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Skeletal muscle oedema and muscle fibre necrosis during septic shock. Observations with a porcine septic shock model

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Abstract In domestic pigs, intermitted application of *Escherichia coli*-endotoxin was used to create an animal model for a prolonged hypo- and hyperdynamic septic shock-like state and to investigate mechanisms of multiple organ failure. Here, we describe the changes in skeletal muscle after 18 h (2 animals) and 48 h (6 animals) of septic shock. Two pigs for each observation period that received physiologic saline solutions instead of endotoxin served as controls. The earliest lesions were endothelial cell damage with endomysial oedema and swelling of mitochondria in muscle fibres. With increasing degree of endothelial cell damage, pericytes showed degenerative changes with cytoplasmic fragmentation and karyolysis. After 48 h of shock, endomysial oedema was increased with fibrinogen present. Muscle fibre diameters were increased and swollen mitochondria and segmental necrosis of muscle fibres were frequently observed. However, phagocytic reaction or regenerative changes were not detected. In this respect, skeletal muscle lesions in septic shock differ from ischemic damage, which is characterized by early phagocytosis. Tumour necrosis factor alpha (TNFα) was increased greatly and significantly in the serum of the pigs that received endotoxin. The lesions described may be the result of both direct damage to muscle fibres by the endotoxin and/or the increased levels of TNFα and indirect damage because of the increased diffusion distance, due to the endomysial oedema. The loss of blood proteins into the endomysium may also play a role in generating hypoproteinemia in patients with septic shock.

Key words Septic shock · Skeletal muscle
Porcine shock model

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Introduction

Despite the significant advances in intensive care, mortality due to sepsis-associated adult respiratory distress syndrome and multiple organ failure (MOF) remains high. Shock-induced changes in lung and kidney have been studied extensively both clinically and in experimental models, but little is known of alterations in other organ systems. Skeletal muscle is a well perfused and voluminous organ and in shock it may be assumed that its endothelium reacts similarly to the endothelium of the lung and kidney. Skeletal muscle may play a major role in the process that causes hypovolemia, and we investigated the changes in muscle in septic shock in an experimental porcine model.

This model was initially established to investigate the factors and mechanisms that initiate and modulate MOF. The experimental design imitates a prolonged hypo- and hyperdynamic septic shock-like state for 48 h which is similar to the complete clinical syndrome [13]. Because tumour necrosis factor α (TNFα) has been shown to be an important factor in septic shock [2, 12, 24], serum levels of TNFα were monitored and correlated with the changes found.

Material and methods

The animal model has been described previously [11, 12]. Briefly, domestic pigs (28–32 kg) were in quarantine and treated with antibiotics to prevent pulmonary infections for at least 1 week. The animals were divided into three groups. The 48 h test group (six animals) received endotoxin intravenously (0.5 µg/kg *Escherichia coli*-endotoxin WO111:B4 over 60 min; Difco Laboratories, Detroit, Mich., USA) at two time points, after 1 h and 22 h. The 18 h test group (two animals) received 0.25 µg/kg endotoxin at the beginning of the experiment and at 5 and 10 h. The control group (two animals for each experimental period) received physiological saline solution. The animals were sacrificed by an intravenous injection of 10% potassium chloride. Tissue samples from the psoas muscle of all animals of all groups were obtained using standard procedures for biopsies of human muscle [21].

The present study was performed in accordance to the NIH guidelines for the use of experimental animals and the German

Law for the Protection of Animals (Tierschutzgesetz). Permission was also granted by the local authorities.

Tissue samples of the animals were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Sections 5 µm thick were cut and stained with haematoxylin and eosin. For transmission electron microscopy (TEM), tissue specimens were fixed in 3% cacodylate-buffered glutaraldehyde for 30 min. After post-fixation in 0.1 M cacodylate-buffered osmium, the specimens were dehydrated in ethanol and embedded in epon. Semi-thin (1 µm) sections were stained with methylene blue-azure II. Ultra-thin sections (70 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a TEM (Philips EM400T). From five blocks from each observation period, serial sections were cut to search for endothelial fenestrations.

