

Ultrastructural Changes in the Diaphragm of Aged Emphysematous Hamsters

Herwin A. Machiels, A. Jeroen Verheul, Huib J. Croes, Theo Hafmans and P.N. Richard Dekhuijzen

Department of Pulmonary Diseases, University Medical Centre Nijmegen, The Netherlands

Abstract

In emphysematous hamsters the diaphragm is continuously exposed to an increased workload. Previous lightmicroscopical observations showed only minor changes in relatively young emphysematous hamsters. However, ageing affects the regenerating capacity of muscles. We hypothesised that the diaphragm of aged emphysematous hamsters shows an increase of ultrastructural alterations in comparison to age-matched normal hamsters. This could contribute to a reduced force-generating capacity, which occurs in emphysematous hamsters.

After intratracheally instillation of elastase or saline the animals were sacrificed at the age of 15-16 months for *in vitro* measurements of isometric contractile properties of the diaphragm. The hamsters were perfused *in situ* with glutaraldehyde for electronmicroscopical investigations.

In emphysematous hamsters, twitch and tetanic forces were reduced by ~28% and ~14%, respectively ($P < 0.001$). Compared to control hamsters the diaphragm showed distinct muscle fibre damage (Z-line dislocation, disarray of myofibrils, misaligned sarcomeres, focal degeneration, focal necrosis and segmental necrosis) and more signs of sub-optimal regeneration (fibre splitting and forking) and abortive regeneration.

In conclusion, the diaphragm of aged emphysematous hamsters exhibits increased ultrastructural abnormalities, which are likely to contribute to a reduced force generating capacity.

Key words: age, diaphragm, emphysema, hamster, injury, ultrastructure.

Basic Appl Myol 12 (5): 201-208, 2002

Skeletal muscle dysfunction in patients with chronic obstructive pulmonary disease (COPD) involves both peripheral and respiratory muscles. Alterations in muscle structure, metabolism and function have been described [1, 10]. Dysfunction of these muscles negatively influences patient's functional status and exercise tolerance [4, 18], health care utilisation [11], and possibly survival [10].

Elastase-induced emphysema in hamsters is frequently used as an animal model to study morphological and functional changes in the diaphragm due to COPD [29, 36]. *In vitro* diaphragm muscle force-generation of elastase-induced emphysematous hamsters has been reported to be similar to saline treated animals [15, 36]. In other studies, however, a downward shift was observed in absolute force-frequency relationships [19, 22, 23, 27] and force-velocity relationships compared to control animals [21]. The discrepancy in results might in part be explained by the age of the animals since the susceptibility to muscle injury is assumed to increase with age

[9, 16, 39]. Secondly, it has been reported that the regenerating capacity of skeletal muscles is impaired in aged animals [7, 8, 17, 35].

In addition, due to the increased resistive load on the emphysematous diaphragm one would expect morphological changes as have been observed after chronic resistive loading in normal hamster diaphragm [20, 31, 33, 40]. These alterations include a greater variation in fibre size, necrotic muscle fibres, sarcomeric disruption and Z-line streaming. Indeed, van Balkom *et al.* reported alterations in the fibre size of diaphragm muscle in 2 years old emphysematous hamsters and suggested muscle changes at the ultrastructural level [37]. However, no ultrastructural injury was reported in ~33 weeks-old hamsters that were instilled with elastase at the age of 7-9 weeks [29].

We hypothesised that the aged emphysematous hamster diaphragm, which is chronically overloaded, shows increased ultrastructural abnormalities due to muscle fibre damage and an impaired or sub-optimal regenerating ca-

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capacity in comparison to age-matched 'healthy' control hamsters. Therefore, an ultrastructural study of the diaphragm in aged emphysematous hamsters (instilled at the age of 40 weeks) and age-matched normal hamsters was performed. To confirm changes in contractile properties in these hamsters, diaphragm twitch and maximal tetanic tensions were also measured in both groups.

Methods

Animals, induction of emphysema and study design

Male Syrian hamsters (n=36) were used for the experiments. The animals were housed under a 12:12 light-dark cycle in a specific-pathogen-free area (SPF unit) according to the FELASA recommendations [30]. Animals were fed *ad libitum*. The studies were approved by the local Animal Ethical Committee.

