SPONTANEOUS AND EXPERIMENTAL MYOFIBRILLAR HYPOPLASIA AND ITS RELATION TO SPLAYLEG IN NEWBORN PIGS

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INTRODUCTION

Splayleg is a clinical term describing the inability of newborn pigs to stand and walk properly. This seems to be due to the fact that their limbs are splayed forwards or sideways as a result of muscular weakness (Thurley, Gilbert and Done, 1967). In consequence, these newborn pigs are unable to go and suckle. Moreover, constant contact with the floor rapidly leads to necrosis of the skin overlying pressure points. The final result is an unacceptably high neonatal mortality and increasing financial loss to the pig industry.

A variety of lesions and biochemical alterations have been proposed as the underlying pathological changes in this syndrome. The most consistent change is the presence of a so-called myofibrillar hypoplasia (Thurley and Done, 1969), in which the sarcoplasm is partly filled with glycogen instead of with contractile elements (Bradley, Ward and Bailey, 1980). However, myofibrillar hypoplasia also has been found in many clinically unaffected newborn pigs (Bradley *et al.*, 1980; Lukas, Pivnik, Straka, Rampochova, Stephanova, Kaman and Vitouch, 1982). The term congenital myofibrillar hypoplasia therefore should not be used as a synonym for porcine splayleg. Some authors have described degenerative changes in the muscles of newborn pigs with splayleg (Bergmann, 1976; Zelena and Jirmanova, 1979), but others have failed to detect any digenerative lesions (Bradley *et al.*, 1980; Bucek, Lukas, Pivnik and Kaman, 1982). The descrepancies in the literature reflect the obscurity of the pathogenesis of this syndrome.

Aetiologically, porcine splayleg is usually considered to be a multifactorial hereditary condition (Ward, 1978b), with a threshold and influenced by strong maternal effects (Sellier and Ollivier, 1982). Environmental effects may influence the threshold (Kohler, Cross and Ferguson, 1969). Porcine splayleg is thus a typical example of a multifactorial disease in which the appearance of clinical signs is highly unpredictable. As a result, an accurate estimate of the risk of splayleg at the level of the individual pig as well as at the level of the herd is still not possible.

These observations stress the need for increased efforts to clarify the

pathogenesis and develop new strategies for the eradication of porcine splayleg. One way to tackle this problem would be to reproduce experimentally the syndrome under controlled circumstances. Another way would be to study natural cases for the presence of aetiologically significant changes.

In the present study, myofibrillar hypoplasia of newborn pigs was reproduced experimentally and compared with natural cases.

<div align="center">MATERIALS AND METHODS</div>

Newborn Pigs with Splayleg–Group A

Twenty-five Belgian Landrace pigs with clinical splayleg, ranging in age from 24 h to 4 days, were used for pathological examination. The animals came from 12 different herds with splayleg problems. The percentage of affected newborn pigs in these herds ranged from 20 to over 60 per cent.

Recovered Pig–Group B

One 10-day-old recovered animal was also used in this study.

Control Newborn Pigs–Group C

For the selection of reliable control material, a line of purebred Belgian Landrace sows was continuously monitored over a period of 5 years for the absence of splayleg in their offspring. Samples were taken on several occasions in the last 3 years to check for the presence of myofibrillar hypoplasia (MFH) in the semitendinosus muscle of the newborn pigs. All these tests were negative. Five pigs from this strain, ranging in age from 24 h to 3 days, were used for pathological examination.

Experimental Newborn Pigs–Group D

For the experimental study, three pregnant sows from the MFH-controlled strain were used. They were treated with 100 mg of dexamethasone (Fortecortine, Bayer) by intramuscular injection every 24 h during the last 7 days of gestation. The sows were allowed to farrow spontaneously and produced litters of 8, 7 and 8 pigs born alive. Out of these three litters, four animals were used for pathological study. The others were examined clinically. All newborn pigs were allowed to suck their dam. At birth, none of these animals had clinical splayleg.

Collection of Samples

Twenty-four pigs of group A, four pigs of group C and three of group D (one from each litter) were used for morphological study. Laparotomy was performed on these animals under deep ether anaesthesia. The abdominal aorta was clamped at the level of the renal arteries and a catheter was introduced into the aorta behind the clamp. After a brief flushing with 9 per cent NaCl solution, the entire hindquarters were fixed by perfusion with 2 per cent paraformaldehyde and 2·5 per cent glutaraldehyde in 0·1 M cacodylate buffer. After fixation, specimens were taken from the M. biceps femoris, M. adductor, M. gracilis, M. semitendinosus and M. semimembranosus for histological study. Specimens from the same muscles and from the M. longissimus Dorsi of a 2-days-old splayleg pig, a 10-days-old recovered pig, a 3-days-old control pig and a 3-days-old experimental pig were taken for transmission electron microscopy (TEM).

