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Gender dimorphism influences extracellular matrix expression and regeneration of muscular tissue in mdx dystrophic mice

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Abstract Mdx mouse, the animal model of Duchenne muscular dystrophy, lacks dystrophin and develops an X-linked recessive inflammatory myopathy characterized by degeneration of skeletal muscle fibers and connective tissue replacement. The present work aimed to assess whether gender dimorphism in mdx mice would influence skeletal muscle pathology at ages corresponding to main histological changes in the microenvironment of muscular tissue: myonecrosis, regeneration, and fibrosis. At the height of myonecrosis (6 weeks postnatal), skeletal muscles of male mdx mice showed increased sarcolemmal permeability, numerous inflammatory foci, and marked deposition of the extracellular matrix components (ECM) type I collagen and laminin. In contrast, age-matched mdx females showed mild ECM deposition, discrete myonecrosis, but increased numbers of regenerating fibers expressing the satellite cell marker NCAM. In contrast ovariectomized mdx females showed decreased numbers of regenerating fibers. Older (24 and 48 weeks postnatal) mdx females showed extensive fibrosis with increased sarcolemmal permeability and marked deposition of ECM components than corresponding males. These results suggest a role for female hormones in the control of myonecrosis probably by promoting regeneration of muscular tissue and mitigating inflammation especially at ages under the critical influence of sex hormones.

Keywords Muscular dystrophy · Extracellular matrix · Mdx · Gender dimorphism

Introduction

Duchenne muscular dystrophy (DMD) is a devastating X-linked recessive disorder in which progressive muscle degeneration is caused by a defect in the gene coding for dystrophin, a large cytoskeletal protein present in all types of muscle tissues and certain neurons (Mehler 2000; Ehmsen et al. 2002). Dystrophin contributes to structural stability of muscle membranes through interaction with a series of proteins forming the dystrophin complex that associates with the extracellular matrix (ECM) (Carlson 1998; Michele and Campbell 2003). Mdx mouse is considered a suitable animal homologue for DMD due to its genetic and biochemical defect, as both lack dystrophin and develop spontaneous necrosis of skeletal muscle fibers (Bulfield et al. 1984). Soon after birth the mdx mouse presents cycles of skeletal muscle necrosis and regeneration that become very active over the next 5 weeks but progress at a lower rate throughout the life of the animal. Yet, persistent fibrosis and accumulation of connective tissue is a characteristic feature of older mdx mice (Lefaucher and Sebille 1996; McGeachie and Grounds 1999; Lagrota-Cândido et al. 2002).

Dystrophin deficiency in the mdx mouse is associated with increased muscular destruction that can be detected by elevated creatine kinase (CK) levels in the serum or evidenced by staining of damaged muscle fibers with extracellular marker dyes (Wehling et al. 2001). These observations have provided additional evidence for the mechanical defect hypothesis in which lack of dystrophin causes tearing of the sarcolemma leading to muscle cell death (Wehling et al. 2001). However, differences in the pathology of various dystrophin-deficient animals indicate that the resultant phenotype depends upon interactions between the primary genetic lesion and a set of compensatory responses from a variable environmental

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network (Infante and Huszagh 1999; Engvall and Wewer 2003).

Although several approaches have been envisaged to counteract the effects of this progressive disease in humans, currently there is no cure available. This is partly due to a lack of understanding of the precise molecular and functional role of dystrophin in the skeletal muscle and also the epistatic factors that ultimately influence the clinical phenotype (Infante and Huszagh 1999). An interesting but often overlooked aspect is the reported gender difference in muscle pathology of the canine muscular dystrophy (CXMD), in which homozygous females are less affected than corresponding males (Valentine et al. 1988). Recent evidence suggests that estrogen may influence the degree of disruption and postdamage inflammatory response in the skeletal muscle (Schneider et al. 1999; Tiidus 2001). Likewise, administration of diethylstilbestrol to DMD boys reduced CK serum levels (Cohen et al. 1972; Cohen and Morgan 1976). This work aimed to assess whether gender dimorphism in mdx mice would influence the process of regeneration in skeletal muscles during distinct phases of muscular dystrophy.

Materials and methods

The study was approved by the University Ethics Committee on Animal Care and was performed in accordance with the Brazilian Guidelines for the Care and Use of Laboratory Animals (COBEA).

Animals

Mdx dystrophic and age-matched C57BL/10J control non-dystrophic mice were maintained in the animal house of the Department of Cellular and Molecular Biology at Fluminense Federal University. Mice kept at constant temperature (20°C) with a light cycle of 12:12 h received acidified water and an enriched diet, supplemented with vitamins, ad libitum. In an attempt to minimize distress and avoid starvation due to muscular dystrophy, water was provided in a bottle with a longer sip and pelleted food in a receptacle placed on the floor of the cage. Male and female mdx mice were selected at ages corresponding to the main phases of the disease: myonecrosis (6 weeks), regeneration (12 weeks), and fibrosis (24 and 48 weeks).

Gonadectomy

C57 and mdx mice of both sexes were randomized at 21 days of age for gonadectomy performed under ketamine–xylazine anesthesia. Females were subjected to bilateral ovariectomy (Ovx) or sham ovariectomy (S-Ovx) via bilateral flank incisions. The ovaries were pulled out from the peritoneal cavity and the junction between the fallopian tube and the uterine horn was ligated. Males were subjected to castration (Odx) or sham castration (S-Odx) for which they were placed supine and the testes removed or left intact via a low-to-middle abdominal incision. Some C57 and mdx mice of both genders were left intact to be used as controls. Both gonadectomized male and female groups were pair-fed to the sham control animals to rule out confounding factors that might be attributed to differences in food intake (McCormick et al. 2004). Mice were killed 4 weeks after surgery.

