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The therapeutic effect of myostatin oral vaccine in Duchenne muscular dystrophy mice model

듀센 근디스트로피 동물모델에서의 마이오스타틴 경구용 백신의 치료 효과

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The Department of Brain and Neuroscience

Graduate School of Seoul National University

Jeehun Lee

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지도 교수 황 용 승

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The therapeutic effect of myostatin oral vaccine in Duchenne muscular dystrophy mice model

By

Jeehun Lee, M.D.

Directed by Prof. Yong Seung Hwang

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Doctoral Committee:

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Professor _____

Professor _____

Professor _____

Abstract

***Introduction*:** Duchenne muscular dystrophy is the most common muscular dystrophy inherited in an X-linked manner. There is no effective therapy to cure the causative patho-mechanism. As an alternative therapy, the blockade of myostatin with monoclonal antibody was reported to improve the dystrophic features in animal models, but failed to prove efficacy in human adults with muscular dystrophy. Recently, myostatin oral vaccine which presents the myostatin antigenic moiety linked to *Lactobacillus casei* was developed. This vaccine is expected to effectively inhibit myostatin and is anticipated to be used for the treatment of muscular dystrophy.

Objectives: The author hypothesized that myostatin oral vaccine can produce circulatory antibodies and can reduce the level of myostatin, which in turn can bring about the functional and histologic improvements of dystrophic features in the mouse model of Duchenne muscular dystrophy (mdx mouse).

Materials and Methods: Six-week-old male mdx mice (C57BL/10ScSn-Dmd$^{mdx/J}$) were randomized into control or treated groups. Mdx mice of the control and treated groups began to be fed with the feed containing 3% of *Lactobacillus casei* with

pgsA (for the control group) or *Lactobacillus casei* with pgsA-myostatin (for the treated group). Serial measurement of body weight and serum creatine kinase was done at two weeks' intervals. At end point, the Rota-rod test was executed. After scarification, measurements of individual muscle weight, serum creatine kinase, serum anti-myostatin IgG antibodies were done. Histologic analysis was done with the gastrocnemius and soleus muscles.

Results: A significant difference was observed in the change of body weight between the control and treated mdx groups (P=0.001). Serum creatine kinase of the treated mdx mice decreased to about 14% of the control group (P=0.001). Serum anti-myostatin antibody titer increased about 5.5 fold more than that of the control group (P=0.001). In the Rota-rod test, the treated mdx group showed 5.8 fold longer duration than the control group at 20 rpm (P=0.003). Histology revealed decreased size variation and fibrosis with remarkable hypertrophy of myocytes. There were no remarkable adverse effects associated with myostatin oral vaccine.

Conclusions: Myostatin oral vaccine which was made via the presentation of myostatin antigenic moiety conjoined to the pgsA vector to the surface of *Lactobacillus casei* successfully blocked myostatin via producing circulatory antibodies in mdx mice. The myostatin blockade resulted in the functional and

histologic improvements of dystrophic features in mdx mice.

Keywords: Duchenne muscular dystrophy, mdx mouse, myostatin, vaccine

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Introduction

Duchenne muscular dystrophy (DMD; MIM 310200) and Becker muscular dystrophy (BMD; MIM 300376) are allelic X-linked recessive disorders caused by a mutation of the DMD gene located at Xp21[1)]. DMD is the most common muscular dystrophy affecting 1 in 3,500 live born males in which approximately a third of cases occur as de novo mutations [1)]. In most cases, the disease is diagnosed on the basis of gait abnormalities at 4–5 years of age, or incidentally found elevations of hepatic enzymes in earlier ages. By 8–10 years of age, deterioration of the patient's condition necessitates wheelchair use. In their early teens, untreated patients are usually wheelchair bound and, in some cases, neurological and cardiac symptoms are apparent. With the progression of muscle degeneration and worsening clinical symptoms, patients usually die in the late teens or early twenties as a result of respiratory or cardiac failure [2)].

The life expectancy of DMD patients has increased with the development of palliative therapy. However, no curative treatment has been developed so far. At present, only oral prednisone is considered as beneficial treatment extending independent ambulation and decreasing scoliosis[3)]. The possible mechanism of

action of prednisolone involves limiting the inflammatory response associated with muscle cell death[4].

Many therapeutic approaches have been tried to improve the symptoms of muscular dystrophy. Recent therapeutic trials can be divided into 1) repair or replacement of the mutated gene, 2) rescue translation, 3) substitution of other proteins, and 4) amelioration of downstream pathological mechanisms [5].