At the age of ~40 weeks the hamsters were anaesthetised with a mixture of halothane and N₂O, vaporised in air. A polyethylene cannula was inserted into the trachea with the tip located above the carina. The animals were intratracheally instilled with either porcine pancreas elastase (24 U/100 g body weight (EPC, Owensville, MI) in 0.50 ml 0.9% NaCl/100 g body weight) (n=18) or an equal volume of 0.9% saline (n=18), as described in detail previously [19,23,36,38]. To improve the distribution to the peripheral parts of the lung, 3 ml of room air was injected through the tube. The hamsters were monitored carefully until spontaneous breathing was restored.

Six months after instillation the hamsters (age 15-16 months) were sacrificed in order to measure contractile properties of the diaphragm *in vitro* (control, n=7; emphysema, n=7). Twelve animals were sacrificed to dissect the diaphragm for ultrastructural studies (control, n=6; emphysema, n=6), and in ten animals the degree of emphysema was verified (control, n=5; emphysema, n=5).

Measurement of contractile properties

The animals were randomly allocated to the experimental groups for measuring contractile properties. All experiments were performed according previously described methods [19,38]. Briefly, the hamsters were anaesthetised with pentobarbital sodium (Nembutal, 70mg/kg *i.p.*) and mechanically ventilated with 100% O₂ (flow of 0.5 ml/g bodyweight/min; respiration frequency 70 breaths/min). The diaphragm and adherent ribs were quickly excised and immediately submerged in cooled oxygenated Krebs solution at pH ~7.40. Muscle strips (~2.5 mm wide) were dissected from the midcostal region with the insertions at the costal margin and central tendon left intact. The muscle bundle was mounted vertically and suspended in a tissue bath containing Krebs solution, perfused with a 95% O₂-5% CO₂ mixture and maintained at 26°C. The ends of the muscle bundle were attached to both the micro-manipulator and to the lever arm of a dual-mode length-force servo-control system (Cambridge Technologies, Cambridge, USA), respectively. The system was controlled by the software program Poly 5.0 (Inspector

Research Systems Inc, Amsterdam, The Netherlands). In the isometric mode, force outputs were digitised using a data-acquisition board (DASH1602, Keithley, Taunton, USA) at a sampling frequency of 2.0 kHz [25].

The muscle was stimulated directly by platinum plate electrodes, with rectangular current pulses (0.5 ms) generated by a stimulator (Instrumental and Electronics Dept., University Medical Centre Nijmegen, The Netherlands). Muscle preload force was adjusted to achieve optimal fibre length (L_0) for maximal twitch force (P_t). After 15 min of thermo-equilibration both P_t and maximal tetanic force (P_0) were determined with a 2 min. interval between subsequent stimulations.

Verification of emphysema

The presence and severity of emphysema was evaluated in normal (n=5) and emphysematous (n=5) hamsters. After anaesthesia the lungs were excised and inflated with 4 % formalin (pH 7.4) to a pressure of 25 cm H₂O for 2 hours and subsequently post fixation lung volume was determined by fluid displacement. The lungs were fixed without external pressure in formalin 4% for at least 5 days. Subsequently, the left lung was embedded in paraffin and sagittal sections (6 µm thickness) were cut and stained with hematoxylin and eosin. To determine the extent of emphysematous changes in the lung, alveolar CSA was measured of at least 100 alveoli using a Sprynt-based, PC-Image digital analysis system (Bos Inc.) [38].

Electronmicroscopy

In another set of experiments the diaphragm was fixed and prepared for light- and electronmicroscopical examination. The hamsters (controls, n=6 and emphysematous hamsters, n=6) were deeply anaesthetised with pentobarbital (6 mg/100 gram body weight). A laparotomy was performed and the abdominal aorta was exposed. An infusion needle was inserted within the aorta in the upstream direction, the caval vein was cut and the abdomen was closed again. The hamsters were perfused with 100 ml 0.9% saline followed by 400 ml of a 2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.3, 25 cm H₂O). After perfusion, the diaphragm was removed, part of the right costal region cut in small slices and immersed overnight in the same fixation fluid. Following rinsing in the same buffer, the tissues were osmicated for one hour in 1% osmium tetroxide in 0.1 M PB, rinsed in PB, dehydrated through graded series of ethanols and embedded in Epon 812.

