

# The soy isoflavone genistein blunts nuclear factor kappa-B, MAPKs and TNF- $\alpha$ activation and ameliorates muscle function and morphology in *mdx* mice

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## Abstract

Several lines of evidence suggest a detrimental role of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation in the dystrophic process. We showed in previous studies that its inhibition through drugs with antioxidant properties, have beneficial effects in *mdx* mice. We tested whether genistein, a well-known isoflavone, inhibitor of NF- $\kappa$ B, MAPK and TNF- $\alpha$  and readily available for clinical use, could have a beneficial effect in *mdx* mice in comparison with methylprednisolone, the gold standard treatment for DMD patients. Five-week old *mdx* mice received for five weeks: genistein (daily or 3-times/week), methylprednisolone or vehicle. Genistein treatment: (1) increased forelimb strength and strength normalized to weight; (2) reduced serum creatine-kinase levels; (3) reduced markers of oxidative stress; (4) reduced muscle necrosis and enhanced regeneration. The positive results were more evident with the daily administration of genistein and were comparable to the effect of corticosteroids. Our data support the novel hypothesis that, as other more specific therapeutic approaches are still under development, this soy-derived compound is a promising option to be further investigated in dystrophic process.

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**Keywords:** Duchenne muscular dystrophy; Genistein; *mdx* mice; Natural supplement; NF- $\kappa$ B

## 1. Introduction

Duchenne muscular dystrophy (DMD) is the most common lethal X-linked recessive disorder, affecting 1 in 3500 live male births. DMD children show early symptoms of muscle degeneration, frequently develop contractures, and lose the ability to walk by 13 years of age. With disease

progression, most patients succumb to death from respiratory failure and cardiac dysfunction in early adulthood [1]. The primary cause of this disease stems from the lack of the protein dystrophin, which is essential for the structural and functional integrity of muscle membrane. Dystrophin absence results in membrane damage, allowing massive infiltration of immune cells, chronic inflammation, necrosis, and severe muscle degeneration [2]. Normally, muscle cells possess the capacity to regenerate in response to injury signals. However, this ability is lost in DMD, presumably due to an exhaustion of satellite cells during ongoing degeneration and regeneration cycles [2]. Although the absence of the protein represents the primary cause of DMD, it is the secondary processes involving persistent

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inflammation and impaired regeneration that likely exacerbate disease progression. A detrimental role is played by the microenvironment of dystrophic muscles consisting of increased oxidative stress, elevated numbers of inflammatory cells that act as a complex interface for cytokine signaling [3].

The *mdx* mouse, a genetically homologous DMD model, is frequently used to study the disease pathogenesis, despite relevant clinical and pathological differences. Compared to the human disease, the murine model shows slower disease progression and similar repetitive degeneration and regeneration cycles occurring between 2 and 12 weeks of age, muscle weakness at a later stage, but no proliferation of connective tissue in limb muscles [4–6].

Reactive oxygen species (ROS) injury has been postulated to contribute to dystrophic muscle damage for a number of years [7]. The increased action of oxidative stress in DMD muscle is indicated in part by enhanced lipid peroxidation, and induction of antioxidant enzymes [7]. Free radical scavengers have been also showed to improve the dystrophic pattern of *mdx* muscles [8–12].

Oxidative stress/lipid peroxidation represents one of the mechanisms activating nuclear factor (NF)- $\kappa$ B and the consequent pathogenetic cascade in *mdx* mice [8–10]. NF- $\kappa$ B is a major transcription factor modulating the cellular immune, inflammatory and proliferative responses [13] and its activation can lead to increased expression of inflammatory molecules such as IL-1 $\beta$ , COX-2 and TNF- $\alpha$ , all of these involved in the pathogenesis of muscular dystrophy and promotion of muscle wasting [8–10,13,14]. NF- $\kappa$ B activation is regulated by the I- $\kappa$ B kinase (IKK) complex composed of catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit IKK $\gamma$ /NEMO [14]. At molecular level, the activation of NF- $\kappa$ B is regulated by cellular kinases, including mitogen-activated protein kinases (MAPKs) [15], involved in different pathological aspects of the dystrophic process [10,16–18]. On the other hand, NF- $\kappa$ B also regulates myogenesis, and a robust amount of evidence demonstrated that NF- $\kappa$ B inhibition stimulates muscle regeneration in different experimental models of muscle necrosis and in *mdx* mice [19–21].

Corticosteroids are considered the gold standard of treatment in DMD [22], and although frequently used in clinical practice their precise mechanism of action is still unknown [23]. Interestingly, these drugs also inhibit the pathways of NF- $\kappa$ B [14,24] and MAPKs [25]. These mechanisms could partially explain their efficacy in DMD.

Our group previously reported NF- $\kappa$ B activation in muscles of DMD patients and *mdx* mice and showed that its inhibition, through compounds with also antioxidant properties, parallels to an amelioration of functional, morphological and biochemical parameters in the murine model [8–10,26].

Genistein is a soy isoflavone that exerts many biological functions when administered in its active form, the aglycone one. The isoflavone is able to inhibit ROIs production [27], NF- $\kappa$ B and STAT-1 activation [28–30] and other

pathways involved in DMD pathogenesis such as TNF- $\alpha$  [31], MAPKs [32] and other proinflammatory mediators [28,32,33]. Moreover it has also been shown to promote cell proliferation in different cell types [34]. Genistein seems to act through different mechanisms depending on cell type and experimental concentrations [34–36]. A tyrosin-kinase inhibitor effect has been widely demonstrated in cancer cells, but requires high concentrations [34]. Genistein is also a natural selective estrogen receptor (ER) modulator and most of the above mentioned effects seem to be mediated by its binding to ER alpha/beta, with higher affinity for ER beta [34–37]. It has been shown to have positive effects on postmenopausal bone loss in controlled clinical trials [38]. This isoflavone has a very safe profile [39] with a nearly dose-linear pharmacokinetic characteristics [40] and is available in US and Europe as natural supplement for postmenopausal health.

