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Is the normal content of sulfhydryl groups attributable to sparing from dystrophic pathology in dystrophin-deficient muscles?

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Deficiency of dystrophin in skeletal muscles is supposed to be responsible for all the symptoms associated with Duchenne dystrophy (DMD) and Becker dystrophy (BMD). The dystrophin-deficient mdx mice, however, are clinically almost asymptomatic. Hence, other factor(s) might be responsible for the muscle pathology in DMD and BDM. As sulfhydryl groups are involved in maintaining the structure of membranes and the protein-phospholipid interactions, total, protein-bound and free sulfhydryl groups (–SH) in DMD, BMD, limb-girdle dystrophy (LGMD) and the mdx mice muscles have been determined. A significant decrease of total and protein-bound –SH groups content and an increased proportion of free –SH groups in DMD and BMD was found. In LGMD the changes of total and protein-bound –SH groups content were less expressed. In the mdx mice muscles the content of –SH groups was generally normal, only a higher than normal proportion of free-SH groups content in old and senile animals, especially in their diaphragm, was present. To test the sarcolemmal integrity, albumin/creatine kinase (CK) influx/efflux was determined. In early stages of DMD and BMD the albumin influx was increased. In advanced stages of these diseases albumin influx was not observed. In LGMD albumin penetration was present only in a few fibres. CK efflux in vitro *was increased both in early and advanced DMD cases. In BMD and LGMD CK efflux was increased only in early stages of the diseases. In mdx mice an increased influx/efflux of albumin/CK was stated in adult animals. The changes persisted in the mdx hind limb muscles up to the senile age of the animals. In the mdx diaphragm of senile animals albumin did not penetrate the muscle cell and no increase of CK efflux was observed. It is suggested that changes in the distribution of –SH groups take part in the molecular disorganisation of the sarcolemma in course dystrophinopathies. Normal content of the sulfhydryl groups is supposed to be attributable to sparing from dystrophic pathology in dystrophin-deficient mdx mice muscles.*

key words: dystrophinopathies, sulfhydryl groups, sarcolemmal integrity

INTRODUCTION

Deficiency of dystrophin and the dystrophin-related proteins in muscles of Duchennne dystrophy (DMD) and Becker dystrophy (BMD) patients is supposed to be responsible for all the pathological events, including increased sarcolemmal permeability [31]. There are, however, many controversies about the function of dystrophin in the muscle [29]. Dystrophin deficiency does not seem to be the primary cause of muscle pathology in dystrophinopathies.

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It has already been settled that the sulfhydryl groups (–SH) are intimately involved in maintaining the membrane structure and its function. In this study their concentration in dystrophinopathies was examined, and the data were compared to those in dystrophin-positive muscles of limb girdle dystrophy (LGMD) and dystrophin-deficient muscles of clinically almost asymptomatic mdx mice. As markers of cell membrane integrity, penetration of albumin [21, 23] and creatine kinase (CK) efflux *in vitro* [19] were tested.

MATERIAL AND METHODS

Twenty patients with Duchenne dystrophy (5 to 18 years of age), 5 patients with Becker dystrophy (aged 9 to 12 years), 10 patients with limb-girdle dystrophy (aged 12 to 21 years), and 20 age-matched healthy controls were examined. The diagnosis of the diseases was established by clinical, genetic, electromyographic, histological, biochemical and immunocytochemical dystrophin examinations. The diagnostic muscle biopsies were obtained after informed consent of the patients or their families.

Male mutant C57BL/10 mice (14 days to 12 months of age) and control age-matched normal mice of C57BL/6J strain were used. The animals (5 in each group) were fed with a standard laboratory diet. At 14, 30, 90 and 365 days of age the extensor digitorum longus muscle (EDL) for the morphological studies and the gastrocnemius muscle for the biochemical examination were excised. Diaphragm was used for both types of analyses.

Cryostat frozen sections (8 μm) of the muscles were stained according to the standard techniques. For immunocytochemical studies monoclonal anti-human serum albumin (Sigma), diluted 1:1000, and TRITC labelled immunoglobulins were used.

For the biochemical examinations human and animal muscles were frozen quickly in dry ice and preserved at –72°C until used. After thawing, all procedures were conducted at +4°C. Total, protein-bound and free sulfhydryl groups were estimated with the Ellman's reagent, according to the method of Sedlak & Lindlay [32]. The concentration of the proteins in the homogenised samples was estimated by the method of Peterson [30]. Creatine kinase (CK) efflux experiments *in vitro* were conducted according to the procedure of Jones et al. [19]. The enzyme activity was determined by an enzyme-linked assay method using the Cormay Diagnostic Kit.

