Pakistan J. Zool., vol. 48(2), pp. 447-452, 2016.

Effect of Maternal Undernutrition on Connective Tissue Content of Fetal Skeletal Muscle of Hill and Lowland Breeds of Sheep

Berjan Demirtas*
Vocational School of Veterinary Medicine, Istanbul University, 34320 Avcilar, Istanbul, Turkey.

ABSTRACT

The early fetal environment such as maternal nutrition plays an important role in the development of skeletal muscle that is composed of both muscle and connective tissues. Hill ewes are adapted to harsh environments and can cope better with poor nutrition compared to lowland ewes. The present study investigated the effect of maternal undernutrition on primary fiber number of muscle as well as the connective tissue content in fetal skeletal muscle of both hill and lowland breeds of sheep. Hill (Welsh Mountain) and lowland (Dorset) ewes were used in 2 different experiments with one year interval. Experimental design, nutritional treatments and methods were same in both experiments. Ewes were mated and assigned as either control or undernourished group for each experiment. In each experiment controls were fed 100% of their daily nutritional requirement throughout experiment whereas undernourished ewes received 70 % of their daily nutritional requirement from day 22 to day 65 of gestation. All fetuses were taken out on 65th day of gestation by overdose injection of pentabarbitone. Fetal weight, fetal crown-rump length were measured. The semitendinosus muscle was dissected and sections were stained for alkali ATPase and hematoxylin eosin stain. Muscle cross sectional area, the percentage of muscle and connective tissue, the number of primary fibers and nuclei were measured. In lowland breed, the fetal weight and fetal weight: fetal crown-rump length ratio were significantly greater in undernourished group ($P<0.05$). Furthermore, underfed fetal skeletal muscle of lowland breed had significantly larger cross sectional area and higher percentage of connective tissue ($P<0.05$). It is concluded that maternal undernutrition seems to increase the connective tissue content of fetal muscle of lowland but not that of hill breed of sheep.

Article Information
Received 9 December 2015
Revised 1 February 2016
Accepted 3 February 2016
Available online 1 March 2016

Key words
Maternal, ewe, fibroblast, myofiber, fetus, nutrition

INTRODUCTION

Skeletal muscle is composed of muscle tissue and connective tissue. Muscle tissue is made up of muscle cells known as muscle fibers whereas connective tissue is mainly made up of fibroblasts (which secrete collagen, elastin, reticulum fibers and ground substance such as glycoprotein and proteoglycans) and other cells including adipocytes, plasma cells, lymphocytes, macrophages and mast cells (Karunaratne *et al.*, 2005).

The early fetal environment plays an important role in development of skeletal muscle. Fetal skeletal muscle development involves muscle tissue development and connective tissue development. Muscle fiber hyperplasia occurs during the fetal period and is completed by birth in many agricultural animals including sheep (Ashmore *et al*., 1972; Stickland and Goldspink, 1973; Russel and Oteruella, 1981; Karunaratne *et al*., 2005; Demirtas and Ozcan, 2012b). The fetal stage is crucial for skeletal muscle development because there is no net increase in muscle fiber numbers after birth (Sticland, 1978; Zhu *et al*., 2004; Du *et al*., 2010a). At the initial stage of muscle fiber formation, primary muscle fibers form then secondary muscle fibers form later around primary fibers. Secondary muscle fibers account for the majority of total skeletal muscle fibers (Du *et al*., 2010a). Connective tissue of skeletal muscle that forms the endomysium, perimysium and epimysium provides structural frame for muscle tissue.

Muscle fiber number is affected by prenatal conditions such as maternal nutrition, with undernourished fetuses having significantly fewer secondary muscle fibers (Ward and Stickland, 1991; Dwyer *et al*., 1995; Zu *et al*., 2004; Du *et al*., 2010a; Demirtas and Ozcan, 2012a). Primary myofibers are more genetically determined, therefore less affected from maternal environment (Maltin *et al*., 2001). However, there is some evidence that primary fibers may also be affected from prenatal conditions in pigs (Rehfeldt *et al*., 2001).