Detection of myofiber damage

Creatine phosphokinase (CK) isoform CK-MM was measured in sera samples of mdx and control mice with a commercial kit (Bioclin, Brazil) and CK activity expressed as international units per liter (IU/l).

Evans Blue dye (EBD) was used as an *in vivo* marker of myofiber damage according to the protocol described by Hamer et al. (2002). Briefly, 100 μ l EBD (Sigma, MO, USA) dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM phosphate buffer, pH 7.0) and sterilized by filtration (membrane pore size 0.2 μ m) was injected intraperitoneally (1 mg EBD/10 g body weight), and mice were killed 24 h later (Hamer et al. 2002). Muscles were snap frozen in OCT (Leica, Nussloch, Germany), and 10- μ m-thick cryosections were fixed in acetone for 2 min, air-dried, quick-dipped in xylene, mounted with balsam, and observed under a 10 \times objective fluorescence microscope (Hund Wetzlar-H500) equipped with an Olympus-PMC35DX camera. The percentage of EBD-stained myofibers was determined in serial sections also stained by hematoxylin-eosin.

Histological staining and morphometric analysis

Gastrocnemius muscles from 6-, 12-, 24-, and 48-week-old mdx mice were carefully removed, fixed in formalin-buffered (pH 7.2) Millonig fixative for 3 days, and 5- μ m-thick wax-embedded material was stained with syrius red.

Images from entire cross-sections of at least five mdx mice at each time point (6, 12, 24, and 48 weeks of age) were acquired with a microdigital camera mounted on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a 20 \times objective. Degenerating and necrotic fibers were identified by a homogenous pale eosinophilic sarcoplasm, whereas regenerating fibers were identified by their strong sarcoplasmic basophilia and centrally located nuclei. The total surface area and areas occupied by myonecrosis, adipose tissue, or fibrosis were determined and results expressed as percentage of pathological area in the cross-section.

Immunohistochemistry

Skeletal muscles were carefully removed and fixed for 3 h in Carnoy's fixative. Five-micron-thick paraffin-embedded sections were collected on poly-L-lysine (Sigma) precoated slides. Sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in PBS for 5 min. After washing with PBS containing 0.1% BSA fraction V (Sigma), optimal concentrations of polyclonal anti-laminin (Pasteur Institute, France) and monoclonal anti-type I collagen (Sigma) diluted in PBS with 1% BSA were applied to each section followed by incubation at room temperature in a moist chamber for 60 min. Thereafter, sections were incubated with the appropriate peroxidase-conjugated whole IgG specific for rabbit (Zymed, CA, USA) or mouse (Sigma) secondary antibodies for 40 min and washed thoroughly with PBS. Peroxidase activity was revealed with aminoethylcarbazole (Sigma) in the presence of H₂O₂. All sections were lightly counterstained with Mayer's hematoxylin. Digital image analysis of the immunoperoxidase staining for laminin (LN) and type I collagen (TI-C) was performed in endomysial areas from whole cross-sections using a 40 \times objective, and the percentage of total area with positive immunolabeling for LN or TI-C was determined with the Scion Program (National Institutes of Health, Image Program, USA).

Muscle samples from three mice per age and group were embedded in OCT (Tissue-Tek; Miles, Elkhart, USA) for detection of Sca-1⁺ multipotent stem cell population and also NCAM muscle satellite cells responsible for postnatal growth and regeneration of skeletal muscle (Torrente et al. 2001; Asakura 2003; Charge and Rudnicki 2004). Five-micron-thick sections were fixed in acetone and incubated with monoclonal IgG anti-CD56 (NCAM) clone 12F11 or monoclonal IgG specific to Sca-1 (anti-Ly-6A/E) clone E13-161.7 (BD-Pharmingen Biosciences, San Diego, CA,

USA). Peroxidase-conjugated anti-rat antibody (Southern Biotechnology, Birmingham, AL, USA) was used as a secondary antibody. All sections were lightly counterstained with Mayer's hematoxylin. Negative and positive controls were included in each staining batch for all antibodies. For each mouse, images from three entire cross-sections of skeletal muscles were analyzed with a 20 \times objective. For quantification of positive cells, 400 myofibers were counted from two individual sections (at least 200 muscle fibers per section) and the percentage of positive cells was calculated as the number of positive cells (positive cells + myofiber number) \times 100 (Cramer et al. 2004)