In light of this findings the aim of our study was to test the novel hypothesis that genistein might attenuate the skeletal muscle damage with positive functional effects in *mdx* mice. We also compared the effects with those of methylprednisolone, an effective drug in clinical practice, but with significant side effects.

## 2. Materials and methods

### 2.1. Animals

Male *mdx* and wild-type C57BJ/10ScSn (WT) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and bred in our animal facilities. Mice were housed in plastic cages in a temperature-controlled environment with a 12-h light/dark cycle and free access to food and water. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of health (NIH Publication No. 85-23, revised 1996). Five-week old *mdx* and WT mice have been treated for 5 weeks with intraperitoneal injections with genistein [n:8, 2 mg/kg i.p. 3-times/week (genistein 1) or n:8, 2 mg/kg i.p. daily (genistein 2)], methylprednisolone (n:8, 0.75 mg/kg i.p. daily) or vehicle (n:8, dimethylsulfoxide [DMSO]/NaCl 0.9%; 0.1:1, v/v; 0.2 mg/kg i.p. daily). At the end of the experiments, animals were anaesthetized with intraperitoneal administration of sodium pentobarbital (80 mg/kg). Then, blood, collected by intracardiac puncture, was drawn to analyze creatine kinase (CK) levels and the biceps, quadriceps and extensor digitorum longus (EDL) muscles were removed bilaterally and immediately frozen in liquid nitrogen-cooled isopentane and stored at  $-80^{\circ}\text{C}$  for histological and biochemical evaluations.

### 2.2. Animal examinations

Mice were weighed and examined for forelimb strength at baseline and after 5 weeks of treatment. Strength limb assessment, using a method suggested in the recently

published Standard Operating Procedures (SOPs) promoted by TREAT-NMD (DMD M.2.2.001) [4], consisted of five separate measurements using a grip meter attached to a force transducer that measures peak force generated (Stoelting Co., Wooddale, IL, USA). The mouse grabs the trapeze bar as it is pulled backward and the peak pull force in grams is recorded on a display. The three highest measurements for each animal were averaged to give the strength score. As at this age the mice are significantly growing, the strength score normalized to body mass was also considered [5,8].

### 2.3. Serum creatine kinase (CK) evaluation

Blood samples were centrifuged at 6000 rpm and the serum was stored at  $-80^{\circ}\text{C}$  until the day of analysis. Serum CK was evaluated at  $37^{\circ}\text{C}$  using a commercially available kit (Randox Laboratories LTD., Crumlin Co., Antrim, UK). The results were expressed as U/L.

### 2.4. Histological studies

Ten-micrometer-thick transverse cryostat sections were obtained from the midpoint of the biceps muscle body. The whole muscle cross-sections (corresponding to a mean area of  $2.15\text{ mm}^2$  in biceps), stained with hematoxylin–eosin (H&E), were examined by a blinded observer, using the AxioVision 2.05 image analysis system equipped with AxioCam camera scanner (Zeiss, Munchen, Germany). The following four areas were recognized with patchy distribution: (i) normal fibers, identified by the presence of peripheral nuclei; (ii) centrally nucleated fibers, identified by normal size but with central nuclei; (iii) regenerating fibers, identified by small size, basophilic cytoplasm and central nuclei; (iv) necrotic fibers, identified by pale cytoplasm and phagocytosis. The results were expressed as the ratio of the area occupied by normal fibers, centrally nucleated fibers, regenerating fibers or necrotic fibers divided by the total surface area as a percentage.

### 2.5. Immunohistochemistry

Immunohistochemical detection of specific antigens was performed on  $7\text{-}\mu\text{m}$ -thick transverse cryostat sections from EDL muscles. A rabbit polyclonal antibody against myogenin (1:60; sc-576, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a mouse monoclonal antibody against developmental myosin heavy chain (dMHC) (1:20; NCL-MHCd, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) were used to study regeneration. Myogenin is expressed by differentiating myoblasts and considered to be an ‘early’ marker of regeneration. DMHC was used as a marker of ‘later’ regeneration. Visualization of antibody staining was achieved using a peroxidase detection system (ABCComplex/HRP kit, Dako, Milan, Italy) and the 3,3-diaminobenzidine tetrahydrochloride as chromogen. In the case of dMHC, the AEC substrate chromogen (Zymed

Laboratories, San Francisco, CA) was used for color development.

### 2.6. NF- $\kappa$ B DNA-binding activity by Electrophoresis Mobility Shift Assay (EMSA)

Isolation of nuclear proteins in approximately 50 mg of frozen quadriceps muscle was performed according to elsewhere detailed methods [8]. Twenty micrograms of nuclear extract were incubated for 30 min at room temperature with 50 fmol of biotin-end-labeled 45-mer double-strand NF- $\kappa$ B oligonucleotide from the HIV long terminal repeat, 5'-TGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGAGGCGTGGG-3' containing 2 (underlined) NF- $\kappa$ B binding sites. Both complimentary oligos were end-labeled separately and then annealed prior to use. Binding reaction mixtures were prepared in a final volume of 20  $\mu\text{L}$  HEPES buffer containing 1 mg double-strand poly dI/dC, 10% glycerol, 100 mM  $\text{MgCl}_2$  and 1% Nonidet P-40. The shift was performed by LightShift™ Chemiluminescent EMSA Kit (Pierce, Milan, Italy), according to the manufacturer's instructions. Competitive assays were also performed by addition of 50-fold excess of unlabeled probe to nuclear extract at room temperature for 10 min before the addition of the labeled probe. Bound complexes were separated on 7.5% nondenaturing polyacrylamide gels, blotted onto nylon membrane and visualized on Kodak X-ray film (Kodak, Milan, Italy) by autoradiography. The results are expressed as relative integrated intensity compared with normal controls and internal positive controls, considering exposure time, background levels, and known protein concentration of an Epstein-Barr virus nuclear antigen-1 extract, with its consensus sequence provided with the Light-Shift Chemiluminescent kit (Pierce), which was used as EMSA control.