The statistical significance of the biochemical studies was assessed using the Student's test and Duncan's multiple range test (GLM). A value of $p > 0.05$ was considered as non-significant.

RESULTS

The concentration of the –SH groups in the muscle was species-dependent. In normal human muscles the total –SH group ($p < 0.001$) and protein bound –SH groups content ($p < 0.001$) was higher, the free –SH groups concentration was lower ($p < 0.01$) as compared to normal mice muscles. In the latter in hind limb muscles the total and protein bound –SH groups content was higher than it was in the diaphragm ($p < 0.05$).

In DMD and BMD cases the protein bound and the total amount of the –SH groups was decreased in nearly all of these cases; in all DMD patients a higher proportion of free –SH groups in the total –SH pool was present. In LGMD a decrease of total and protein-bound –SH groups was observed. In mdx mice muscles changes in the –SH groups pool were a rare finding, and were observed as an increase of free-SH groups content in old animals, especially in the diaphragm (Table 1).

Table 1. Muscle sulfhydryls in dystrophinopathies (μM/mg protein)

	n	Age	CK (IU)	Total –SH groups	Protein-bound –SH group	Free –SH groups
Duchenne dystrophy	20	7.5 ± 4.1	1708–4898	64.2 ± 20.7*** (18)	30.9 ± 27.3*** (14)	33.4 ± 27.7*** (20)
Becker dystrophy	5	10.6 ± 1.3	855–3157	62.1 ± 30.9*** (4)	51.8 ± 31.5*** (4)	10.2 ± 8.1 (1)
Limb-girdle dystrophy	10	15.0 ± 4.3	288–3614	121.9 ± 24.6**	111.2 ± 32.9**	4.7 ± 2.9
Healthy controls	20	12.0 ± 3.0	0–34	153.8 ± 32.1	148.1 ± 30.7	5.7 ± 3.2
Mdx mice						
hind limb muscles	20	14–365 days		88.5 ± 17.0	78.5 ± 17.6	10.0 ± 1.6*
diaphragm	20	14–365 days		102.4 ± 30.7	86.3 ± 26.6 (5)	16.1 ± 6.3*** (10)
Normal mice						
hind limb muscles	20	14–365 days		97.7 ± 15.5	89.3 ± 16.1	8.4 ± 2.6
diaphragm	20	14–365 days		85.5 ± 17.9	77.2 ± 16.8	10.3 ± 2.3

Values are means ± SD. Significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Number of cases with abnormal values (over the normal mean ± SD × 2) is given in parentheses

The immunocytochemical analysis in normal human muscles indicated that albumin did not penetrate the muscle cell and was located in the endomysium. In the early stages of DMD albumin positive fibres were randomly distributed within the muscle fibres both in necrotic and opaque fibres. In advanced DMD stages extensive proliferation of connective tissue was present and albumin accumulated in the interstitial spaces of the dystrophic muscle. The muscle fibres were albumin negative (Fig. 1). In early stages of BMD albumin positive fibres, although less frequent when compared to DMD, were also observed. In more advanced stages of this disease rare positive albumin fibres were present, albumin was stained in the interstitial spaces as in DMD (Fig. 2). In LGMD few albumin positive fibres were also observed, some staining in the endomysium was present (Fig. 2). In the mdx mice, after 1 month of age, albumin penetrated EDL and diaphragm cells (Fig. 3, 4). In the latter, in senile animals, increased albumin staining in the interstitial spaces was observed (Fig. 4).

In the *in vitro* experiments CK efflux from muscle cells was significantly increased in DMD, BMD, and