To repair or replace the mutated gene, two main approaches are used; gene supplementation by viral vector delivery and cell therapies. These methods have shown some improvement in animal studies. However, there are many obstacles to overcome such as the dosage of transferred gene, problems of cell-mediated immune response, or limitation of local spread [3].

Recently, rescue translations such as read-through of stop codon mutations or skipping mutation have shown remarkable development. Nonsense mutations are responsible for about 15% of DMD cases and premature translation termination by the ribosome before a functional protein is generated. Barton-Davis et al. demonstrated the in vivo pharmacological correction of premature-stop codon mutations with aminoglycoside antibiotics[6].

PTC124 (Ataluren, PTC therapeutics, South Plainfield, NJ, USA) is a drug

containing 284 Da, 1,2,4-oxadiazole linked fluorobenzene and benzoic rings designed from a high throughput screen with a function to read-through premature-stop codon mutations as it continues translating the mRNA but respecting the normal termination codon thereby allowing the formation of a functional protein[7, 8]. There was no statistical significance within the 48 week duration of a double-blinded, placebo-controlled trial. However, such studies have contributed to the development and establishment of important safety and efficacy outcomes in DMD patients[9].

Exon skipping provides an alternative, post-transcriptional approach that allows direct manipulation of the dystrophin protein. Antisense oligonucleotides (AONs) are chemically synthesized as single-strand DNAs, designed to first hybridize with the complementary sequence in the messenger RNA, then alter RNA processing by excluding one or more exons from the messenger RNA and finally bring the genetic code back into the frame to restore a partially functional dystrophin protein[9-11]. AON based treatments have been rapidly emerging as the frontline therapeutic approach in DMD. However, the method imposes several problems; optimization of systemic AON delivery, inability to target the heart in the mdx mouse at doses corresponding to those suggested for clinical applications, and

feasibility of AON therapy in a rare condition where mutation-specific oligonucleotides would need to be used[9, 12-14].

Muscles have an inherent ability to regenerate via resident progenitor stem cells. Regeneration process in many muscle diseases is incomplete and results in remodeling of muscles with fibrosis and fatty infiltration. Stimulating growth and regeneration may provide functional benefits by increasing the size and strength of minimally affected muscles or by improving the quality of the composition of muscles[15]. IGF-1 promotes regeneration by stimulating proliferation and differentiation of muscle progenitor cells[16, 17]. Mdx mice expressing an IGF-1 transgene (mdx/mIGF$^{+/+}$) have shown to increase muscle mass, force, and reduced fibrosis compared with their mdx controls[18-20].

Myostatin is a member of the TGF-β superfamily and an endogenous negative regulator of muscle growth[21]. Loss of function mutations in myostatin result in massive muscle hypertrophy in a variety of species, including humans. Mdx mice lacking myostatin have increased muscle mass, grip strength, and improved muscle histological features with decreased fibrosis[22]. Myostatin blockade can be achieved by a variety of mechanisms. Inhibition of myostatin with a neutralizing monoclonal antibody and with a modified myostatin pro-peptide,

delivered by an adenovirus vector, improved the dystrophic features of mdx mice[23, 24]. The phase I/II trial of a myostatin inhibitor in adult muscular dystrophy patients demonstrated safety without significant adverse effects of a neutralizing antibody to myostatin, MYO-029. Improvement of muscle strength was not proved in the trial, but the increased muscle mass and fiber diameters could be the evidence of biological activity of MYO-029[15].

The delivery of antigens by safe, non-invasive vectors, such as commensal lactobacilli, was developed initially for enhancing mucosal immunity. A study established displaying of antigens on lactic acid bacteria via a novel expression vector using the pgsA gene product as an anchoring matrix[25, 26]. PgsA is a synthetase complex of *Bacillus subtilis*, as a fusion partner for the expression of heterologous antigens on the surface of *Lactobacillus casei*. The methods have been applied to severe acute respiratory syndrome[27] and porcine epidemic diarrhea[28]. The method was also applied to induce antitumor effects in the human papillomavirus type 16 E7-expressiong cancer cells[29]. Recently, the oral vaccine against human myostatin carrying the myostatin protein displayed on the surface of the *Lactobacillus casei* was developed. This vaccine can produce circulatory antibodies against myostatin and may improve dystrophic features.