Immunohistochemistry was performed on both paraffin-embedded and epon-embedded muscle. Paraffin sections 5 µm thick were deparaffinized with xylene and rehydrated using decreasing ethanol concentrations. Incubation with the primary antibody (polyclonal anti-fibrinogen antibody A080, Dako, Hamburg, Germany) at a dilution of 1:100 was performed for 60 min at room temperature in a humid chamber. After washing three times with phosphate-buffered saline/bovine serum albumin (PBS/BSA) the sections were covered with the peroxidase-conjugated secondary antibody (swine anti-rabbit, Dako) at a dilution of 1:40. After further rinsing with PBS/BSA, the slides were incubated with diaminobenzidine, rinsed again, counterstained with haematoxylin and mounted with *N*-propyl gallate. Controls were performed by replacing the primary antibody by 1.5% BSA/PBS.

Semi-thin sections of epon-embedded, unosmicated specimens were de-eponised in 10% sodium methoxide for 15 min, washed twice each with methanol and with sodium chloride (NaCl)-TRIS buffer (pH 7.5) for 1 min, incubated for 15 min in hydrogen peroxide, and rinsed again twice in NaCl-TRIS buffer. Thereafter, the sections were incubated for 1 h in fetal calf serum to block unspecific antibody binding, washed again twice with NaCl-TRIS buffer and incubated with the antiserum against fibrinogen (AO80) for 60 min at room temperature. After two washes with PBS the sections were incubated with peroxidase-conjugated secondary antibody (described above) at a concentration of 1:40 for 20 min at room temperature, washed again twice with PBS and then stained with diaminobenzidine and toluidine blue. As a control for unspecific staining, the first antibody was replaced by 1.5% BSA/PBS on one section in each round of immunostaining.

Photomicrographs were made from randomly selected areas sparing the margins of the muscle at a magnification of 240×. The slides were projected on a planimetry table. Five fascicles of each animal were evaluated morphometrically. A personal computer with a planimetry table (Summagraphics, model 1201, Fairfield, Conn., USA) and image analysis software (developed in the Institute of Pathology, RWTH) was used to measure the cross sectional areas and the diameters of the individual muscle fibres as well as the total fascicle areas. The areas occupied by the interstium were determined by subtracting muscle fibre areas from fascicle areas. For statistical analysis, Student's *t*-test was used.

Plasma concentrations of TNFα were measured by the ^{125}I-TNF_{1a}-radioimmunoassay (RPA 532, Amersham, Braunschweig, Germany). Blood samples were taken at each hour from both endotoxin treated and the control animals with polypropylene syringes (Monovetten, Saarstedt-Nümbrecht, Germany) containing ethylenediaminetetracetic acid (2 mg/ml blood) and acetylsalicylic acid (Aspirol, Bayer, Leverkusen, Germany; 0.5 mg/ml blood) dissolved in 0.6 ml 0.9% NaCl. Samples were carefully tilted twice and immediately centrifuged at 2500 *g* for 10 min at 4° C. The supernatant was divided into 1.0 ml portions in polypropylene reaction tubes (model 780.500, Brand, Wertheim, Germany) and stored at −70° C until use for analysis. Radioimmunoassays were then performed according to the supplier's instructions. Plasma concentrations were estimated by use of the standard curve. All samples were measured in duplicate. Cross reactivities of the anti-TNFα serum were: TNF1α (mouse, recombinant) 0.025%; TNFβ (human, recombinant) <0.24 interleukin; (IL)-1α (human, recombinant) <0.44; IL-1β (human, recombinant) <0.17; IL-2 (human, recombinant) <0.18; IL-3 (human, recombinant) <0.17; IL-4 (human, recombinant) <0.19; IL-6 (human, recombinant) <0.029.

Results

In skeletal muscle of 18 h the most marked cellular changes occurred in endothelial cells, which showed damage of various degree. There was intracellular oedema, accompanied by an increase in the number of pinocytotic vesicles, vacuolization, and necrosis (Fig. 1). Pericytes adjacent to necrotic endothelial cells showed similar signs of damage. Widening of endothelial fenestrations was not seen. However, considerable endomysial oedema and vacuolization of some muscle fibres was present (Fig. 2). On morphometrical analysis, the portion of muscle fascicle area occupied by interstitial space was increased from 17.5% in control animals to 23.6% in the endotoxin treated pigs, whereas the mean muscle fibre diameters were increased only slightly (Fig. 3). At this early stage, TEM showed that the oedematous fluid contained fine granular material (Fig. 4a), which probably consisted of proteins. The vacuolization of muscle fibres was caused by swelling of the mitochondria, often resulting in the development of giant cystic mitochondria (Fig. 4a, b). In the control animals, the mitochondria were not damaged (not shown).

