available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Histological changes in masticatory muscles of mdx mice

Alexander Spassov [a,*], Tomasz Gredes [a], Tomasz Gedrange [a], Silke Lucke [a], Dragan Pavlovic [b], Christiane Kunert–Keil [a,c]

[a] Department of Orthodontics, Faculty of Medicine, University of Greifswald, Rotgerber Str. 8, 17475 Greifswald, Germany
[b] Department of Anaesthesiology and Intensive Care Medicine, Faculty of Medicine, University of Greifswald, Friedrich-Loeffler Str. 23c, 17475 Greifswald, Germany
[c] Institute of Pathophysiology, Faculty of Medicine, University of Greifswald, Greifswalder Str. 11C, 17495 Karlsburg, Germany

ARTICLE INFO

Article history:
Accepted 6 February 2010

Keywords:
Mdx mice
Muscular dystrophy
DMD
Histopathology
Masticatory muscles

ABSTRACT

Objective: Duchenne muscular dystrophy (DMD) patients have distorted dentofacial morphology that could be a result of changed force balance of masticatory muscles due to unequal dystrophic changes in various masticatory muscles. Skeletal muscles of DMD patients and those of murine model of DMD – mdx mice – are both characterized by Ca^{2+} induced muscle damage, muscle weakness and characteristic histological changes. Therefore, to determine the pathological changes in this animal model of DMD, we examined the masticatory muscles of the mdx mice for histological abnormalities including nuclei localization, fibre diameters, and collagen expression.

Design: Muscle sections from masseter (MAS), temporal (TEM), tongue (TON) and soleus (SOL) of mdx and control normal mice were stained with hemalaun/eosin or with Sirius Red and morphometrically analysed. Levels of collagen staining in normal and mdx muscles were measured using image analysis and the mean optical density (mod) was determined.

Results: Dystrophin deficient masticatory muscles contained 11–75% fibres with centralised nuclei. In mdx mice an increased mean fibre diameter was observed as compared to the age-matched control muscles (control vs. mdx; MAS: 33.44 ± 0.49 μm vs. 37.76 ± 0.68 μm, $p < 0.005$; TEM: 32.93 ± 0.4 μm vs. 42.93 ± 0.68 μm, $p < 0.005$; SOL: 33.15 ± 0.29 μm vs. 40.62 ± 0.55 μm, $p < 0.005$; TON: 13.44 ± 0.68 μm vs. 15.63 ± 0.18 μm, $p < 0.005$). Increased expression of collagen was found in MAS (mod control vs. mdx: 1.34 vs. 3.99, $p < 0.005$), TEM (mod control vs. mdx: 3.11 vs. 4.73, $p < 0.01$) and SOL (mod control vs. mdx: 2.36 vs. 3.49, $p < 0.01$).

Conclusion: Our findings revealed that mdx masticatory muscles are unequally affected by the disease process. The masticatory muscles of the mdx mice could present a useful model for further investigating the influence of dystrophin deficiency on muscles function.

1. Introduction

Duchenne muscular dystrophy (DMD) is a progressive and fatal disease of muscle degeneration caused by mutations in the gene encoding for the cytoskeletal protein dystrophin.[1] The increased size of the calves and certain other limb muscle groups, also known as pseudohypertrophy, is a well known feature of DMD.[2] Histological changes found in this condition are degeneration, variation in fibre size, with the enlargement or atrophy of fibres and the internal migration of nuclei[3] and subsequently the loss of fibres. Studies performed in the animal model of DMD, the mdx mice, reveal acute phases of

* *Corresponding author*. Tel.: +49 3834 867119; fax: +49 3834 867113.
E-mail address: alexspas@uni-greifswald.de (A. Spassov).
0003–9969/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.archoralbio.2010.02.005

muscle necrosis at the age of 3–4 weeks, followed by regeneration and apparent stability with ageing.[4] Degeneration and regeneration processes in mdx muscles are associated with histopathological features, such as centronucleated fibres, increased collagen expression and fibrosis, variation in muscle fibres size and the presence of phagocyte cells.[5–7] Mdx skeletal muscles often present varying patterns of histopathology. For example adult mdx skeletal muscles revealed either absence (oesophagus), very mild (trunk and limb muscles), or severe (diaphragm) histopathological changes.[8]