The objectives of this study are 1) to prove the serologic evidence of myostatin antibody, and 2) to delineate the functional and histologic improvements in the mdx mouse, the animal model of DMD, using myostatin oral vaccine.

Materials and Methods

Myostatin oral vaccine

Myostatin oral vaccine was provided by the Bioleaders (Dae-Jeon, Republic of Korea). The company has the patent of 'Surface display motif (PgsA), promoter, and expression vector' and 'Cell surface expression vector of myostatin and microorganisms transformed thereby'. The production of myostatin oral vaccine and the confirmation of myostatin protein expression on the surface of *Lactobacillus casei* were executed by the Bioleaders. The methods of 'modification and amplification of myostatin', 'cloning of the modified myostatin genes', and 'Confirmation of myostatin protein expression on the surface of *Lactobacillus casei* (by western blot analysis)' were cited from the thesis for degree of doctor of philosophy written by Long-Chun Xu[30].

1. **Modification and amplification of myostatin[30]**

Myostatin oral vaccine was produced by the Bioleaders (Dae-Jeon, Republic of Korea). The manufacturing processes were as follows. In order to express the

myostatin mature segment gene in *Lactobacillus casei*, the gene was first modified by adding BamHI and LpnI at the 5' and 3' terminals, respectively. Polymerase chain reaction (PCR) was employed to amplify the gene using the forward primer 5'-CG*G GAT CC*G AGG TCA GAG TTA CAG AC-3' and reverse primer 5'-GC*T CTA GAG GTA CC*T TAT TAT CAT GAG CAC CCG CAA CG-3'. PCR was performed in 100ul volume containing 20ng template, 20nmoles dNTP, 50pmoles of forward and reverse primers, and 5U EX Tag polymerase (Takara, Korea). Thermocycling conditions consisted of 35 cycles of 50 second denaturation (94℃), 50 second annealing (55℃), and 50 second extension (72℃) with the final extension at 72℃ for 7 minutes. The reaction was performed in the thermocycler (CoreBio 96 plus, CoreBio L&B, Korea).

2. **Cloning of the modified myostatin genes**[30)]

Bacterial strains, cloning, and construction of surface display plasmids, *Escherichia coli* JM83 and *Lactobacillus casei,* were used. Plasmid pPSG1, harboring the pgsBCA genes (GenBank accession no. AB016245) of *Bacillus*

subtilis, was used to construct a minimal surface expression vector.

To generate the surface display vector pHAT: pgsA, which contains the pgsA gene under the control of high constitutive expression and promoter, the pgsA gene was amplified from pPSG1 using the primers 5'-CAT ATG AAA AAA GAA CTG AGC-3' and 5'-GGA TCC AGA TTT TAG TTT GTC-3'. The amplified DNA fragment was cloned into pHCEIIB (TaKaRa) to generate pHCEIIB-psgA. Subsequently, using the fragment containing the HCE promoter, pgsA gene, multicloning site was excised from pHCEIIB-pgsA and cloned into pAT19 to generate pHAT: pgsA. To generate constructs containing the myostatin protein gene, the myostatin active domain was amplified from pCR®T7/NT-TOPO®: myostatin using primer pairs 5'-CG*G GAT CC*G AGG TCA GAG TTA CAG AC-3' and 5'-GC*T CTA GAG GTA CC*T TAT TAT CAT GAG CAC CCG CAA CG-3', respectively. The PCR-amplified fragments were inserted into the *Bam*HI/K*pn*I-digested pHAT: pgsA to yield pHAT: pgsA-AMm (myostatin active domain). The construct was designed such that the expressed protein would have the AMm fused at the carboxy-terminal of the pgsA anchor protein.

