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The Impact of Gastrocnemius Muscle Cell Changes in Chronic Venous Insufficiency

T. Qiao,* C. Liu and F. Ran

Department of Vascular Surgery, Drum Tower Hospital, Affiliated Hospital of Medical College, Nanjing University, Nanjing 210008, China

Objective. *To investigate the pathological and metabolic changes in the gastrocnemius muscle in patients with chronic vein insufficiency (CVI).*
Method. *Thirty-six patients with varicose veins were investigated by ambulatory venous pressure (AVP) and duplex ultrasonography. Twelve age and height-matched controls were used for comparison. Patients and controls consented to participate in this study. Twenty-one patients with primary vein varicose (group AI) and 15 patients (group AII) with primary deep venous valve incompetence (DVI) underwent biopsies of the gastrocnemius muscle during operation. Adductor biopsies obtained from the same limbs served as a control group (group B) and specimens from controls subjects without venous disease served as the second control group (group C). All the specimens were investigated by superoxide dismutase (SOD), nitric oxide (NO), Na^+–K^+-ATPase, Ca^{2+}-ATPase and lactic acid (LD) determinations. Samples were subjected to light and electron microscopy following H & E staining, special ATPase, cytochrome oxidase/succinate dehydrogenase (COX/SDH) stains.*
Results. *Normal muscle architecture was seen following H & E, ATPase and COX/SDH staining and normal cell metabolism was observed in specimens of groups B and C. In group A, pathological changes were encountered in the gastrocnemius muscle including disseminated myofibril atrophy, cell denaturation and necrosis, inflammatory cell infiltration, proliferation and dilation of interfascicular veins. ATPase staining (pH 9.4) demonstrated grouping of atrophic fibres, especially type I myofibril grouping, accompanied by moderate to severe atrophy of type II muscle fibres. However, no patient had selective type I fibre atrophy. Enhanced enzymatic activity in single or multiple myofibrils was demonstrated by COX/SDH staining in approximately half of the specimens in group AII. In group AII, electron microscopy showed swelling, myelin figure denaturation of mitochondria, disruption of the myofibrils and increased lipid droplets in the gastrocnemius muscle. Increased concentration of LD was found in most specimens from group A patients. There were also reductions of SOD, NO, biochemical activity of Na^+–K^+-ATPase, Ca^{2+}-ATPase with increasing concentration of LD in these patients, most prominently in group AII. We found correlation between AVP assessments and the biochemical measurements as well as morphological appearances of the gastrocnemius muscle.*
Conclusion. *Venous hypertension results in pathophysiological changes in the gastrocnemius muscles of patients with DVI, associated with decreased calf pump function.*

Keywords: Gastrocnemius muscle; Chronic venous insufficiency; Pathophysiology; Calf muscle pump.

Introduction

The factors which result in blood flow from the leg to the heart are heart pump function, competent venous valves and the calf muscle pump. The calf muscle pump is considered to be 'the second heart of body'.[1] Restoration of calf pump function is a problem often faced by vascular surgeons. Most published articles have concentrated on venous haemodynamics and restoring or improving venous valve function. These techniques have limited use in clinical practice. In general, the morphological and metabolic changes in calf muscle have been ignored in earlier research in this field. Some patients continue to suffer symptoms (cramps in the leg, tiredness, hot throbbing pain, swelling, eczema and ulceration), which are not improved by surgical treatment. The purpose of this pilot study was to investigate the pathophysiological changes of calf muscle in patients with chronic venous disease.

