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PII: S0960-8966(13)00983-8
DOI: http://dx.doi.org/10.1016/j.nmd.2013.10.003
Reference: NMD 2796

To appear in: Neuromuscular Disorders

Received Date: 2 May 2013
Revised Date: 7 October 2013
Accepted Date: 18 October 2013

Please cite this article as: McC. Howell, J., Walker, K.R., Creed, K.E., Dunton, E., Davies, L., Quinlivan, R., Karpati, G., Phosphorylase re-expression, increase in the force of contraction and decreased fatigue following Notexin-induced muscle damage and regeneration in the ovine model of McArdle disease, Neuromuscular Disorders (2013), doi: http://dx.doi.org/10.1016/j.nmd.2013.10.003

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Phosphorylase re-expression, increase in the force of contraction and decreased fatigue following Notexin-induced muscle damage and regeneration in the ovine model of McArdle disease.

J. McC. Howell 1,2, K. R. Walker 3, K.E.Creed 1, E. Dunton 1, L. Davies 1,
R. Quinlivan 5, and G. Karpati 4†

1 School of Veterinary and Life Sciences, Murdoch University, Perth, Western Australia,
2 Australian Neuro-Muscular Research Institute, CNND, University of Western Australia, Perth, Western Australia,
3 Centre for Medical Research, University of Western Australia, Perth, Western Australia,
4 Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada.
5 Centre for Neuromuscular Diseases, National Hospital for Neurology and Neurosurgery, London, United Kingdom

Proofs and reprint requests are to be sent to:
Emeritus Professor John Howell AO PhD DVSc FRCPath
School of Veterinary and Life Sciences
Murdoch University
Perth 6150
Western Australia
Fax. (+61) 08 9360 6630
Email. J. Howell@murdoch.edu.au

† Deceased
Abstract

McArdle disease is caused by a deficiency of myophosphorylase and currently a satisfactory treatment is not available. The injection of notexin into, or the layering of notexin onto, the muscles of affected sheep resulted in necrosis followed by regeneration of muscle fibres with the expression of both non-muscle isoforms of phosphorylase within the fibres and a reduction of the amount of glycogen in the muscle with an increase in the strength of contraction and a decrease in fatiguability in the muscle fibres. The sustained re-expression of both the brain and liver isoforms of phosphorylase within the muscle fibres provides further emphasis that strategies to enhance the re-expression of these isoforms should be investigated as a possible treatment for McArdle disease.

Keywords: Myophosphorylase; Glycogen phosphorylase; McArdle disease; Ovine model of McArdle disease; Notexin; Phosphorylase isoforms.

1. Introduction

McArdle disease is transmitted as an autosomal recessive metabolic myopathy caused by a deficiency of muscle glycogen phosphorylase. As a consequence glycogen metabolism is blocked inhibiting aerobic and anaerobic glycolysis [1-3]. Patients with this condition experience exercise intolerance with premature fatigue, myalgia and stiffness leading to contracture and rhadomyolosis. Muscle weakness predominately
affecting the upper limbs and paraspinal muscles may occur as a late consequence in some patients. Acute rhabdomyolysis can be severe and results in collapse and acute renal failure. Severe generalised weakness at, or soon after birth, with respiratory insufficiency and death in infancy has been reported [4, 5]. Other than aerobic conditioning which can improve exercise tolerance [6], there is no satisfactory treatment to reverse McArdle disease. More than 100 different mutations of the myophosphorylase gene (PYGM) have been reported in human patients with McArdle disease: (n=133 PYGM mutations - Human Gene Mutation Database (HGMD http://www.hgmd.org) 9.3.12). There are three glycogen phosphorylase isoenzymes (brain (pygb) liver (pygl) and muscle (pygm)) each encoded by a different gene. In mature skeletal muscle, the muscle isoform of glycogen phosphorylase is predominantly expressed while in regenerating muscle fibres the non-muscle isoforms of glycogen phosphorylase pygb and pygl are expressed [6, 7].

Since 1997, a unique flock of Merino sheep carrying a mutation in the PYGM has been maintained at the Murdoch University Veterinary School farm in Western Australia and has been available for study. In affected animals (double recessive) as determined by genotyping, there is an absence of glycogen phosphorylase activity in muscle fibres and the sheep exhibit similar clinical effects and morphological changes to those seen in humans. Expression of the brain and liver isoforms of glycogen phosphorylase has been shown to be present in developing and regenerating muscle fibres but are not expressed in mature muscle fibres [8]. The mutation in the sheep model is a mutation of the acceptor splice site in intron 19 of PYGM [9]. The splice site mutation leads to the
activation of a cryptic acceptor splice site which in turn causes an eight-base deletion from the mRNA at the 5’ end of exon 20 resulting in a frameshift and a downstream premature stop codon removing the last 31 amino acid residues from the myophosphorylase protein.

McArdle disease has also been described in Charolais cattle [10] but the ovine model is the first animal model of the condition in a species with a similar body mass throughout life to that of humans. Affected sheep provide a useful animal model for the preclinical testing of putative therapy regimes and for the evaluation of the risks and benefits of such therapies. The ovine model has been used for pre-clinical gene therapy trials [8], and phosphorylase activity has been detected at the sites of injection of myophosphorylase adeno and adeno-associated viral vectors carrying myophosphorylase cDNA or Lac Z cDNA [11]. The phosphorylase activity was a mixture of expression of the human muscle glycogen phosphorylase resulting from the presence of human myophosphorylase cDNA and re-expression of the ovine brain and liver isoforms in regenerating muscle fibres.