### 2.7. Biochemical assays (hydrogen peroxide and total peroxidase)

Thirty milligrams of quadriceps muscle specimen were homogenized in 20 volumes of ice-cold buffer containing 0.5 M KCl, then centrifuged at 5500g for 10 min. Protein concentration of tissue homogenates was determined by Lowry assay. An aliquot of 200  $\mu\text{L}$  of muscle homogenate was used to perform hydrogen peroxide (HP) and peroxidase assays by spectrophotometry using Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes Inc., Eugene, OR, USA).

### 2.8. Isolation of cytoplasmatic proteins and determination of JNK and TNF- $\alpha$

Muscle samples from quadriceps were homogenized in lysis buffer (1% Triton; 20 mM Tris/HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1% aprotinin; 15  $\mu\text{g}/\text{mL}$  leupeptin). Protein samples (30  $\mu\text{g}$ ) were denatured in reducing

buffer (62 mmol Tris/HCl, pH 6.8, 10% glycerol; 2% SDS; 5%  $\beta$ -mercaptoethanol; 0.003% bromophenol blue) and separated by electrophoresis on a SDS (12%)–polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris/HCl, pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein and blocked with 5% non-fat dry milk in TBS–0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.1% Tween, and incubated with a primary phosphorylated antibody for phospho-JNK (Cell Signalling, Beverly, MA) and TNF- $\alpha$  (Chemicon, CA, USA) in TBS–0.1% Tween overnight at 4 °C. After being washed three times for 10 min each in TBS–0.1% Tween, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, UK) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham, UK). The protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Italy). The results were expressed as relative integrated intensity compared with controls and subtracting respective backgrounds. Equal loading of protein was assessed on stripped blots by immunodetection of  $\beta$ -actin with a rabbit monoclonal antibody (Cell Signaling, Beverly, MA) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, UK).

### 2.9. Drug

Genistein aglycone was a kind gift of Primus Pharmaceuticals Inc., Scottsdale, AZ, USA. Methylprednisolone was purchased by Sigma (Milan, Italy).

All substances were prepared fresh daily and administered at a volume of 100  $\mu$ L. The vehicle used to dilute Genistein aglycone was a DMSO/NaCl solution.

### 2.10. Statistical analysis

Results are expressed as mean  $\pm$  SD. Statistical evaluation was performed by using one-way analysis of variance followed by Dunnett's post hoc tests and paired Student's *t*-test with the use of the InPlotPrism software version 3.0 (GraphPad Software, San Diego, USA). *p* values <0.05 were considered significant.

## 3. Results

### 3.1. Effects of genistein on functional performances

Genistein inhibiting several mechanisms known to be involved in the dystrophic process might lead to greater functional capacity of treated dystrophic muscles. To verify this hypothesis we performed functional tests, measuring muscle strength of treated and untreated *mdx* mice.

Body mass was not significantly different among the animal groups at baseline. After treatment, only methylprednisolone induced in WT mice an increase of body mass compared to WT mice treated with vehicle ( $32 \pm 0.7$  vs  $27 \pm 0.4$  g;  $p < 0.01$ ). All groups showed at the end of the experiment an increased body mass compared to baseline (Fig. 1A).

At baseline, strength and strength normalized to body mass were significantly lower in untreated *mdx* mice groups compared to WT groups ( $p < 0.05$ ). At the end of experiments, genistein-treated (both therapeutic schemes) and methylprednisolone-treated *mdx* mice showed a better performance of muscle function evaluated as forelimb strength (respectively, +25%,  $p < 0.05$ ; +49%,  $p < 0.01$ ; and +43%;  $p < 0.01$ ) compared to vehicle-treated *mdx* mice. Notably, only daily genistein-treated and methylprednisolone-treated *mdx* mice showed higher strength normalized to body mass (+40%,  $p < 0.01$  and +37%,  $p < 0.01$ ) compared to vehicle-treated *mdx* mice. Analogously all treated groups showed an increment of strength compared to baseline ( $p < 0.01$ ), but when normalized to body mass among the *mdx* groups only the mice treated with daily genistein and methylprednisolone showed a significant amelioration in strength ( $p < 0.01$ ) (Fig. 1B and C). Treatments did not cause any significant change in the forelimb strength, strength normalized to body weight in WT animals.

Although the use of the transducer produced by Stoelting Co. lead to values similar to those of Connolly et al. and to those previously obtained and published by our group [5,8], it is worth mentioning that these values are possibly overestimated based on the large amount of data from other groups using different devices [41–43] and that found a large consensus in the scientific community, as mentioned in the SOPs in DMD\_M.2.2.001 (<http://www.treat-nmd.eu/research/preclinical/dmd-sops>). In spite of the possible overestimation of the absolute values, the rather normal distribution of the data in all the experimental groups allowed us to rule out the occurrence of bias in estimating genistein effects.

### 3.2. Effects on muscle damage and serum biomarker

Pathological consequences of the absence of dystrophin are the recurrent cycles of necrosis and regeneration. The imbalance between muscle damage and exhaustible regeneration leads to an abnormal muscle architecture. Wild type animals showed a normal architecture of the biceps muscles which was not modified by treatments.