Although many studies have compared various mdx muscles, including masticatory muscles, only few have been concentrated on the progression of the disease among muscles involved in mastication and swallowing. Existing animal and clinical studies imply a different timing for the onset of the dystrophic process in various masticatory muscles[9,10] which may influence their coordinated activity during the masticatory process.[11] Characteristic malocclusions and orofacial dysfunctions in DMD patients are most probably related to the altered masticatory and orofacial functions due to muscle dystrophy.[12] In DMD patients the maximum biting force and mouth opening distance are significantly reduced as compared to controls.[13] Furthermore, the lip incompetence, mouth breathing, macroglossia and tongue thrusting are common findings in these patients.[14] Therefore, it is of importance to investigate histomorphometric changes in masticatory muscles in animal studies to be able to comprehend the muscle response to dystrophic changes. In humans, orofacial deformations occur at advanced stages of the disease. The importance of such investigations has grown, since the life expectancy of DMD patients has been extended.

Most previous studies on mdx mice have almost exclusively investigated the masseter. However, mastication involves a group of muscles acting in synchrony, which must be taken into consideration, when studying the influence of dystrophy on the orofacial musculature.

In 9-week-old mdx mice almost all skeletal muscles of the head, neck and trunk show degeneration and necrosis of muscle fibres. However, there is little information about histological parameters beyond 12 weeks in mdx mouse masticatory muscles, when the majority of fibres have already regenerated. Earlier studies investigated predominantly younger mdx mice.

The aim of this present study was to obtain more information than is available from earlier studies. Therefore we analysed and compared histological parameters, such as fibre diameter, centralized nuclei, collagen expression and presence of inflammatory foci in three functionally important masticatory muscles: tongue (TON), masseter (MAS), temporal muscle (TEM), and compared them to the skeletal muscle soleus (SOL) in 100-day-old mdx mice. The data obtained will serve as background for functional measurements in future studies.

2. Materials and methods

2.1. Animals

Mice of the inbred strains C57Bl/10ScSn (control) and C57/Bl10ScSn-*Dmd*^{mdx}/J (mdx) were originally obtained from Harlan Winkelmann (Borchen, Germany) and Charles River (Sulzfeld, Germany). Both strains were bred in the Department of Pathophysiology of the Medical Faculty at the University of Greifswald. Age-matched pairs of mdx and control animals (each $n = 6$, 100 days old) of either sex and of the same body weight (about 30 g) were killed using ether inhalation in a manner approved by the institutional animal ethics committee.

2.2. Morphology

Masseter, temporal, tongue and soleus muscle were collected and mounted on cork supports using cryomatrix (Germany). The samples were snap-frozen in melting petroleum ether (Merck, Darmstadt, Germany) and stored at −80 °C. Samples (5 μm) were cryo cross-sectioned from the middle of all test muscles and the corpus linguae, and placed on slides. The slides were air dried, fixed with acetone for 10 min at room temperature and either stained with hemalaun/eosin for the visualisation of nuclei, or stained in Sirius Red F3B (Niepötter Labortechnik, Bierstadt, Germany) for the visualisation of collagen. A blind test was conducted at the same time using identical staff, equipment, and chemicals.

2.3. Image acquisition and analysis

Five digital pictures from each section of both staining methods were acquired at random of different places of the tissue (20-fold magnification; 3CCD colour camera; Hitachi HV-C20M; Hitachi Denshi Ltd., Japan, and Axiolab, Carl Zeiss, Göttingen, Germany). For standardisation of the measurement in each picture the optical density of white background colour was attuned to 250 as described previously.[15] Image analysis of up to 100 muscle fibres in each section was performed in two steps: (1) determination of the muscle fibre size according to the minimal ''Feret's diameter'' – the minimum distance of parallel tangents at opposing borders of the muscle fibre[16] and (2) determination of the percentage of muscle fibres containing centralized nuclei. Apart from this, the content of collagen between fibres was measured by determination of the quantity of pixels in each picture that had a positive reaction for collagen (mod: mean optical density). For fibre size measurement the smallest and the largest fibre diameter was determined and from these values the mean diameter was calculated for each fibre.[17,18] Semi-automated analysis was performed using the image analysis program KSRun (Imaging system KS400, release 3.0; Zeiss, Vision GmbH, Munich, Germany) or ImageJ 1.42 for Windows (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2009).

2.4. Statistical analysis

Statistical analysis was performed using the SigmaPlot Software (Systat Software, Inc., 1735, Technology Drive, Sn Jose, CA 95110, USA). The obtained values for the groups were compared using Student's unpaired *t*-test. Data are given as means ± S.E.M. $p < 0.05$ was considered statistically significant.