The results reported in the present paper examine the re-expression of the ovine brain and liver isoforms of phosphorylase in skeletal muscle of sheep with McArdle disease following the injection of notexin, a myotoxic phospholipase derived from the venom of the Australian Tiger Snake *Notechis scutatus scutatus*. It has previously been reported that necrosis and hyalinization of muscle fibres with neutrophil infiltration begins soon after injection of notexin; regeneration commences by 3 days post injection; myotube
formation is visible by 5 days and by 21 days fully differentiated muscle fibres are found [12,13]. However, the basal lamina is left intact and the vascular supply is not impeded by notexin [14]. In the experiments reported here, notexin was administered to sheep with McArdle disease in one of two ways, either as a single injection into the muscle or as an application layered onto the surface of the muscle. The changes in the glycogen content and muscle function were observed in biopsies taken at 10, 21, 30 or 31, 60, 90 and 120 days after notexin administration (Table 1), to ascertain whether muscle function can be improved by stimulation of phosphorylase in muscles by notexin.

2. Materials and Methods

2.1 Approvals for Surgical and Experimental procedures

All trials and procedures involving animals were carried out with the approval of the Murdoch University Animal Ethics Committee, the University of Western Australia or IMVS Institutional Biosafety Committee. All injections or applications of notexin and biopsies were performed under general anaesthesia. In young lambs up to 3 months old, isoflurane was given by mask and in older animals Alfaxan-CD-RTU (Jurox Pty. Ltd.) was used intravenously for induction, followed by isoflurane via intubation or mask.

2.2 Preparation of notexin
The notexin was obtained as freeze-dried powder from Venom Supplies Pty Ltd, Tanunda, South Australia. Prior to injection, the notexin was diluted to a concentration of 5µg/ml in sterile saline.

2.3 Biopsies

In order to observe the changes in the effects produced by the administration of notexin at various times, the injections or layering were made in or onto several muscles (Table 1) all of which were long muscles and it was possible to leave an area of undamaged muscle around each site and therefore when biopsies were taken, the risk of changes being present as a result of scarring or regeneration from adjacent sites was avoided. Incisions were made in the direction in which the fibres were running such that the biopsies were 2.0 to 3.0cm long and 0.5cm in diameter. They were transversely cut at approximately 0.5cm intervals into a series of blocks and each of the blocks was immediately frozen in liquid nitrogen-cooled isopentane. The blocks of muscle remained frozen at -80°C until they were required for sectioning or for glycogen analysis. Sections for histochemistry were cut on a cryostat (Tissue-Tek (Beyer) or Leica CM 1510) and stained.

Biopsies of semitendinosus were also taken for measurements of the force of contraction and the fatiguability of treated and untreated muscle 21 days after the injection of notexin. The measurements from notexin treated muscles from sheep with McArdle disease were compared with those from untreated muscle and with muscle from untreated normal sheep.
Control uninjected or unlayered sites (Table 1)

During 4 trials (2, 3, 5 and 8) a total of 42 biopsies were taken from 20 affected animals from sites in the muscles where notexin had neither been injected nor layered and a total of 53 blocks were examined. In trial 8, 6 normal sheep had one biopsy taken from a dorsal and one biopsy taken from a ventral site in the left semitendinosus to provide control muscle.

Notexin treated sites (Table 1)

Samples of muscle were taken for histological and histochemical studies from the sheep with McArdle disease that had been injected with notexin (Trials 1,2,3,4,5) or layered with notexin (Trials 6,7,8). Biopsies were taken at intervals of 10, 21, 30/31, 60 and 90 days after injection or layering, with additional biopsies at 120 days after the layering. Only one biopsy was taken from each injected or layered site. One sheep from trial 3 (Table 1) died from an unrelated cause (killed by a fox) before the 90 day biopsy was due.

2.4 Injections (Table 1)

For all injections, the skin and fascia over the area of muscle chosen for the injection site were incised and loose, nonabsorbable sutures of black monofilament nylon (Ethicon Inc.) were placed into the muscle at least 0.5 cm above and below the area of muscle identified as the injection site, where they acted as markers for future biopsies. The injection was of 100µl of the 5µg/ml solution of notexin. A total of 27 sheep with McArdle disease were used ranging in age from 3 days to 3 years (Table 1). The age of
the sheep depended on availability of sheep at the time of the trial. The muscles injected were, in Trial 1 and 5: the left semitendinosus; in Trial 2 and 3: the right semitendinosus; in Trial 4, the right semitendinosus and right peroneus Tertius. The left peroneus tertius and the left semitendinosus were taken for comparison.

2.5 Layering (Table 1)

Because injection may, itself, damage the muscle, the skin and fascia, over the area of muscle chosen for the layering were carefully incised, without damaging the muscle. The notexin solution was gently layered onto the surface of the muscle from a syringe. Care was taken to avoid anything other than the notexin touching the surface of the muscle. Marker sutures were not put into the muscle but the skin was closed with sutures and the healed skin incision was subsequently used as a marker for future biopsies.

Notexin was layered onto the semitendinosus, peroneus tertius, gluteobiceps, ulnaris lateralis and extensor carpi radialis muscles of 13 sheep with McArdle disease aged between 2 and 15 months depending on availability (Table 1). The volume of notexin solution layered was 100µl on one site on each muscle in trial 6 and on two sites on each muscle in trial 7; in trial 8, 200µl of notexin solution was layered onto a site at each of the dorsal and ventral ends of the semitendinosus muscle. In addition in trial 8, 200µl of the notexin solution was layered onto the surface of the right semitendinosus muscle of 6 normal sheep aged 2 months.
2.6 Histology, histochemistry and immunohistochemistry