Studies carried out in porcine skeletal muscle indicate that the smallest littermate due to the low nutrient situation in utero contains more connective tissue elements (Chelland, 2001; Karunaratne *et al*., 2005). There is some evidence that sheep fetuses possess more perirenal adipocytes when maternal restricted diet is

* Corresponding author: berjan@istanbul.edu.tr
0030-9923/2016/0002-0447 $ 8.00/0

applied (Symonds *et al*., 2003; Bispham *et al*., 2005). Meat animals are grown up for their skeletal muscle. Connective tissue is an important parameter to the meat industry as an increased amount of connective tissue may affect meat toughness with lower meat quality whereas increased muscle fiber number indicates better meat quality (Karunette *et al*., 2005; Du *et al*., 2010a).

Hill sheep breeds managed extensively for many generations result in better adaptation for harsh environments and are superior in survival whereas lowland sheep breeds managed intensively for greater production of meat are inferior to survive in harsh environments compared to the hill breeds (Dwyer *et al*., 2005).

The objective of this study was to investigate the effect of maternal undernutrition in early gestation on skeletal muscle development, especially primary muscle fiber and connective tissue content of 65 day old fetus in both hill and lowland breeds of sheep.

MATERIALS AND METHODS

Animals and nutritional treatment

All procedures were conducted in Royal Veterinary College with local ethics approval of the Royal Veterinary College under the UK Animals Scientific Procedures Act.

Welsh mountain (Hill breed, n=14) and Dorset (Lowland breed, n=12) ewes were used respectively in 2 different experiments. Experimental design, nutritional treatments, tissue collection and methods were the same in both experiments. All procedures took place within the normal breeding season of Welsh Mountain and Dorset ewes with one year interval.

Ewes with the same body condition score (3.5, moderate) were used in both experiments. This score was obtained twelve weeks before mating according to the criteria of the Meat and Livestock Commission (1993). All ewes were housed in individual pens covered with wheat straws. Ewes were allowed free access to water and fed a complete pelleted diet providing 100% of their daily nuritional requirements based on the criteria of the Meat and Livestock Commission (1993). The complete diet provided 10.8 MJ metabolizable energy (ME) and 149.8 g crude protein per kg dry matter.

After estrus synchronization with progestagen impregnated sponges (60 mg medoxyprogesterone-acetate, Veramix; Upjohn Ltd) and a prostaglandin F2 analogue, Estrumate (0.5 ml i.m.; Schering Plough Animal Health), ewes were presented to the ram 48 h later. The day of mating constituted the first day of gestation (day 0). Pregnancy was confirmed by measuring plasma progesterone levels with ELISA on day 16 of gestation.

At 22 day of gestation (dg) ewes were randomly assigned to either control (C) or undernourished (U) group. Control group received the 100 % of their daily requirements according to the maternal body weight and gestational stage as reported in the Meat and Livestock (1993) guidelines. Undernourished group received the 70% of their daily requirements from 22 dg until 65 dg.

Tissue collection and analysing

All ewes were killed on day 65 of gestation by overdose injection of sodium pentabarbitone (NVS, Stoke on Trent) and fetuses are taken out. For all fetuses, the semitendinosus muscle (ST) was dissected, weighed and a complete mid belly transverse slice rapidly frozen in liquid nitrogen. 10 μm sections were cut on a cryostat and stained for alkali-stable ATPase after preincubation at pH 10.4 (Guth and Samaha, 1970) and hematoxylin-eosin stain. Muscle cross sectional are was measured. The number of primary fibers and nuclei were counted in frame areas which were approximately 2% and 3% of total cross sectional muscle areas, respectively. Although the fibers did not show any differentiation with ATPase staining at this stage, primary fibers were counted as large and central fibers. The percentage of light and dark parts of muscle area was measured using Kontron image analysis. Light area represented connective tissue, dark area represented muscle tissue. Nutritional groups were compared using Student's t test.