For histochemical study, a further three pigs were killed without perfusion fixation of the hindquarters. One was a 2-days-old pig with splayleg, one was a 3-days-old control pig and one was a 10-days-old recovered animal. Specimens from these three animals were taken and frozen at −70 °C. The specimens were from the M. biceps femoris, M. adductor, M. gracilis, M. semitendinosus and M. semimembranosus.

Processing of the Samples

For histopathological study, longitudinal and cross-sections of muscle were embedded in paraffin wax, cut 4 μm thick and stained with haematoxylin and eosin (HE), periodic acid-Schiff and toluidine blue.

For histochemical study, cryostat sections of muscle were stained with acridine orange for DNA and RNA (Beerman and Cassens, 1977), with acetyl-thiocholine-ammonium sulphide for acetylcholinesterase (Koelle, 1951), methylene blue-sodium succinate for succinic dehydrogenase (Lillie, 1965) and ATP-cobalt chloride for acid preincubated ATPase (Guth and Samaha, 1970). All sections of the same muscle were stained in one batch.

For ultrastructural cytochemical study, one series of samples was post-fixed in 2 per cent OsO_4, dehydrated with acetone in a vacuum chamber and embedded in Spurr-medium as described earlier (Ducatelle, Coussement, Debouck and Hoorens, 1982). A second series of samples of the same tissues was not post-fixed in OsO_4, but dehydrated and embedded in the same manner as the first series. A third series of samples was treated with 0·8 per cent potassium hexacyanoferrate (II) before dehydrating and embedding (Degheele D., pers. commun.). A fourth series of samples was both post-fixed with OsO_4 and treated with potassium hexacyanoferrate (II).

Semi-thin sections were cut and stained with toluidine blue. Ultra-thin sections were cut and contrasted as follows: from each specimen one grid was contrasted with uranyl acetate, one was not contrasted, one was contrasted with both uranyl acetate and lead citrate and one was etched with 1 per cent periodic acid and contrasted with 5 per cent aqueous phosphotungstic acid (Quatacker, 1975).

RESULTS

On light microscopic examination, the muscle cells of the control pigs (group C) were seen to be in close apposition to one another. They were filled with myofibrils showing a regular cross-striation which was perpendicular to the long axis of the cells. There was almost no extramyofibrillar space (EMS). The cell nuclei were all at the periphery of the cells [Fig. 1(a)] and were 8 to 11 μm long and approximately 4 μm wide. On PAS staining, fine bands of PAS-positive material were seen between the myofibrils. These were more pronounced in the younger animals than in the 2- and 3-days-old pigs.