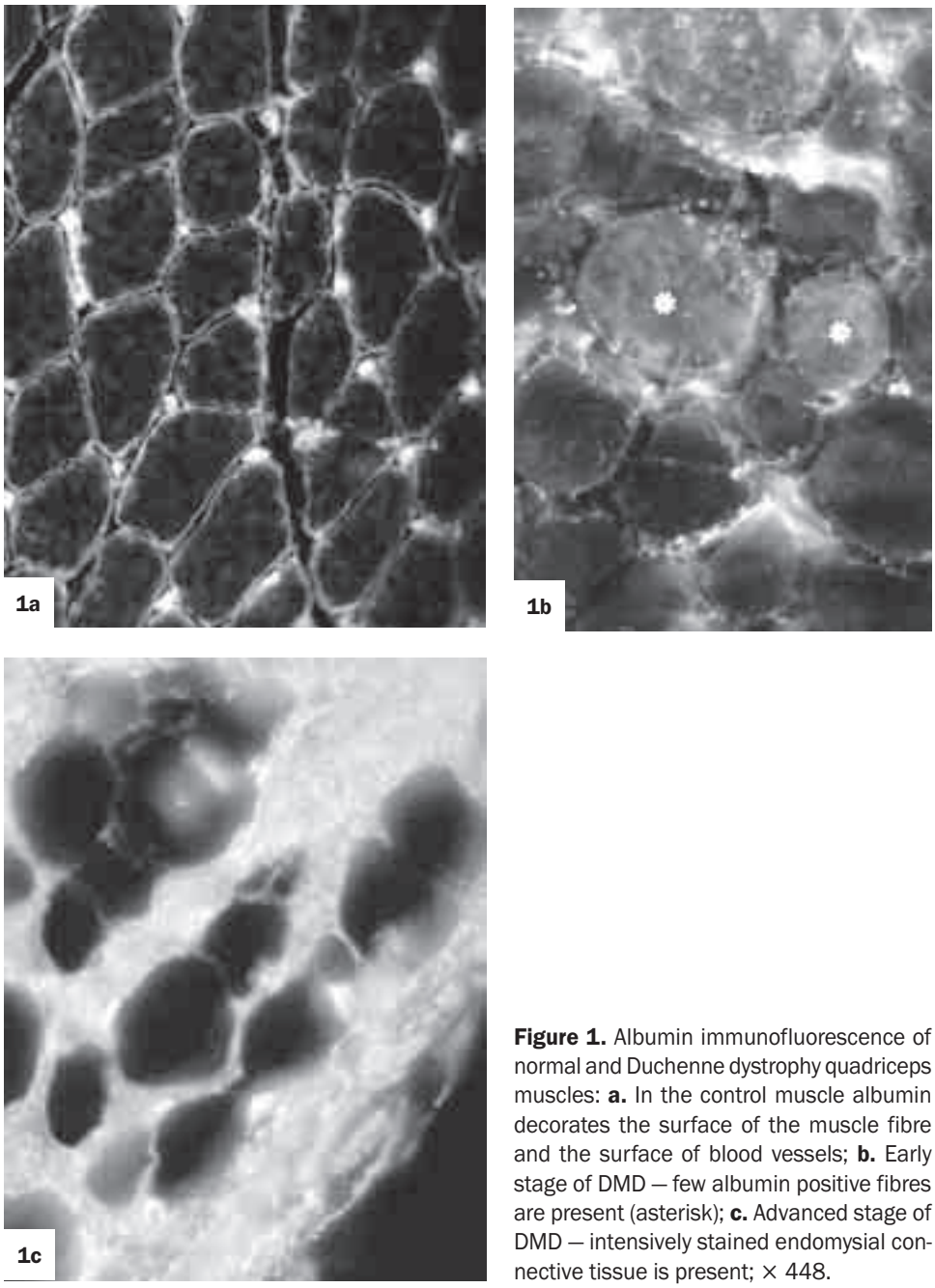
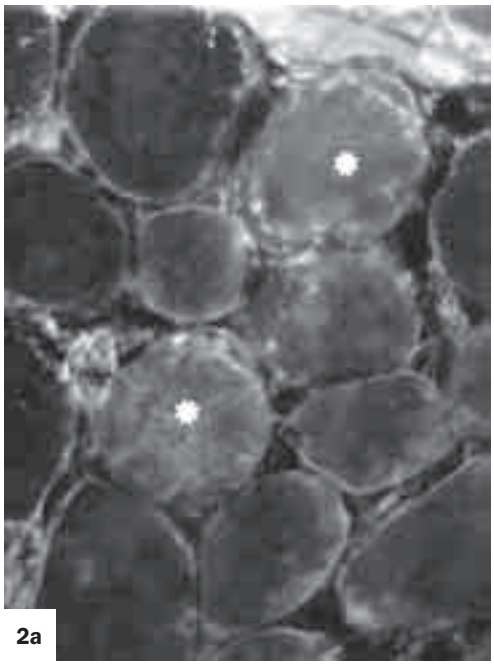


Figure 1. Albumin immunofluorescence of normal and Duchenne dystrophy quadriceps muscles: **a.** In the control muscle albumin decorates the surface of the muscle fibre and the surface of blood vessels; **b.** Early stage of DMD — few albumin positive fibres are present (asterisk); **c.** Advanced stage of DMD — intensively stained endomysial connective tissue is present; × 448.



