Detection of giant fibres in undernourished fetal sheep muscle

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Abstract

During an investigation the occurrence of giant muscle fibres was noted in fetal sheep skeletal muscle. The opportunity was, therefore, taken to clarify the histochemical and ultrastructural characteristics of these fibres and to understand if environmental stress such as maternal undernutrition during gestation affects the occurrence of these giant fibres in fetal sheep muscle. Welsh mountain ewes were randomly assigned to either the control group or the nutrient restricted group. The restricted animals received 50% of their daily nutritional requirement from the time of conception until 70 days of gestation and then 100% of their daily nutritional requirement thereafter. The control animals were fed 100% of their daily nutritional requirement for the entire period of gestation. The ewes were killed at 126±1 day of gestation by an intravenous injection of pentobarbitone. For singleton fetuses from each group the semitendinosus muscle was dissected, stained for the examination of histochemical and ultrastructural properties both by light microscope and transmission electron microscope. All singleton fetuses from both control and restricted groups were shown to possess giant fibres in their muscles. The giant fibres were round in shape and larger than adjacent normal fibres. They showed generally strong ATPase-alkaline positive reaction. Regular hexagonal array of myofilaments was absent in the giant fibres. Sarcomer length was shorter than normal fibres. However, there was no significant difference in the number of giant fibres between the control and restricted groups (P>0.05). Maternal undernutrition did not seem to have any effects on the occurrence of giant fibres in fetal sheep muscle. Giant fibres are present in fetal muscle and may result from defects in the developing muscle fibres leading to structural and metabolic anomalies within the fibres.

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Introduction

Giant fibres are mostly studied in pigs (Handel and Stickland, 1986; Sosnicki, 1987; Severini and Loschi, 1997; Fazarinc et al., 2002; Schubert-Shopmeyer et al., 2008). Apart from pig, giant fibres were also observed in poultry (Sosnicki et al., 1988[a,b]; Soike and Bergmann, 1998; Remington et al., 2000; Mammoli et al., 2004) and cattle (Sink et al., 1986). Giant fibres are muscle fibres characterized by peculiar traits when observed under a light microscope (Miraglia et al., 2006). They are generally round in shape and larger than normal fibres. Because of their enlarged size, they have a compressing effect on surrounding muscle fibres. The histochemical profile of giant fibres as observed by various studies has varied significantly. Giant fibres have been classified as alkaline ATPase positive (Sink et al., 1986), negative (Solomon and Eastridge, 1987) or both (Handel and Stickland, 1986; Sosnicki et al., 1988[a]).

Although different explanations for the origin of giant fibres have been hypothesized, the aetiology of giant fibres is not yet fully understood. Some regard it as a post-mortem phenomenon of normal skeletal muscle (Sink et al., 1986; Remington et al., 2000), while others suggest an artefact due to muscle biopsy procedure (Carpenter and Karpati, 1984; Gibala et al., 1995; Roth et al., 1999; Roth et al., 2000). Giant fibres were frequently observed in muscles from stress-susceptible pigs (Bader, 1987; Solomon et al., 1998; Fiedler et al., 1999). Moreover, reduced adaptive abilities to environmental conditions can cause stress and trigger the formation of giant fibres in pig (Karsrpazky et al., 2010).

Meat animals are raised for their skeletal muscles. Muscle fibre formation occurs prenatally and is affected by genetic and intrauterine environmental factors. There is no net increase in total number of muscle fibres in many mammalians after birth (Zhu et al., 2004; Rehdelt and Kuhn, 2006). Maternal undernutrition reduces muscle fibre number in fetal skeletal muscle (Ward and Stickland, 1991; Dwyer et al., 1995; Du et al., 2010).

Animals which contain low number of muscle fibres in their muscles also produce carcases with poor meat quality (Stickland et al., 2000; Rehfeldt and Kuhn, 2006; Du et al., 2010). The occurrence of giant fibres was also shown to correlate with poor meat quality in pigs and poultry in terms of pale, soft, exudative (PSE) conditions (Klosowska et al., 1979; Fiedler et al., 1999; Fiedler et al., 2004; Schubert-Shopmeyer et al., 2008). Furthermore, piglets born with low number of muscle fibres caused by maternal reasons tend to have giant fibres and show poor meat quality in their carcases (Rehfeldt, 2005).