Serial transverse sections of muscle were cut at a thickness of 6 and 10μm. Sections from each block were placed on silanated slides that had been treated with acetone for 1 minute at room temperature. Sections from each block were stained by haematoxylin and eosin (6μm) and for glycogen phosphorylase (10μm) [15]. For all other staining procedures, 2 sections from each block were placed at the top and 2 at the bottom of silanated slides that had been treated with acetone for 1 minute at room temperature. Those at the top were treated with the histochemical or immunological reagents and those at the bottom acted as controls being treated with a phosphate buffered solution for phosphorylase or diluted sheep serum for immunological stains. Serial sections were stained to demonstrate glycogen by the periodic acid Schiff (PAS) technique and to identify regenerating fibres with developmental myosin heavy chain (MHCd) [16]. Desmin antibodies were used to identify the localization of desmin, which has been reported to be over expressed during muscle regeneration [17]. In Trial 8 sections from 6 normal sheep of similar age to the McArdle sheep were used as controls for H+E and phosphorylase staining [15]. All staining was performed following standard procedures. All the monoclonal antibodies were from Novocastra Laboratories UK. Cryostat sections from each block were examined by light microscopy for the presence or absence of phosphorylase positive stained fibres with an Olympus BX51F microscope and the number of positive fibres was counted at a magnification of 20. The number of positive fibres was counted in each section by two investigators and the mean of the two counts was recorded as the score.
2.7 Protein Expression Analysis

2.7.1 Generation of antibodies

Polyclonal antibodies were generated against the ovine brain (pygb), liver (pygl) and muscle (pygm) glycogen phosphorylase isoforms. The pygl and pygm antibodies were raised in rats by immunising with 500µg of pygl or pygm protein purified from ovine liver and muscle. To create isoform specific antibodies the sera were further affinity purified and cross absorbed to remove any cross reaction to the other glycogen phosphorylase isoforms. The pygb polyclonal antibody was produced in rabbits by the Institute of Medical and Veterinary Science (IMVS) Veterinary Services Division (Adelaide, South Australia) from a 15-residue peptide CPPALQTPPPSLPRD from the ovine pygb protein sequence and affinity purified.

2.8 Biochemical Glycogen Analysis

In order to obtain an accurate estimate, biochemical analysis of the glycogen level in the muscle samples was performed on samples in which greater than 0.9g of muscle remained after other procedures. Approximately 250mg of muscle was weighed to 3 decimal places, homogenized in 30mM HCl. Lactate analysis on the homogenate was carried out on an Olympus AU 400 autoanalyser with an enzymic method kit [Ref. No.0SR6193] designed for the machine. Glycogen in the homogenate was hydrolised to glucose by a double enzyme method [18] and glucose was measured on the Olympus AU 400 with a glucose kit (Cat.No 0SR6121). With this method, the total
glycogen was calculated by halving the lactate value and adding it to the glucose value and expressing it as grams of glycogen per 100g muscle [18].

2.9 Native Activity Gels

The type of glycogen phosphorylase isoenzymes in the muscle extracts was determined by native gel activity assays as previously described [11]. Protein concentrations were quantified by the dye binding method of Minamide et al. [19]. The newly formed glycogen was visualised by staining according to the method of DiMauro et al. [4]. The position of the phosphorylated and non-phosphorylated isoforms had previously been established in homogenates from normal and dephosphorylated samples of the same muscles [8].

2.10 Western Blotting

20μg of tissue lysates were denatured in Laemmli SDS buffer at 95°C for 5 minutes and electrophoresed in triplicate on 7.5% TRIS-HCl polyacrylamide gels (Biorad) at 120 V for 1.5 hours under denaturing conditions. The polyacrylamide gels were electroblotted onto PVDF membranes (Pierce) according to Towbin et al., [20] at 200 mA for 3 hours. The membranes were then blocked for 3 hours in skimmed milk - phosphate buffered saline/0.1% Tween-20 (PBS-T). The blocked membranes were incubated overnight at 4°C with primary polyclonal antibody diluted in 5% skimmed milk/PBS-T (pygb (1:10,000 dilution), pygl (1:1,000) and pygm (1:1,500)). This was followed by two 2 minute and two 20 minute washes in PBS-T. The blots were then incubated in HRP-conjugated secondary antibodies (Sigma A0545 for the pygb rabbit
antibody and Pierce 31470 for the pygl and pygm rat antibodies) for 1 hour at room temperature, followed by washing as previously described. Visualisation was by chemiluminescent detection with ECL-Plus (GE Healthcare), according to the manufacturer’s instructions.

2.11 Muscle strength and fatigue

The force of contraction and the extent of fatigue was measured in 47 strips of muscle from 17 normal sheep, 52 strips from 22 untreated sheep with McArdle disease, and 11 strips from 6 sheep with McArdle disease 21 days after the injection of 100μl of a solution containing 5μg/ml of notexin. Up to four strips of muscle (2x2x20 mm) were cut from each biopsy parallel to the way in which the fibres were running. Each strip was then tied with cotton at each end and passed through two Ag/AgCl ring electrodes. One end was held by a fixed hook in a 10ml organ bath, the other end was attached to a force transducer (Grass FT 03) which recorded the contraction and the signal was transferred to a pen recorder (Grass polygraph) which was connected to a MacLab/4E (AD Instruments) data acquisition system and the data was analysed on a total peripheral PC computer.

The organ bath contained modified Krebs solution (mM NaCl 120, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.0, NaH₂PO₄ 1.0, NaHCO₃ 25.0, glucose 11.0) at 35 °C and bubbled with 5% CO₂ in O₂. The strips were stimulated at 1, 20 and 50 Hz for 2 seconds with pulses of 70V and 10 milliseconds at intervals of 2 minutes. For each strip, the twitch/tetanus ratio
was calculated for 20 and 50 Hz. The force at 2 seconds as a % of the initial peak was taken as the degree of fatigue.