One-micron thick sections were collected, toluidine blue-stained and screened lightmicroscopically. Examination of these semithin sections was carried out by two well-experienced independent observers. Twenty five muscle fibres in each section (five sections per animal) were investigated. Subsequently, the semithin sections were photographed with a Dialux 20 Leitz (Digital camera Coolpix 990, Nikon). The observed abnormalities appeared to be in concordance for both investigators. Based on the abnormalities found

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Based on the abnormalities found at the lightmicroscopical level, ultrathin sections for electronmicroscopy were cut, double contrasted with uranyl-acetate/lead-citrate and photographed in a transmission electronmicroscope (JEOL 1010).

Measurement of blood oxygen tension in vivo

Arterial blood samples were taken directly after placing the infusion needle in the abdominal aorta in order to determine pH, pO₂ and pCO₂ during both control and emphysema conditions (Ciba Corning 238 pH/ Bloodgas Analyzer, Halstead, UK).

Data treatment and statistics

Cross-sectional area of the muscle bundle was calculated by dividing diaphragm bundle weight (in g) by length (cm) times specific density (1.056 g/cm³). Force was expressed per cross-sectional area (in N/cm²). All data are presented as means $\pm$ SE. Differences in contractile parameters between the treatment groups were analysed using a Student's t-test. Statistical analysis was performed using the SPSS package, v 10.0 (Chicago, IL). Comparisons were considered significant at the P<0.05 level.

Results

Body weight

Body weight was similar in both normal and emphysematous hamsters at the time of instillation of either saline or elastase (control: 149 $\pm$ 3 g, emphysema: 150 $\pm$ 3 g) and at the time of morphological and functional studies (control: 150 $\pm$ 3 g, emphysema: 150 $\pm$ 3 g).

Degree of emphysema and arterial blood gas values

The post-fixation lung volume, determined by fluid displacement, increased from 7.8 $\pm$ 0.4 ml in normal hamsters to 11.6 $\pm$ 0.7 ml in emphysematous hamsters (P<0.05). The alveolar CSA was increased (5147 $\pm$ 335 μ m² in emphysematous hamsters vs. 2698 $\pm$ 91 μ m² in normal hamsters, P<0.05). Arterial blood samples taken directly after laparotomy showed a similar pH (emphysema vs. control: pH 7.24 $\pm$ 0.02 vs. 7.25 $\pm$ 0.02, P>0.05), a lower pO₂ in emphysematous hamsters (9.3 $\pm$ 0.3 kPa vs. 10.2 $\pm$ 0.6 kPa, P<0.05) and hypercapnia in emphysematous hamsters (pCO₂ 9.8 $\pm$ 0.8 vs. 6.8 $\pm$ 1.0 kPa, P<0.05).

Contractile properties

Emphysema significantly reduced P_t and P_o compared to the control group (P_t: 6.5 $\pm$ 0.2 vs. 9.0 $\pm$ 0.2 N/cm², P<0.001; P_o: 22.4 $\pm$ 0.9 vs. 26.7 $\pm$ 0.7 N/cm², P<0.001).

Lightmicroscopy

Control group

In the control group the muscle fibres of the diaphragm exhibited no or occasionally minor alterations in the semithin sections. The empty, widened blood vessels resulted from the perfusion fixation. Within the perimysium

large blood vessels and capillaries were localised in between the connective tissue together with a few axons and several lipocytes. The endomysium was characterised by numerous capillaries in close contact with the muscle cells. Few fibres exhibited elongated central nuclei, sometimes characterised by chain formation. A few splitting fibres were present. No necrotic fibres were observed. Small dark-stained muscle fibres with accumulations of mitochondria close to the cell membrane alternated with thicker light-stained fibres. In several fibres central mitochondrial accumulations branched parallelly along the myofibrils (*i.e.* streaking). Several fibres also showed longitudinal rows of lipofuscin deposits as well as small light-stained vacuoles. Incidentally some thin atrophic fibres were present (Fig. 1A).

Emphysematous group

In the diaphragm of emphysematous hamsters there were more severe structural alterations compared to the control group. In general $\pm$ 20% of the fibres (*i.e.* 4-6 fibres from $\pm$ 25 fibres) appeared to be abnormal in the semithin sections.