Statistical analysis

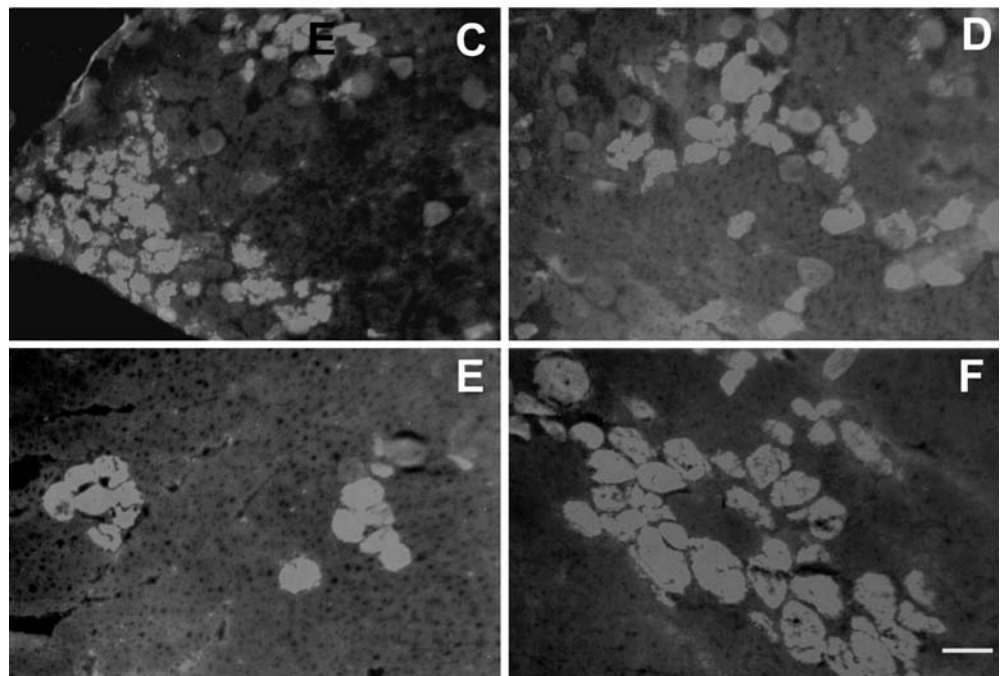
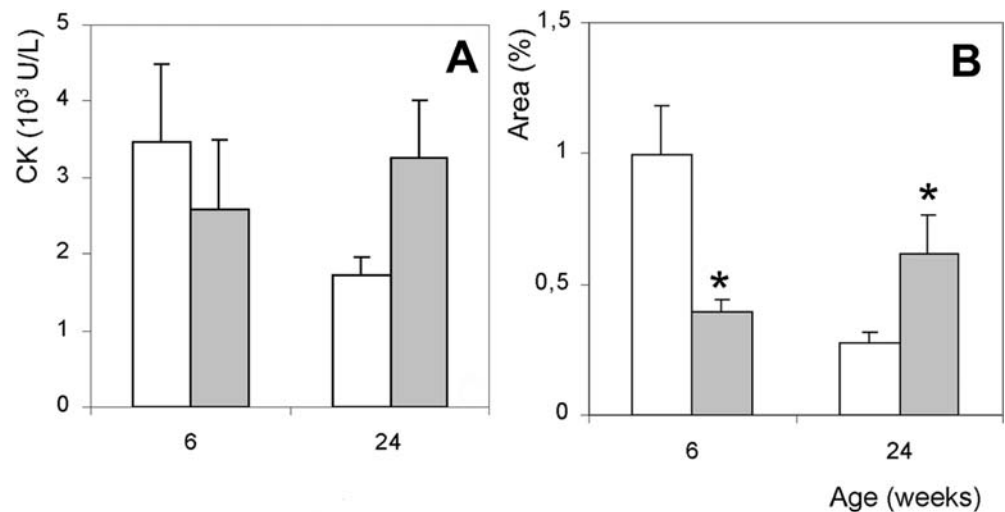
Microsoft Excel software (Microsoft, Seattle, WA) was used to calculate mean and standard deviations. The unpaired Student's *t*-test was applied to assess the level of statistical significance.

Results

Assessment of muscular damage

In agreement with the literature, mdx mice showed increased CK activity in their serum (Fig. 1A) in relation to non-dystrophic C57BL10 mice at all ages studied ($P < 0.001$). During the height of myonecrosis (6 weeks), mdx males presented, in comparison with age-matched mdx females, increased levels of serum CK, but without statistical significance ($P > 0.05$). However, mdx males at 12 weeks showed an approximately 2.1-fold increase of CK levels, thus indicating a greater muscular destruction than age-matched females. In contrast, older (48 weeks) mdx males showed an approximately 4.5-fold decrease of CK activity than corresponding (Fig. 1A) age-matched females.

Fig. 1A–F Detection of myofiber damage in mdx muscle. **A** Serum creatine kinase (CK) activity from mdx muscle at 6 and 24 weeks. Results are expressed as mean \pm standard error (SE) of five to nine animals per group. *Open columns* male, *filled columns* female. **B** Results are expressed as mean \pm standard deviation (SD) of percentage/area unit labeled with Evans Blue dye (EBD) in gastrocnemius skeletal muscle from mdx mice at ages 6 and 24 weeks. * $P < 0.001$ for comparisons between males and females. **C–F** Transverse frozen sections (10 μ m) of the gastrocnemius muscle from male (**C**, **E**) and female (**D**, **F**) mdx mice at 6 (**C**, **D**) and 24 weeks (**E**, **F**) after intraperitoneal injection of EBD. Bar 100 μ m