3. **Confirmation of myostatin protein expression on the surface of**

***Lactobacillus casei* (by western blot analysis)[30)]**

To confirm the expression of the myostatin gene, immunoblotting was performed following the method described by Laemmli[31)]. Recombinant *Lactobacillus casei* cells were cultured in MRS broth (Difco) overnight at 30℃, and then cell fractionation and protein extraction were performed. Protein samples were mixed with SDS-PAGE sample buffer (62.5mM Tris-Cl [pH 6.8], 10% glycerol, 2% sodium-dodecyl-sulfate [SDS], 5% 2-mercaptoethanol, 0.05% bromophenol blue and heated to 100℃ for 5min. The denatured protein samples were loaded onto SDS-polyacrylamide gel (8%) and electrophoresed. The proteins separated by SDS-PAGE were electrophoretically transferred to a polyvinylidine difluoride (PVDF) membrane (Roche, Germany). After transfer, the membrane was immersed in blocking buffer (50mM Tirs-Cl, 5% skimmed mild, pH8.0) with gentle shaking for 1 hour. For immunodetection of fusion proteins, rabbit anti-pgsA (1:1,000) or mouse anti-myostatin (1:500, R&D) monoclonal antibodies were used. After washing with washing buffer (20mM Tris-Cl, 150mM NaCl, 0.05% Tween 20), the antigen-antibody complex was visualized using avidin-biotin solution (Vector laboratories, Burlingame, CA), and substrate solution (containing 30mg

diaminobenzidine, 100 mM Tris-HCL, 4mM NaCl, pH 7.5), and 0.03% hydrogen peroxide (H_2O_2).

Animal model

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRI). The study number is C-B1-309-1. SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and abide by the Institute of Laboratory Animal Resources (ILAR) guide.

1. Mice maintenance

Male and female mdx mice (C57BL/10ScSn-Dmd$^{mdx/J}$) were purchased from the Jackson Laboratory (Maine, USA). After breeding, the male mdx mice underwent genotyping following the method recommended by the Jackson Laboratory.

2. Experimental groups

The male mdx mice were divided into two groups; control mdx mice group (control mdx group) and treated mdx mice group (treated mdx group). Each group contained 10 mice respectively.

3. Immunization

At the age of 6 weeks, mice of the control and treated groups began to be fed with the feed containing 3% of *Lactobacillus casei* with pgsA (for the control mdx group) or *Lactobacillus casei* with pgsA-myostatin (for the treated mdx group). The concentration of *Lactobacillus casei* was $1.2X10^9$ colony forming units per gram. The immunization continued for 10 weeks.

4. Collecting samples

4.1 Body weight

Serial body weight was measured at 2 weeks' intervals.

4.2 Serum creatine kinase

Serial serum creatine kinase was measured at 2 weeks' intervals with blood obtained from the tail vein. Serum creatine kinase was also measured from the whole blood obtained after the scarification at the end of the experiment. Serum creatine kinase was measured using the indirect CK colorimetric assay kit and

standards (Sigma, St. Louis, MO, USA).

4.3 **Scarification and sample collection**

After the 10 week immunization, the mdx mice (the control mdx group and the treated mdx group) were sacrificed under general anesthesia with ketamine (0.1-0.5mg/kg via intra-peritoneal injection until full sedation was obtained). Whole blood was collected from the direct puncture of the left ventricle. Serum was separated after centrifugation at 3000rpm for 5 minutes. It was then stored in the -80℃ refrigerator. The gastrocnemius and soleus muscles were collected after perfusion with normal saline.

4.3 Tissue preparation and muscle morphology

The collected muscles were fixed in the formalin solution for at least 24 hours. After fixation, the weight of each muscle was measured and paraffin blocks were made. Serial sections (thickness of 4 μm) were cut and processed for hematoxylin and eosin (H&E) staining for histology. Pictures were taken using an Olympus Magnifire or Nikon Coolpix 950 Digital camera. Morphometric measurement and analysis were made on these digital images using Image J program (Wayne Rasband, National Institutes of Health, USA, http://imagej.nih.gov/ij).

4.4 Measurement of anti-myostatin antibody (IgG)

Myostatin (R&D) was diluted with 50mM carbonate buffer (Sigma, pH 9.0) into 1μg/ml. The diluted myostatin was infused into 96 well plates with the amount of 50μl and coated with gentle shaking overnight. After three times of washing, PBS with 1% BSA was added into each well with the amount of 200μl and incubated for 1 hour at room temperature. After three times of washing, 100μl of experimental serum was added to each well with the concentration of 10μl/ml and incubated for 1 hour at room temperature. After then, three times of washing with PBS containing 0.05% tween 20, 100μl of 1:1,000 diluted HRP conjugated anti-mouse antibody was added to each well. After 1 hour of incubation, of washing with PBS containing 0.05% tween 20 was done three times. After adding 50μl of TMB solution into each well, the samples were incubated until color appeared. Adding 50μl of stop solution, the samples were read under the microplate reader (BioRad) at OD450.

5. Rota-rod test

The Rota-rod apparatus (Samkwang, Korea) was used for the functional evaluation of the mice. Rotation speed was 20rpm. Each mouse had six training sessions per day for three days in the 9^{th} experimental week. At the

end of the experiment, the maximum period among the 3 times of the training was selected as the record.