Quantitative morphological evaluation of biceps muscle from genistein-treated (genistein 1 and 2) *mdx* mice showed a significant decrease in necrotic area ( $p < 0.01$ ) and an increase in regenerating fiber area ( $p < 0.05$ ) compared with vehicle-treated *mdx* mice (Fig. 2B, C, F and G). Similarly, methylprednisolone-treated biceps of *mdx* mice revealed a decrease in necrotic area ( $p < 0.05$ ) and a trend towards an increase in regenerating area (Fig. 2D, F and G). Hematoxylin and eosin staining did not reveal significant



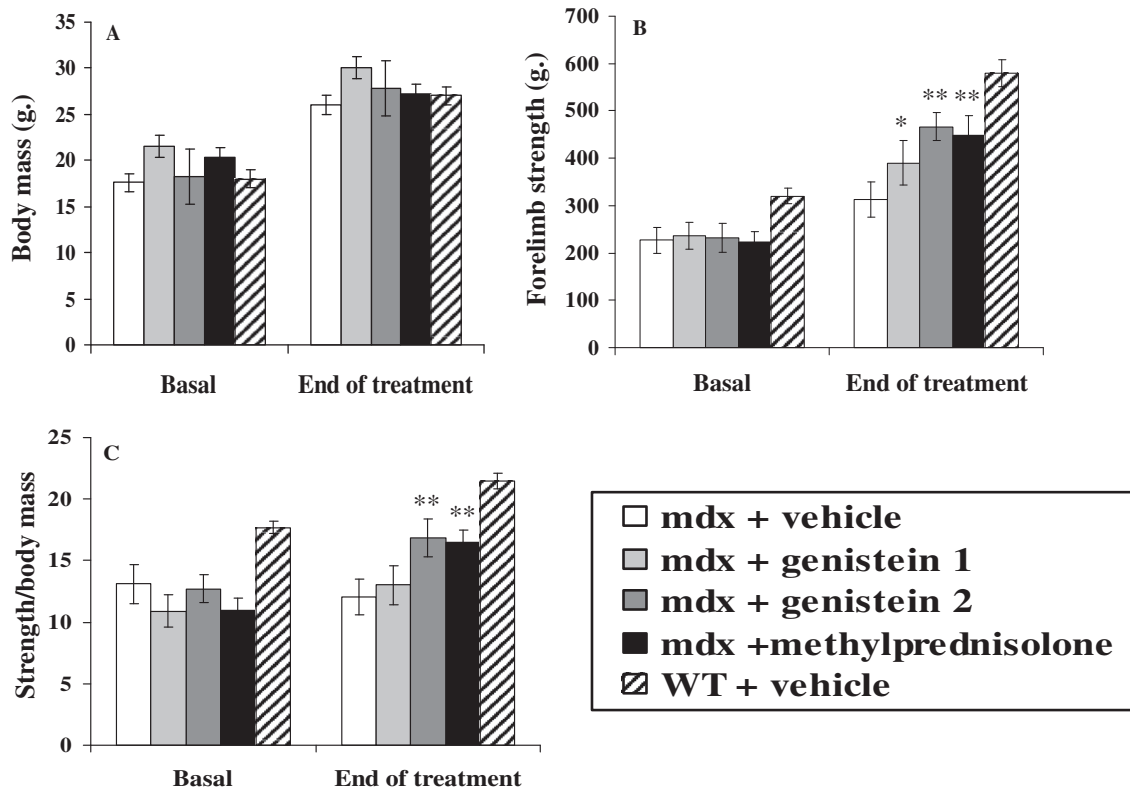


Fig. 1. Body mass (A), forelimb strength (B), forelimb strength normalized to body mass (C) in *mdx* mice treated with genistein 1, genistein 2, methylprednisolone and vehicle and in WT treated with vehicle.  $n = 8$  in each group. \* $p < 0.05$ , \*\* $p < 0.01$  vs vehicle-treated *mdx* mice. For other statistical comparisons see text.

differences in muscle architecture between treated and untreated WT mice. To better detail the effect of treatments on the different phases of muscle regeneration, we tested the expression of early and late markers as myogenin and dMHC. Immunohistochemical analysis revealed a significant increase in the number of myogenin-positive and nuclei per  $\text{mm}^2$  in genistein-treated muscle, compared to vehicle-treated *mdx* mice (genistein 1,  $p < 0.05$ , genistein 2,  $p < 0.01$ ) (Fig. 3A and C). Although there was a positive trend, genistein treatment did not significantly modify the number of dMHC fibers per  $\text{mm}^2$  (Fig. 3B and D). Methylprednisolone treatment did not modify these parameters. As expected, WT animals showed a very low number of myogenin-positive nuclei and of dMHC-positive fibers per  $\text{mm}^2$  that was not modified by treatments ( $< 0.2 \text{ mm}^{-2}$ ).

As biomarker of whole animal muscle damage, serum CK measurements were performed on *mdx* and WT mice. *Mdx* mice showed a significant increase in serum CK levels (*mdx* + vehicle =  $4100 \pm 79 \text{ U/L}$ ;  $p < 0.01$  vs WT + vehicle =  $220 \pm 33 \text{ U/L}$ ). Treatment with genistein (either scheme 1 or 2) or methylprednisolone resulted in a noticeable reduction of the serum enzyme levels (*mdx* + genistein 1 =  $2500 \pm 173 \text{ U/L}$ ; *mdx* + genistein 2 =  $2100 \pm 123 \text{ U/L}$ ; *mdx* + methylprednisolone =  $2090 \pm 80 \text{ U/L}$ ;  $p < 0.01$  vs *mdx* + vehicle) (Fig. 4). WT animals were used as control and the measurement of CK levels did not show significant difference between untreated and treated WT mice.

### 3.3. Analysis of molecular pathways and signaling modulated by genistein treatment

To define the molecular mechanisms underlying the reduced muscle necrosis, increased regeneration and improved functional parameters after genistein treatment, we monitored the activity and expression of factors potentially involved at different levels in DMD pathogenesis and modulated by genistein.

NF- $\kappa$ B DNA binding activity revealed by EMSA was markedly increased in *mdx* mice administered with vehicle compared to WT mice (*mdx* + vehicle =  $82 \pm 17 \text{ AU}$ ;  $p < 0.01$  vs WT + vehicle =  $2 \pm 0.2$ ). Treatment did not induce any changes in WT mice. Treatment with genistein and methylprednisolone led to a reduction of NF- $\kappa$ B binding activity in dystrophic mice ( $p < 0.05$ ), even more evident with daily administration ( $p < 0.01$ , Fig. 5).