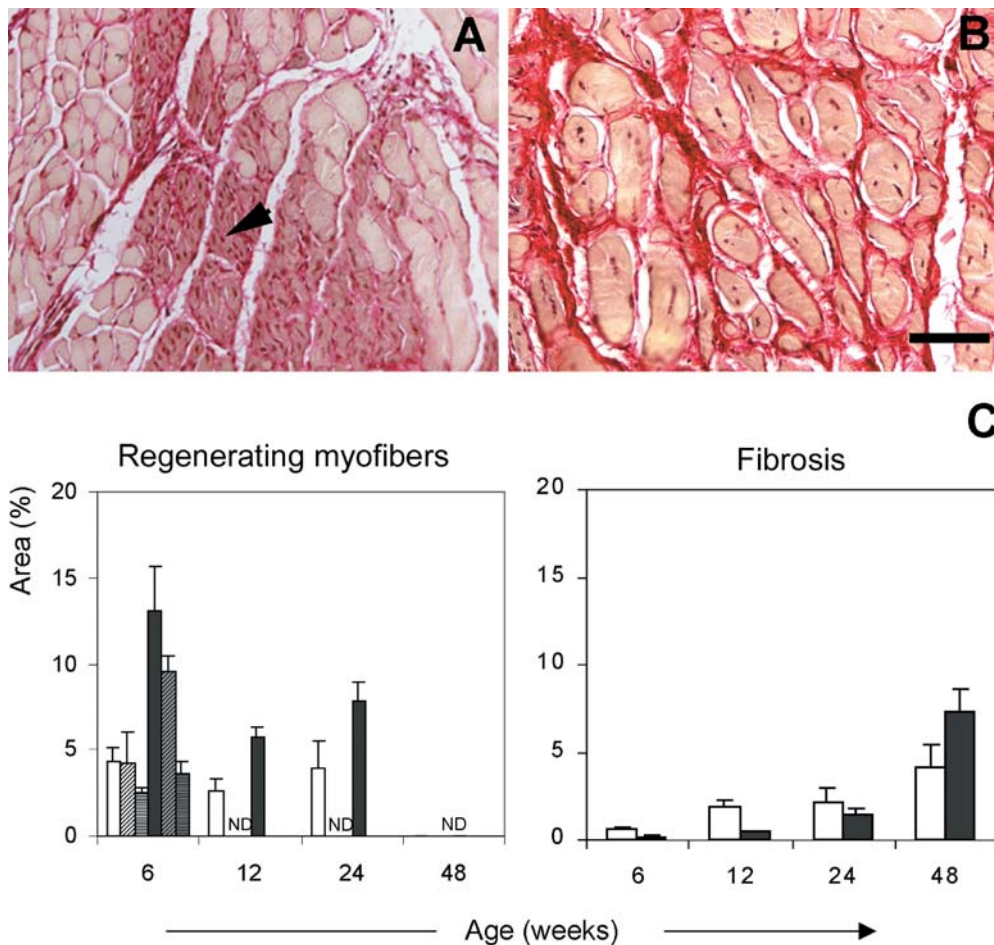


Fig. 2A–C Histological analysis of a 6- μ m-thick wax-embedded sections of gastrocnemius muscle from mdx dystrophic mice stained by picrosirius. **A** Numerous regenerating fibers with centrally located nuclei and basophilic cytoplasm (arrowhead) were more evident in female mdx mice at 6 weeks; few inflammatory foci and little connective tissue. **B** Overdeposition of connective tissue (asterisk) in the period of fibrosis in a 48-week-old female mdx mouse. Original magnification $\times 200$. Bar 100 μ m. **C** Frequencies of regenerating myofibers and fibrosis in mdx skeletal

muscle. Results are expressed as mean \pm SE. A 20 \times objective was used for the analysis of three entire cross-sections of gastrocnemius skeletal muscle from three animals per group at ages 6, 12, 24, and 48 weeks. * $P < 0.05$ for comparison between males and females. Open columns male, light diagonal shading castrated (Odx) males, light horizontal shading sham-Odx males, filled columns female, dark diagonal shading ovariectomized (Ovx) females, dark horizontal shading sham-Ovx females, ND not determined

Sarcolemmal permeability

Experiments using fluorescent EBD as an *in vivo* indicator of sarcolemmal disruptions (Straub et al. 1997) showed that mdx males at the height of myonecrosis (6 weeks) presented more extensive damage of gastrocnemius muscle, as evidenced by increased myofiber fluorescence, than corresponding age-matched mdx females (Fig. 1C, D). In addition, histomorphometric analysis showed that fluorescent myofibers corresponded to 0.995% of analyzed muscle fragments in young mdx males but only 0.395% in age-matched females (Fig. 1B). In contrast, older (24 weeks) mdx males presented less areas stained with EBD (0.280%) than corresponding age-matched females (0.613%) (Fig. 1E, F). At both ages the difference between dystrophic males and females was highly significant ($P < 0.001$).

Morphometric analysis of pathological features of dystrophic muscles

Histological analysis of muscle samples showed consistent differences in muscular tissues between mdx females and age-paired males (Fig. 2). Dystrophic females at 12 weeks presented fewer areas with inflammation and myonecrosis and more areas with regenerating fibers than age-paired mdx males (Fig. 2A). Indeed, regardless of the stage of the disease (6, 12, and 24 weeks) mdx females consistently showed (Fig. 2C) an increased percentage of regenerating fibers.

Castrated males (Odx-mdx) did not present any change in the pattern of skeletal muscle regeneration and fibrosis as evidenced by histomorphometry (Fig. 2C). In contrast, Ovx-mdx at 6 weeks showed a decrease in the percentage of regenerating (9.5%) fibers in relation to control (13.1%) age-matched females. Interestingly, both sham

Table 1 Determination of stem cell (Sca-1⁺) and satellite cell (NCAM) in C57BL10 non-dystrophic mice. It was counted under a 20× objective; 400 myofibers from two individual sections for each group. The results are expressed as percentage of positive cells in relation to myofiber numbers

	Male (%)	Female (%)
Sca-1	24.52±6.6	22.88±8.65
NCAM	1.17±0.32	1.62±0.65

gonadectomized female S-Ovx (3.6%) and male S-Odx (2.5%) showed a marked reduction in the number of regenerating fibers.