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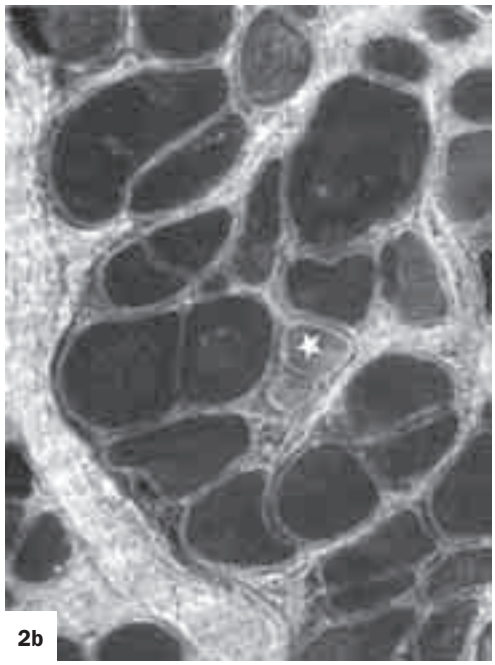

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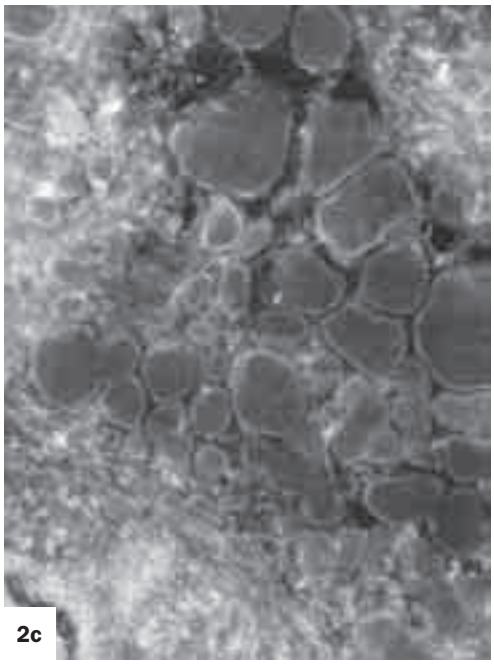

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Figure 2. Albumin immunofluorescence of Becker and limb-girdle dystrophy quadriceps muscles: **a.** Early stage of BDM — very few albumin positive muscle fibres are present (asterisk), × 448; **b.** Later stage of BMD — albumin positive fibres are rare. Albumin decorates the endomysial connective tissue, × 448; **c.** LGMD — very few albumin positive fibres and slightly stained connective tissue are present; × 224.

LGMD, especially in early stages of these diseases (Fig. 5). In mdx mice the increased CK efflux started in adult animals and persisted in the gastrocnemius muscle up to senile age of the animals; in the diaphragm the increased CK efflux returned to normal values after 8 months of age (Fig. 6).

No correlation between the decrease in the –SH groups in DMD, BMD and LGMD with the age of the patients, CK activity in blood/CK efflux *in vitro*, and albumin influx was detected. In mdx mice there was also no correlation between the changes in the albumin influx/CK efflux and the concentration of the muscle –SH groups.

DISCUSSION

Loss of membrane integrity is thought to be the consequence either of direct membrane damage, lack of sufficient energy supply, or changes in the membrane structure. Dystrophin deficiency is generally indicated as the primary cause of the lost membrane integrity

