at 140 days gestation. ND = Not detected.

Amino acid	Single	Twin	P-value	Twin vs Single (%)
Total amino acids	31.4 ± 1.6	30.1 ± 0.7	0.456	-
Essential amino acids				
L-Arginine	0.82 ± 0.06	0.45 ± 0.04	<0.001	↓ 46%
L-Glutamine[1]	6.80 ± 0.18	5.91 ± 0.29	0.017	↓ 13%
L-Histidine	0.38 ± 0.07	0.12 ± 0.03	0.003	↓ 67%
L-Isoleucine	0.04 ± 0.01	0.03 ± 0.01	0.006	-
L-Leucine	0.09 ± 0.02	0.08 ± 0.01	0.618	-
L-Lysine	0.19 ± 0.02	0.18 ± 0.02	0.847	-
L-Methionine	0.12 ± 0.01	0.18 ± 0.02	0.012	↑ 55%
L-Phenylalanine	0.09 ± 0.01	0.08 ± 0.01	0.505	-
L-Threonine	3.18 ± 0.33	3.26 ± 0.13	0.834	-
L-Valine	0.26 ± 0.03	0.15 ± 0.01	0.001	↓ 52%
Non-essential amino acids				
L-Alanine	3.80 ± 0.24	2.74 ± 0.17	0.003	↓ 28%
L-Aspartic acid	0.94 ± 0.04	0.87 ± 0.04	0.153	-
L-Asparagine	0.06 ± 0.01	0.03 ± 0.01	0.248	-
L-Citrulline	ND	0.11 ± 0.05	0.036	-
L-Cystathionine	0.31 ± 0.08	0.30 ± 0.14	0.950	-
L-Glutamic acid	3.02 ± 0.21	3.30 ± 0.28	0.443	-
L-Glycine	3.12 ± 0.23	4.32 ± 0.30	0.007	↑ 38%
L-Ornithine	0.24 ± 0.02	0.29 ± 0.03	0.264	-
L-Proline	0.68 ± 0.07	0.41 ± 0.05	0.007	↓ 40%
L-Taurine	7.14 ± 0.83	7.27 ± 0.58	0.901	-
L-Tyrosine	0.13 ± 0.02	0.09 ± 0.01	0.025	↓ 35%

[1] L-Glutamine is a conditionally essential amino acid in neonates.

Collectively these results highlight the potential importance of intracellular FAA levels for regulation of muscle growth, and provide some insights into which individual AA may be limiting for muscle growth in fetal lambs. Further research is required to confirm if the findings reported herein apply to other conditions with differential muscle growth, as well as detailed investigation on the cause-effect relationships between intracellular FAA and muscle growth. Increased knowledge of the impact of nutrition and other environmental factors on the mechanisms regulating fetal growth is essential in order to develop new strategies to enhance production performance in sheep farming.

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REFERENCES

Afolayan, R.A.; Fogarty, N.M.; Ingham, V.M.; Gilmour, A.R.; Gaunt, G.M.; Cummins, L.J.; Pollard, T. 2007: Genetic evaluation of crossbred lamb production. 3. Growth and carcass performance of second-cross lambs. *Australian Journal of Agricultural Research* **58**: 457-466.

Cetin, I.; Ronzoni, S.; Marconi, A.M.; Perugino, G.; Corbetta, C.; Battaglia, F.C.; Pardi, G. 1996: Maternal concentrations and foetal-maternal concentration differences of plasma amino acids in normal and intrauterine growth-restricted pregnancies. *American Journal of Obstetrics and Gynaecology* **174**: 1575-1583.

De Blasio, M.J.; Gatford, K.L.; McMillen, I.C.; Robinson, J.S.; Owens, J.A. 2007: Placental restriction of foetal growth increases insulin action, growth, and adiposity in the young lamb. *Endocrinology* **148**: 1350-1358.

Firth, E.C.; Rogers, C.W.; Vivkers, M.; Kenyon, P.R.; Jenkinson, C.M.C.; Blair, H.T.; Johnson, P.L.; Mackenzie, D.D.S.; Peterson, S.W.; Morris S.T. 2008: The bone-muscle ratio of fetal lambs is affected more by maternal nutrition during pregnancy than by maternal size. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* **294**: R1890-R1894.

Haussinger, D. 1996: The role of cellular hydration in the regulation of cell function. *Biochemical Journal* **313**: 697-710.

Kenyon, P.R.; Blair, H.T.; Jenkinson, C.M.C.; Morris, S.T.; Mackenzie, D.D.S.; Peterson, S.W.; Firth, E.C.; Johnston, P.L. 2009: The effect of ewe size and nutritional regimen beginning in early pregnancy on ewe and lamb performance to weaning. *New Zealand Journal of Agricultural Research* **52**: 203-212.

Kim, S.W.; Wu, G. 2004: Dietary arginine supplementation enhances the growth of milk-fed young pigs. *Journal of Nutrition* **134**: 625-630.

Kim, S.W.; Wu, G. 2009: Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids* **37**: 89-95.

Kwon, H.; Spencer, T.E.; Bazer, F.W.; Wu, G. 2003: Developmental changes of amino acids in ovine foetal fluids. *Biology of Reproduction* **68**: 1813-1820.

Kwon, H.; Ford, S.P.; Bazer, F.W.; Spencer, T.E.; Nathanielsz, P.W.; Nijland, M.J.; Hess, B.W.; Wu, G. 2004: Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and foetal plasma and foetal fluids. *Biology of Reproduction* **71**: 901-908.

Liao, X-H.; Majithia, A.; Huang, S.; Kimmel, A.R. 2008: Growth control via TOR dinase signalling, an intracellular sensor of amino acid and energy availability, with crosstalk potential to proline metabolism. *Amino Acids* **35**: 761-770.

McCoard, S.A.; McNabb, W.C.; McCutcheon, S.N.; Harris, P.M.; Peterson, S.W. 2000: Muscle growth, cell number, type and morphometry in single and twin foetal lambs during mid to late gestation. *Reproduction, Fertility and Development* **12**: 319-327.

McCoard, S.A.; McNabb, W.C.; Birtles, M.J.; Harris, P.M.; McCutcheon, S.N.; Peterson, S.W. 2001: Immunohistochemical detection of myogenic cells in foetal and neonatal lambs. *Cells, Tissues and Organs* **169**: 21-33.

Pacheco, D.; Treloar, B.P.; McCoard, S.A. 2010: Brief Communication: The effect of growth hormone on the intracellular amino acid profiles in the mammary gland of lactating cows. *Proceedings of the New Zealand Society of Animal Production* **70**: 67-70.

Suryawan, A.; Jeyapalan, A.S.; Orellana, R.A.; Wilson, F.A.; Nguyen, H.V.; Davis, T.A. 2008: Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. *American Journal of Physiology, Endocrinology and Metabolism* **295**: E868-875.

Tan, B.; Yin, Y.; Liu, Z.; Li, X.; Xu, H.; Kong, X.; Huang, R.; Tang, W.; Shinzato, I.; Smith, S.B.; Wu, G. 2008: Dietary L-Arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. *Amino acids* **37**: 169-175.

Wu, G. 2009: Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**: 1-17.

Yao, K.; Yin, Y-L.; Chu, W.; Liu, Z.; Deng, D.; Li, T.; Huang, R.; Zhang, J.; Tan, B.; Wang, W.; Wu, G. 2008: Dietary arginine supplementation increases mTOR signalling activity in skeletal muscle of neonatal pigs. *Journal of Nutrition* **138**: 867-872.