Although hypertrophied fibers and fibrosis are considered a characteristic histological feature (Lagrotta-Cândido et al. 2002) of older (48 weeks) mdx skeletal muscles (Fig. 2B) it is possible that such a pattern may be influenced by gender dimorphism. Indeed, older mdx females presented (Fig. 2C) a significant increase ($P<0.05$) in the whole area occupied by fibrosis (7.30%) in comparison to age-matched mdx males (4.06%). Furthermore, skeletal muscles of older mdx females consistently showed increased deposition of fatty tissue (data not shown).

Analysis of stem cells and satellite cells in the skeletal muscle

Gastrocnemius muscles from male and female 6-week-old non-dystrophic C57BL10 mice were used to investigate whether gender dimorphism would influence the regenerative capability and numbers of myogenic progenitor cells. Both male and female C57BL10 non-dystrophic mice showed a similar expression of stem cell antigen (Sca-1⁺ cells) and satellite cell marker (NCAM; Table 1). In contrast, mdx females presented increased numbers of

activated myoblasts as evidenced by NCAM immunolabeling and many niches of Sca-1⁺ cells (Fig. 3).

Influence of ECM components on myofiber regeneration and fibrosis

Since mdx females presented more regenerating fibers than corresponding males, it appeared relevant to assess the pattern of LN expression in both male and female dystrophic skeletal muscles considering the influence of LN on muscle formation and regeneration (Mundegar et al. 1995; Patton et al. 1999).

A faint LN immunolabeling was consistently observed delineating myofibers of gastrocnemius muscles of non-dystrophic C57BL10 males, though age-matched C57 females presented a mild increase of LN expression at all ages studied (Fig. 4E). In contrast, mdx mice showed intense immunolabeling in the endomysium close to sites of myonecrosis and nearby regenerating myofibers (Fig. 4A). Image analysis showed that gastrocnemius muscle from 6- and 12-week-old mdx males presented more areas labeled with anti-LN (26.65% and 20.74%, respectively) than age-paired females (16.65% and 18.31%, respectively). In contrast, mdx females at ages 24 and 48 weeks exhibited a significant increase ($P<0.001$) in the extension of areas labeled for LN (Fig. 4E). A gradual reduction of LN expression with age was also noticed in gastrocnemius muscles of mdx males (Fig. 4A, C, E). LN expression was intense in 24-week-old females mainly in areas with regenerating myofibers.

Expression of TI-C was evidenced solely at contours of endothelial cells in C57BL10 normal skeletal muscles (data not shown). As expected (Seixas et al. 1994) marked deposition of TI-C was consistently present mostly around lesioned myofibers associated with in-

Fig. 3A–D Expression of NCAM and Sca-1 in skeletal muscles from C57BL10 non-dystrophic mice. Immunolabeling for NCAM (A, B; arrowheads) and Sca-1 (C, D; arrows) in gastrocnemius muscles from male (A, C) and female (B, D) C57BL10 at 6 weeks of age. Bar 100 μ m