RESULTS

In the first experiment with a hill breed (Welsh Mountain), 11 (C, n=5; U, n=6) had singleton pregnancies. In the second experiment with a lowland breed (Dorset), 11 (C, n=5; U, n=6) had singleton pregnancies. To avoid any nutritional effects on fetal number, only singleton fetuses were used. Twin-bearing ewes from experiment 1 (C, n=2; U, n=1) and experiment 2 (C, n=1; U, n=0) were excluded from the study.

In the first experiment with a hill breed, there were no significant differences in fetal weight, fetal weight: fetal crown-rump length (CRL) ratio between C and U groups ($p>0.05$, Table I). ST weight, ST length, primary fiber number and nuclei number were not significantly different between groups ($p>0.05$, Table I). A similar trend was observed for total muscle cross sectional area, the percentage of muscle tissue and the percentage of connective tissue in fetal ST between C and U groups in a hill breed ($p>0.05$, Fig. 1).

In experiment 2 with a lowland breed, fetal weight and fetal weight: fetal CRL ratio were significantly higher in U group ($P<0.05$, Table II). ST weight, ST

Table I.- Comparison of some parameters between control and undernourished groups in a hill breed of sheep (Welsh Mountain).

Parameter	Control (n=5)	Under-nourished (n=6)	P-value
Ewe weight (kg)	50.2±0.37	50.83±0.31	NS
Fetal weight (g)	94.77±6.2	100.06±5.07	NS
Fetal CRL (cm)	15.6±0.13	15.85±0.11	NS
Fetal weight: Fetal CRL (g cm^{-1})	6.09±0.4	6.3±0.3	NS
Fetal ST weight	0.14±0.012	0.13±0.014	NS
Fetal ST length (*in situ*, mm)	21±0.27	20.83±0.11	NS
Primary fiber number	8202.4±971.9	9931.1±1857	NS
Nuclei number	101123.3± 16803.4	84947.6± 11644.4	NS

Values are the mean ± SEM
NS: no significant difference (P>0.05)
CRL: crown-rump length
ST: semitendinosus muscle

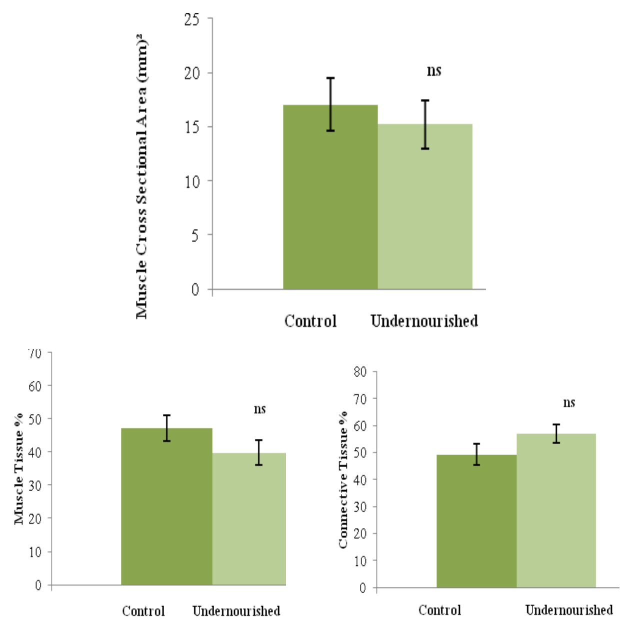


Fig. 1. Total muscle cross sectional area, muscle tissue % and connective tissue % of fetal semetendinosus muscle in control and undernourished hill breed of sheep (Welsh Mountain). For each parameter, the difference is non-significant (ns, P>0.05).

length, primary fiber number and nuclei number were not significantly altered between C and U groups (p>0.05, Table II). However, total muscle cross sectional area and the percentage of representing connective tissue were significantly higher whilst the percentage of representing muscle tissue was decreased significantly in U group in a lowland breed of sheep (P<0.05; Fig. 2).