2.12 Statistics

Results are expressed as mean ± SD and are compared with Students T-test.

3. Results

3.1 Histology

3.1.1. Haematoxylin and Eosin

3.1.1.1. Biopsies taken from uninjected or unlayered sites (Table 1)

The histological appearance of the muscle fibres was within normal limits for affected sheep. (Fig.1A).

3.1.1.2. Notexin Injected sites (Table 1)

All the muscle biopsies contained areas of muscle fibres which were of normal size and areas in which the fibres were of varying size. In some fibres internalised nuclei were present. The changes were numerous in the 10 day biopsies and less frequent in the later biopsies. However internalised nuclei were still present in the 90 day biopsies.
3.1.1.3. Notexin Layered sites (Table 1)

All the muscle biopsies contained areas of histologically normal sized muscle fibres and areas in which the fibres were of varying size and staining intensity (Fig 2). A small number of fibres were seen to contain internalised nuclei. The changes in the 10 day biopsies became less frequent in the later biopsies. However mononuclear cells and internalised nuclei were still present in the 90 and 120 day biopsies.

3.1.1.4

The above results indicate that notexin injection or layering, produced a transient effect on muscle cells with a peak at 10 days.

3.1.2 Glycogen phosphorylase

3.1.2.1 Control, uninjected and unlayered sites in the muscles of sheep with McArdle disease (Table 1 Fig 1 B)

A total of 42 biopsies were taken from uninjected or unlayered sites in the muscles of 20 affected sheep. They were taken from the left semitendinosus muscle in all but 4 of these sheep and in these 4 they were taken from the right semitendinosus. The number of biopsies taken from uninjected or unlayered sites after 10, 21, 30, 60, 90 and 120 days after notexin administration to other sites or other muscles in the same animals are shown in Table 1. Phosphorylase positive fibres were absent from 49 of the 53 blocks examined. One of the 4 positive blocks was from a biopsy taken from a sheep 21 days after treatment, 2 were from a sheep biopsied 30 days after treatment. Two, 6, 7 and 15
phosphorylase positive fibres were seen and the latter was from a biopsy taken 120 days after treatment to other muscles and may have been related to local trauma unrelated to the trial.

3.1.2.2 Notexin Injections (Table 1)

All the sheep injected with notexin had phosphorylase positive fibres in the biopsies taken from the site of injection 10, 21, 30 or 31 days after injection. In trial 4 two muscles were biopsied at the same time point increasing the number of blocks examined for each sheep biopsied. A range from 1 to 5,700 positive fibres were present. The maximum number of positive fibres (5,700) was seen in a block cut from a biopsy taken from a 3 month old animal 10 days after injection (Fig 3A).

3.1.2.3 Notexin Layering (Table 1)

Phosphorylase positive fibres were seen in all 7 of the sheep that were biopsied 10 days after notexin was layered on the muscle (Fig 2B, Fig 3B).

3.1.2.4
Phosphorylase activity was therefore present after injection or layering, was at a maximum at 10 days and declined subsequently. The decline was more marked after layering.

3.1.3 Desmin and developmental myosin heavy chain.
3.1.3.1 Control, uninjected and unlayered sites

Sections from all control, uninjected and unlayered site blocks in trial 2 were stained by the PAS, desmin and developmental myosin techniques. PAS positive material was present but there were no desmin or developmental myosin positive fibres.

3.1.3.2 Notexin injections

In trial 2 desmin and developmental myosin heavy chain immunocytochemistry was performed on serial sections from the biopsies taken 10, 30 and 60 days after the injection of notexin in which numerous fibres had been seen to contain phosphorylase (Table 2). Desmin and developmental myosin heavy chain were found to be present at all time points.

3.1.3.3

Desmin expression was absent in untreated muscle but was induced by notexin injection.

3.2 Biochemical Glycogen Analysis of homogenised muscle

In order to obtain a more accurate estimate, a biochemical method was used. The glycogen content of untreated muscle from the semitendinosus of 6 normal sheep in trial 8 together with the glycogen content of the semitendinosus from 8 and the triceps muscle from 4 sheep with McArdle disease is presented in Table 3. The glycogen content is significantly higher than normal in the muscle of the non-treated McArdle sheep (p<0.001).
After injection of notexin the glycogen content of the muscle was reduced. 10 days after the injection, the glycogen content of the muscle of 11 affected sheep was significantly less than in untreated animals (p<0.001) (Table 3). The glycogen remained lower than in normal sheep at 21 and 31 days after the injection.

After 10 days layering with notexin, the glycogen content of 22 muscles was also less than in non-treated McArdle sheep and 30 days later was well down (p<0.001)(Table 3).

Glycogen content was therefore significantly higher in muscles of McArdle sheep than in normal sheep, and was reduced in notexin-treated sheep.

3.3 Native Activity Gels

Native gel activity assays were performed on biopsies taken from the sheep in trials 1 and 2 following notexin administration. Biopsies were selected in which there were numerous phosphorylase positive fibres but little or no granulation tissue. The gels revealed that the re-expressed phosphorylase was both the brain and liver isoforms. The identity of these isoforms was further confirmed by Western Blot analysis with isoform specific antibodies (Fig 4). It should be noted that in these McArdle sheep the two non-muscle isoforms but not the muscle isoform were re-expressed in the regenerating muscle fibres. The native gel assays and Western blots were performed using total protein quantitation rather than total fibre number and therefore the amounts of the brain and liver isoforms detected in the biopsies could not be correlated with the
total number of phosphorylase positive fibres counted in sections from the muscle biopsy blocks.

The re-expressed phosphorylase was the brain and liver isoforms not the muscle isoform.