In the muscles of the pigs with clinical splayleg (group A) in contrast, there was an interstitial space of variable width. The space was very wide in the splayleg animals at 24 h of age, whereas in the 3- and 4-days-old splayleg animals it was much reduced. Myofibrils filled only part of the sarcoplasm in all splayleg pigs, leaving a significant EMS [Fig. 1(b)]. The extent of the EMS varied from pig to pig and from muscle to muscle. Nevertheless, the M. adductor often appeared to be most severely affected. There was no difference in the size and shape of the muscle cells and the muscle cell nuclei between splayleg animals and control animals. In contrast, the cross-striation of the muscle cells was remarkably different. In the splayleg animals, this cross-striation was often irregular and not perpendicular to the longitudinal axis of the cells. There also was more PAS-positive material in the muscle cells of the pigs with splayleg. This was most striking in the 24 h age groups. In semi-thin sections of tissue blocks treated with potassium hexacyanoferrate, the EMS was well demonstrated and appeared to be filled completely with a homogeneous material.
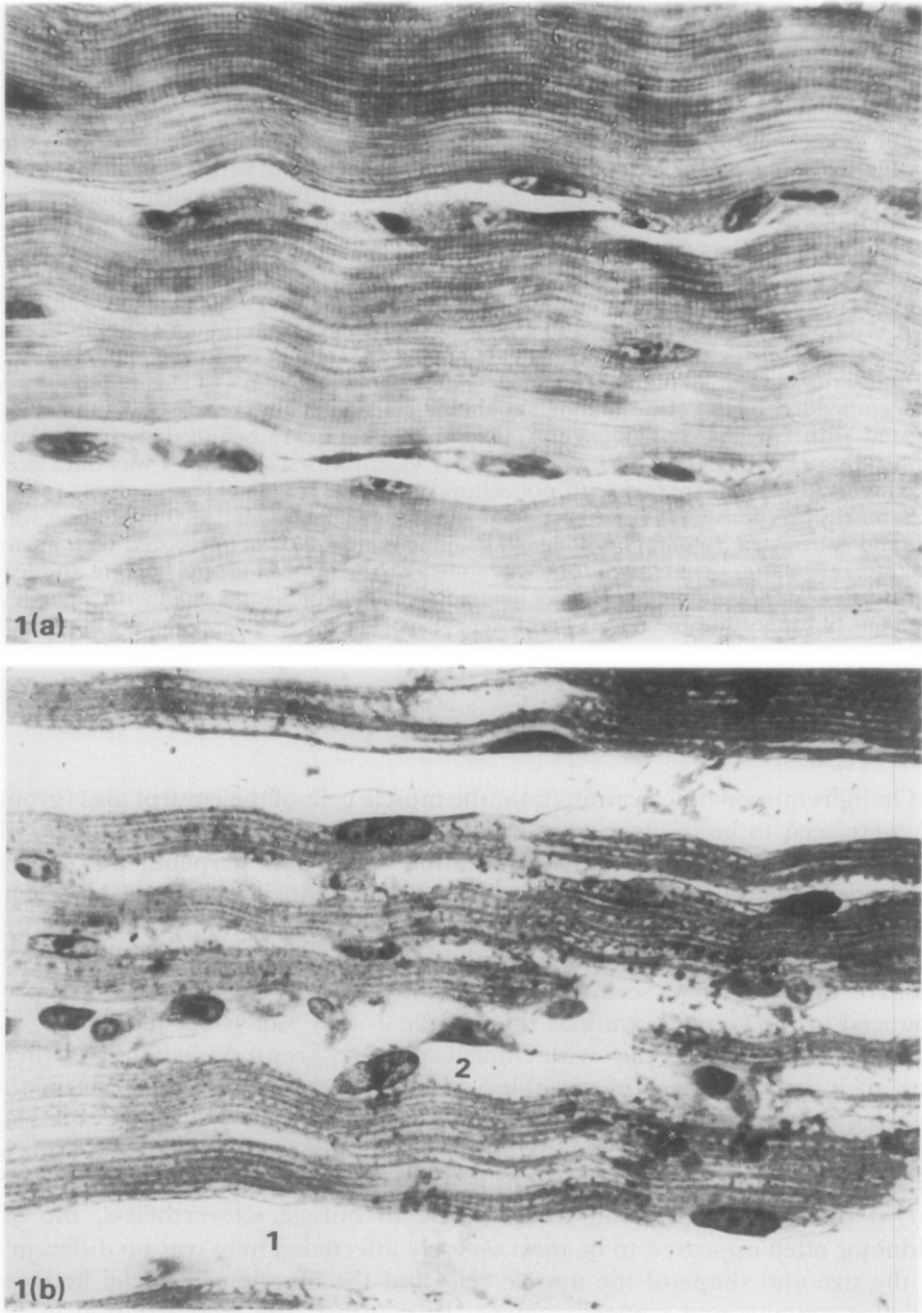
Fig. 1. Morphology of M. adductor in longitudinal section. Toluidine blue staining. × 1000.
   1(a) Control newborn pig (24 h) with well developed muscle fibres with little extramyofibrillar space and interstitial space. Cell nuclei are at the periphery. Cross-striation is regular and marked.
   1(b) Newborn pig (24 h) with splayleg. Note the wide intercellular space (1) and extramyofibrillar space (2). The cross-striation is not very well defined.

The pig that had recovered from splayleg had muscles that were similar in light microscopic aspects to those of the control pigs [Fig. 1(c)].

The experimental pigs (group D) all had myofibrillar hypoplasia, which on the whole seemed to be slightly less pronounced than in the natural splayleg cases. In the experimental pigs the intercellular space was moderately increased compared with the controls. The cross-striations of the myofibrils were more regular than in the pigs, with splayleg [Fig. 1(d)]. These lesions seemed to be most prominent in the M. adductor. On PAS staining, the EMS contained only a faint, scattered PAS-positive reaction. In semi-thin sections of blocks treated with potassium hexacyanoferrate, the EMS appeared empty.

Histochemical and enzyme-histochemical examination of muscles yielded the following results.

Succinic dehydrogenase activity was slightly higher in splayleg muscles.

Acid pre-incubated ATPase activity was high in type 1 muscle cells and low in type 2 muscle cells. The newborn pig with splayleg had only few type 1 fibres in its muscles; the control animal had more type 1 muscle fibres. These were homogeneously scattered between the type 2 fibres. The animal that had recovered from splayleg had more type 1 fibres.