After 48 h, the interstitial oedema was increased markedly from 23.6% of total muscle fibre area after 18 h to 35.6% (Fig. 3). Even though the standard deviations of the two groups overlap slightly, the differences proved to be significant in the *t*-test ($P<0.05$). The mean muscle fibre diameters were increased considerably from 95.8 µm in control animals to 104.7 µm (maximum 126.3 µm) in septic pigs. As shown in Fig. 5, the normal dome-shaped distribution of size classes in control animals was altered. The occurrence of globular precipitates in the endomysal oedema suggested an increase in protein content (Fig. 6). Immunohistochemically, a strong anti-fibrinogen reactivity was found in the interstitial oedema in both semi-thin and paraffin sections (Fig. 7), which was similar to the immunoreactivity of the blood in cross sectioned blood vessels. No other structures were stained by the antiserum. As after 18 h, the endothelial fenestrations were not found to be widened. Some muscle fibres showed segmental necrosis (Fig. 8), with transition from areas with cystic dilated mitochondria to necrotic fibres (Fig. 9). It is of interest to note that even after 48 h of septic shock, a phagocytic reaction was not found.

Immediately after the endotoxin injections, plasma TNFα increased almost twofold. The following endotoxin injections were accompanied by a less prominent TNFα peak (Fig. 10). The serum concentrations in the animals which received endotoxin at two time points during a 48 h period were similar, showing a considerable increase after the first injection and a smaller increase after the second one.