During an investigation into maternal nutrition on fetal sheep muscle (Demirtas and Ozcan, 2012) the occurrence of giant muscle fibres was noted in fetal sheep muscle. The opportunity was, therefore, taken to clarify the histochemical and ultrastructural characteristics of these fibres and to understand if environmental intrauterine stress such as maternal undernutrition can cause the possible formation of giant fibres in fetal sheep muscle.

The aim of the present investigation was to characterize giant fibres in fetal sheep muscle and to understand if maternal undernutrition has any effect on the occurrence of giant fibres.

Materials and Methods

Animals: This study was conducted in UK and all procedures were carried out in the laboratories of Royal Veterinary College, London with local ethics approval of the Royal Veterinary College in accordance with the regulations of the UK Home Office Animals (Scientific Procedures) Act, 1986.

Welsh mountain ewes of uniform age, weight and body condition score were mated with the same rams during the normal breeding season (November). Fourteen ewes were randomly assigned to either the control group (C, n: 7) or the restricted group (R, n: 7). The ewes were mated after estrus synchronization. The day of mating constituted the first day of gestation (dg). Progesterone levels in blood samples were measured with ELISA at 16 dg. Ewes with low levels of progesterone were mated with the same ram again. The ewes were scanned with ultrasonography at 60 dg. Since singleton fetuses were used, twin-bearing ewes (C, n: 2; R, n: 2) were excluded from the study.

Nutritional treatment: A hierarchical feeding system exists within a group of sheep; in order to permit specific regulation of individual nutritional intake, the animals used in this study were housed in individual pens. The floors of all pens were covered with wood shavings. The animals were allowed free access to water and were fed a complete pelleted diet. The diet consisted of barley, wheat, cooked cereal meal, micronized full-fat soya, grass meal, molasses, chopped straw, calciumcarbonate, dicalcium phosphate, salt, and a sheep vitamin/mineral supplement. It provided 10.81 MJ/kg of metabolizable energy and 149.8 g/kg of crude protein, and it contained 88.4% dry matter. The control animals were fed a complete pelleted diet according to Agricultural Food and Research Council (AFRC, 1993) recommendation. The restricted animals received 50% of their daily nutritional requirement (DNR) from 0 dg until 70 dg and then 100% of their DNR thereafter. The control animals were fed 100% of their DNR for the whole of gestation. Post-mortem studies were carried out at 126±1 dg. The animals were killed by intravenous injection of pentobarbitone.

Sample collection, preperation and histochemistry: Singleton fetuses from each group (C, n:5; R, n:5) were used. The semitendinosus muscle (ST) was dissected and a complete mid belly transverse slice was rapidly frozen in liquid nitrogen. Ten-micrometre sections were cut on a cryostat and stained for alkali-stable ATPase after preincubation at pH 10.4 (Guth and Samaha, 1970). For each fetus, total cross-sectional area of the muscle was determined. The numbers of giant fibres and total fibre numbers were counted in the frame areas.

The frame transversed areas the muscle to take into account differences in the deep and superficial parts of the muscle; this was approximately

3% of the total cross-sectional muscle area. These data were used to estimate the total number of fibres, total number of giant fibres and percentage of giant fibres. Kontron image analysis (KS300, Zeiss, Germany) was used for all measurements.

Transmission Electron Microscopy (TEM): Adjacent part of each muscle was fixed in Karnovsky fixative (4% parafolmaldehyde, 5% glutaraldehyde in 0.1 sodium cacodylate buffer; pH 7.4). The specimens were rinsed in 0.1 M cacodylate buffer, post fixed for 1 hour in 1% osmium tetroxide in cacodylate buffer, dehydrated in increasing concentrations of ethanol (30-100%), rinsed in propylene oxide and embedded in resin. The resin-embedded sections were cut using an ultra microtome. Thin transverse sections (90 nm) were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with TEM (EM 10B, Zeiss, Germany). Giant fibres in each muscle were examined ultrastructurally by TEM.