Cholinesterase activity was limited to the neuromuscular endplates. These endplates (essentially the post-synaptic part) were demonstrated as brown to black dots situated on the edge of the muscle fibres. The endplates were found in groups of 4 to 10. There was no difference in the staining or in the localization of these endplates between the affected, the recovered and the non-affected pig.

Acridine orange staining produced a green fluorescence of muscle cell nuclei and an orange fluorescence of the cytoplasm. The nuclear fluorescence was similar for all muscles of all three pigs (groups A, B and C). The cytoplasmic fluorescence was intense in the muscles of the pig with splayleg, indicating a high concentration of RNA in the sarcoplasm [Fig. 2(a)]. In contrast, the recovered pig had a low sarcoplasmic fluorescence. The control pig had a faint positive sarcoplasmic fluorescence [Fig. 2(b)].

At the ultrastructural level, the muscles of the control pig appeared normal. The cytoplasm of the muscle cells was almost completely filled with myofilament bundles which showed a regular banding with well delineated Z-lines, approximately 8 nm wide. The A-band was approximately 1·65 µm wide. Between the myofilament bundles, small groups of γ-glycogen particles and rows of lipid droplets were regularly seen. Occasional lysosomes were seen to contain a few γ-glycogen particles. With phosphotungstic acid staining, the endomysium was seen to consist of coiled collagen fibres.