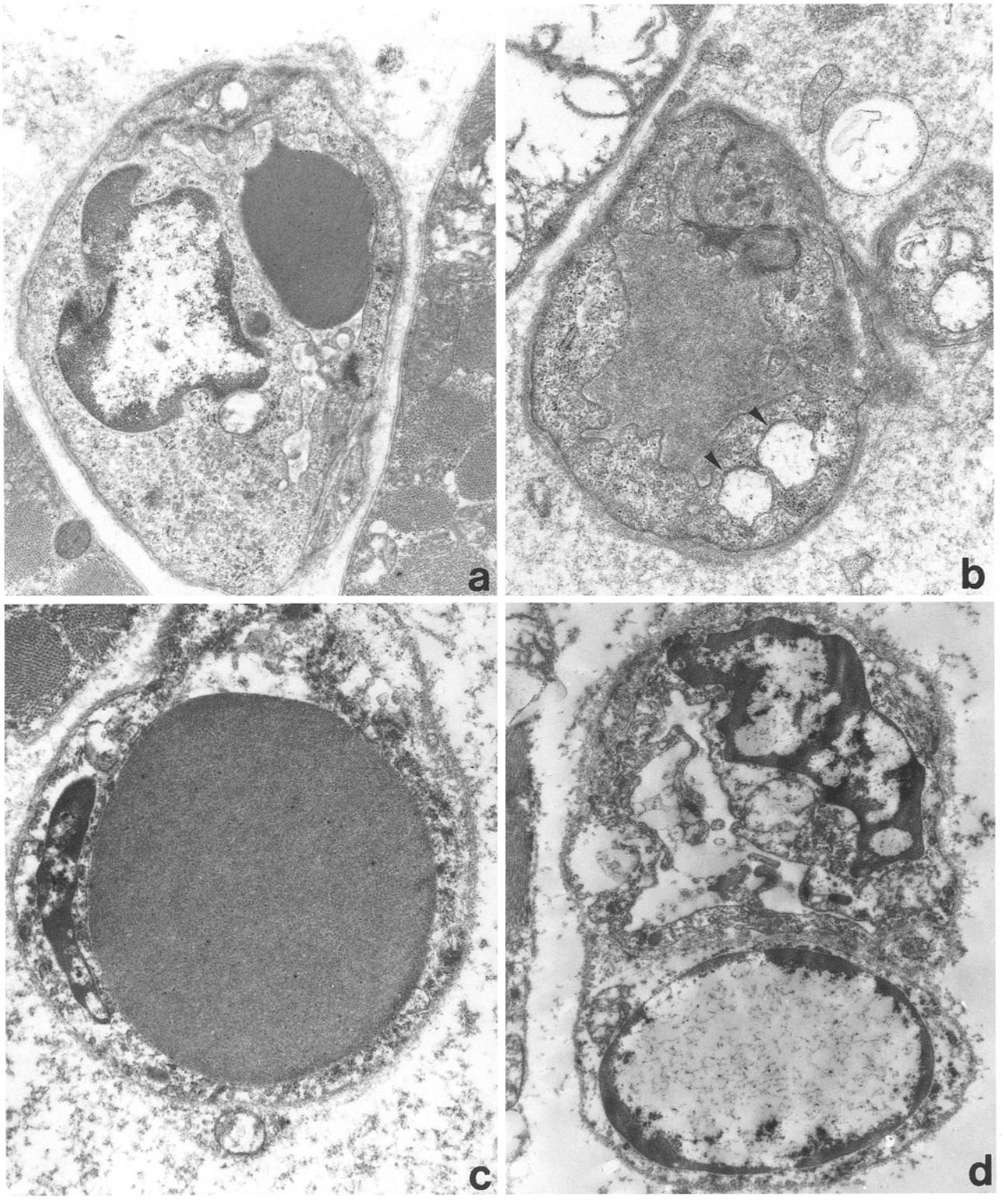
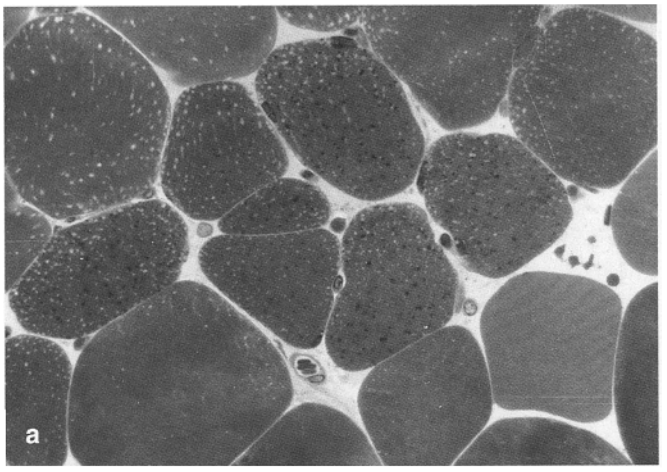
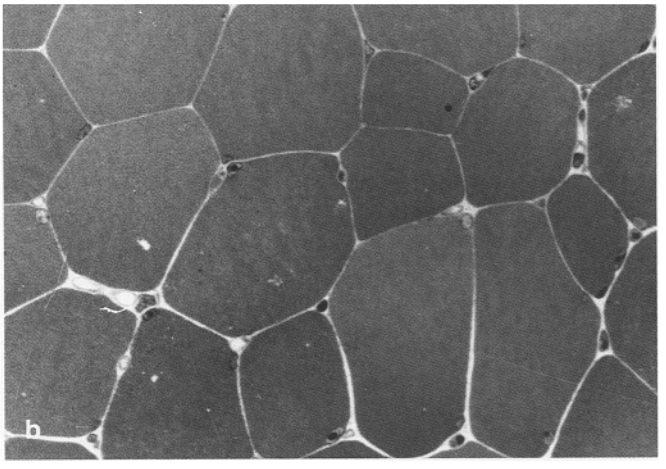


Fig. 1 Various degrees of damage to the capillaries in muscle after 18 h septic shock: intracellular oedema (**a**), vacuolization (*arrowheads*; **b**, necrosis of endothelial cells (**c**), and pericytes (**d**). [Transmission electron microscopy (TEM), ×18,000]

Discussion

The morphological pattern of skeletal muscle changes during the first 48 h of septic shock was characterized by three phenomena. First, there were various degrees of damage to the capillary walls, ranging from intracellular oedema and vacuolization to necrosis of endothelial cells