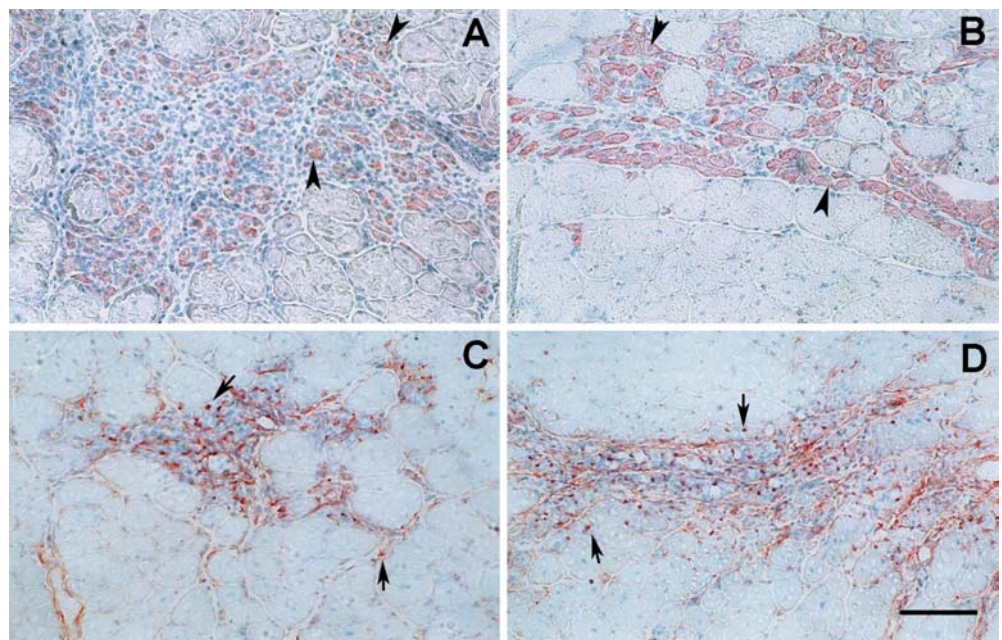
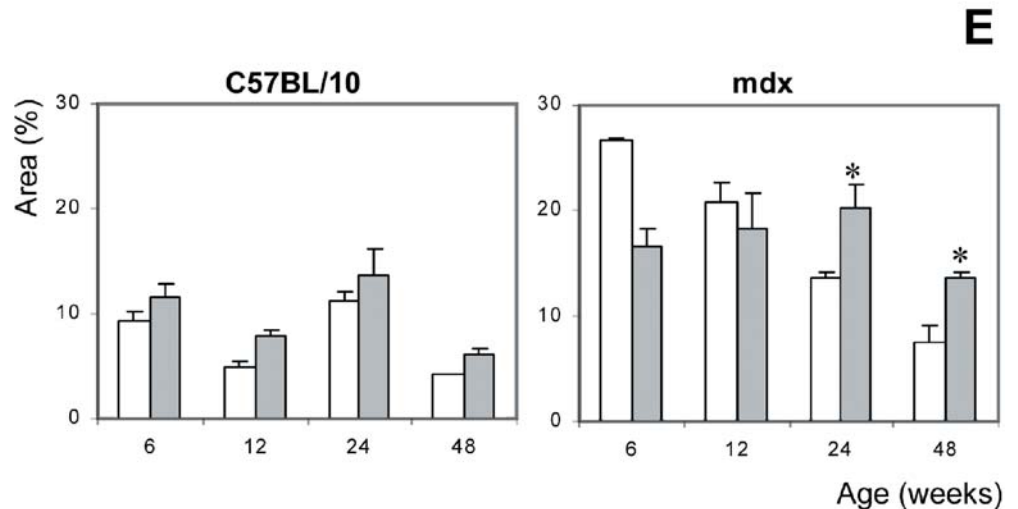
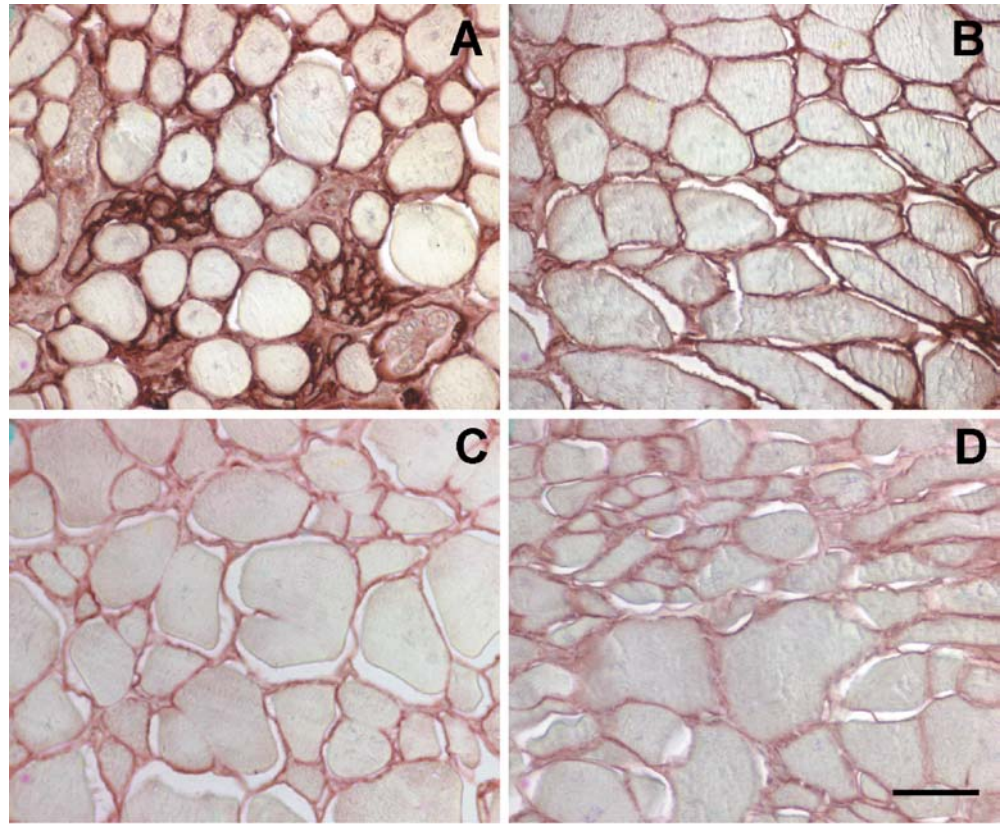


Fig. 4A–E Laminin (LN) expression in the mdx gastrocnemius muscle. Immunolabeling of LN in sections ($5\ \mu\text{m}$) of gastrocnemius muscle from male (**A, C**) and female (**B, D**) mdx mice at ages 6 (**A, B**) and 24 weeks (**C, D**). **E** Quantitative image analysis of LN expression. Results are expressed as mean (\pm SD) percentage/area labeled with monoclonal anti-LN. * $P < 0.001$ for comparison between males (*open columns*) and females (*filled columns*)