Electron microscopy of the muscles of the splayleg animal revealed that the myofilament bundles filled only part of the cytoplasm. The A-band had a mean length of 1·50 µm. The Z-band consisted of Z-band material, streaming Z-lines and irregular Z-lines approximately 10·8 nm wide. M-lines were often ill-defined. Splitting of myofilament bundles was frequently observed. Ribosome-studded thick (myosin) filaments were often seen obliquely tangential to a myofilament bundle (Fig. 3). Outside the myofilament bundles, the sarcoplasm contained an EMS which was delineated by the sarcolemma. After
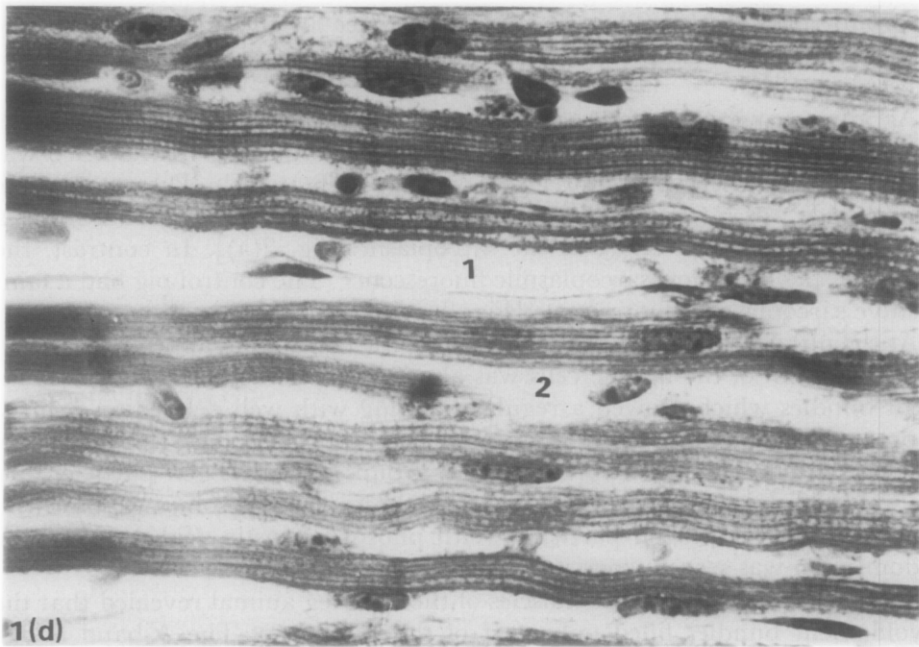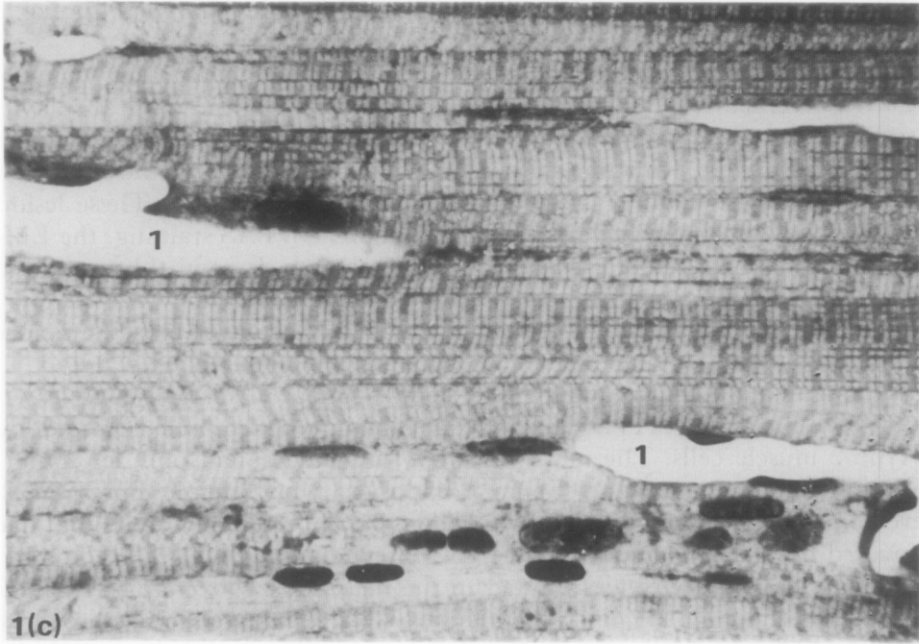
1(c) Ten-day-old pig, recovered from splayleg. The interstitium contains only empty capillaries
      (1). Muscle cell cross-striation is clearly defined.
1(d) Pig born (24 h) after experimental dexamethasone treatment of the sow. The interstitial
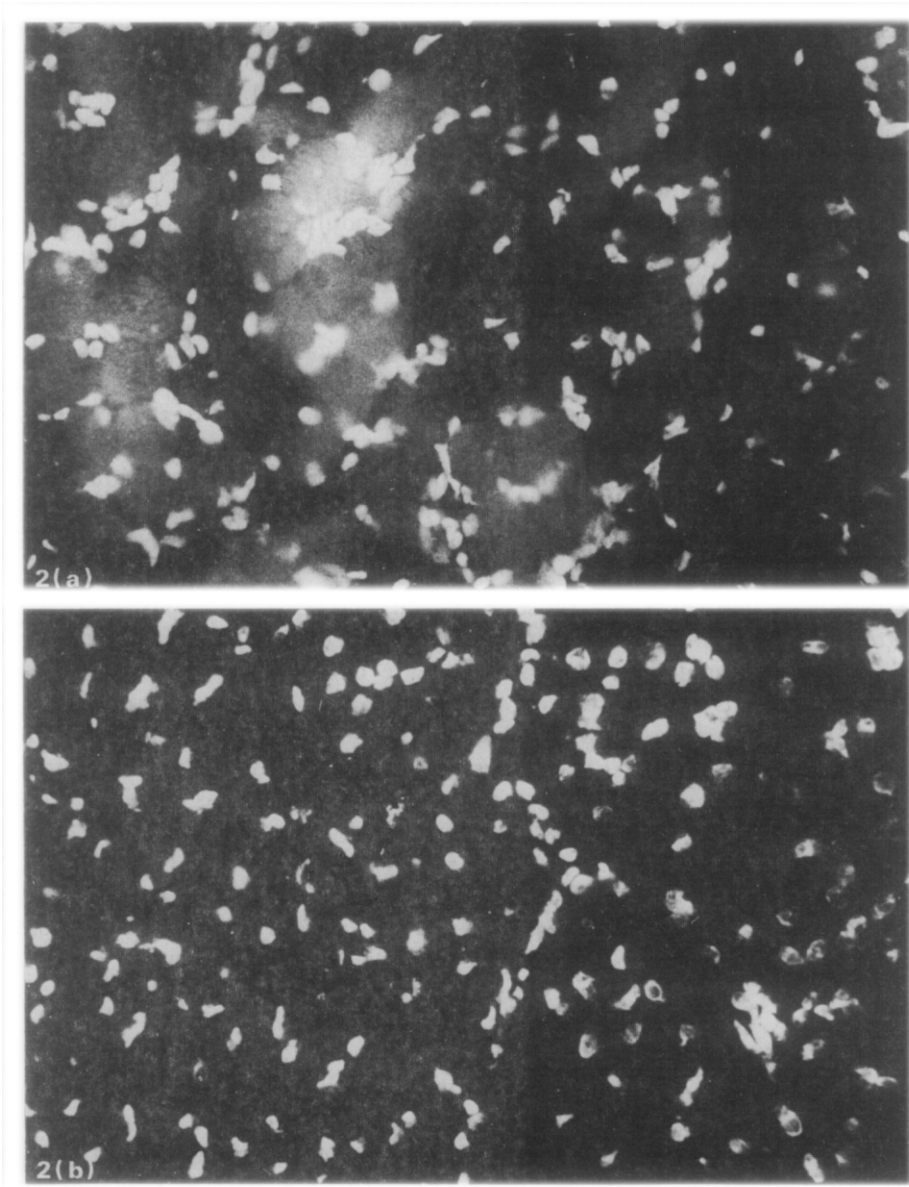   space (1) and the extramyofibrillar space (2) are wide.