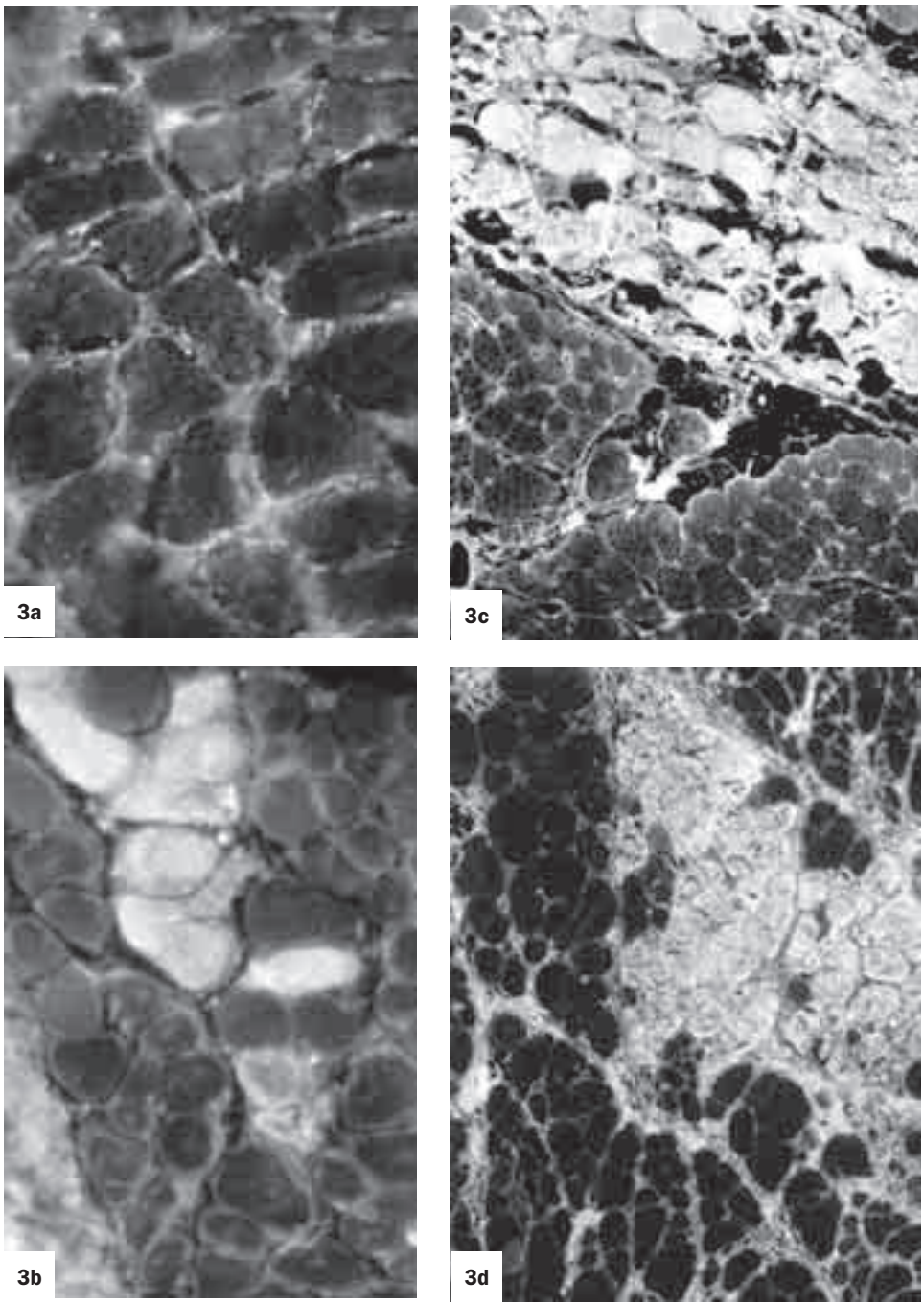

Figure 3. Albumin immunofluorescence of normal and mdx mice EDL muscle: **a.** In the control muscle albumin decorates the surface of the muscle fibre and the surface of blood vessels; **b.** Mdx mice, 1 mo. of age. Very few albumin positive muscle fibres; **c.** Mdx mice, 3 mo. of age. Albumin positive muscle fibres are present in large clusters; **d.** Mdx mice, 12 mo. of age. Albumin positive fibres in clusters and stained connective tissue are present; × 448.

and the increased membrane permeability in dystrophinopathies. Controversies with regard to the function of dystrophin [29] suggest, however, that other mechanism(s) is/are responsible for the muscle pathology in dystrophinopathies.

It is already accepted that in DMD and BMD an increased membrane permeability is present. It is indicated by an increased intracellular calcium content [2, 3, 7, 12, 13], increased penetration of albumin [7, 11], of porcion yellow [4], the elevated CK activity in