*Corresponding author. Dr Tong Qiao, MD, Department of Vascular Surgery, Drum Tower Hospital, Affiliated Hospital of Medical College, Nanjing University, Nanjing 210008, Jiangsu Province China.
E-mail address: qiaotongmail@yahoo.com.cn

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Materials and Methods

Patients

Forty-eight patients (including 12 control cases) gave their consent to participation in this study. There were 26 men and 22 women, median age 44.5 years (range 26–65). In the venous disease cohort, the medical history of venous disease ranged from 5 months to 20 years and the severity appeared to increase with time. There were 22 patients of CEAP class 2 and 14 patients of CEAP classes 4–6. Participating patients completed a study questionnaire and where previous deep vein thrombosis was suspected ascending venography was performed before this research in order to exclude any patient with post-thrombotic syndrome. The patients with venous disease were mobile without evidence of arterial insufficiency, which was excluded by Doppler ankle pressure measurement. There was no limitation of ankle movement on clinical examination. The patients did not wear compression bandages or stockings during the study period. Twelve age and height-matched patients from the trauma or orthopaedics departments who had no clinical evidence of venous disease comprised the control group. Normal function of the arterial and venous system was confirmed in all volunteers by duplex ultrasound imaging. The control group also had full ankle movement on clinical examination.

All patients with venous disease underwent biopsy of the gastrocnemius muscle during their operation (group A). Group A was divided into group AI (21 varicose veins patients) and group AII (15 DVI patients). In these patients biopsies were also obtained from thigh adductor muscle and served as a control group (group B). A second control group comprised 12 biopsy specimens from gastrocnemius muscle of patients without vascular disease from patients undergoing surgical treatment in the trauma and orthopaedics department (group C).

This study was approved by the medical committee of hospital, and informed consent was obtained from each patient.

Examinations

Duplex ultrasound scan

The limbs were evaluated by duplex ultrasound scanning using a SONOSITE 180 scanner (Sonosite, USA), with 2.5–7.5 MHz probes. Patients stood for the examinations and reflux was defined as retrograde flow persisted for greater than 0.5 s following a manual distal compression and release or Valsalva manoeuvre.[2,3] Sites examined included the common femoral, femoral, popliteal, greater and small saphenous veins.[4,5] The venous disease patients were classified into superficial venous disease (reflux in the superficial veins with competent popliteal valves), and deep venous incompetence (DVI) (popliteal reflux on duplex examination) groups.

Ambulatory venous pressure (AVP) measurement

All patients with venous disease patients and eight volunteers in the control group agreed to undergo AVP measurement. Venous pressure was measured by inserting a needle into a vein on the dorsum of the foot. The needle was connected through a pressure transducer and an amplifier to a potentiometric pen recorder and changes in venous pressure recorded during rest and standard tiptoe exercise.[6] The resting pressure (P0) was measured in the standing position; post-exercise pressure (PEP), the lowest pressure after standard exercise, and 90% venous pressure refilling time (RT90) were measured to assess the calf muscle pump function with and without the effect of a tourniquet placed below the knee.

Pathophysiological examinations

Fresh muscle biopsies was divided into three samples, avoided crushing when cutting the epimysium. Samples were stored in $-10\,^{\circ}C$ while clamped in an isometric device to prevent contraction artefact and were analysed for superoxide dismutase (SOD), nitric oxide (NO), Na^{+}–K^{+}-ATPase, Ca^{2+}-ATPase and lactic acid (LD) within 6 h of collection according to the instructions of the appropriate reagent kit (Jiancheng Medical Institute, China).

The second samples were immediately frozen at $-160\,^{\circ}C$ in isopentane cooled in liquid nitrogen. Frozen sections were cut 6 μm in thickness in a cryostat and stained with haematoxylin–eosin (H & E), myofibrillar protein adenosine triphosphatase (ATPase) at a pH of 9.4, cytochrome oxidase/succinate dehydrogenase (COS/SDH) and were subjected to light microscopy examination. The third group of samples were examined by electron microscopy.

Statistical analysis

Values are represented by the mean and SD. The significance of differences in the parameters measured in all samples was assessed by analysis of variance. Differences were considered significant if P was less than 0.05.

Results

Duplex ultrasound investigation demonstrated that there were 21 patients with isolated superficial venous disease of CEAP clinical class 2 and 15 patients with deep venous incompetence, 14 of CEAP class 4–6, one of CEAP class 2. Twelve controls from trauma and orthopaedics department have not any superficial or deep venous reflux.