Statistical analysis: Nutritional groups were compared using Windows SPSS 16.0 with the independent samples t-test. All values are presented as means±standard error of mean (SEM). *P* value ≤ 0.05 was set for statistical significance.

Results

Giant fibres in fetal ST were observed in all the C and R animals (fig 1). In light microscope, the giant fibres were round in shape and larger than the adjacent normal fibres. They had a compressing effect on the surrounding fibres and a homogenous appearance. They showed generally strong ATPase-alkaline positive reaction (Fig. 1). This resulted in the giant fibres being darker than the other normal positive fibres. Based on ATPase staining the giant fibres were classified as type II or fast fibres. The giant fibres were distributed generally near the periphery of the fasciculus but they were not observed in every fasciculus. In two of the restricted animals with a high incidence of giant fibres (1.94% and 2.09%) they were distributed throughout the muscle. The transverse sections showed that regular hexagonal array of myofilaments was absent in the giant fibres (Fig. 2-right). The longitudinal sections showed that sarcomere length was shorter than normal fibres (Fig. 3-right). The giant fibres were clearly hypercontracted fibres. There was no significant difference in the giant fibre number between the C and R groups (Table 2), but in the two R animals a high incidence of giant fibres was observed (1.94% and 2.09%; Table 1). The total fibre number was significantly lower in the R group than the C group (Table 2). However, since these data were published in a previous paper (Demirtas and Ozcan, 2012), it is not discussed here.

Table 1   Total fibre number, giant fibre number and percentage of giant fibre in fetal *semitendinosus* muscle

Group	Total fibre number	Giant fibre number	Giant fibre %
Control	445052	3531	0.79
Control	497528	5127	1.03
Control	453354	3757	0.83
Control	449824	3575	0.80
Control	650650	5014	0.77
Restricted	404430	2078	0.51
Restricted	326117	1590	0.49
Restricted	400063	4289	1.07
Restricted	359357	7470	2.09*
Restricted	457855	8873	1.94*

*represents high incidence of giant fibre in two restricted group.

Table 2   Comparison of total fibre number and giant fibre number in fetal *semitendinosus* muscle between control and restricted groups

	Control n:5	Restricted n:5	*P* value
Total fibre number	499280±38956	389560±22286	P=0.04
Giant fibre number	4201±358	4860±1444	P=0.67

All values are mean±SEM.
SEM: Standard error of mean

Discussion

To our knowledge our finding of giant fibres in fetal sheep muscle is the first. Giant fibres are mostly observed in postnatal farm animals such as pig (Handel and Stickland, 1986; Sosnicki, 1987; Severini and Loschi, 1997; Fazarinc et al., 2002; Schubert-Shopmeyer et al., 2008), poultry (Sosnicki et al., 1988 [a,b]; Soike and Bergmann, 1998; Remington et al., 2000) and cattle (Sink et al., 1986).

All fetuses from both control and restricted groups in this study were shown to possess giant fibres in their muscles. In previous reports, Handel and Stickland (1986) reported that 85 percent of animals possessed giant fibres.

The giant fibres here were not different from previous studies recorded in literature regarding their rounded or ovoid shape, larger cross-section than other normal fibres and compressing effect on surrounding fibres (Fazerinc et al., 2002; Velotto et al., 2010). The giant fibres had a strong reaction with ATPase-alkaline

staining (Fig. 1). This is consistent with Sink et al. (1986). The abnormally high ATPase activity (Fig. 1) shows very chronic hypercontraction within the giant fibres. Sink et al. (1986) proposed that supercontraction might also compress myofibrils within myofibre and this compression could explain the presence of stronger ATPase reaction of giant fibres as a higher density of myofibrillar ATPase per unit area. Handel and Stickland (1986) suggested that the extent of hypercontraction within giant fibres was sufficiently chronic to induce compensatory myofibrillar proliferation and enhance ATPase activity. The severe