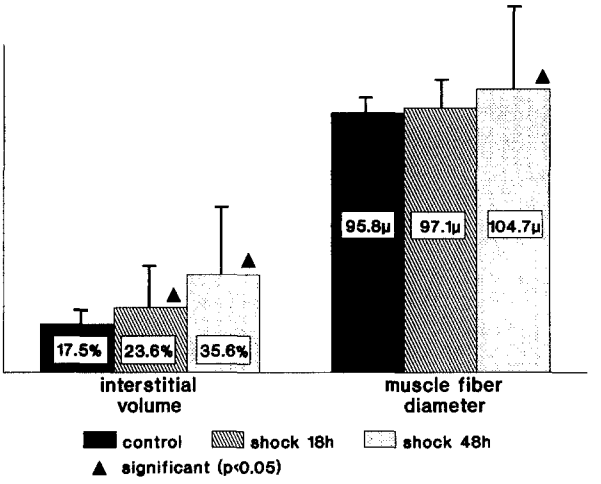
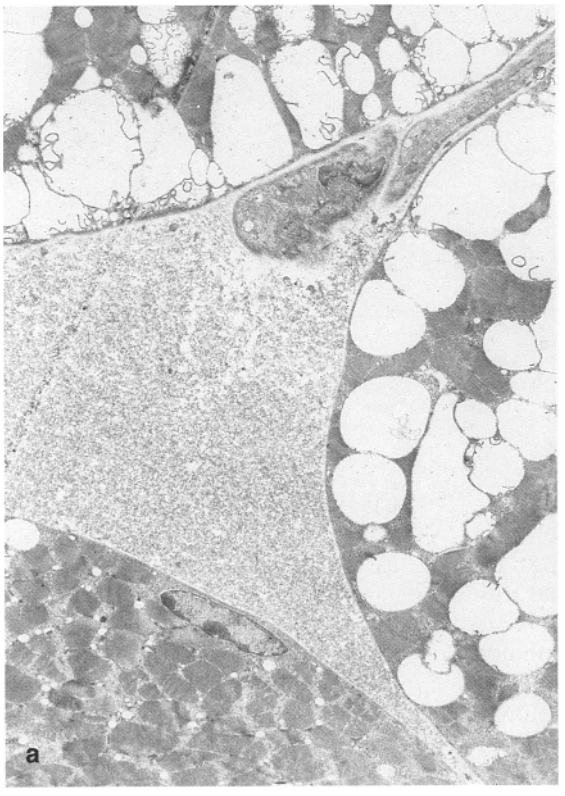


Fig. 3 Morphometric analysis of muscle fibre diameters (*right*) and interstitial space (*left*). Muscle fibre diameters are given in micrometers, interstitial space in percent of the fascicle area

Fig. 2a Endomysial oedema as well as vacuolization of muscle fibres after 18 h of shock (semithin section, ×400). **b** Normal control muscle (semi-thin section, ×400)



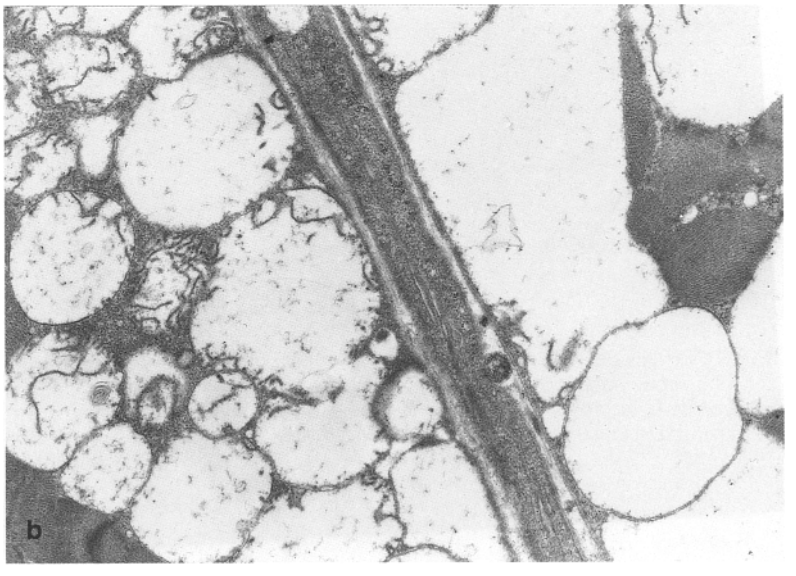


Fig. 4a Endomysial oedema with electron-dense fine granular material as well as vacuolization of muscle fibre mitochondria after 18 h of septic shock (TEM, ×5,040). **b** Considerable cystic dilatation of skeletal muscle fibre mitochondria at the same stage of shock (TEM, ×14,400)