AVP measurement

The measurements of P0, PEP and RT90 in limbs of each group were shown in Table 1. No significant difference was found in the P0 between the three groups. In limbs with DVI, PEP was higher than it in control group ($P<0.05$). After application of a tourniquet to occlude the superficial veins, the PEP remained significantly higher in the DVI group than both the control and PVV group. The venous refilling time (RT90) was reduced in both the PVV and DVI groups compared with the control group ($P<0.05$). Application of a tourniquet returned the RT90 in PVV group close to that in the control group. However, the RT90 in the DVI group remained of short duration, as would be expected.

Pathophysiological examinations

The biochemical changes in the all muscle biopsy specimens are summarised in the Table 2. The parameters of SOD, NO, biochemical activity of Na^+–K^+-ATPase, Ca^+-ATPase in all muscle specimens of groups B and C were in normal range. In the specimens of 36 patients in A group, the concentration of LD was raised compared to B and C group specimens. In group AII, the concentrations of SOD and NO in muscle cells were significantly reduced, and biochemical activity of Na^+–K^+-ATPase, Ca^+-ATPase was lower in than the control groups.

As are shown in Fig. 1(a) and (b), control biopsy specimens removed from the B and C groups revealed no significant pathological changes on H & E staining, ATPase or COX/SDH and the ultrastructure of myofibrils is clear and orderly under both light and electron microscopy. In the gastrocnemius muscle of patients with venous disease (group A) pathological changes were found including disseminated myofibril atrophy, cell denaturation and necrosis, inflammatory cell infiltration, proliferation and dilation of interfascicular veins. Examples are shown in Fig. 2(a)–(c). Fig. 3(a) and (b) shows that in 13 of 15 specimens in group AII and five of 21 specimens in group AI, ATPase stain (pH 9.4) demonstrated grouping of myofibrils, especially for type I myofibrils, accompanied by moderate to severe atrophy of type II myofibrils. At a pH of 9.4, type I fibres were pale in ATPase staining and type II fibres exhibit darkly staining. We did not find any specimens with selective type I fibre atrophy. Fig. 3(c) shows enhanced enzymatic activity in single or multiple myofibrils with the COX/SDH stain which was found in eight of

Table 1. Ambulatory venous pressure values in each group (mean, SD)

	Control group ($n=8$)	PVV group ($n=21$)	DVI group ($n=15$)
P0 (kPa)	11.7 SD 1.4	12.3 SD 2.3	12.5 SD 3.3
PEP (kPa)	4.0 SD 0.6	5.7 SD 0.7	7.5 SD 1.4*
A	4.2 SD 0.8	4.1 SD 0.6	7.1 SD 1.6†
RT90 (s)	30.6 SD 8.0	17.7 SD 5.3*	10.6 SD 2.3†
A	32.6 SD 7.3	28.4 SD 6.8	14.2 SD 3.6†

A, with tourniquet.
* $P<0.05$ *vs* control group.
† $P<0.05$ *vs* control and PVV groups.

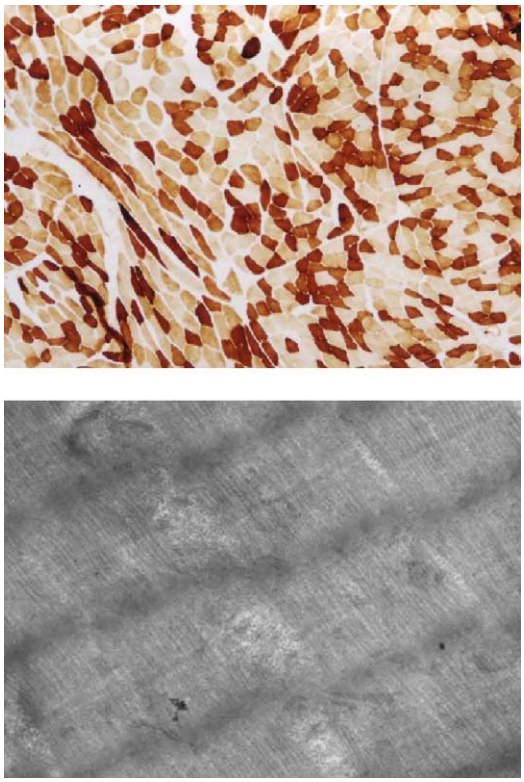

Fig. 1. (a) COX stain: normal muscle fibre from normal gastrocnemius muscle of group C. Type I and type II muscle fibres are approximately equal in size and are polygonal in configuration. The distribution of fibre types resembles that of a checkerboard (40×). (b) Normal muscle from normal gastrocnemius muscle of group C. Electron photomicrograph clearly shows the ultrastructure of skeletal muscle (20,000×).