3.4 Muscle Physiology
Measurements were made of the force of contraction (Fig 5A, B) and the fatiguability (Fig 5C) of the semitendinosus muscle from normal sheep and from notexin treated and untreated semitendinosus muscle from sheep with McArdle disease.

The contractile response to a single stimulus (twitch), which depends on the dimensions of the strip, varied widely in each of the 3 groups (Fig 5A). Once the maximum twitch contraction had been found by varying the muscle length, the response to 20 Hz and 50 Hz was measured. The tetanus/twitch ratio, which is the relative increase in contraction with repetitive stimulation, was significantly greater in normal (2.62 ± 0.99 n=44 and 5.40 ± 2.01 n=47) than affected muscles (1.70 ± 0.44 n=49 and 2.12 ± 0.92 n=47, p<0.001) with stimulation at 20 Hz and 50 Hz respectively (Fig 5B). The ratio for both frequencies (1.84 ± 0.25 n=11 and 4.45 ± 1.55 n=11) increased in muscles from affected sheep after the injection of notexin but this was only significant for 50Hz, when the value approached that found in normal muscles ( p<0.001 Fig. 5B).

With continuous repetitive stimulation the contraction rose to a peak after about 0.2 seconds then declined to a steady level at 2 seconds in muscle from normal sheep. Fatigue was taken as the level at 2 seconds as a % of the peak. Fatigue was
significantly greater in affected muscles (50.3 ± 21.3 %) than in normal muscle (88.1 ± 12.5 %) at 50 Hz stimulation (Fig 5C) and was significantly less in notexin injected muscles (88.7 ± 14.6 % p <0.001)). However, there was little difference in fatigue at 20Hz in normal, affected or injected muscles (67.3 ± 14.6, 66.9 ± 20.9 and 71.9 ± 20.9, Figure 5C). A biopsy taken from a second notexin injected site (Table 1, Trial 5) for histochemical examination at the same time as the biopsy taken for strength and fatigue measurements contained 2 to 600 (mean of 161.5) phosphorylase positive fibres.

The force of contraction was therefore shown to be greater in normal than in McArdle muscle and fatigue was less. Notexin produced some reversal of the responses.

4. Discussion

Numerous studies have been made of the effects that follow the injection into muscle of notexin, a phospholipase A₂ toxin extracted from the venom of the Australian Tiger Snake (Notechis scutatus scutatus), and the technique has often been used in the study of muscle regeneration [21]. Much of the work has been done in rodents and it was shown that within 12 -24 hours after the injection of notexin, necrosis occurred with oedema and the infiltration of lymphocytes, polymorphs and macrophages. Three days post injection the oedema had subsided, the necrotic fibres had been removed by phagocytes and regenerating myoblasts were identified. Myotubes were found at 5 days and immature muscle fibres were seen at 7 – 14 days. Fully differentiated muscle fibres were seen by 21 – 28 days [12, 13, and 22]. "Even after 6 months, however, the
nuclei of many muscle fibres remained in a central position” [13]. Following the injection of notexin the muscle may contain a mixed population of destroyed, partially damaged and undamaged muscle fibres. The histological changes reported in the present study in the muscle biopsies from sheep either injected or layered with notexin were in accord with the findings given above and the mosaic pattern of affected and unaffected fibres was similar to that described by Harris, Johnson and Karlsson in rats, [12].

The re-expression of phosphorylase in the regenerating muscle fibres and satellite cells of humans with McArdle disease has been known for many years [7,23,24 and 25]. Our previous studies, in which viral vectors containing myophosphorylase or Lac Z expression cassettes were injected into the muscles of McArdle sheep, resulted in muscle fibre damage, regeneration and the re-expression of the non-muscle brain and liver isoforms of phosphorylase within the muscle fibres [11]. In the notexin trials described above, muscle biopsies were taken from the muscles of 20 McArdle sheep that had neither been layered nor injected with notexin. Sections were taken from 53 blocks and a total of only 30 positive fibres were found in 4 of the blocks. Glycogen phosphorylase was not seen in biopsies taken from uninjected or unlayered sites in the muscle in 16 of the 20 McArdle disease sheep (Table 1). It was absent in 49 of the 53 blocks examined from the 20 sheep. In the 5 blocks with phosphorylase positive fibres, the range was limited to 2 to 15 positive fibres. These could have been the result of regeneration following accidental damage to muscle that may have occurred in any of the sheep during procedures such as transport or weighing.
In contrast, numerous phosphorylase positive fibres were found in the 64 biopsies taken from the 27 affected sheep at 10, 21 and 30 or 31 days after the injection of notexin, with the highest count being 5,700 in a biopsy taken 10 days after the injection in an animal aged 3 months. Phosphorylase positive fibres were also found in the biopsies taken from 9 of 13 sheep at 60 days, 5 of 7 sheep at 90 days and 6 of 7 sheep 120 days after the injection of notexin. The total number of positive fibres was reduced (Table 1). It was demonstrated that this phosphorylase was the brain and liver isoforms (Fig 4). In 1978 Harris and Johnson [13] recorded that internalised nuclei, indicative of regeneration, were still present in rat muscle fibres 6 months after damage caused by notexin and in the present study the presence of glycogen phosphorylase at 60, 90 and 120 days after the injection of notexin in the McArdle affected sheep is considered to be directly related to the emergence of regenerating fibres following necrosis caused by notexin.