Increased Z-line streaming and disarray of myofibrils (smudging of striation) were present. In contrast to the control group, more fibres exhibited rows of central large nuclei with prominent nucleoli. There were more split and forked fibres (Fig. 1B). In several fibres focal degeneration occurred and even distinctly aberrant fibres were observed with sarcoplasmic inclusions and rods formation. Focal necrosis, resulting in indented and split fibres, was observed in a variety of myofibres. In cross-sections several segmental necrotic fibres were found (Fig. 1C). Myophagia, *i.e.* the invasion of a myofibre by mononucleated cells, was only observed in the emphysematous group (Fig. 1D). There were more signs of streaking, but no differences were found in the presence of vacuoles. Lipofuscin granules were increased and there were more atrophic fibres.

Electronmicroscopy (Table 1)

Control group

In the control group the diaphragm sections showed no or only minor electronmicroscopical alterations (Fig. 2A). Incidentally, central nuclei as well as fibre splitting were found. Nuclei were small and slender with a quiescent aspect. Satellite cells were found only sporadically. Atrophic fibres were observed only incidentally.

Emphysematous group

Subtle as well as pronounced alterations were consistently found in emphysematous hamsters compared to control animals. These alterations included Z-line dislocation and dissolution as well as Z-line streaming (Fig. 2B). Furthermore, disarray of myofibrils and misaligned sarcomeres (register-shifting) were more common in emphysema.

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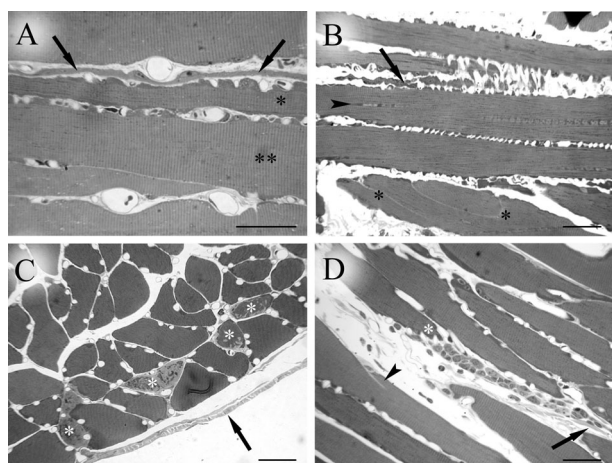


Figure 1. Lightmicroscopical figures. *A.* Control group: single-fibre atrophy (arrows) with loss of striation in between normal appearing myofibres. * indicates thin dark fibre (more mitochondria). ** indicates thick light fibre. *B.* Emphysema group: at the left forking (asterisk) of fibre due to splitting; arrow points to atrophic fibre and arrowhead to fibre with central nuclei. *C.* Emphysema group: several cross-sectioned fibres showing different stages of necroses (asterisks); arrow points to the peritoneal side of the diaphragm. *D.* Emphysema group: abrupt ending of intact fibre (asterisk); between arrow and asterisk the remaining ghost of an extension of the same fibre invaded by mononuclear cells and macrophages indicating myophagia (compare EM Fig 3C); arrowhead: split fibre. _____ = 50 μ m.

Apart from this, numerous satellite cells as well as regenerated fibres with centronucleation were found (Fig. 2C). Fibre splitting and forking were found frequently (Fig. 2D). Even ring fibres (peripheral rim of circular myofibrils surrounding longitudinal fibres) were observed.

Remarkably, abortive regeneration was only observed in the emphysematous group, as demonstrated by partially normal developing myofibrils in the periphery of the fibre (Fig. 3A). Central degeneration with Z-line abnormalities and formation of rods in aberrant myofibrils were present.