Table 2. Results of biochemical analysis of muscle in the different clinical groups (mean, SD)

Group	Na^+–K^+-ATPase (μmol/mg pro)	Ca^{2+}-ATPase (μmol/mg pro)	LD (μmol/g pro)	NO (μmol/g pro)	SOD (U/mg pro)
AI	1.03 SD 0.22	0.67 SD 0.13	1004 SD 312*	25 SD 2.4	74 SD 15
AII	0.49 SD 0.14†	0.45 SD 0.11†	1225 SD 372*	18 SD 2.2†	43 SD 9.9†
B	1.11 SD 0.31	0.96 SD 0.25	615 SD 15432	32 SD 3.2	70 SD 21
C	1.08 SD 0.29	0.81 SD 0.16	567 SD 127	28 SD 3.0	88 SD 17

AI, gastrocnemius muscle, PVV group; AII, gastrocnemius muscle, DVI group; B adductor muscle, PVV and DVI groups; C gastrocnemius muscle, control patients.
* $P<0.05$ *vs* B, C group.
† $P<0.01$ *vs* AI, B, C group.

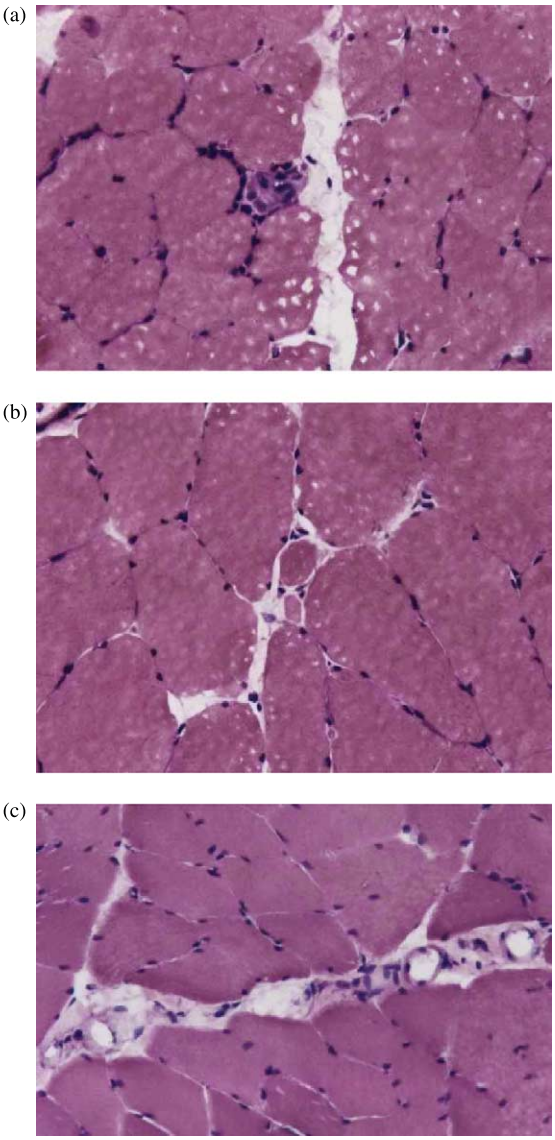

Fig. 2. (a) Gastrocnemius muscle from a group A (venous disease) patient, H & E stain: a single myofibril shows atrophy and lymphocyte infiltration (200×). (b) Gastrocnemius muscle from a group A (venous disease) patient, H & E stain: multiple myofibril atrophy (400×). (c) Gastrocnemius muscle from a group A (venous disease) patient H & E stain: dilation of interfascicular vein (200×).