6. Statistical analysis

Mann-Whitney U test and Kolmogorov-Smirnov test were used to determine statistical significance of results using PASW statistics 18 (Chicago, IL). The data are expressed as mean ± SEM.

Results

During the 10 weeks of immunization, there were no remarkable adverse effects associated with *Lactobacillus casei* with pgsA and *Lactobacillus casei* with pgsA-myostatin. The physical properties, functional studies, and histologic results are presented respectively.

The body weight and elementary muscle weight

A significant difference was noted in the change of body weight during 10 weeks of vaccination between the control and treated mdx groups (control group = 6.33 ± 1.50g, treated group = 10.25 ± 1.77 g, P= 0.001; Table 1, Figure 1). The weight of the gastrocnemius and soleus muscles were not significantly different between two groups (P=1.0, P=0.03, respectively; Table 1, Figure 2).

Serum creatine kinase

Serum creatine kinase at the end-point of the study was significantly different between the control mdx group and treated mdx group (P= 0.001, Table 1, Figure 3). However, the serial values of serum creatine kinase in the treated group were not significantly different between the groups.

Serum anti-myostatin antibody (IgG)

Serum anti-myostatin antibody titers at 10 weeks of experiment in each group were significantly different (P= 0.001, Table 1 and Figure 4). The titer of the treated mdx group was 5.5 fold more than that of the control mdx group.

Rota-rod test

After 3 days of the training program, the mdx mice were tried three sessions of the Rota-rod test at the end of the experiment before scarification. The maximum duration of stay was selected as the record.

The treated mdx group showed 5.8 fold longer duration than the control mdx group at 20 rpm (P= 0.003, Table 1 and Figure 5).

Histology

The typical histologic characteristics of muscular dystrophy such as size variation of muscle fibers, infiltration of inflammatory cells, and fibrosis were decreased in the treated mdx group (Figure 6). The cell surface area calculated with Image J program was significantly higher in treated mdx group than that of control mdx group (Table 2, P= 0.001) (Figure 7).

Discussion

The delivery of antigens by displaying it on the lactic acid bacteria via a novel expression vector, pgsA gene product, as an anchoring matrix was proved to be safe and effective in previous studies[27-29]. The delivery of myostatin antigen via *Lactobacillus casei*-pgsA was tried for the first time in this study. The vaccinated mdx mice showed a significant increase in body weight, remarkable reduction of serum creatine kinase, a prolonged stay in the Rota-rod test, and a decrease of dystrophic feature in histology. These improvements were well correlated with the titers of serum anti-myostatin antibody (IgG).

Myostatin is a member of the transforming growth factor-beta (TGF-β) family of signaling molecules and the blockade of it brings excessive growth and increased muscle power[21, 32]. In an animal study, inhibition of myostatin was reported to improve the histology and performance of mdx mice[23]. And genetic deletion of myostatin in mice resulted in excessive muscle growth[21]. Additionally,

myostatin knock-out mice revealed an increased body weight of approximately 30% during the young adult period which could be accounted by an increase in muscle mass. The increase of muscle mass was due to hyperplasia (mean fiber diameter of gastrocnemius was 22% larger in mutants than wild-type littermates) in fetal age as well as hypertrophy (the total cell number was 86% higher in mutant animals compared to wild type animals) after birth[21].

The function of myostatin was postulated via animal models of myostatin over-expression. The over-expressed mice revealed a massive decrease in total body weight (30%) within 16 days[33]. Double muscle cattle such as Belgian Blues and Piedmontese have been documented for at least 200 years[34]. Belgian blues have an 11-nucleotide deletion in the third exon and Piedmontese cattle contain a missense mutation in exon 3 inherited autosomal recessive inheritance presenting the heterozygote effect[35]. Therefore, the function of myostatin is postulated as inhibition of muscle hyperplasia and hypertrophy.

The human myostatin gene maps to chromosome 2q32.2 and in mouse to chromosome 1 27.8 cM[21, 36]. When myostatin binds to propeptide, it becomes ineffective (inactive form) and if the propeptide splits from myostatin, myostatin changes into an active form. There has been great interest in the myostatin-binding

proteins because the control of the binding protein can alter the function of myostatin. The known myostatin binding proteins are as follows; myostatin propeptide, activin receptor, metalloproteases, follistatin and follistatin related proteins[32].