Table II.- Comparison of some parameters between contol and undernourished groups in a lowland breed of sheep (Dorset).

Parameter	Control (n=5)	Under-nourished (n=6)	P-value
Ewe weight (kg)	63.78 ± 0.47	64.18 ± 0.38	NS
Fetal weight (g)	93.38 ± 2.5	109.1 ± 3.12	P<0.05
Fetal CRL (cm)	15.4 ± 0.26	15.6 ± 0.46	NS
Fetal weight: Fetal CRL (g cm^{-1})	6.08 ± 0.2	7.02 ± 0.04	P<0.05
Fetal ST weight	0.12 ± 0.005	0.15 ± 0.018	NS
Fetal ST length (*in situ*, mm)	21 ± 0.4	21.12 ± 0.32	NS
Primary fiber number	9172.6 ±1405.7	10104.5±1213.4	NS
Nuclei number	70827.8 ± 3901.2	95174 ±12868	NS

Values are the mean ± SEM
NS: no significant difference (P>0.05)
CRL: crown-rump length
ST: semitendinosus muscle

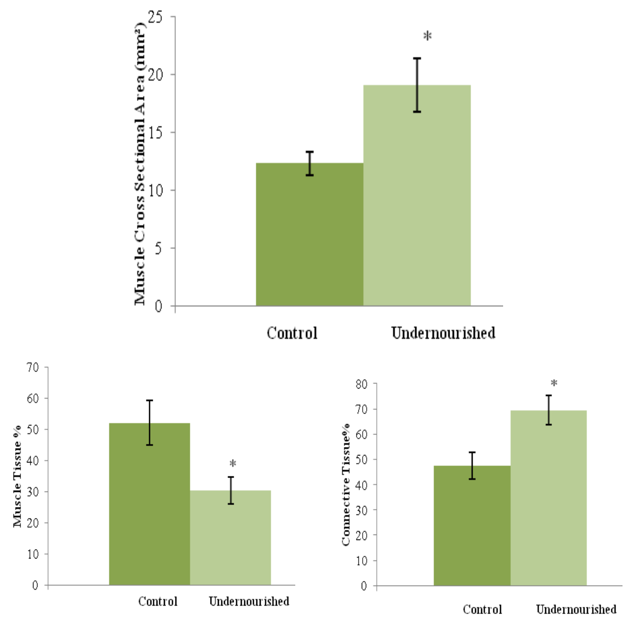
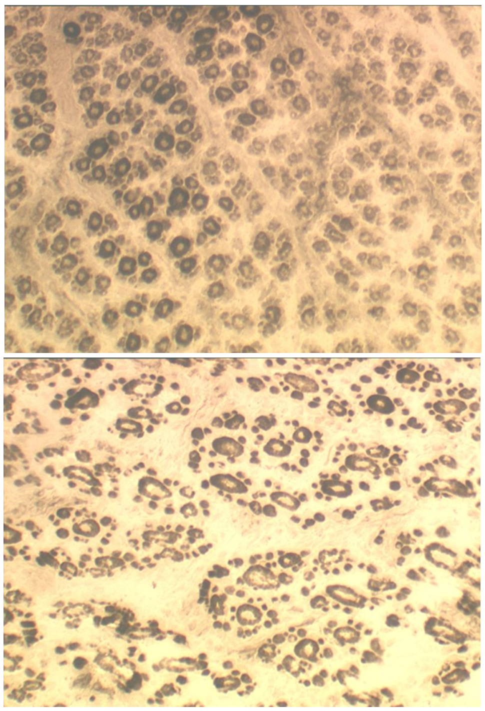


Fig. 2. Total muscle cross sectional area, muscle tissue % and connective tissue % of fetal semetendinosus muscle in control and undernourished lowland breed of sheep (Dorset). For each parameter, the difference is significant (*P<0.05).