15 specimens in group AII. Electron microscopically, swelling, myelin figure denaturation of mitochondria, disruption of the myofibrils and increased lipid droplets in gastrocnemius were apparent in 10 of 15 specimens in group AII and three of 21 specimens in group AI. (Fig. 4(a)–(c)).

Discussion

The efficiency of calf muscle pump relies on normally functioning venous valves, powerful contraction of calf muscle, full ankle joint movement and normal muscular fasciae. Any malfunction in this system may contribute to calf pump dysfunction, influencing the venous haemodynamics and resulting in venous hypertension.[7] Patients with chronic venous disease have venous reflux, weakness of calf muscle strength and calf pump dysfunction.[8] The aim of this study was to investigate the relationship of venous reflux and pathophysiological muscle changes.

In normal human skeletal muscle, type I and type II muscle fibres are approximately equal in size and are polygonal rather than angular in configuration. The distribution of fibre types resembles that of a checkerboard, with light and dark fibres arranged in an evenly mixed mosaic pattern.[9] We found changes characterised by denervation and reinnervation in gastrocnemius muscle from patients with venous disease, but in no other biopsy specimens. Electron microscopy showed denaturation of mitochondria and disruption of the myofibrils in the AII group. In muscle cells mitochondria are scattered among and around the myofibrils and provide the ATP needed to power muscular contractions. We suspected that the ATP demands of a contracting skeletal muscle might not be met by the impaired mitochondria in patients with CVI.

Na^+–K^+-ATPase, found in plasma membranes of most animal cells, catalyses ATP-dependent transport of Na^+ out of a cell in exchange for K^+ entering the muscle cell. Ca^{2+}-ATPases, in endoplasmic reticulum (ER) and plasma membranes of muscle cells, catalyses

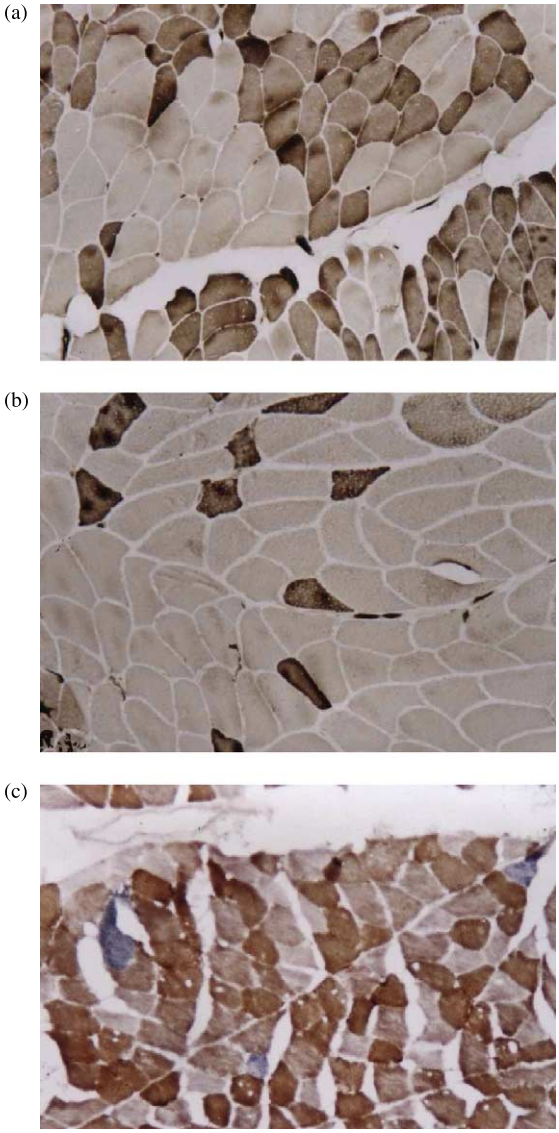

Fig. 3. (a) Gastrocnemius muscle from a group AII (DVI), ATPase stain: type I myofribrils grouping (200×). (b) Gastrocnemius muscle from a group AII (DVI), ATPase stain: type I myofibrils grouping, accompanied by moderate to severe atrophy of type II myofibrils (200×). (c) Gastrocnemius muscle from a group AII (DVI), COX/SDH stain: enhanced enzymatic activity in single or multiple myofibrils (100×).