Additionally, different stages of myonecrosis were observed in the emphysematous group. The area of degeneration was filled up with a few organelles and glycogen in between disintegrated myofilaments (sarcoplasmic mass) (Fig. 3B). Signs of focal necrosis (fibres with serrated borders) were found without macrophages in their vicinity. Segmental necrotic fibres were found in several sections, as well as local signs of myophagia. After the disappearance of all myofibrillar and sarcotubular components the basal lamina appeared to be still persistent. The remaining space between these laminae was stuffed by invading macrophages (Fig. 3C). However, no gen-

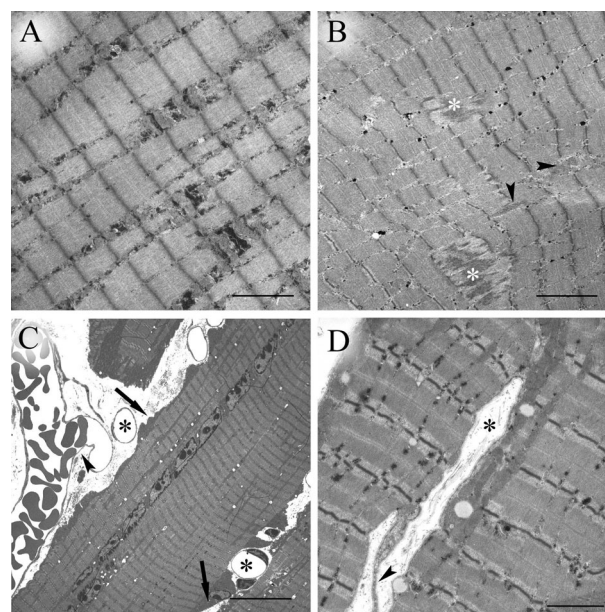


Figure 2. Electronmicroscopical figures. *A.* Control group: generally well aligned sarcomeres with in between mitochondria and small areas of dense glycogen granules. _____ = 5 μ m. *B.* Emphysema group: at asterisks two areas of Z-line streaming, generally there is a reduced density of mitochondria in this abnormal fibre region. At arrowheads a few remaining mitochondria. _____ = 5 μ m. *C.* Emphysema group: about ten nuclei with distinct nucleolisation located midcentrally within a regenerated fibre with electron-lucent fat globules. At arrows subsarcolemmal accumulations of mitochondria, empty capillaries (asterisks) and a small venule filled with erythrocytes and its capillary branch (arrowhead). _____ = 20 μ m. *D.* Emphysema group: longitudinal splitting of a fibre, at arrowhead an abnormal long papillary projection of the fibre surface membrane. At asterisk the invaginating plasma membranes and basal laminae accompanied with collagen fibrils. _____ = 5 μ m.

eral influx of inflammatory cells was observed within the interstitial space.

No obvious differences were observed in the mitochondrial population, with exception of an increased variation in mitochondrial size. There were more and larger lipofuscin granules in the emphysema group as already indicated in the semithin sections. There were no differences with regard to lipid droplets. In addition, many extrusions of vacuolar structures (extrusion vacuoles) were present, eventually resulting in sequestrations within the extracellular spaces and accompanied by folding of the sarcolemma (Fig. 3D). Atrophic fibres were increased and isolated replications of the basal laminae were often found.

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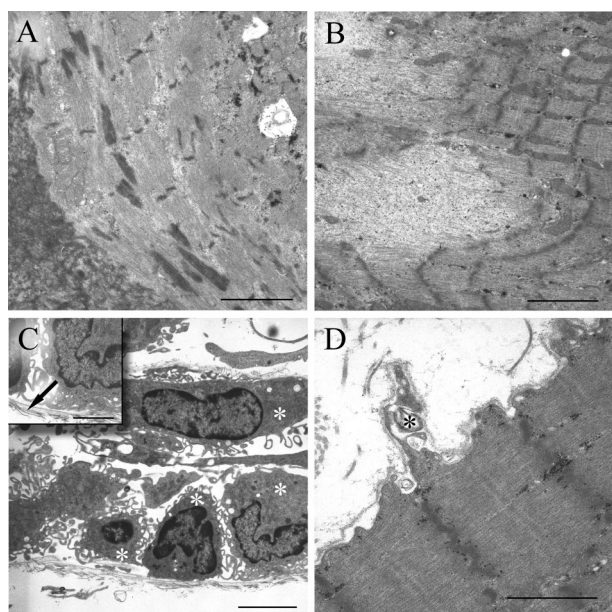