The 100µl volume of injection may have contributed to the initial muscle damage and the ethicon marker sutures, which remained in place in the injected animals until the biopsies were taken, may have produced recurring damage to the muscle fibres resulting in ongoing regeneration. In order to rule out the possible influence of these factors, notexin was layered onto the muscle surface without the use of marker sutures and with no damage to the muscle, the incision scar in the skin acting as a marker for the biopsy sites. The layering of notexin did result in muscle degeneration, regeneration and the re-expression of phosphorylase (Table 1). Phosphorylase positive fibres were found in biopsies taken from all 7 sheep 10 days after the muscle had been layered with
notexin with a range in the number of positive fibres of 11 to 1135 with 2 of the blocks containing more than 1,000 positive fibres. The number of sheep in which positive fibres were found and the number of such fibres diminished thereafter. In the biopsies taken from 13 sheep 30 days after the layering of notexin, phosphorylase was found in 7 and the highest number of phosphorylase positive fibres was 167. Only 2 of the 7 sheep biopsied at 60 days and 2 of the 5 biopsies at 120 days contained phosphorylase positive fibres. Phosphorylase positive fibres were not found in the 5 sheep biopsied 90 days after layering. These results are similar to the biopsy results from the untreated affected sheep where a maximum of 7 positive fibres were found (Table1). Counts in excess of this number were taken to indicate glycogen phosphorylase expression as a result of the layering of notexin. The results from the later biopsies in the layering trials indicate that some of the positive fibres seen in the 60 and 90 day biopsies from the injected animals, may have been due to the continuing presence of the marker sutures rather than being due solely to the effects of notexin.

McArdle disease is an autosomal recessive disorder caused by mutations in the gene for the muscle isoform of glycogen phosphorylase (PYGM). As a result an insufficient amount of functional pygm protein is produced in the muscles. However, while the genes encoding for the brain and liver isoforms of phosphorylase are not mutated in McArdle patients, they are not normally expressed in mature muscle fibres. We have shown that the brain and liver isoforms of the phosphorylase gene may be made to re-express in the diseased muscle of sheep with McArdle disease by the activation of muscle regeneration following viral vectors carrying Lac Z cDNA (Howell et al., 2008)
[11] or by notexin (Howell et al 2003) [26] and as reported in the present study. In this paper we also show a reduction in glycogen storage in fibres where these non-muscle isoforms are expressed indicating potential functional benefit from these normally latent isoforms [11]. Furthermore we have also shown that, following the injection of notexin, the strength of contraction of these muscle fibres is increased and the fatiguability is decreased. We believe that the investigation of the systemic delivery of agents that are capable of inducing and maintaining the re-expression of the brain and liver isoforms in muscle should be actively pursued as they appear to provide a promising target for the treatment of McArdle disease as it has been shown that minimal myophosphorylase activity ameliorates the typical McArdle disease phenotype by augmenting muscle oxidative capacity [27].

**Acknowledgements**

The authors thank Kim Thomas and Don Hook for the care and management of the sheep, Professor Helen Chapman and Drs T. Tan, H. Rebhan for Veterinary care and assistance, Maja Waschk and A. Everaardt for technical assistance and Professor David Pethick, Diana Wilson and Rini Margawani for the Glycogen Analysis. The work was supported by grants from Muscular Dystrophy Association of the United States of America and the Muscular Dystrophy Association of Western Australia.
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[18] Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen measurement in tissues. Anal Biochem 1974; 60(2); 405-12


Description of Figures.

Figure 1. Cryostat sections of a biopsy taken from an uninjected site in the left semitendinosus muscle of a castrated male affected sheep aged 5 months in trial 3. The biopsy was taken 30 days after other sites had been injected with notexin.
A: Haematoxylin and eosin. The muscle fibres are evenly stained and there is no increase in cellularity.
B: Phosphorylase. Cells in the wall of the vessel in the top left hand corner of the sections are positive for phosphorylase. The muscle fibres are not.

Figure 2. Adjacent cryostat sections from the notexin layered site in the right gluteobiceps muscle of a 4 month old affected male lamb 10 days after the layering of notexin. A transverse section of a nerve fibre is present at the right hand side of both A and B.

A. Haematoxylin and eosin. Variable fibre size and a small number of fibres contain internalised nuclei.
B. Phosphorylase. The numerous darkly stained fibres are phosphorylase positive fibres.
Figure 3. Percentage of sections with fibres positive for phosphorylase (red) or with 1000 or more positive fibres (green) in biopsies, taken after the injection (A) or layering (B) of notexin from various muscles shown in Table 1.

Figure 4. Western blot analysis of 20µg of muscle biopsy lysate from notexin injected affected lambs.
A pygb antibody (1:10,000 dilution)
B pygl antibody (1:1,000 dilution)
C pygm antibody (1:1,500 dilution)

Key:
Lane 1: Uninjected McArdle lamb semitendinosus (negative control)
Lane 2: Injected 3 day old McArdle lamb 10 day biopsy, 2500 phosphorylase positive fibres.
Lane 3: Injected 4 day old McArdle lamb 10 day biopsy, 2000 phosphorylase positive fibres.
Lane 4: Injected 3 day old McArdle lamb 30 day biopsy, 200 phosphorylase positive fibres.
Lane 5: Injected 6 day old McArdle lamb 30 day biopsy, 300 phosphorylase positive fibres.
Lane 6: Control adult semitendinosus muscle lysate (20µg).
Lane 7: Control adult heart lysate (20µg).
Lane 8: Control adult brain lysate (20µg)
Lane 9: Control adult liver lysate (20µg)

Figure 5. Muscle strength and fatigue in semitendinosus biopsies. A: Responses to single stimuli (twitch) in normal (green), affected McArdle (red) and affected muscles treated with notexin (yellow). B: Amplitude of responses to 20 and 50 Hz relative to the twitch amplitude in the same muscle (twitch/tetanus ratio) C: Amplitude after 2 second stimulation as a % of the initial peak (fatigue). Normal muscles and those injected with notexin were compared statistically with muscle from affected untreated sheep. n.s. = not significant, *** = highly significant (P<0.001).
Table 1: Biopsies Taken From Muscles of Sheep with McArdles Disease