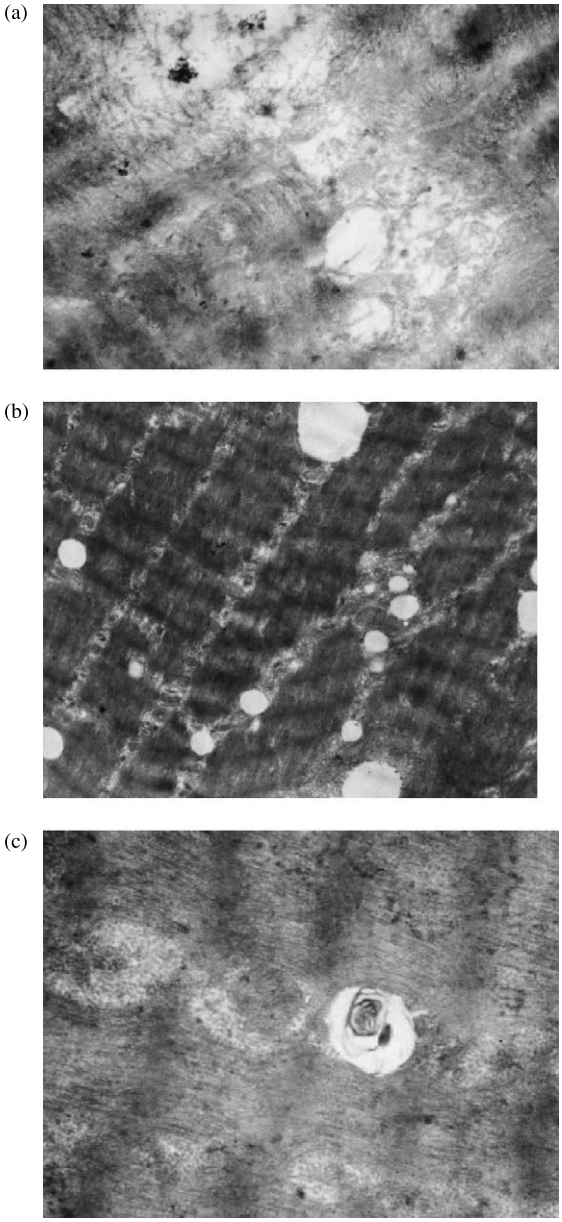

Fig. 4. (a) Gastrocnemius muscle from a group AII (DVI). Electron microscopy shows swelling, disruption of the myofibrils and mitochondrial denaturation (20,000×). (b) Electron microscopy of gastrocnemius muscle from group AII (DVI) showing increased lipid droplets in myofibrils (30,000×). (c) Electron microscopy of gastrocnemius muscle from group AII (DVI) showing myelin figure denaturation of mitochondria (30,000×).

ATP-dependent transport of Ca^{2+} away from the cytosol, either into the ER lumen or out of the cell. We found reduced ATPase activity in group AII specimens. This might lead to impaired ability to maintain or restore Na^+ and K^+ balance across the sarcolemma during repeated muscle contractions. An abnormal intracellular Ca^{2+} concentration would result in defective muscle function, impacting the muscle excitation-contraction coupling (E–C-C), causing prolonged physiological Ca^{2+}-elevation, slowing of relaxation, leading to mitochondrial damage and disorganisation of myofibrils and muscle weakness.[10] This might be the cause of calf muscle fatigue in CVI patients. It has been shown that failure of Ca^{2+} release is strongly implicated in muscle fatigue, depressed Na^+–K^+-ATPase enzyme activity of up to 17% has been reported during fatigue, while training studies

have shown between 13 and 16% increase in Na^+–K^+-ATPase activity.[11–14]