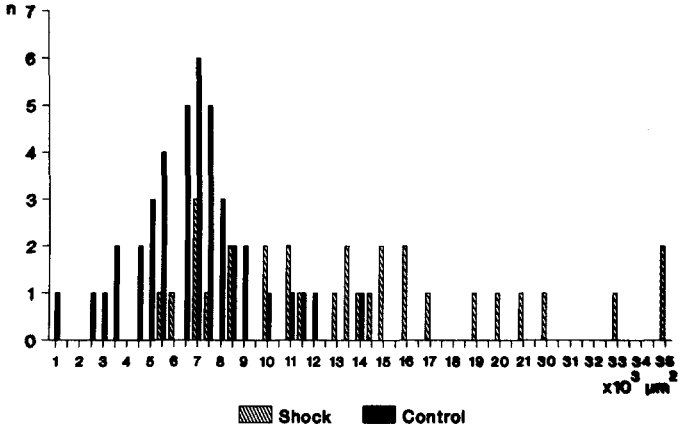


Fig. 5 Muscle fibre diameters (size classes) in a control and a pig treated with endotoxin. The normal distribution of size classes is altered in the septic state

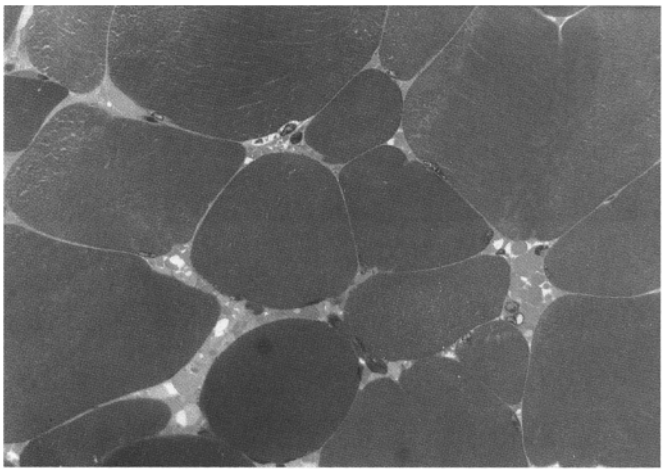
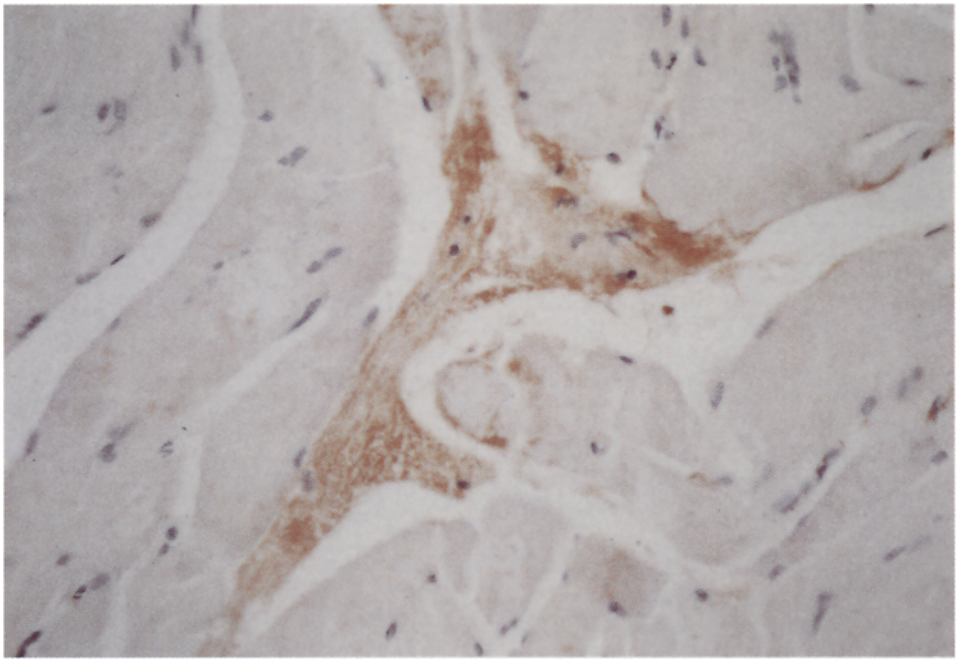


Fig. 6 Formation of globular precipitates in the endomysial oedema suggesting increased protein content after 48 h of septic shock (semi-thin section, ×400)

Fig. 7 Immunohistochemical detection of fibrinogen in the interstitial space (×240)



and pericytes. The second component was an increasing endomysial oedema rich in proteins such as fibrinogen. Third, there were changes in the muscle fibres themselves, ranging from mitochondrial swelling to cystic degeneration and vacuolization. Segmental necrosis of muscle fibres was present after 2 days of shock, but a phagocytic reaction was absent.