Figure 3. A. Emphysema group: detail of an abortive regeneration of a fibre showing at the left degenerated filamentous structures, at the right several rods as well as newly produced myofibrils with irregular formed Z-lines. _____ = 5 μ m. B. Emphysema group: focal degeneration with disintegrated myofibrils, loss of Z-lines and very few organelles with glycogen granules represents a sarcoplasmic mass in a myofibre. _____ = 5 μ m. C. Emphysema group: in end stage of necrosis persistence of basal lamina (arrow), invaded macrophages (asterisks) are distinctly present. _____ = 10 μ m; small frame _____ = 5 μ m. D. Emphysema group: a long papillary projection, extrusion of vacuolar remnant (asterisks), the excluded content still remains within the enclosing basal lamina. _____ = 2 μ m.

Discussion

This study shows that the aged emphysematous hamster diaphragm exhibits multiple morphological alterations in comparison to age-matched control hamsters. Light-microscopically, the emphysematous diaphragm showed more signs of damage and regeneration compared to the control diaphragm. The ultrastructural observations supported in a more detailed manner the former observations. The pathological changes may in part contribute to reduced contractile properties as found in the present study.

The lack of ultrastructural alterations in young elastase instilled emphysematous hamster diaphragm can in part be explained by the age of the animals used since aged animals are more susceptible to muscle injury compared to young animals [9, 16, 39]. Moreover, the diaphragm of young animals might exhibit a greater adaptive response when exposed to an increased load, resulting in less pathological changes [31]. On the other hand, older animals exhibit an impaired muscle regenerating capacity [7, 8, 17, 35]. For example, regeneration in skeletal

muscle of senescent rats has been reported to be slow and markedly impaired without restoration to normal muscle structure [34].

In the elastase-induced emphysema model the diaphragm contracts continuously against an increased elastic and resistive load due to hyperinflation and air-flow limitation [26]. This resembles to some extent the models of resistive loading induced by tracheal banding as applied in experimental animal settings. In these models ultrastructural changes have been described, e.g. sarcomere disruption, Z-line streaming, and membrane damage [33, 40].

In situ fixation has shown not to induce changes in muscle structure [29]. It is unlikely that the observed changes between the two groups are caused by tissue fixation and processing, since all animals were treated according to the same protocol.

Moreover, physical inactivity does not seem to play a role in the differences observed between the two groups. Although we did not quantify the activity levels of the animals, daily inspection showed no differences in activity level between the two groups. Finally, myopathy due to malnutrition is unlikely since the animals did not lose weight.

The results of our model of elastase induced emphysematous hamsters are partly in concordance with the observations of Orozco-Levi *et al.* [28]. They showed that sarcomere disruption is present in normal human diaphragm muscle, but is found more frequently in COPD patients. To our knowledge, the above-mentioned alterations have not yet been described in the emphysematous hamster diaphragm.

Abnormalities of the contractile apparatus, like register-shifting and Z-line distortions, were consistently found in the diaphragm of emphysematous hamsters. In addition, the observed sarcoplasmic masses and focal degeneration indicate various stages of degeneration. Focal as well as segmental necrosis and eventually myonecrosis were found frequently. In age-matched control hamsters only a few of these alterations were found incidentally (see Table 1).

Our findings indicate that the aged emphysematous diaphragm, exposed to increased loading, exhibits signs of myofibre damage. Usually, muscle damage is followed by a phase of muscle regeneration. Indeed, as observed in peripheral skeletal muscle [6], the presence of numerous satellite cells in this study indicates a regenerative response of the emphysematous diaphragm muscle. However, frequent fibre splitting and forking suggest sub-optimal regeneration, partly underlined by the presence of abortive regeneration [13, 34]. In focal necrosis there is still continuity of the fibre and necrotic material is broken down in autophagic vacuoles. The affected fibres appeared indented, split and show serrated borders as already reported [2]. In addition, it is assumed that both central nuclei and fibre splitting are myopathic changes or represent a defective regeneration [13].

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Table 1. Semiquantitative representation of the electronmicroscopical observations.