Fig. 2. Acridine orange staining of M. adductor in cross-section. × 320.
2(a) Intense nuclear and sarcoplasmic fluorescence in the adductor muscle of a pig with splayleg, indicating a high content of DNA in the nucleus and RNA in the sarcoplasm.
2(b) In this control pig, a much reduced sarcoplasmic fluorescence is present indicating less RNA.

potassium hexacyanoferrate treatment, the EMS was seen to be filled almost completely with γ-glycogen particles (Fig. 4). In the sarcoplasm surrounding the peripherally located muscle cell nuclei, various organelles were present. These were mainly mitochondria, cisternae of endoplasmic reticulum, lysosomes and occasional Golgi cisternae. Lysosomes were also found between the glycogen particles of the EMS. In the EMS, groups of glycogen particles were regularly seen to be sequestrated in membrane-bounded organelles neighbouring the lysosomes (Fig. 4). Occasional fusion of lysosomes with the glycogen-containing organelles was seen. T-tubules were small and irregular. The endomysium was composed of coiled collagen fibres as in the control animal. There was a wide extracellular space containing scarce collagen fibrils.

In the 10-day-old pig that had recovered from splayleg, there was almost no EMS. Glycogen was demonstrated with the potassium hexacyanoferrate technique as small rims of γ-particles between the myofilament bundles and under the sarcolemma. Occasional secondary lysosomes and residual bodies were present. The A bands had a mean length of 1·65 μm and Z-lines were approximately 9 nm wide. Occasional lipid droplets were present between the myofilament bundles.

The ultrastructure of the muscles of the pig born after dexamethasone treatment of the sow was in many aspects similar to that of the muscles of the pig with natural splayleg. There was a wide EMS and so the myofilament bundles filled only part of the sarcoplasm. A-bands had a mean length of 1·55 μm and Z-lines were approximately 10 nm wide but were nevertheless clearly defined, as were the M-lines. Occasional splitting of myofilament bundles was seen. With potassium hexacyanoferrate, it was shown that the EMS only contained a few scattered γ-glycogen particles. In contrast, membrane-bounded organelles in the EMS were seen to contain many γ-glycogen particles (Fig. 5). With phosphotungstic acid staining, the limiting membranes of these organelles were shown to be characteristic of lysosomal membranes. Outside the EMS, other organelles appeared normal. T-tubules were small and irregular. The extracellular space was moderately widened.

DISCUSSION

Splayleg in newborn pigs has been a subject of intensive study for many years (for reviews see: Ward, 1978a, b; Coussement, Castryck, Ducatelle and Hoorens, 1984). Efforts have been made to detect histological, histochemical or biochemical changes which would explain the clinical signs and the pathogenesis of this syndrome. So far, no histochemical or biochemical parameters have been detected which are unequivocally associated with porcine splayleg.