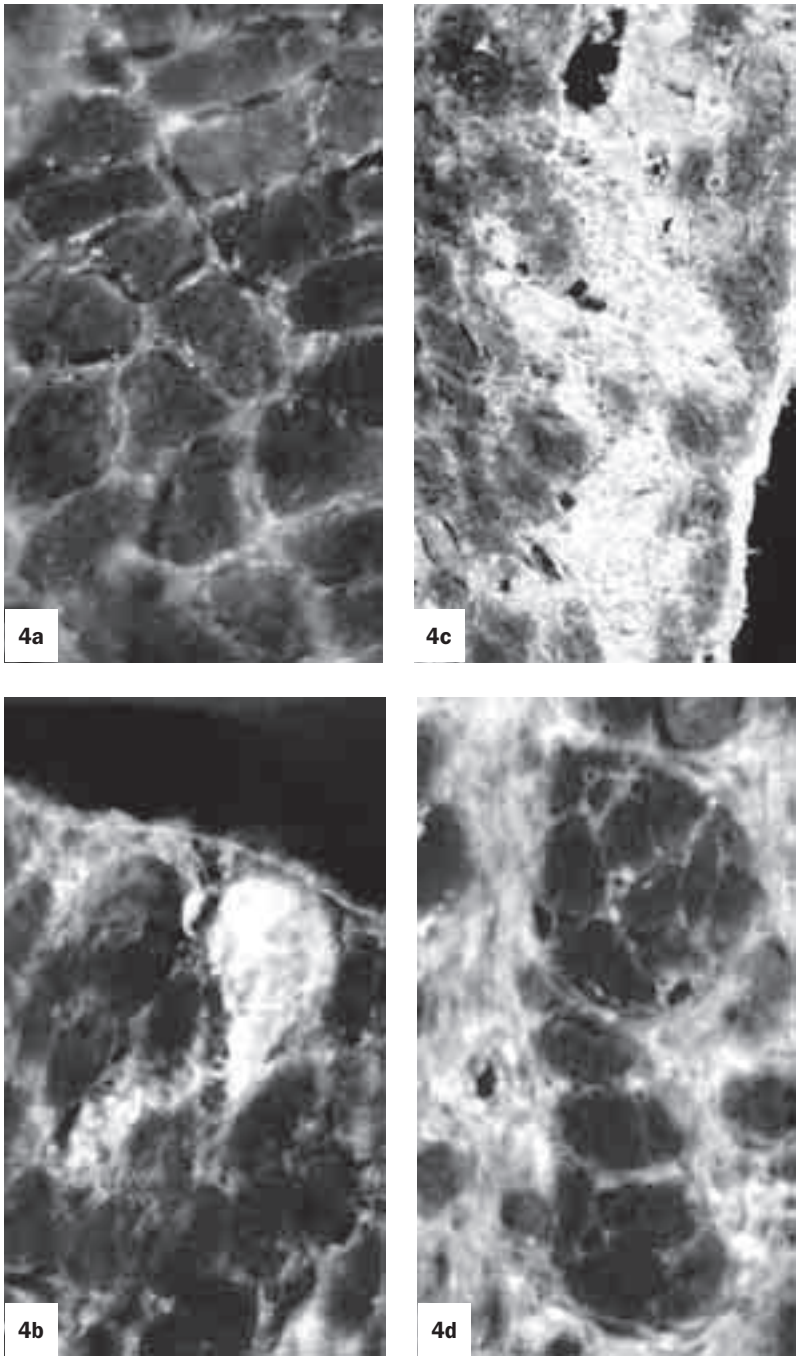

Figure 4. Albumin immunofluorescence of normal and mdx mice diaphragm: **a.** Albumin labelled the surface of the muscle fibre; **b.** Mdx mice, 1 mo. of age. Single positive muscle fibre is present; **c.** Mdx mice, 3 mo. of age. Small clusters of albumin positive fibres are present; **d.** Mdx mice, 12 mo. of age. Albumin intensively stains the connective tissue.

the blood [15, 31] and increased CK efflux *in vitro* [22, 27, 28].

Muscle pathology in dystrophinopathies is, however, a multi-factorial process. Among several factors, except the deficiency of dystrophin and dystrophin-related proteins, changes in muscle –SH groups are to be taken into account.

–SH groups are known to participate in regulating and altering transport processes across biological membranes [1]. They are highly concentrated in integral membrane

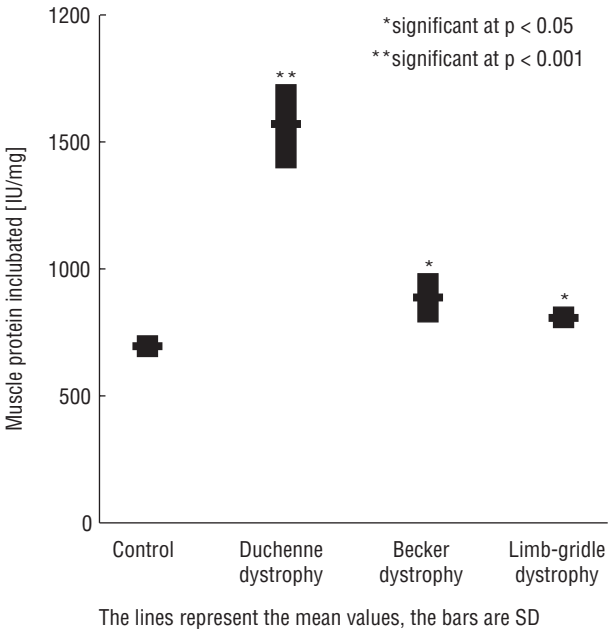

Figure 5. Creatine kinase release *in vitro* from skeletal muscles in Duchenne dystrophy and controls.