contraction of fibres could explain the larger size of giant fibres than normal fibres. This hypercontraction of giant fibres was also reflected ultrastructurally in this study. In TEM, the transverse and longitudinal sections confirmed that the giant fibres were hypercontracted fibres. The disarray of myofilaments, disrupted myofibrillar banding as extremely widened Z lines, loss of A, I and M lines, and shortened sarcomere length are suggested as hypercontracted fibres (Fig. 3-right). These electron microscopic results are consistent with Handel and Stickland (1986).
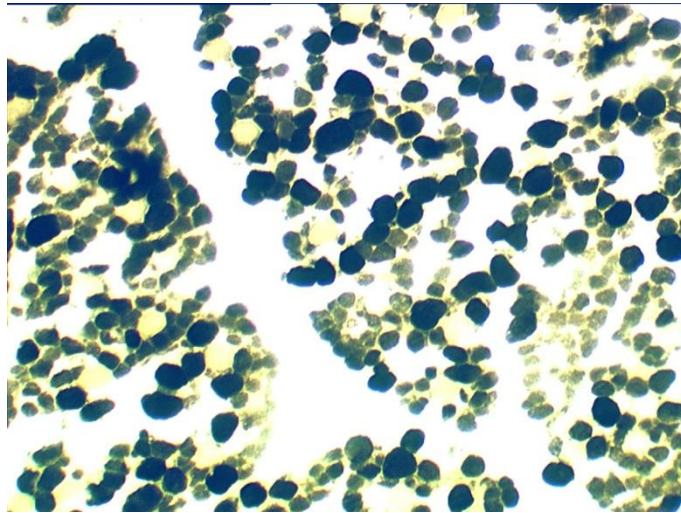

Figure 1   Myosin ATPase staining (PH 10.4) of fetal *semitendinosus* muscle from 126±1 dg (X20 objective). The giant fibres show strong ATPase staining.
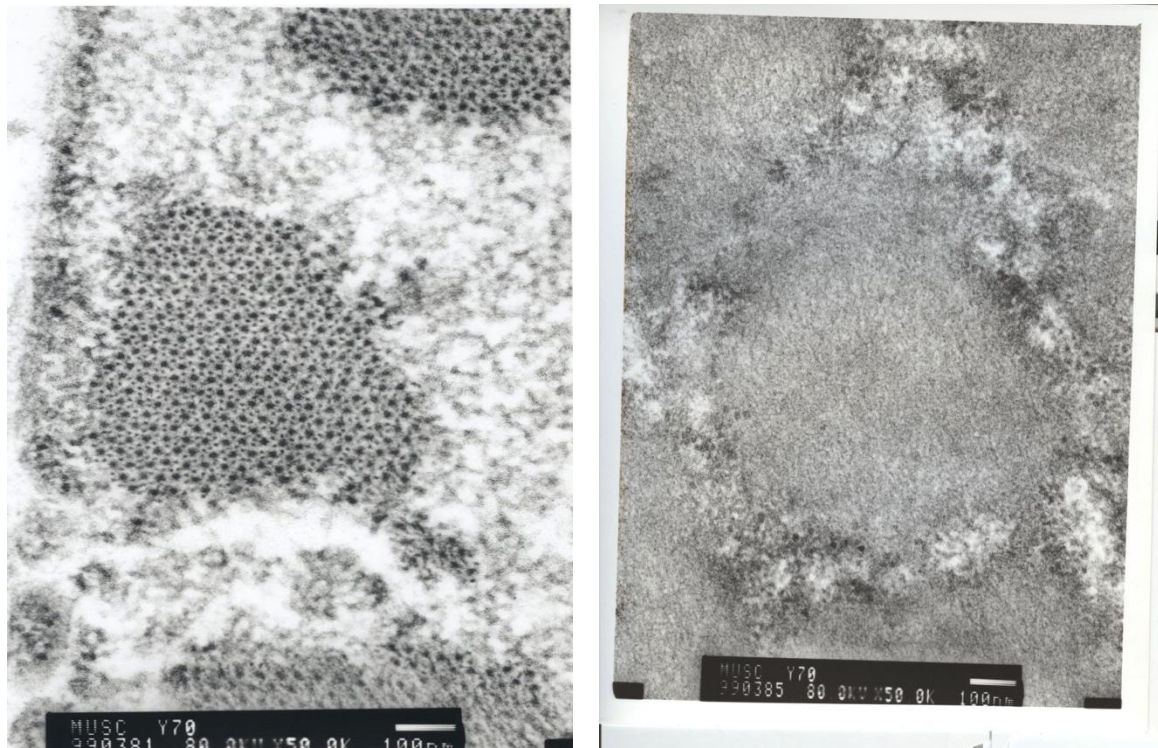