The study of porcine splayleg is seriously hampered by its variable clinical expression, which is influenced by such aetiological factors as genetics, environment and maternal effects (Sellier and Ollivier, 1982). One morphological characteristic which is often associated with splayleg is myofibrillar hypoplasia (MFH) (Thurley and Done, 1969). Myofibrillar hypoplasia is usually considered to be an immaturity of muscle (Bradley *et al.*, 1980; Bucek *et*
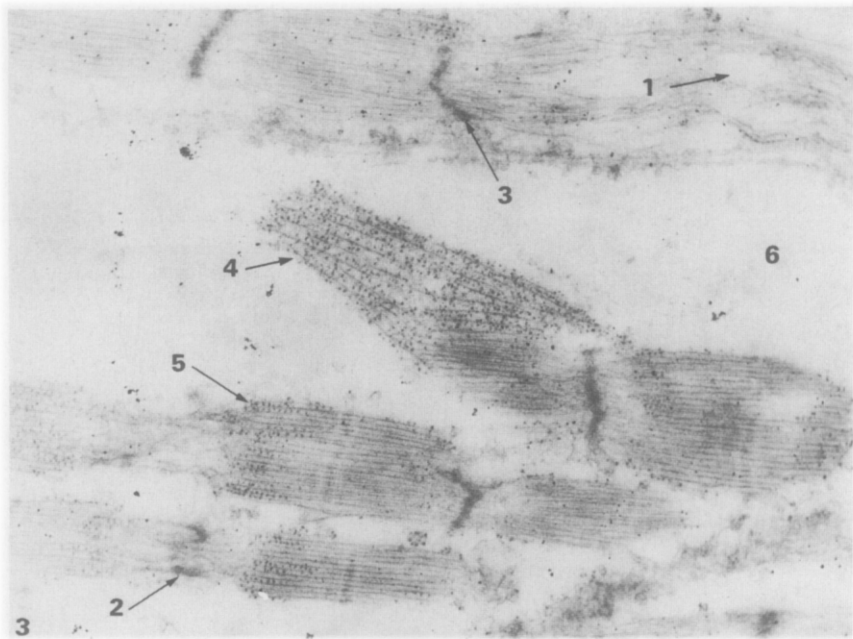
Fig. 3.   Longitudinal section of myofilaments in the M. adductor of a pig with splayleg at 24 h after birth. Z-lines are either absent (1), form irregular Z-band material (2) or show as irregular Z-lines (3). Ribosomes are present on obliquely apposed myosin filaments (4) and on the more fully organized myofilaments (5). The extramyofibrillar space is wide (6). Lead citrate and uranyl acetate contrast. × 29 450.

Fig. 4.   Longitudinal section of the M. adductor of a pig with splayleg 24 h after birth. After $K_4Fe(CN)_6$ treatment of tissue blocks, the extramyofibrillar space is shown to be filled with electron-dense γ-glycogen particles (1). The extracellular space remains almost empty (2). Lysosomes (3) are seen in close apposition to glycogen-filled phagosomes (4).
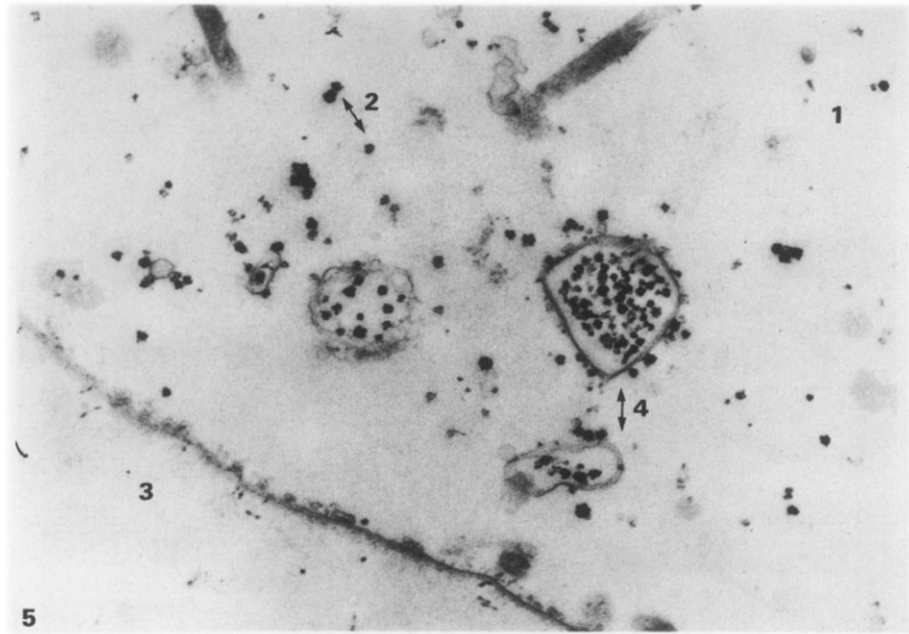
Fig. 5. Longitudinal section of the M. adductor of a pig born after experimental dexamethazone treatment of the sow and killed 24 h after birth. In spite of the $K_4Fe(CN)_6$ treatment of tissue blocks in an identical manner to Fig. 4, the extramyofibrillar space (1) contains only very few scattered glycogen particles (2). The extracellular space is empty (3). Glycogen particles are, nevertheless, accumulated in the autophagosomes (4).

al., 1982), although some controversy exists on the presence or not of degenerative changes (Bergmann, 1976; Zelena and Jirmanova, 1979). The major problem with MFH as a criterion for splayleg is its occurrence in clinically normal pigs (Bradley *et al.*, 1980), including newborn pigs from herds which are free from the splayleg syndrome (Lukas *et al.*, 1982).