$\text{H}_2\text{O}_2$  content and total peroxidase activity were unchanged in WT animals after genistein 1, genistein 2 or methylprednisolone treatment compared with vehicle-treated mice. *Mdx* mice showed markers of oxidative stress damage when compared to WT animals, characterized by a significant increase in the  $\text{H}_2\text{O}_2$  content (*mdx* + vehicle =  $100 \pm 0.2$  vs WT + vehicle =  $25 \pm 3 \mu\text{M}/\text{mL}/\text{mg}$  tot prot;  $p < 0.01$ ), accompanied by a concomitant decrease in activity of total peroxidase (*mdx* + vehicle =  $17 \pm 0.2$  vs WT + vehicle =  $42 \pm 3 \mu\text{M}/\text{min}/\text{mg}$  tot prot;  $p < 0.01$ ). Genistein administration in *mdx* mice resulted in a reduc-

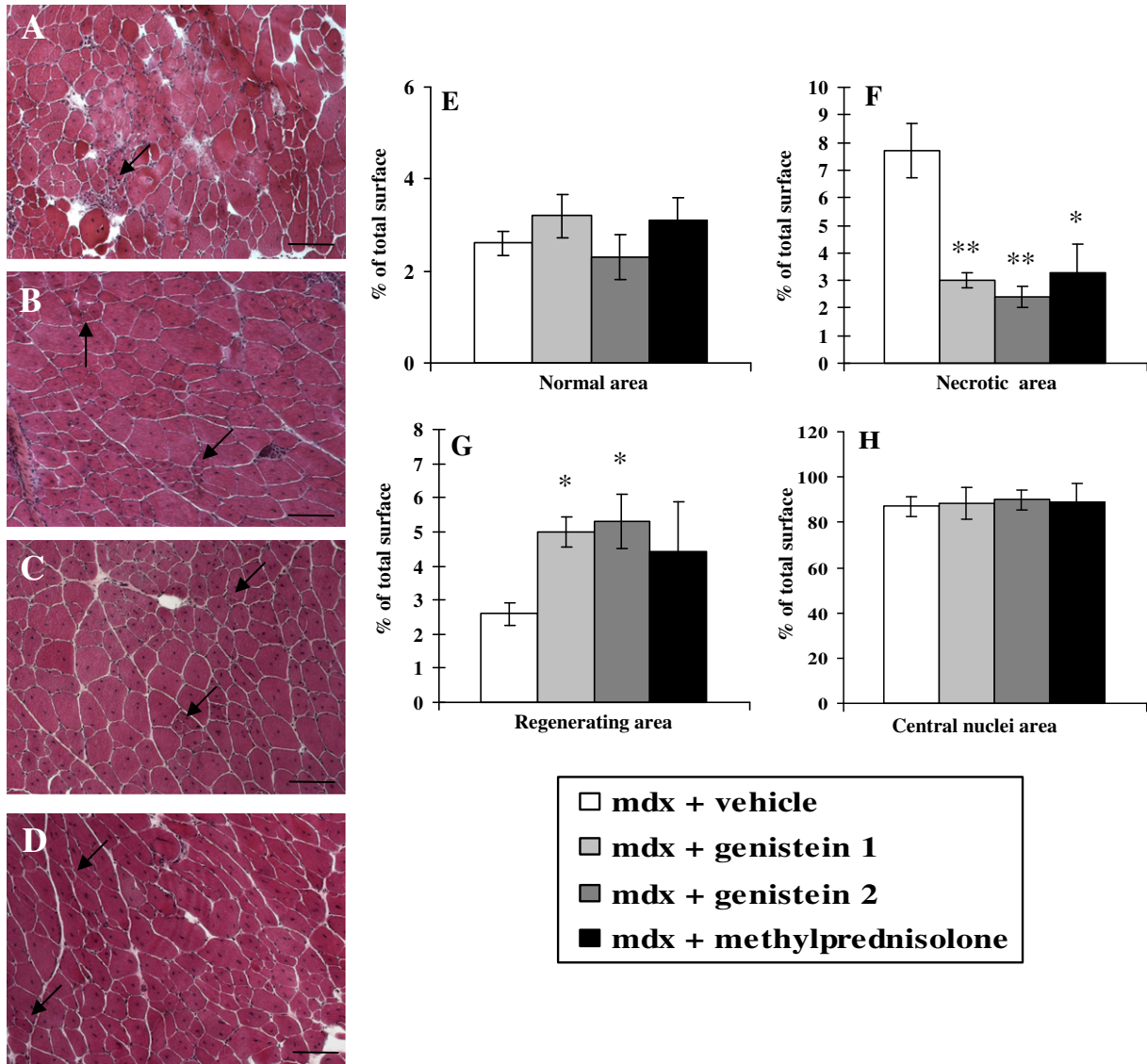


Fig. 2. On the left: histological appearance of biceps muscles in (A) vehicle-, (B) genistein 1-, (C) genistein 2- and (D) methylprednisolone-treated *mdx* mice. Muscle biopsies from vehicle-treated *mdx* mice showed necrotic features with inflammation (A, arrow), specimens from genistein 1-, genistein 2- and methylprednisolone-treated *mdx* mice showed restored muscle morphology with regenerating fibers (B–D, arrow). Hematoxylin–eosin staining. Scale bar 100  $\mu$ m. On the right: surface area of biceps muscles occupied by normal area (E), necrotic area (F), regenerating area (G) and centrally nucleated fibers area (H) in the different animal groups.  $n = 8$  animals in each group. \* $p < 0.05$  and \*\* $p < 0.01$  vs vehicle-treated *mdx* mice.

tion of  $H_2O_2$  level ( $p < 0.01$ ) and an increase in the total peroxidase activity (genistein 1  $p < 0.05$ , genistein 2  $p < 0.01$ ) (Fig. 6).