Observed parameter	Emphysematous hamster	Normal hamster
Disorganised contractile apparatus*	+++	+/-
Satellite cells	+++	+/-
Central nuclei	+++	+
Splitting and forking	+++	+
Abortive regeneration	+	-
Sarcoplasmic masses	+	-
Focal degeneration	+++	+/-
Necrotic fibres	++	-
Lipofuscin	+++	++
Extrusion vacuoles	+++	-
Atrophic fibres	++	+/-
Replication basal lamina	+++	+/-

- = not detected, +/- = incidentally, + = few, ++ = several, +++ = numerous

* = Z-line dislocation and dissolution, Z-line streaming, disarray of myofibrils and register-shifting.

There were numerous extrusion vacuoles [14] with autophagic remnants discarded in the extracellular space as observed in the emphysematous group. This implicates a high lysosomal activity, related to *e.g.* focal degeneration and focal necrosis. We also observed more lipofuscin granules in the emphysematous hamsters compared to the control group. It is well-known that with ageing the size and number of lipofuscin granules increases [14]. An increased presence of lipofuscin may also be reflective of a higher lysosomal activity of the involved fibre [24]. In emphysematous hamster diaphragm there are no data on these typical lysosomal waste products. However, an enlarged number of lipofuscin granules has been previously reported to occur in diaphragm muscle from COPD patients [24].

Atrophic myofibres were incidentally detected in the control hamsters, but were detected frequently in the emphysematous hamsters. Muscle fibre atrophy can occur due to several factors [3]. Since the diaphragm is continuously active disuse atrophy is unlikely to occur in this study. However, repeated cycles of degeneration followed by incomplete regeneration or repair, could be a cause of fibre atrophy in this study [3]. Furthermore, the increased presence of isolated replications of basal membranes is in accordance with numerous atrophic fibres or degenerating and necrotic fibres as already reported [14]. The remnants of basal laminae are quite resistant and remain present for a long period [13].

In this study both subtle and pronounced disturbances of the contractile system were present, including sarcolemmal folding and numerous extrusion vacuoles. Areas of focal degeneration and focal as well as segmental necrosis were obvious. After focal fibre necrosis, cellular remodelling might weaken the affected fibres, but apparently does not destroy them [2]. All these events are likely to result in an impairment of muscle contractility.

Muscle changes at the ultrastructural level have already been suggested to play a role in a decreased force generating capacity as occurs in emphysematous hamsters [37]. The reduction of P_t and P_o in the present study was in line with previous reports [22, 23, 37].

In rabbits an impaired *in vitro* muscle contractility appeared to be associated with diaphragm injury after inspiratory resistive loading [20]. Diaphragm injury caused by ventilatory failure, induced by resistive loading, has been linked to changes in myofibrillar complexes, which appear to be susceptible to calpain-mediated degradation [5, 32, 33]. Besides calpain, free radicals are suggested to contribute to diaphragm muscle injury. Recently, Jiang *et al.* have shown that free radical scavengers can prevent the development of diaphragm injury and, in part, the reduction of *in vitro* diaphragm contractility [20].

Additionally, oxidative stress has also been implicated in the pathogenesis of muscle injury due to overloading. Impaired force generation in the emphysematous hamster diaphragm is related to an increase in the ratio of oxidised to reduced glutathione [19]. Inhibition of free radical generation by the xanthine oxidase inhibitor allopurinol has been shown to attenuate exercise-induced morphological damage such as irregularities in myofibrillar organization, intrafibre oedema and mitochondrial swelling in the soleus muscle [12]. In regard to future studies the emphysematous hamster model seems suitable to investigate the role of oxidative stress on diaphragm injury.

In summary, the diaphragm of aged emphysematous hamsters is characterised by increased ultrastructural abnormalities as a result of chronic increased loading. In these hamsters the regenerating capacity of the emphysematous diaphragm seems to be impaired compared to younger animals as used in previous studies, suggesting an inadequate adaptation. The observed pathological alterations are likely to contribute to the reduced force generation of the diaphragm.

Acknowledgements

The authors wish to thank Paul H.K. Jap, PhD, for his expertise in the field of light- and electronmicroscopy, and Leo Ennen for his expert biotechnical assistance.

This study was supported by a grant from GlaxoSmithKline, The Netherlands.

Address correspondence to:

P.N.R. Dekhuijzen, MD, PhD, Department of Pulmonary Diseases, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands, tel. xx-31-24-3614579, fax xx-31-24-3610324, Email R.Dekhuijzen@long.umcm.nl

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