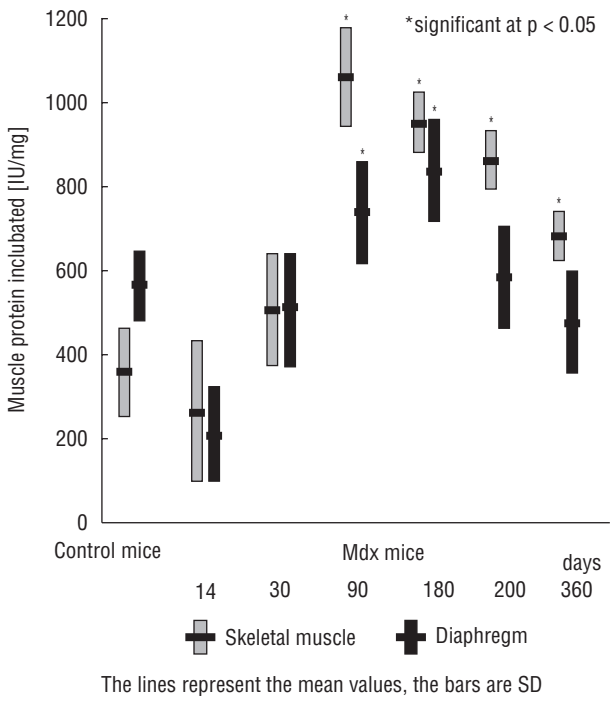

Figure 6. Creatine kinase release *in vitro* from mdx mice muscles and controls.

proteins and are intimately involved in tight protein-phospholipid interactions, they modulate also the membrane proteins (enzymes, transport proteins, antigen, etc.), including their architecture and motion. Lipid peroxidation in the membranes is inhibited also by a process requiring sulfhydryl groups [14]. Oxidation of protein –SH groups, evoked by free radicals [6], may damage viable tissues and lead to perturbations of membrane permeability and membrane located enzymes [20] and are critical for ATP-dependent Ca binding properties [8].

It is already known that in DMD, except for the lack of dystrophin and dystrophin-related proteins, structural membrane changes are localised at the polar-apolar interface of the sarcolemma [26]. Membrane abnormalities at the molecular level in DMD are indicated also by alterations in lipid-protein organisation at the surface of erythrocytes, as the consequence of decreased content of –SH groups which are strongly immobilised at the surface of the membranes and deep within the lipid bilayer [10]. As the –SH groups play a role in hydrophobic bonding of proteins [5] the changes at the polar-apolar interface may be the consequence of changes in –SH groups concentration.

The presented data indicate that the sarcolemmal changes in dystrophinopathies are more widespread. The increased membrane permeability does not seem to be the consequence of lack of support against mechanical stress because of dystrophin deficiency [18], but rather may be the consequence of oxidative stress and the action of excessively generated reactive oxygen free radical species, which are known to be active in DMD and BMD [9, 16, 17, 24]. In this report no direct relationship between dystrophin deficiency, the –SH groups changes and the albumin penetration and CK efflux/CK activity in blood was stated. It should be mentioned here that the albumin influx is of limited value for membrane permeability testing, as it is also a useful marker for analysing the process of muscular atrophy [33]. The activity of CK, on the other hand, is artificially increased because of the action of activators/inhibitors, which are known to be present in DMD [25].

Changes in the content of sulfhydryl groups may be the cause of the molecular disorganisation of sarcolemma; they may change the motion of the molecules and the cell metabolic status and participate in the muscle pathology in dystrophinopathies. The normal concentration of the –SH in dystrophin-deficient and almost clinically asymptomatic mdx mice may indicate that they have a protective action against muscle pathology.

REFERENCES

1. Abramson JJ, Salama G (1988) Sulfhydryl oxidation and Ca2+ release from sarcoplasmic reticulum. Mol & Cell Biochem, 82: 81–84.
2. Bertorini TE, Cornelio F, Bhattacharya SK, Palmieri GMA, Dones I, Dworzak F et al. (1984) Calcium and magnesium