Figure 3 illustrates the higher amount of connective tissue in cross section of fetal ST of the undernourished lowland breed (Dorset) of sheep.



A

B

Fig. 3. Myosin ATPase staining (PH 10.4) of fetal semitendinosus muscle at 65dg from control (A) and undernourished (B) lowland breed illustrating the higher amount of connective tissue of fetal muscle in the undernourished ewe (20x Objective).

DISCUSSION

In this study, it was found that maternal undernutrition increased the fetal weight, fetal weight: fetal CRL ratio, total muscle cross sectional area and the connective tissue content in fetal ST of lowland (Dorset) but had no any significant effect on fetal weight or fetal ST parameters of hill breed (Welsh Mountain) of sheep.

In the first experiment with a hill breed there was no significant difference in fetal parameters between C and U groups. Dwyer *et al*. (2005) reported that hill ewes had larger caruncles in uterus as a result of breed adaptation to cope with poor nutrition during pregnancy thereby increasing the efficiency with which nutrients are transferred to the fetus. Mild and severe maternal undernutrition didnot have any effect on fetal parameters such as fetal body weight and fetal CRL in Welsh mountain ewes (Demirtas and Ozcan, 2012a). Rooke *et al*. (2010) also reported that undernutrition in early to mid gestation didnot have any effect on birth weight of Blackface lamb, also known as a hill breed. Therefore, the lack of effect of undernutrition on fetal parameters in our first experiment with Welsh Mountain was consistent with these previous studies. Fetuses of hill breed are better adapted to harsh prenatal environment for survival. In experiment 2 with lowland ewes, fetal weight and fetal weight: fetal CRL ratio were significantly higher in U group than C group. Fetal weight of near term lowland fetuses at 125 dg was not affected by maternal nutrition (Asworth *et al*., 2011). Rooke *et al.* (2010) reported that birth weights of lowland lambs from undernourished mothers were lighter. Our study was conducted from fetuses at 65dg where placental hyperplasia was very high in contrast to the previous studies. Steyn *et al.* (2001) reported that undernutrition increased the growth of fetal side of placenta in undernourished lowland ewes. The increase in fetal weight of U group in this study with lowland breed might be result of short term compensation for fetus to survive. These fetuses from U group may lose weight in the later stages of gestation after placental hyperplasia terms at 80 dg weight.

In experiment 1 with a hill breed sheep, there was no significantly difference in total muscle cross sectional area and the percentage of connective tissue content between C and U groups. However, in experiment 2 with Dorset, a low land breed, there was a significant increase in total muscle cross sectional area and the percentage of connective tissue content in fetal muscle in U group. Undernutrition seemed to cause higher connective tissue content in place of muscle content. The increased connective tissue content was also observed in nutritionally disadvantaged smallest porcine fetal muscle (Clelland, 2001; Karunaratne *et al*., 2005). Muscle cells and connective tissue cells are derived from a common mesencymal stem cells (Sordella *et al*., 2003), which can be committed to a myogenic lineage or connective tissue cell types such as fibroblasts and adipocytes. Clase *et al.* (2000) reported that connective tissue fibroblasts in the limb define the regions in which muscle fibers do not differentiate. Switching the committed stem cells from myogenesis to fibrogenesis or adipogenesis are competitive process and reduce muscle fiber number while increasing fat and connective tissue in muscle (Yan *et al*., 2013; Du *et al*., 2010b; Tong *et al*., 2009). In our study at 65 dg primary fiber number is not affected in U group. This is consistent with primary fibers being more genetically determined and are not affected by maternal undernutrition (Stickland *et al*., 2000). Primary fibers form till 50 dg, whereas secondary fibers begin to form around 60 dg at first rapidly then slower up to 140 days of gestation (Ashmore *et al*., 1972). Therefore secondary muscle fiber formation thus total muscle fiber formation is not completed at 65 dg. The higher connective tissue content might be the result of downregulation of myogenesis (*i.e*. secondary muscle fiber development) while upregulation of fibrogenesis and adipogenesis. Fat and connective tissue components have also been suggested as a default pathway when muscle is unable to form (Kablar *et al*., 2003).