As a consequence of the increased NF- $\kappa$ B DNA binding activity and  $H_2O_2$  content and total peroxidase activity a significant increase of TNF- $\alpha$  and p-JNK expression in *mdx* compared to WT animals was detected (respectively, *mdx* + vehicle =  $7.9 \pm 0.9$  vs WT + vehicle =  $1.5 \pm 0.1$ ;  $p < 0.01$  and *mdx* + vehicle =  $6.9 \pm 0.9$  vs WT + vehicle =  $1.8 \pm 0.1$ ;  $p < 0.01$ ). In WT mice the different treatments did not modify TNF- $\alpha$  or p-JNK expression. By contrast, genistein-treated and methylprednisolone-treated *mdx* mice showed a significantly reduced expression of these inflammatory molecules when compared to vehicle-treated *mdx* mice ( $p < 0.01$ ) (Fig. 7A and B).

#### 4. Discussion

We confirmed in dystrophic muscle previous evidence, mainly reported *in vitro*, that genistein is able reduce oxidative stress and to inhibit proinflammatory pathways such as NF- $\kappa$ B, TNF- $\alpha$ , MAPKs. Moreover, we demonstrated the novel hypothesis that genistein effects ameliorate the *mdx* mice functional and morphological pattern over the 5-week treatment period. In our study, genistein-treated *mdx* mice showed *in vivo* higher strength and strength normalized to body mass than vehicle-treated animals. At morphological level, genistein-treated *mdx* mice showed a significant decrease of the necrotic area and an increase in the area occupied by regenerating fibers, being both effects more evident with the daily regimen.

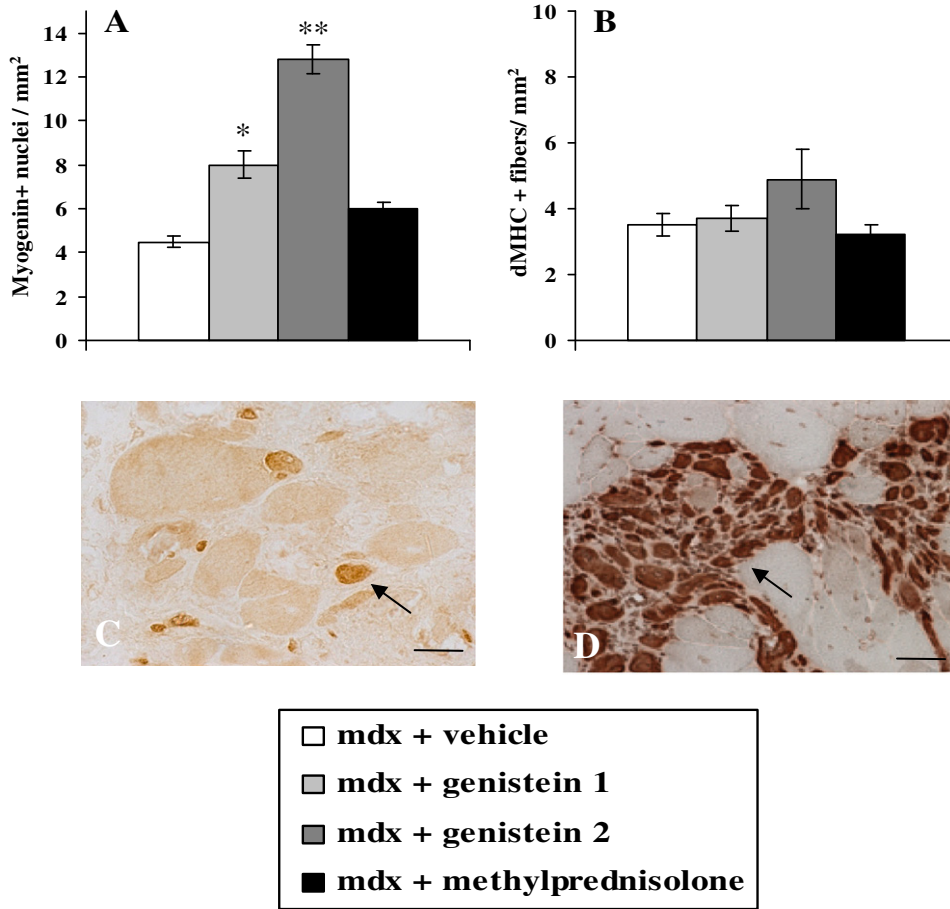


Fig. 3. In the upper part, numbers of myogenin-positive nuclei (A) and dMHC-positive fibers (B) per mm<sup>2</sup> in biceps muscles of different animal groups. In the lower part, representative images of myogenin-immunolocalization (nuclei in C; see arrows) and dMHC-positive fibers (D, arrow) in genistein-treated *mdx* mice (*n* = 8 in each group). \**p* < 0.05 vs vehicle-treated *mdx* mice; scale bar 100 μm.

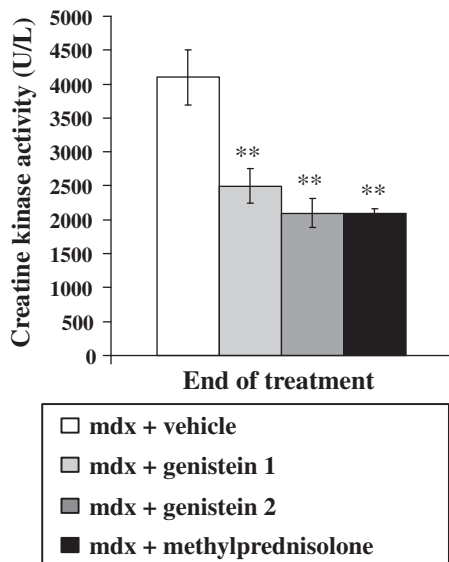


Fig. 4. Effects of genistein 1, genistein 2, methylprednisolone and vehicle treatment on serum CK levels in *mdx* mice. *n* = 8 in each group. \*\**p* < 0.01 vs vehicle-treated *mdx* mice.