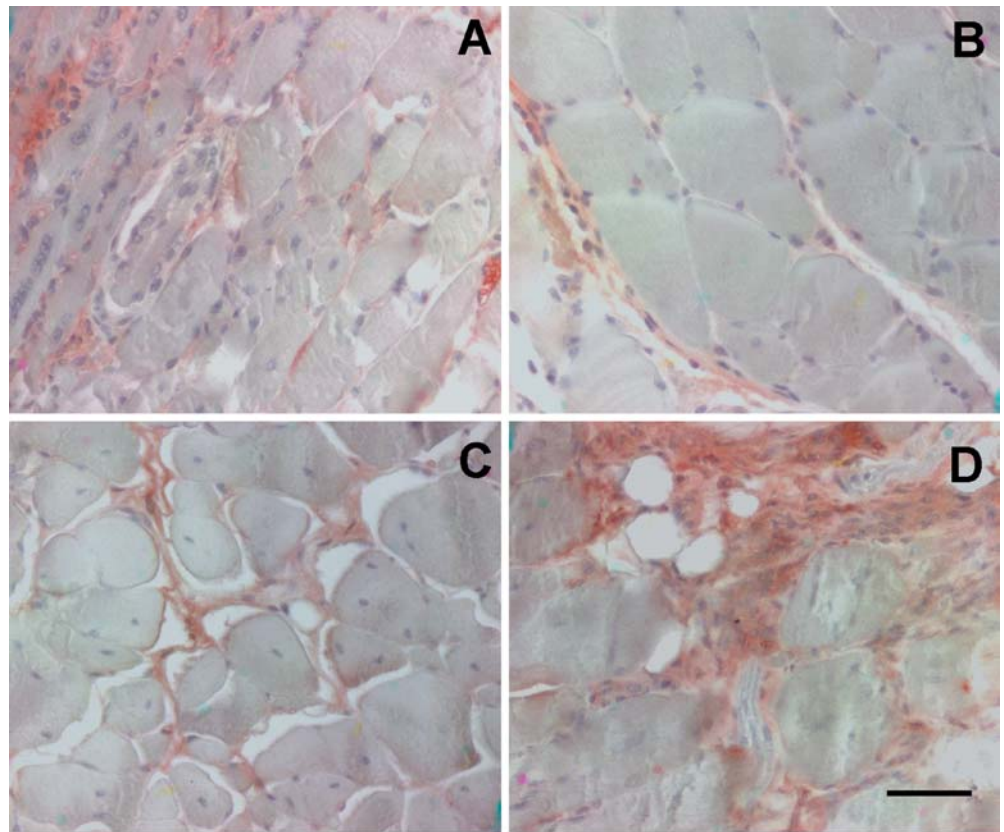


inflammatory reaction in younger mdx and with fibrosis in older mdx mice (Fig. 5). Image analysis showed that the relative proportion of TI-C expression diminished significantly ($P < 0.001$) with age in the skeletal muscles of males, though skeletal muscles of age-matched mdx females presented numerous areas labeled with TI-C (Fig. 5E). Indeed, a consistent deposition of fat and fibrosis was observed, as evidenced by syrius dye in older mdx females.

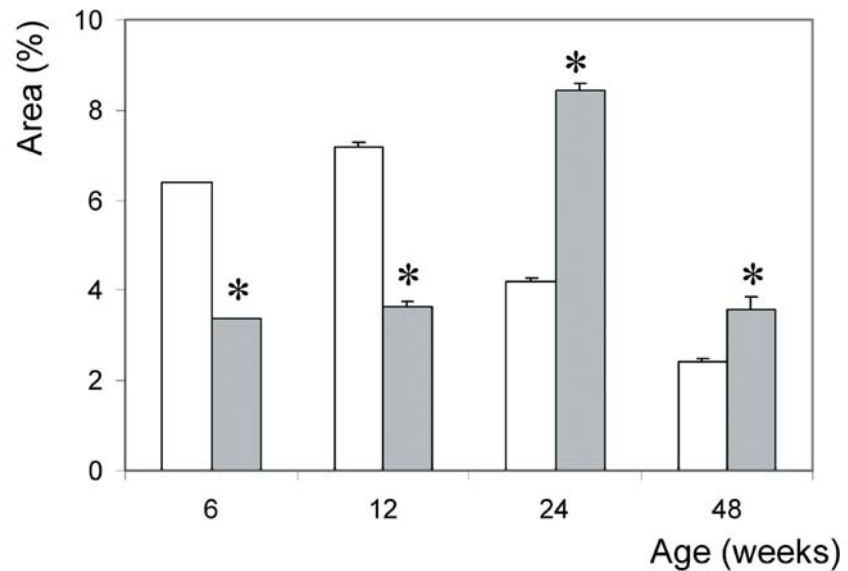
Discussion

Lack of dystrophin prevents the assembly of the entire complex of proteins (Ervasti et al. 1990; Ehmsen et al. 2002) and increased cell membrane permeability as evidenced by elevated serum levels of CK or by the presence of extracellular marker dyes within lesioned muscle fibers (Wehling et al. 2001; Hamer et al. 2002). Using these parameters we observed that sarcolemmal integrity of young female mdx mice was more preserved than corresponding age-matched dystrophic males, especially at early stages of the myopathy. Indeed, mdx females

Fig. 5A–E Type I collagen expression in the mdx gastrocnemius muscle. Immunolabeling of type I collagen in sections (5 μm) of gastrocnemius muscle from male (**A, C**) and female (**B, D**) mdx mice at ages 6 (**A, B**) and 24 weeks (**C, D**). Bar 100 μm . **E** Quantitative image analysis of type I collagen expression. Results are expressed as mean (\pm SD) in percentage/area labeled with monoclonal anti-type I collagen antibody. * $P < 0.001$ for comparisons between males (*open columns*) and females (*filled columns*)



E



showed a milder degree of muscle disruption than corresponding males at the time characterized by extensive myonecrosis and numerous inflammatory foci. Similar results were found in the canine model of muscular dystrophy (CXMD) where dystrophin-deficient homozygous CXMD females presented lower CK levels than

the hemizygous males (Valentine et al. 1988). However, the extent of myofiber damage was more intense in elderly (24 weeks) mdx females.

Besides diminished inflammatory reaction, extensor skeletal muscles of mdx females at 6, 12, and 24 weeks also presented an increased percentage of areas with a

predominance of regenerating myofibers than age-matched males. This could be partly explained by a potent mitogen activity of estrogen, a steroid hormone that binds to members of the nuclear receptor superfamily, influencing proliferation and differentiation of mammalian cells (Kahlert et al. 1997; Schneider and Sannes 2001). This has been confirmed by *in vivo* experiments showing that estradiol implants activate muscle satellite cell proliferation (Johnson et al. 1998). Although mouse skeletal myoblasts express estrogen receptors, *in vitro* estradiol did not seem to influence myoblast proliferation. It is argued that estrogen influences myoblast growth indirectly through induction of growth factor production, such as IGF-1 and IL-6 (Kurek et al. 1996; Johnson et al. 1998; Verthelyi 2001).