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Muscles Sampled From Each Sheep</th>
<th>Age at Time of Injection or Layering</th>
<th>Number of Biopsies From Each Sheep</th>
<th>Biopsy Time after treatment</th>
<th>Number of Sheep with Phos +ve Fibres</th>
<th>Number of Blocks with Phos Expression</th>
<th>No. Fib +ve for Phos</th>
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<td></td>
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<td></td>
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</tr>
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<td>2</td>
<td>left semitendinosus</td>
<td>1 at 3 yr 3 at 2 yr</td>
<td>1</td>
<td>10 days</td>
<td>0/4 (0%)</td>
<td>0/9 (0%)</td>
<td>0</td>
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<tr>
<td>3</td>
<td>left semitendinosus</td>
<td>3 months</td>
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<td>10 days</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0</td>
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<td>5</td>
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<td>5 months</td>
<td>1</td>
<td>21 days</td>
<td>1/6 (16%)</td>
<td>1/6 (16%)</td>
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<tr>
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<td>1 at 3 yr 3 at 2 yr</td>
<td>2</td>
<td>31 days</td>
<td>0/4 (0%)</td>
<td>0/7 (0%)</td>
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<td>3 months</td>
<td>1</td>
<td>30 days</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0</td>
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<td>30 days</td>
<td>2/6 (33%)</td>
<td>2/12 (16%)</td>
<td>2 &amp; 6</td>
</tr>
<tr>
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<td>0/4 (0%)</td>
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<td>1</td>
<td>90 days</td>
<td>0/3 (0%)</td>
<td>0/3 (0%)</td>
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<td>120 days</td>
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<td>13/18 (72%)</td>
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<td>9/11 (82%)</td>
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<td>1</td>
<td>10 days</td>
<td>4/4 (100%)</td>
<td>10/14 (71%)</td>
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<td>9 months</td>
<td>1</td>
<td>10 days</td>
<td>4/4 (100%)</td>
<td>9/12 (75%)</td>
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<td>1</td>
<td>21 days</td>
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<td>16/23 (70%)</td>
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<td>9/9 (100%)</td>
<td>16/31 (52%)</td>
<td>1&lt;-&gt;300</td>
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<td>1</td>
<td>30 days</td>
<td>4/4 (100%)</td>
<td>18/24 (75%)</td>
<td>1-500</td>
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<td>2</td>
<td>30 days</td>
<td>4/4 (100%)</td>
<td>14/23 (60%)</td>
<td>6-1780</td>
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<td>15/38 (39%)</td>
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<td>2/4 (50%)</td>
<td>8/27 (30%)</td>
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<td>1/3 (33.3%)</td>
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<td>1</td>
<td>90 days</td>
<td>4/4 (100%)</td>
<td>7/14 (50%)</td>
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<td>1</td>
<td>90 days</td>
<td>3/4 (75%)</td>
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<td>right gluteobiceps (anterior &amp; posterior)</td>
<td>4 months</td>
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<td>10 days</td>
<td>5/5 (100%)</td>
<td>35/41 (85%)</td>
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<td>15 months</td>
<td>1</td>
<td>30 days</td>
<td>1/2 (50%)</td>
<td>3/10 (30%)</td>
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<td>right extensor carpi radialis (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>30 days</td>
<td>4/5 (80%)</td>
<td>19/32 (59%)</td>
<td>5-167</td>
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<td>8b</td>
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<td>2 months</td>
<td>1</td>
<td>60 days</td>
<td>2/6 (33%)</td>
<td>3/27 (11%)</td>
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<td>15 months</td>
<td>1</td>
<td>60 days</td>
<td>0/2 (0%)</td>
<td>0/7 (0%)</td>
<td>0</td>
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<tr>
<td>7</td>
<td>left peroneus tertius (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>60 days</td>
<td>0/5 (0%)</td>
<td>0/39 (0%)</td>
<td>0</td>
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<tr>
<td>7</td>
<td>left ulnaris lateralis (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>60 days</td>
<td>2/5 (40%)</td>
<td>4/44 (9%)</td>
<td>3-57</td>
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<tr>
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<td>right extensor carpi radialis (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>60 days</td>
<td>0/5 (0%)</td>
<td>0/39 (0%)</td>
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<td>left gluteobiceps (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>90 days</td>
<td>0/5 (0%)</td>
<td>0/35 (0%)</td>
<td>0</td>
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<tr>
<td>7</td>
<td>right peroneus tertius (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>90 days</td>
<td>0/5 (0%)</td>
<td>0/67 (0%)</td>
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<td>7</td>
<td>right ulnaris lateralis (dorsal &amp; ventral)</td>
<td>4 months</td>
<td>2</td>
<td>120 days</td>
<td>1/5 (20%)</td>
<td>1/71 (1.4%)</td>
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</table>

Untreated muscle biopsies in trials 2,3,5 8a were taken from uninjected sites in affected animals. Trials 6,7,8b were taken from muscles following layering with 100µl of a 5µg/ml solution of notexin.
<table>
<thead>
<tr>
<th>Number of Biopsies from each sheep</th>
<th>Biopsy time point (days after injection)</th>
<th>Number of blocks of muscles examined</th>
<th>Number of sheep with desmin positive fibres</th>
<th>Number of blocks and range with desmin positive fibres</th>
<th>Number of sheep with MHCd positive fibres</th>
<th>Number of blocks and range of MHCd positive fibres</th>
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<tr>
<td>1</td>
<td>10 days</td>
<td>16 desmin 18 MHCd</td>
<td>4/4 (100%)</td>
<td>13/16 (79-1200)</td>
<td>4/4 (100%)</td>
<td>10/18 (82-800)</td>
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<td>1</td>
<td>30 days</td>
<td>24 desmin 24 MHCd</td>
<td>4/4 (100%)</td>
<td>17/24 (3-1000)</td>
<td>4/4 (100%)</td>
<td>12/24 (7-250)</td>
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<tr>
<td>1</td>
<td>60 days</td>
<td>20 desmin 20 MHCd</td>
<td>3/3 (100%)</td>
<td>9/20 (2-500)</td>
<td>1/3 (33%)</td>
<td>3/20 (8-150)</td>
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</table>