In previous studies, we obtained beneficial effects on functional, biochemical and morphological parameters in

*mdx* mice by pharmacological inhibition of the pathological cascade involving oxidative stress, NF-κB, and other inflammatory mediators [8–10].

Increased oxidative stress is considered one of the causes contributing to dystrophic muscle damage. Genistein can act as free radical scavenger and can stimulate several antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and reductase as showed in different cell types [34–36]. We herein confirmed an increase in markers of oxidative stress in *mdx* mice muscle and demonstrated for the first time that genistein treatment increased the peroxidase activity in a dose-dependent manner resulting in a reduction of H<sub>2</sub>O<sub>2</sub> content in dystrophic muscle.

Recently, other studies strengthen our findings showing that NF-κB inhibition, through IKK-β depletion [20] or ablation of 1 allele of the p65 NF-κB subunit [21] improved muscle function and morphology in *mdx* mice and after muscle damage. Furthermore, specific pharmacological inhibition of IKK resulted in amelioration of muscle function and pathology in *mdx* mice [21]. The potent pro-inflammatory mediator TNF-α is an effective NF-κB inducers through a positive feedback loop. We postulated that this feedback perpetuates the detrimental effects of the NF-κB/TNF-α signaling pathways activation in the

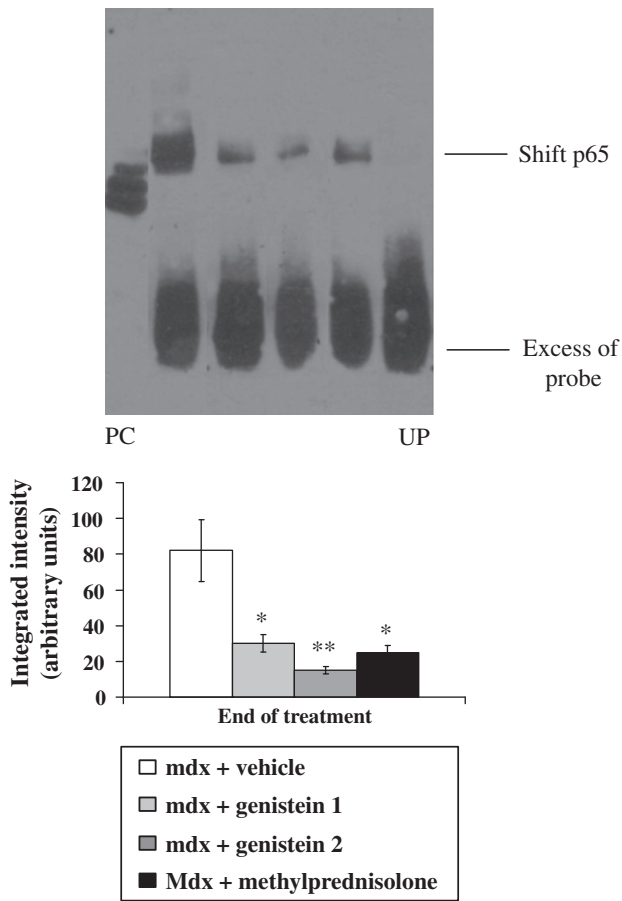


Fig. 5. Electrophoretic mobility shift assay of muscular NF- $\kappa$ B binding activity. In the upper part, representative autoradiograms; in the lower part, graphs with quantitative data.  $n = 8$  in each group. \* $p < 0.05$  and \*\* $p < 0.01$  vs vehicle-treated *mdx* mice. Positive quality control (PC): shift of Epstein Barr virus nuclear antigen-1 protein extract control reacting with specific labeled oligonucleotides; UP: unlabeled probe.

context of the dystrophic process [8–10]. Several lines of evidence suggested an involvement of TNF- $\alpha$  in DMD pathogenesis, showing beneficial effects in *mdx* mice [8–10,12,44]. Genistein has been shown in previous studies to inhibit NF- $\kappa$ B/TNF- $\alpha$  signaling pathways through ER-dependent and -independent mechanisms [33–36]. In our experiments we confirmed the inhibitory effects of genistein on NF- $\kappa$ B/TNF- $\alpha$  signaling pathways. We hypothesize that genistein could act with multiple mechanisms: (a) through a well-known scavenger effect and therefore a subsequent reduction of ROS-mediated NF- $\kappa$ B/TNF- $\alpha$  signaling activation [14]; (b) through ER binding, as ER are well represented in mouse skeletal muscle and shown to be up regulated after genistein exposure [34,45]; (c) through a demonstrated *in vivo* reciprocal antagonism between ER and NF- $\kappa$ B activity [30].

Oxidative stress has been shown to activate some components of the MAPK cascades in *mdx* muscle [15,18] and this might contribute to the disease progression [10,17–18]. The JNK effector kinase, associated with the DGC, has been reported to be constitutively activated in

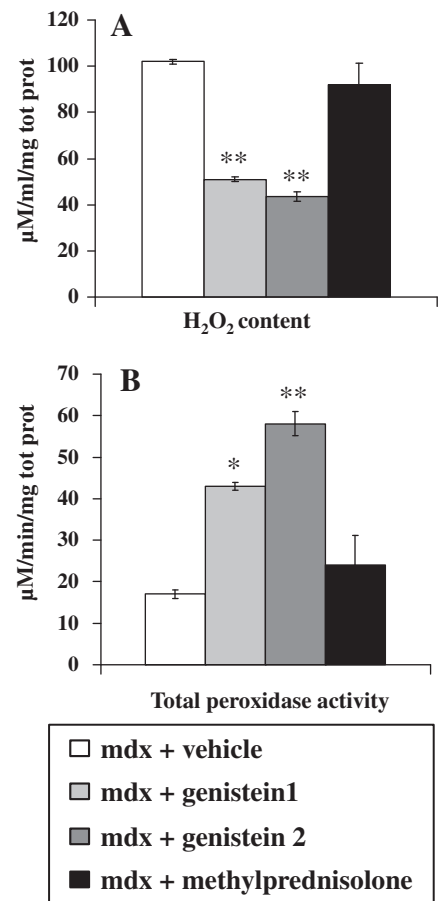


Fig. 6. Levels of muscular H<sub>2</sub>O<sub>2</sub> (A) and total peroxidase activity (B).  $n = 8$  in each group. \* $p < 0.05$ , \*\* $p < 0.01$  vs vehicle-treated *mdx* mice.