Figure 2   TEM section showing regular hexagonal arrangement of normal fibre (left). The giant fibres (right) have no regular hexagonal arrangement of myofibrils.
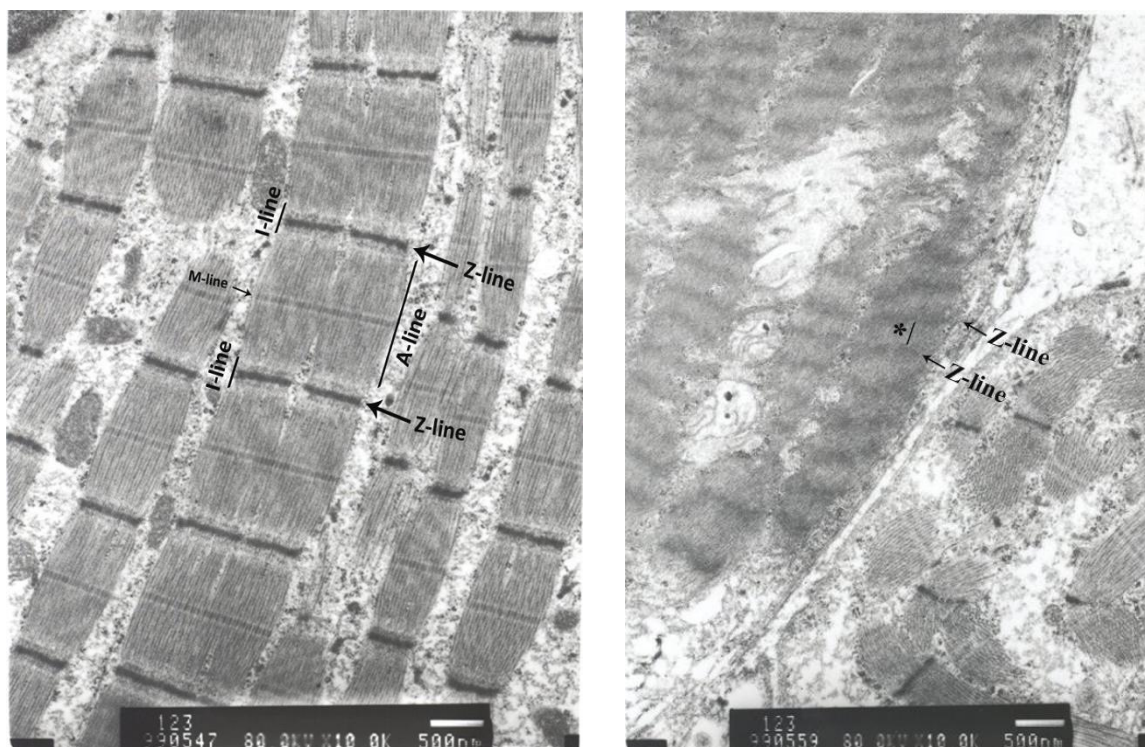
Figure 3 TEM section showing sarcomere length of normal fibre (left). ***:** Sarcomere length is shorter in the giant fibres (right) than the normal fibre.

Giant fibres have been associated with degenerative features (Sosnicki et al., 1988b; Sosnicki et al., 1989; Sosnicki et al., 1991) and certain muscular dystrophies (Sosniki, 1987). The present investigation did not observe any signs of degenerative changes in the samples. This is also consistent with Handel and Stickland (1986). Some authors (Carpenter and Karpati, 1984; Cherel et al., 1995; Roth et al., 2000) suggested that giant fibres were artefact due to biopsy. In our samples the post-mortem study was conducted on different days. Sosnicki et al. (1991) concluded that these hypercontracted fibres could not be artefact. Remington et al. (2000) observed giant fibres in postrigor but not in prerigor muscles. In the present study the samples were collected and processed immediately after slaughter before rigor mortis.