In summary, nutrient restriction in early gestation has increased the fetal weight and total muscle cross-sectional area by increasing connective tissue content of fetal muscle in a lowland breed but does not have any significant effect on fetal growth or fetal muscle parameters in a hill breed at 65 days of gestation. This increase in connective tissue of muscle may continue till the birth where secondary muscle fibers are completed to form. This study has focused on the fetal skeletal muscle at 65 dg but further investigation should be carried out in the skeletal muscles of near term fetuses and newly born lambs of both breeds of sheep.

ACKNOWLEDGEMENT

I would like to thank Prof. Dr. N.C. Stickland from Royal Veterinary College, University of London for providing animals.

REFERENCES

Ashmore, C.R., Robinson, D.W., Ratrayy, P.V. and Doerr, L., 1972. Biphasic development of muscle fi bers in the fetal lamb. *Exp.Neuol.,* **37:** 241-255.

Asworth, C.J., Dwyer, C.M., McLlvaney, K., Werkman, M. and Rooke, J.A., 2011. Breed differences in fetal and placental development and feto-maternal amino acid status following nutrient restriction during early and mid pregnancy in Scottish Blackface and Suffolk sheep. *Reprod. Fertil. Dev.,* **23**: 1024-1033.

Beermann, H., Cassens, R.G. and Hausman, G.J., 1978. A second look at fiber type differentiation in porcine skeletal muscle. *J. Anim. Sci.,* **46:** 125-132.

Bispham, J., Gardner, D.S., Gnanalingham, M.G., Stephenson, T., Symonds, M.E. and Budge, H., 2005. Maternal nutritional programming of fetal adipose tissue development: differential effects on messenger ribonucleic acid abundance for uncoupling proteins and peroxisome proliferator-activated and prolactin receptors. *Endocrinology,* **146:** 3943-3949.

Clase, K.L., Mitchell, P.J., Ward, P.J., Dorman, C.M., Johnson, S.E. and Hannon, K., 2000. FGF5 stimulates expansion of connective tissue fibroblasts and inhibits skeletal muscle development in the limb. *Dev. Dyn.,* **219:** 368–380.

Clelland, A., 2001. *Intra-litter variation in early porcine muscle development.* Ph.D. thesis, The Royal Veterinary College, University of London. Available from: Library of Royal Veterinary College.

Demirtas, B. and Ozcan, M., 2012a. The effect of maternal undernutrition on muscle development in the ovine fetus. *Turk. J. Vet. Anim.* Sci., **36:** 297-303.

Demirtas, B. and Ozcan, M., 2012b. A study on ovine muscle development. *J. Fac. Vet. Med. Ist. Univ.,* **38:** 97-106.

Du, M., Tong, J., Zhao, J., Underwood, K.R., Zhu, M.J, Ford, S.P. and Nathanıels, P.W., 2010a. Fetal programming of skeletal muscledevelopment in ruminant animals. *J. Anim. Sci.,* **88:** E51-E60.

Du, M., Yan, X., Tong, J.F., Zhao, J. and Zhu, M.J., 2010b. Maternal obesity, inflammation, and fetal skeletal muscle development. *Biol. Reprod.,* **82:** 4-12.

Dwyer, C.M., Madgwick, A.J.A., Ward, S.S. and Stickland, N.C., 1995. Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea pig. *Reprod. Fertil. Dev.,* **7:** 1285-1292.

Dwyer, C.M. and Lawrence, A.B., 2005. A review of the behavioural and physiological adaptations of hill and lowland breeds of sheep that favour lamb survival. *Appl. Anim. Behav. Sci.,* **92:** 235-260.

Guth, L. and Samaha, F.J., 1970. Research note: procedure for the histochemical demonstration of actomyosin atpase. *Exp. Neuol.,* **28:** 365-367.