dystrophic muscle [17]. A striking increase in phosphorylation of JNK has been reported in the diaphragm muscles, whereas there was little increase in the limb muscles of 12-month-old *mdx* mice suggesting a correlation with the functional overload and the severity of morphological involvement [17]. Moreover, activation of JNK1 *in vitro* resulted in abnormal myotube viability similar to a dystrophic phenotype and JNK1 inhibitory protein injection in *mdx* muscle dramatically attenuated muscle damage [17]. Furthermore, the modulation of some MAPK cascade components has been postulated to contribute to an amelioration of the morphological pattern in *mdx* mice after free radical scavengers administration [10,12,18]. Genistein has been shown to inhibit MAPKs pathways *in vitro* through its antioxidant effects and also through an ER-dependent mechanism [32,36]. Indeed, the reduced phospho-JNK expression observed after genistein treatment in our study may have contributed to the observed beneficial effects.

Corticosteroids are the only drugs at present with positive effects on DMD natural history, but with possible relevant side effects. Beneficial effects of corticosteroids on muscle functional properties and morphology in *mdx* mice have been previously demonstrated, even if these findings are still debated [22]. Interestingly corticosteroids are



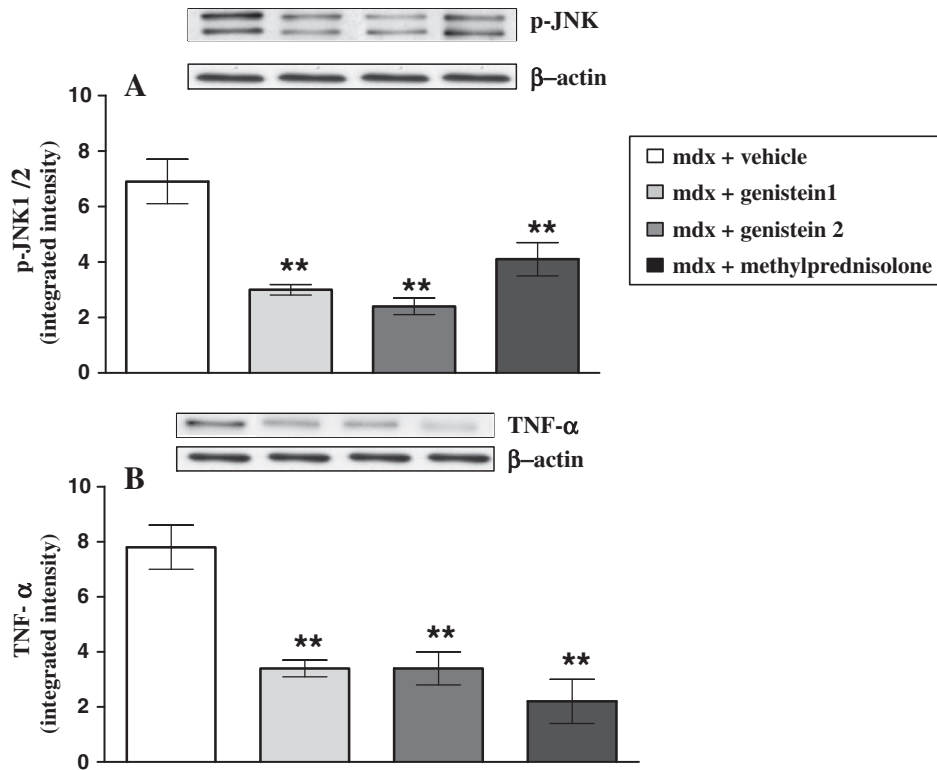


Fig. 7. Western blot analysis of phospho-JNK (A) and TNF- $\alpha$  (B) at the end of the experiment: quantitative data and representative autoradiograms (the upper represents the target protein and the lower the  $\beta$ -actin control protein). Bars (integrated intensity values) represent the mean  $\pm$  SD for each group.  $n = 8$  in each group; \*\* $p < 0.01$  vs vehicle-treated *mdx* mice.

known to inhibit NF- $\kappa$ B, MAPK and TNF- $\alpha$  [46]. In our study, we confirmed our previous findings that corticosteroids inhibit these inflammatory and oxidative stress-induced responses also in this experimental model [10]. Genistein and methylprednisolone showed similar efficacy in inhibiting MAPK pathways, whereas NF- $\kappa$ B activity was blunted more efficiently by genistein at daily regimen, probably due to its additional antioxidant effect. Genistein was also more effective than methylprednisolone in reducing muscle necrosis and in enhancing muscle regeneration, increasing the number of myogenin positive regenerating fibers. This pro-regenerative effect could be caused not only by NF- $\kappa$ B inhibition, but also by the reported effect of genistein on cell cycle in promoting cell proliferation in different cell type including muscle cells [47–49].

Herein we reported data supporting the novel hypothesis that genistein with its multiple effects can be beneficial in dystrophic process. As other more specific therapeutic approaches are still under development, this soy-derived compound, with a high safety profile in humans [38,39,50,51], could be a valuable option to be further investigated. Further experiments might better clarify genistein mechanisms of action such as the ER-dependent mechanisms, using specific ER inhibitors, and the effects on cell-cycle. These studies might strengthen our results and help to predict potential outcome in patients. However our data should be considered with caution as several promising therapeutic approaches failed when translated

to humans, the efficacy of this compound can be only proven by randomized double-blind placebo-controlled clinical trials. Still, this work represents a step forward in understanding how antioxidant and anti-inflammatory molecules act on the pathways involved in DMD pathogenesis.

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