In the present work we observed a slight reduction in the number of regenerating fibers in ovariectomized (Ovx-mdx) mice. This may be due to the fact that initial ovariectomy causes a state of adaptive hypersensitivity to estradiol, which is associated by an upregulation of the MAP kinase and PI-3 kinase pathways and increased usage of a membrane-associated ER alpha (Santen 2003). In addition, recent data confirm the ability of skeletal muscle to synthesize estrogen and contribute to the circulating pool of estrogens that is comparable to that of adipose tissue (Larionov et al. 2003). It is also known that skeletal muscles contains specific and saturable binding sites for naturally occurring and synthetic sex hormones which cannot be displaced by either cortisol or synthetic glucocorticoid. Neither do androgens nor estrogens compete for glucocorticoid sites in skeletal muscle (Snochowski et al. 1980). Interestingly, S-Ovx-mdx mice showed a significant decrease in the regenerative capability of muscle tissue when compared to Ovx-mdx. Such results could be related to production of stress hormones since glucocorticoids induce muscular atrophy by upregulating expression of the myostatin gene (Ma et al. 2003). Furthermore, the great regenerative capacity of mdx females is probably associated with increased stem cell recruitment and activation of satellite cells within the foci of myonecrosis. Such a hypothesis is supported by experiments showing that gender dimorphism did not influence the numbers of stem (Sca-1⁺) and satellite (NCAM⁺) cells in the skeletal muscles of control nondystrophic mice.

Several studies (Amelink et al. 1988; Komulainen et al. 1999; Tiidus et al. 2001) strongly suggest that females have greater protection than males against muscle membrane disruption consequent to intense exercise. Female hormone may thus be considered a presumed protecting factor responsible for differences in the pattern of inflammatory response following muscle damage (Tiidus et al. 2001). Interestingly, gender-specific responses to exercise indicate that men have greater levels of stress protein HSP70 (Paroo et al. 2002) and circulating CK, an indicator of exercise-induced muscle damage, than women. Explanations for gender differences in myofiber damage have mainly been based on the hypothesis that estrogen may improve structural membrane integrity by having a direct receptor-mediated influence on the mem-

brane stability (Amelink et al. 1990) and/or an antioxidant property (Bar and Amelink 1997; Tiidus 2000). Likewise, estrogen also reduces leukocyte infiltration following muscle damage (Amelink et al. 1988; Komulainen et al. 1999) by limiting the availability of endothelial adhesion molecules such as VCAM-1 and ICAM-1 in the inflamed tissue (Schneider et al. 1999; Galea et al. 2002). As reported in other models of injury (Wise and Dubal 2000; Zhai et al. 2000), female hormones may also attenuate inflammatory-related leukocyte infiltration into skeletal muscles in female mdx mice compared with male mdx mice.

In the present work we present evidence that mdx males and females also display a distinct pattern of ECM expression in the microenvironment of muscular tissue. Marked deposition of TI-C and LN was consistently augmented in the skeletal muscles of young (6 and 12 weeks) dystrophic males at ages characterized by increased percentage of areas with myonecrosis and inflammatory foci in relation to age-paired females. Nonetheless, older (24 and 48 weeks) mdx females showed extensive fibrosis and increased deposition of TI-C and LN than corresponding males. It is not ruled out that marked deposition of LN concomitant with increased percentage of myonecrosis in skeletal muscles of young (6 weeks) mdx males could be promoting inflammatory cell migration. We have previously reported that predominant expression of ECM receptors ($\alpha 4$, $\alpha 5$, and $\alpha 6$ integrins) by infiltrating cells near the foci of myonecrosis, indicate an important role for ECM ligands and receptors in directing adhesion and migration of mononuclear cells in the lesioned muscle and toward local draining lymph nodes (Lagrota-Cândido et al. 1999).

The process of regeneration involves degradation of damaged myofibers, activation and migration of myoblast precursor/satellite cells to the site of injury, with subsequent proliferation, differentiation, and fusion to form multinucleated myotubes that mature into myofibers (Foster et al. 1987; Grounds 1991; Yao et al. 1996). Such a process is influenced by the type of LN since *in vitro* assays revealed that C2C12 muscle cell lineage and primary myoblasts adhere to LN-1 and LN-2 isoforms in a similar manner, though myoblast spreading was significantly faster on an LN-2-coated surface (Schuler and Sorokin 1995).

Another aspect to be considered is a role for TGF β -1, a cytokine with anti-inflammatory properties (Ling and Robinson 2002) that is highly expressed in the postpubertal period of females (Rosenkranz-Weiss et al. 1994). Although older females normally present lower TGF β levels (Ashcroft et al. 1997), it is possible that TGF β signaling to intense fibrosis in mdx females at 24 and 48 weeks had already occurred during the postpubertal period. In this sense, data from our laboratory (data not shown) showed that expression of TGF β 1 at early stages of DMD may be critical for initiating muscle fibrosis. In such a way therapeutic strategies using anti-TGF β 1 would attenuate fibrosis and slow progression of the disease (Yamazaki et al. 1994; Bernasconi et al. 1995;

Amemiya et al. 2000). Altogether our results indicate that female hormones are partly responsible for gender-related differences in the muscular lesion of mdx mice, by favoring resolution of myonecrosis and promoting regeneration of skeletal muscles especially at ages under the critical influence of sex hormones.

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