These considerations led the authors to select carefully a strain of sows producing MFH negative pigs for the present study [Fig. 1(a)]. Newborn pigs from these sows were compared with newborn pigs with splayleg by histochemical and cytochemical techniques in order to study the differences in their muscles. This study indicated that, in addition to MFH, several other differences were present in the muscles of the 2 groups of animals (group A–C). These differences included a higher content of sarcoplasmic RNA in the splayleg animals, reflected ultrastructurally by the presence of numerous ribosomes. Myofilaments were considered to be immature in the splayleg animals, as indicated by the presence of ribosome-studded myosin filaments, by the splitting of myofilament bundles, and by the irregular banding of the myofilament bundles. In the pig with splayleg, the EMS was found to be filled with glycogen. This is in accord with published data (Bradley *et al.*, 1980; Bucek *et al.*, 1982).

In the present study, an experimental dexamethasone myopathy was

induced in newborn pigs and is proposed as a model for the study of myofibrillar hypoplasia. In these experimental pigs, the MFH was very similar to that seen in pigs with splayleg. Nevertheless, none of the experimental pigs had clinical splayleg. It was therefore of interest to look for morphological differences between these two groups of animals (A and D), since such differences may be important in explaining the appearance or absence of clinical signs. The most remarkable morphological differences were in the composition of the EMS: the pigs with splayleg had a glycogen-filled EMS, whereas in the pigs born after dexamethasone treatment of the sow the EMS contained little glycogen. In the EMS of the latter many glycogen-filled phagosomes and residual bodies were also found. These observations point to a difference in the metabolism of glycogen in the first two to three days after birth.

In rats, it has been shown that extensive glycogen reserves are present not only in the liver but also in skeletal muscle at birth (Schiaffino and Hanzlikova, 1972). This muscle glycogen fills extensive areas in the sarcoplasm. It is mobilized within the first 24 h to 3 days after birth by a process of segregation and digestion in autophagolysosomes. In the pig, the accumulation of large liver and muscle glycogen stores during late intrauterine life is considered to be of major importance in reducing baby pig mortality (Yen, Eichner, Arnold and Pond, 1982). These glycogen stores can be influenced by the feeding level of the sow (Ojamaa, Elliot and Hartsock, 1980). Large muscle glycogen stores are therefore a normal finding in pigs at birth (Campion, Fowler, Hausman and Reagan, 1981). The newborn pig is a much more mobile animal than the newborn rat and it has almost no other energy reserves but glycogen at birth (Yen et al., 1982). It is therefore to be expected that glycogen in the newborn pig will be mobilized even faster than in the newborn rat. Some observations made in the present study support this hypothesis. Indeed, in the control group the amount of glycogen was already significantly reduced at 24 h after birth. Furthermore, in the experimental group the glycogen levels in the EMS were low and glycogen was concentrated in autophagolysosomes. In the pigs with splayleg, however, glycogen still filled the EMS at 24 h after birth.

A fast lysosomal metabolism of glycogen should, in our opinion, be considered as a normal phenomenon in the muscles of newborn pigs. Consequently, the finding of secondary lysosomes and residual bodies should also be considered as normal. This is in contrast with the interpretation given by Zelena and Jirmanova (1979).

In the present study, the pigs with splayleg were found to have a higher content of RNA and ribosomes in their sarcoplasm. This is in accord with published data (Zelena and Jirmanova, 1979). Ribosome-studded myofilaments, as observed in the newborn pigs with splayleg, have also been described in 95-days-old pig foetuses (Campion et al., 1981). This points to immaturity of muscles in pigs with splayleg. A similar interpretation has been given to the light microscopical aspect of splayleg muscles (Thurley et al., 1969).

In conclusion, dexamethasone treatment of sows during late pregnancy can be used to induce MFH in the newborn pigs. Some remarkable differences were found in the morphology of muscles when comparing these experimental pigs

(MFH +, splayleg −) with natural cases of splayleg (MFH +, splayleg +). The observed differences in the maturity of the myofilaments and in the metabolism of muscle glycogen may help to explain the presence or absence of clinical signs of splayleg in newborn pigs with MFH.

In the present study, splayleg in newborn pigs was not identical to glucocorticoid myopathy, although this was suggested by Jirmanova (1983).

SUMMARY

The relation of myofibrillar hypoplasia to clinical splayleg was studied. A strain of Belgian Landrace sows was selected for this study because they produced pigs which had no myfibrillar hypoplasia. Myofibrillar hypoplasia could nevertheless by induced experimentally in these animals by dexamethasone treatment of the sows during late pregnancy.

The lesion was observed without clinical signs and was compared to the myofibrillar hypoplasia in clinical cases of splayleg. The differences between these 2 groups may account for the appearance of clinical signs. These differences included the maturity of the myofibrils and the degree of autophagosomal glycogen breakdown.

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