Giant fibres were observed in muscles from stress-susceptible pigs, which are prone to the development of pale, soft and exudative (PSE) muscles (Bader, 1987; Solomon et al., 1998). This study did not observe any signs of PSE meat quality from the muscle samples. Linke (1972) observed giant myofibres from both normal and PSE muscle. Handel and Stickland (1986) also proposed that the association between giant fibres and PSE muscle was unreal. Giant fibres were found in wild stress-resistant pig (Handel and Stickland, 1986; Solomon and Eastridge, 1987).

Sink et al. (1986) reported that giant fibre formation could be related to environmental, genetic and metabolic factors. In this investigation both control and restricted groups contained giant fibres. Maternal undernutrition did not seem to have any significant effects on the occurrence of giant fibre. It is suggested that giant fibres, since they are present prenatally, may result from defects in the developing muscle fibres leading to structural and metabolic anomalies within the fibres. These defects such as inadequate amount of sarcoplasmic reticulum, leaky membranosus of sarcoplasmic reticulum or deficiency in the production of ATP by the mitochondria can prevent relaxation of muscle and cause hypercontraction and subsequent formation of giant fibres.

Acknowledgements

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บทคัดย่อ

การตรวจพบเส้นใยกล้ามเนื้อขนาดใหญ่ในตัวอ่อนลูกแกะเนื่องจากภาวะขาดอาหาร

เบอจาน เดมิรัส

รายงานนี้ ศึกษาเส้นใยกล้ามเนื้อขนาดใหญ่ในตัวอ่อนแกะ ด้วยวิธีทางจุลกายวิภาคและฮิสโตเคมี เพื่อให้เข้าใจถึงการเปลี่ยนแปลง ของกล้ามเนื้อในภาวะความเครียด เช่น ภาวะขาดอาหารของแม่แกะในช่วงการตั้งท้อง ทำการศึกษาแม่แกะอุ้มท้องพันธุ์ Welsh mountain ในกลุ่มควบคุมและกลุ่มขาดอาหาร ซึ่งกลุ่มขาดอาหารจะได้รับสารอาหารร้อยละ 50 ของสารอาหารที่ต้องการจนถึง 70 วันของการตั้งท้อง และ 100% ในช่วงเวลาต่อมา ในขณะที่กลุ่มควบคุมจะได้รับสารอาหาร 100% ตลอดระยะเวลาการตั้งท้อง และสัตว์ทุกตัวจะถูกการุณฆาตใน 126±1 วันของการตั้งท้อง จากนั้นนำกล้ามเนื้อของตัวอ่อนของแต่ละกลุ่ม มาศึกษาลักษณะทางจุลกายวิภาค ด้วยกล้องจุลทรรศน์แสงขาว กล้องจุลทรรศน์อิเล็กตรอนและศึกษาทางฮิสโตเคมี ผลพบว่าตัวอ่อนทั้งสองกลุ่ม พบเส้นใยกล้ามเนื้อขนาดใหญ่ในมัดกล้ามเนื้อ มีรูปร่างกลม และขนาดใหญ่กว่าเส้นใยข้างเคียง และมี ATPase-alkaline ที่แข็งแรง แต่ไม่มีการจัดเรียงตัวแบบ hexagonal array ของ myofilaments เหมือนกล้ามเนื้อทั่วไป และมี Sarcomere ที่สั้นกว่ากล้ามเนื้อทั่วไป อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญของจำนวนเส้นใย กล้ามเนื้อขนาดใหญ่ในตัวอ่อนทั้งสองกลุ่ม (p>0.05) สรุปได้ว่าภาวะขาดอาหารในแม่แกะไม่มีผลต่อเส้นใยกล้ามเนื้อขนาดใหญ่ในกล้ามเนื้อ ตัวอ่อนแกะ โดยสาเหตุที่เกิดเส้นใยกล้ามเนื้อขนาดใหญ่อาจจะเกิดจากความผิดปกติในช่วงการพัฒนาของกล้ามเนื้อทำให้เกิดความผิดปกติ ของรูปร่างและเมตาโบลิสมของกล้ามเนื้อ

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