Superoxide dismutase is one of the most important and powerful antioxidants in tissue. Contractions of skeletal muscles produce an increased concentration of superoxide anions and activity of hydroxyl radicals in the extracellular space which alters the skeletal muscle oxidative capacity.[15] Some studies indicated that superoxide radicals play an important role in the pathogenesis of varicose veins, and SOD can inhibit the expression of free radicals and the adhesion molecules, protecting skeletal muscle from impairment.[16,17] We found that biopsies from the AII group had low levels of muscle SOD compared to the other groups. It has been demonstrated that interval and continuous exercise training results in increases in SOD activity and recovery time after exercise was shortened if Vitamin C, E or exogenous SOD was supplied.[18,19] It may be possible to this type of treatment in patients with venous disease in order to improve calf muscle pump function.

Concentration of lactic acid in blood (LAB) is one of the main measures used to evaluate skeletal muscle fatigue in sports medicine and it is mainly influenced by the balance between production and elimination of lactic acid in skeletal muscle (LAM).[20] The concentration of LAM directly evaluates lactic acid metabolism in muscle.[21] We measured the concentration of LAM to estimate the metabolism of gastrocnemius cells and found that the concentration of LAM in A group including PVV and DVI patients was increased compared to control specimens. This would lower the intracellular pH and alter functional characteristics of key enzymes in muscle cells. Lactic acid diffuses out of the muscle fibers and enters the Cori cycle in which lactic acid is metabolised in the liver returning glucose to the muscle cells during recovery,[22] Until LAM levels fall premature muscle fatigue may be the result.

A review of the literature suggests that the effects of nitric oxide (NO) on skeletal muscles fibres can be classified in direct effects and cGMP-mediated effects. These include NO-stimulated glucose uptake, glycolysis and mitochondrial respiration, increasing the shortening velocity of loaded or unloaded contractions. NO has a clear role in regulating basal vascular tone at rest and contributing in part to the blood flow in recovery after exhaustive exercise.[23–26] The data from our study shows that the concentration of NO in biopsies from the CVI group was lower than in the control group.

Impaired biochemical function and morphological have also been reported in samples of skeletal muscle from patients with arterial occlusive disease or disuse atrophy.[27–29] Ischaemia affects each muscle fibre type in different ways according to its particular metabolic and functional properties. In some studies effects on type I fibres predominated and other studies indicated a selective vulnerability of the fast glycolytic fibres.[30,31] Studied by Clyne *et al.* showed decreased levels of aerobic enzymes paralleling decreased Doppler ankle pressure, while claudicants demonstrated increased levels of anaerobic enzymes.[32] We consider that anoxia due to arterial ischaemia or venous congestion would both lead to skeletal muscle impairment characterised by denervation and reinnervation.

We used AVP measurement for quantitative examination of the peripheral venous haemodynamics since venous hypertension is the main factor predisposing to venous insufficiency.[7,8,33] In our series PEP was elevated in patients with a venous disease, with the more severely affected patients having the highest PEP, in keeping with published literature.[34] A shorter RT90 and higher PEP resulted in persistent venous hypertension in limbs with DVI. Patients with the highest PEP also had the greatest disturbance in biochemical measurements and morphological changes in their biopsies. These findings provide further evidence of the interplay between the metabolic and haemodynamic factors that might contribute to the calf pump dysfunction in patients with DVI.

In this study, we classified the clinical presentation of our patients according to the CEAP method.[35] Our patients with the highest CEAP grades has the greatest haemodynamic changes as well as the most severe biochemical and histological changes in their muscle biopsies. Correlation with the CEAP clinical grade confirms that our findings are related to an internationally agreed measure of disease severity.

In conclusion, we have found structural and metabolic abnormalities in the skeletal muscle cells of the calf in patients with venous insufficiency which are most prominent in the most severely affected patients. Some of the symptoms reported by patients in their lower limbs may be attributable to these changes. It has been suggested that pharmacological intervention may be useful in addressing similar muscle changes reported in patients with peripheral arterial diseases.[36–38] In the future we might add to conventional management of venous disease by including physical therapy or therapeutic exercise, muscle nutrition and antioxidant therapy. Clearly these proposals would have to be investigated in clinical trials.

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