Our observations indicate that in the porcine model of recurrent endotoxemia, the capillaries of skeletal muscle undergo damage to the same degree as observed in the lung [13]. These results confirm and extend previous

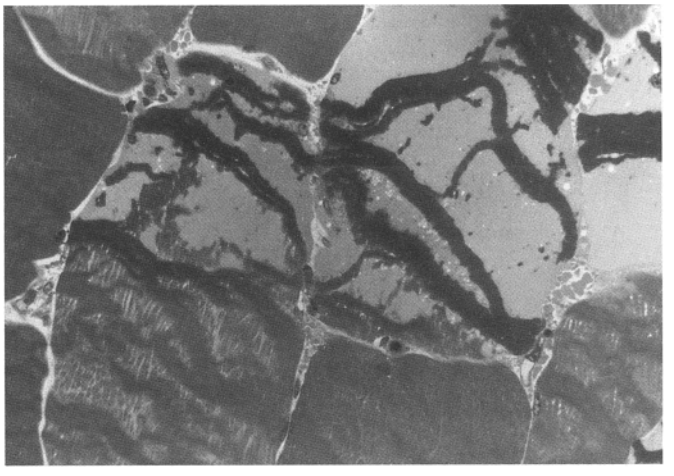


Fig. 8 Segmental necrosis of muscle fibres after 48 h of septic shock (semi-thin section, ×400)

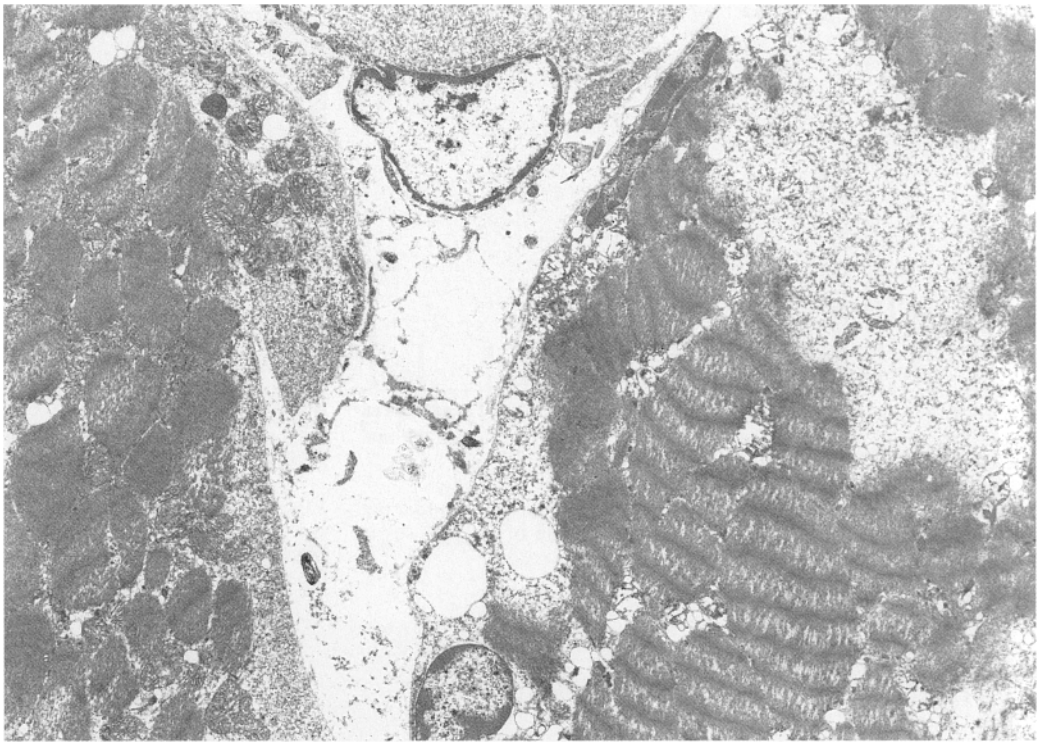


Fig. 9 Segmental necrosis with transition between muscle fibre segments with dilated mitochondria and necrotic segments (TEM, ×8,280)