Kablar, B., Krastel, K., Tajbaknsh, S. and Rudnicki, M.A., 2003. Myf5 and MyoD activation define independent myogenic compartments during embriyonic development. *Dev. Biol.,* **258:** 307-318.

Karunaratne, J.F., Ashton, C.J. and Stickland, N.C., 2005. Fetal programming of fat and collagen in porcine skeletal muscles. *J. Anat.,* **207:** 763-768.

Maltin, C.A., Delday, M.I., Sinclair, K.D., Steven, J. and Sneddon, A.A., 2001. Impact of manipulations of myogenesis in utero on the performance of adult skeletal muscle. *Reproduction,* **122:** 359-374.

Meat and Livestock Comission, 1993. *Meat and livestock commission sheep year book.* Meat and Livestock Commission, Milton Keynes, UK.

Rehfeldt, C., Kuhn, G., Vanselow, V., Furbass, R., Fidler, I., Nurnber, G., Clelland, A.K., Stickland, N.C. and Ender, K., 2001. Maternal treatment with somatotropin during early gestation affects basic events of myogenesis in pigs. *Cell and Tissue Res.,* **306:** 429-440.

Rooke, J.A., Houdiijk, J. G. M., McLlvaney, K., Asworth, C.J. and Dwyer, C.M., 2010. Differential effects of maternal undernutrition between days 1 and 90 of pregnancy on ewe and lamb performance and lamb parasitism in hill and lowland breeds. *J. Anim. Sci.,* **88:** 3833-3842.

Russell, R.G. and Oteruelo, F.T., 1981. An ultrastructural study of the differentiation of skeletal muscle in the bovine fetus. *Anat. Embryol.,* **162:** 403-417.

Sordella, R., Jiang, W., Chen, G.C., Curto, M. and Settleman, J., 2003. Modulation of Rho GTPase signalling regulates a switch between adipogenesis and myogenesis. *Cell,*

113: 147–158.

Steyn, C., Hawkins, P., Saito, T., Noakes, D.E., Kingdom, J.C.P. and Hanson, M.A., 2001. Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. *Eur. J. Obstet. Gynecol. Reprod. Biol*., **98:** 165-170.

Stickland, N.C., 1978. A quantitative study of muscle development in the bovine fetus (*Bos indicus*). *Anat., Histol. Embryol.,* **7:** 193-205.

Stickland, N.C., Demirtas, B., Chelland, A.K. and Ashton, C., 2000. Genetic and nutritional influences on muscle growth in farm animals. *Comp. Biochem., Physiol., A.,* **126:** S141.

Stickland, N.C. and Goldspink, G., 1973. A possible indicator muscle for the fiber content and growth characteristics porcine muscle. *Anim. Prod.,* **16:** 135-146.

Symonds, M.E., Gopalakrishnan, G., Bispham, J., Pearce, S., Dandrea, J., Mostyn, A., Ramsay, M.M. and Stephenson, T., 2003. Maternal nutrient restriction during placental growth, programmingof fetal adiposity and juvenile blood pressure control. *Arch. Physiol. Biochem.,* **111:** 45–52.

Tong, J.F., Yan, X., Zhu, M.J., Ford, S.P., Nathanielsz, P.W. and Du, M., 2009. Maternal obesity down regulates myogenesis and β-catenin signaling in fetal skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.,* **296:** E917–E924.

Ward, S.S. and Stickland, N.C., 1991. Why are fast and slow muscles differentially affected during prenatal undernutrition? *Muscle Nerve,* **14:** 259-267.

Yan, X., Zhu, M.J., Dodson, M.V. and Du, M., 2013. Developmental programming of fetal skeletal muscle and adipose tissue development. *J. Genom.,* **1:** 29-38.

Zhu, M.J., Ford, S.P., Nathanielsz, P.W. and Du, M., 2004. Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. *Biol. Reprod*., **71:** 1968-1973.