Biopsies of right semitendinosus muscles taken from sheep in trial 2 (table 1) following injection of notexin into muscle.
<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
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<tbody>
<tr>
<td>Glycogen content of biopsies from normal and affected sheep</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NORMAL untreated</th>
<th>AFFECTED untreated</th>
<th>AFFECTED injected at 10 days</th>
<th>AFFECTED injected at 21 days</th>
<th>AFFECTED injected at 31 days</th>
<th>AFFECTED layered at 10 days</th>
<th>AFFECTED layered at 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>semitend.</td>
<td>semitend.</td>
<td>semitend.</td>
<td>semitend.</td>
<td>semitend.</td>
<td>gluteobiceps</td>
<td>ext. carpi rad</td>
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<tr>
<td>1.271</td>
<td>3.535</td>
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<tr>
<td>Std Dev</td>
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</tbody>
</table>

The muscle biopsies were homogenised and after enzyme action, the resulting glucose concentration was determined.
Figure 1

A
H+E

B
Phosphorylase

20 μm

20 μm
Figure 2

A  H+E

B  Phosphorylase
% of blocks with phosphorylase

Biopsy time point (days)

A Injected

B Layered

% of blocks with phosphorylase

Biopsy time point (days)
Fig 4

A

B

C
FIG 5

A. Force of contraction (g)

B. Twitch:tetanus ratio

C. Percentage

A. Single

B. 20:1 (Hz)

C. 20 (Hz)

B. 50:1 (Hz)

C. 50 (Hz)

ns

***

ns

ns

***

ns

ns

***
Description of Figures.

Figure 1. Cryostat sections of a biopsy taken from an uninjected site in the left semitendinosus muscle of a castrated male affected sheep aged 5 months in trial 3. The biopsy was taken 30 days after other sites had been injected with notexin. (A: Haematoxylin and eosin. Magnification x 620) The muscle fibres are evenly stained and there is no increase in cellularity. (B: Phosphorylase. magnification x 620) Cells in the wall of the vessel in the top left hand corner of the sections are positive for phosphorylase. The muscle fibres are not.

Figure 2. Adjacent cryostat sections from the notexin layered site in the right gluteobiceps muscle of a 4 month old affected male lamb 10 days after the layering of notexin. A transverse section of a nerve fibre is present at the right hand side of both A and B. A. Haematoxylin and eosin. Magnification x 310) Variable fibre size and a small number of fibres contain internalised nuclei. B. Phosphorylase. Magnification x 310) The numerous darkly stained fibres are phosphorylase positive fibres.

Figure 3. Percentage of sections with fibres positive for phosphorylase (red) or with 1000 or more positive fibres (green) in biopsies, taken after the injection (A) or layering (B) of notexin from various muscles shown in Table 1.
Figure 4. Western blot analysis of 20µg of muscle biopsy lysate from notexin injected affected lambs.

A pygb antibody (1:10,000 dilution)
B pygl antibody (1:1,000 dilution)
C pygm antibody (1:1,500 dilution)

Key:

Lane 1: Uninjected McArdle lamb semitendinosus (negative control)
Lane 2: Injected 3 day old McArdle lamb 10 day biopsy, 2500 phosphorylase positive fibres.
Lane 3: Injected 4 day old McArdle lamb 10 day biopsy, 2000 phosphorylase positive fibres.
Lane 4: Injected 3 day old McArdle lamb 30 day biopsy, 200 phosphorylase positive fibres.
Lane 5: Injected 6 day old McArdle lamb 30 day biopsy, 300 phosphorylase positive fibres.
Lane 6: Control adult semitendinosus muscle lysate (20µg).
Lane 7: Control adult heart lysate (20µg).
Lane 8: Control adult brain lysate (20µg)
Lane 9: Control adult liver lysate (20µg)

Figure 5. Muscle strength and fatigue in semitendinosus biopsies. (A: Responses to single stimuli (twitch) in normal (green). Affected McArdle (red) and affected muscles
treated with notexin (yellow). (B: Amplitude of responses to 20 and 50 Hz relative to the
twitch amplitude in the same muscle (twitch/tetanus ratio) (C: Amplitude after 2 second
stimulation as a % of the initial peak (fatigue). Normal muscles and those injected with
notexin were compared statistically with muscle from affected untreated sheep. n.s. =
not significant, ** = highly significant (P<0.001).
ABSTRACT

McArdle disease is caused by a deficiency of myophosphorylase and currently a satisfactory treatment is not available. The injection of notexin into, or the layering of notexin onto, the muscles of affected sheep resulted in necrosis followed by regeneration of muscle fibres with the expression of both non-muscle isoforms of phosphorylase within the fibres and a reduction of the amount of glycogen in the muscle with an increase in the strength of contraction and a decrease in fatiguability in the muscle fibres. The sustained re-expression of both the brain and liver isoforms of phosphorylase within the muscle fibres provides further emphasis that strategies to enhance the re-expression of these isoforms should be investigated as a possible treatment for McArdle disease.

Keywords: Myophosphorylase; Glycogen phosphorylase; McArdle disease; Ovine model of McArdle disease; Notexin; Phosphorylase isoforms.