content in fetuses at risk and prenecrotic Duchenne muscular dystrophy. Neurology, 34: 1436–1440.
3. Bodensteiner JB, Engel AG (1978) Intracellular calcium, accumulation in Duchenne dystrophy and other myopathies. Neurology, 28: 436–446.
4. Bradley WG, Fulthorpe JJ (1978) Studies of sarcolemmal integrity in myopathic muscle. Neurology, 28: 670–670.
5. Carter JR, Jr. (1973) Role of sulfhydryl groups in erythrocyte membrane structure. Biochemistry, 12: 171–176.
6. Comporti M (1989) Three models of free radical-induced cell injury. Chem-Biol Interactions, 72: 1–56
7. Cornelio F, Dones I (1984) Muscle fiber degeneration and necrosis in muscular dystrophy and other muscle diseases: cytochemical and immunocytochemical data. Ann Neurol, 16: 694–701.
8. Corpus V, Sun AY (1983) Effects of ATP on calcium binding to synaptic plasma membrane. Neurochem Res, 8: 501–520.
9. Degl'Innocenti D, Pieri A, Rosati F, Ramponi G (1999). Oxidative stress and calcium homeostasis in dystrophic skin fibroblasts. Iubmb Life, 48: 311–396.
10. Dellantonio R, Angeleri F, Capriotti M, Lenaz G, Curatola G, Mazzanti L, Bertoli E (1980) Biophysical studies of erythrocyte membranes from patients with Duchenne muscular dystrophy. Ital J Biochem, 29: 121–128.
11. Dupont-Versteegden EE, Kitten AM, Katz MS, Mc Carter RJ (1996) Elevated levels of albumin in soleus and diaphragm muscles of mdx mice. Proc Soc Exp Biol Med, 213: 281–286.
12. Emery AEH, Burt D (1980). Intracellular calcium and pathogenesis and antenatal diagnosis of Duchenne muscular dystrophy. Brit Med J, 9: 355–357.
13. Franco A, Lansman JB (1990) Calcium entry through stretch-inactivated ion channels in mdx myotubes. Nature, 334: 670–673.
14. Gibson DD, Hawrylko J, McCay PB (1985) GSH-dependent inhibition of lipid peroxidation: properties of a potent cytosolic system which protects cell membranes. Lipids, 20: 704–711.
15. Glesby MJ, Rosenmann E, Nylen G, Wrogemann K (1988) Serum CK, magnesium and oxidative phosphorylation in mdx mouse muscular dystrophy. Muscle & Nerve, 11: 852–856.
16. Haycock JW, Mac Neil S, Jones P, Harris JB, Mantle D (1996) Oxidative damage to muscle protein in Duchenne muscular dystrophy. Neuroreport, 8: 357–361.
17. Haycock JW, Mac Neil S, Mantle D (1998) Differential protein oxidation in Duchenne and Becker dystrophy. Neuroreport, 9: 2201–2207.
18. Hoffman EP, Kunkel LM (1989) Dystrophin abnormalities in Duchenne/Becker muscular dystrophy. Neuron, 2: 1019–1029
19. Jones DA, Jackson MJ, Edwards RHT (1983) Release of intracellular enzymes from an isolated mammalian skeletal muscle preparation. Clin Sci, 65: 193–201.
20. Kaneko M, Hayashi H, Kobayashi A, Yamazaki N, Dhalla NS(1991) Inhibition of heart sarcolemmal Ca(2+)-pump activity by oxygen free radicals. Bratislavske Lek Listy, 92: 48–56.
21. Kasper CE (1995) Sarcolemmal disruption in reloaded atrophic skeletal muscle. J Appl Physiol, 79: 607–614.
22. Mc Ardle A, Edwards RHT, Jackson MJ (1994) Time course of changes in plasma membrane permeability in the dystrophin-deficient mdx mouse. Muscle & Nerve, 17: 1378–1384.
23. Mc Neil PL, Khakee R (1992) Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. Am J Pathol, 140: 1097–1109.
24. Murphy ME, Kehrer JP (1989) Oxidative stress and muscular dystrophy. Chem-Biol Interact, 69: 101–178.
25. Niebroj-Dobosz I (1974) The influence of the dilution effect on serum creatine phosphokinase activity in neuromuscular diseases. Clin Chem Acta, 50: 301–304.
26. Niebroj-Dobosz I (1992) Fluorescent probe analysis of muscle plasmalemma in Duchenne's progressive muscular dystrophy. J Neurol, 239: 267–269.
27. Niebroj-Dobosz I, Łukasiuk M (1996) Release of intracellular enzymes from skeletal muscles and diaphragm in mdx mice. Basic & Appl Myol, 6: 377–383.
28. Niebroj-Dobosz I, Hausmanowa-Petrusewicz I (1998) Is the mdx mouse an adequate model of Duchenne's muscular dystrophy? Acta Myol, 2: 11–19.
29. Niebroj-Dobosz I, Fidziańska A, Hausmanowa-Petrusewicz I (2001) Controversies about the function of dystrophin in muscle. Folia Neuropathol, 39: 163–168.
30. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem, 83: 346–356.
31. Rowland LP (1980) Biochemistry of muscle plasma membranes in Duchenne muscular dystrophy. Muscle & Nerve, 3: 3–20.
32. Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound and non-protein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem, 25: 192–205.
33. Wagatsuma A, Yamazaki Y, Mizuno K, Yamada S (2001) Molecular properties and gene expression of albumin in the skeletal muscle following hind limb immobilization in a shortened position. Acta Neuropathol, 101: 540–546.