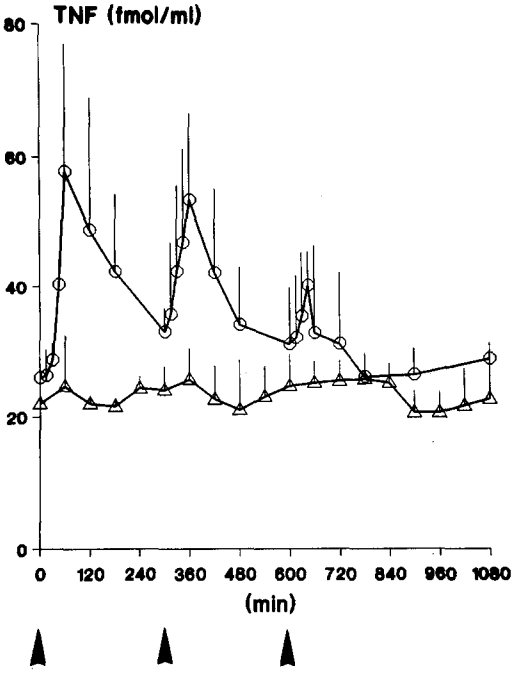


Fig. 10 Tumour necrosis factor-level in the serum during the 18 h experiments (mean values) in control pigs (*triangles*) and endotoxin treated pigs (*circles*). Arrowheads indicate the endotoxin administration

findings that an intracellular and intercellular oedema as well as patchy cell injury with mitochondrial changes accumulate in skeletal muscle of sheep with caecal perforation [8]. Skeletal muscle in haemorrhagic shock in cats [1] and in a canine polytrauma model [20] showed interstitial oedema, but protein accumulation in the oedematous fluid was not seen.

A comparison of our findings after 18 and 48 h of septic shock shows that the initial lesion is endothelial cell injury. Pericytic alterations were observed only in segments of muscles that already showed severe endothelial cell alterations. The endothelial cell swelling may reflect damage to the plasma membrane for reasons which are unclear. It is of interest to note that endothelial fenestrations were not widened. Possible explanations for the endothelial damage include a direct toxic effect of the endotoxin injected or the effects of mediators or oxygen radicals [15]. The defects in microcirculation may have caused the formation of oedema and the extravasation of proteins [4]. Due to the interstitial oedema, the diffusion distance from blood vessel to muscle fibres was increased, possibly contributing to the muscle fibre damage.

Rhabdomyolysis is not an uncommon condition in sepsis, but may escape notice due to the underlying disease [9, 17, 18, 22]. Direct infiltration of the muscle by bacteria or the effects of toxins or mediators are thought to be the cause of muscle fibre damage, limb thus study shows that muscle fibre necrosis can occur witout bacteremia. A low pO_2 which causes changes of ion concentrations both inside and outside the fibres and disturbances of membrane potential may be important factors in the process [19]. It is also known that TNFα , which is increased in this model of septic shock, can induce a decline transmembrane potential in skeletal muscle in vitro [23], resulting in an increase in intracellular calcium. Thus, a direct cytokine effect may contribute to segmental muscle fibre necrosis.

The time course of skeletal muscle fibre necrosis in our model was different from that in acute ischemic muscle fibre injury. In acute ischemia muscle fibre necrosis occurred quickly and was accompanied by an early phagocytic reaction after 18 h [6, 7, 10, 16]. In contrast, a phagocytic reaction was completely absent in our experimental animals. This delay may be related to the impaired blood supply in septic shock which is comparable to partial ischemia. This hypothesis is supported by the results of muscle microembolization experiments causing partial ischemia in rats [14]. In addition, depletion of the immune response by toxin related effects such as alterations in cytokine production or generation of radicals may inhibit the phagocytic reaction [5, 12].

We suggest that protein loss in the muscular interstitial space may play a significant role in the generation of hypoalbuminemia and volume disturbance in septic shock. Damage of skeletal muscle in septic shock may not merely be a concomitant involvement but a crucial step in the progression to multiple organ failure.

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