The development of antibodies that specifically target and inhibit myostatin has greatly advanced the myostatin inhibitory therapies. Monoclonal antibodies against mouse myostatin increased muscle mass of up to 20% via hypertrophy of muscle cells. The inhibition of myostatin did not influence other organs such as heart, and serum parameters of liver, kidney and bone, and glucose metabolism was found to be normal[37].

The application of myostatin inhibition into muscle diseases was first done in 2002[22]. The researchers made a double knock-out mouse of the genes coding myostatin and dystrophin. The mice showed improved dystrophic features (enlarged mean muscle fiber diameter and decreased fibrosis) and also revealed residual features of muscular dystrophy such as degeneration and regeneration of muscle fibers, centrally located nuclei, and elevated serum creatine kinase.

Bogdanovich et al.[23] tried systemic injection of monoclonal antibodies into mdx mice on a weekly basis for a period of 3 months. Individual muscle mass

increased up to 35% and the increase of weight was associated with enlargement of muscle fibers. Despite the inhibition of myostatin, centrally located nuclei were observed, which indicated regeneration of muscle fibers. Additionally, serum creatine was significantly decreased. Using these results, Zammit and Partridge[38] hypothesized that inhibition of myostatin might improve stress resistance due to increased fiber size hence leading to less fiber degeneration.

The remarkable improvement of dystrophic features in mdx mice by myostatin blockade led to the application of monoclonal antibodies against myostatin to humans. Wagner et al.[15] executed a phase I/II trial of MYO-029 in adults with Becker muscular dystrophy, facio-scapulo-humeral muscular dystrophy, and limb-girdle muscular dystrophy. MYO-029, the monoclonal antibodies to human myostatin proved to have safety and tolerability. However, no improvements were noted in muscle strength or function. Although improvements were not shown, muscle mass was found to increase by about 2.4%.

Another approach was used to inhibit myostatin. Qiao et al.[24] tried myostatin propeptide gene delivery by an Adeno-associated virus. In *mdx* mice, a significant increase in muscle mass was observed after AAV-MPRO76AFc injection. The treated *mdx* mice showed larger and more uniform myofibers, fewer

infiltrating mononuclear cells, less fibrosis, and lower serum creatine kinase levels. Also, a grip force test and an *in vitro* tetanic contractile force test showed improved muscle strength.

Surface display of antigens on lactobacillus was developed with a novel expression vector using the pgsA gene product[27]. According to a previous study, recombinant fusion proteins comprised of pgsA and the Spike (S) protein segments SA (residues 2 to 114) and SB (residues 264 to 596) of severe acute respiratory coronavirus were stably expressed in *Lactobacillus casei* [27]. Surface localization of the fusion protein was verified by cellular fractionation analyses, immunofluorescence microscopy, and flow cytometry. Oral and nasal inoculations of recombinant *L. casei* into mice resulted in high levels of serum immunoglobulin G (IgG) and mucosal IgA, as demonstrated by enzyme-linked immune-sorbent assays using S protein peptides. More importantly, these antibodies exhibited potent neutralizing activities against severe acute respiratory syndrome pseudoviruses[27].

Using a similar method, *Lactobacillus casei* with pgsA-myostatin was developed by Bioleaders (Dae-Jeon, Republic of Korea). The author postulated that oral *Lactobacillus casei* with pgsA-myostatin could generate mucosal antibody IgA and circulatory antibody IgG, based on a previous study with severe acute

respiratory coronavirus expressed in *Lactobacillus casei*. Also, the resultant circulatory antibodies were expected to inhibit myostatin, which might improve dystrophic features in mdx mice.

In this study, oral administration of *Lactobacillus casei* with pgsA-myostatin for 10 weeks brought about significant increase of body weight and prolonged stay on the Rota-rod test.

The serial body weight in the treated group showed continuous increase in comparison with the control group. This result is similar to a previous study using monoclonal antibodies or myostatin-null mice[22, 23]. To determine that the increased muscle mass could explain the increase of body weight; the researchers compared the total body weight to carcass weight, and found that the difference in carcass weights between the wild-type and myostatin-null mice was comparable to the difference in total body weights[21].

In this study, the serum CK was decreased to the level of 14.3% of control mdx mice. Elevated creatine kinase concentrations were consistently noted in mdx mice and DMD patients owing to sarcolemmal damage[23]. According to a previous study, the muscles of mdx mice treated with the monoclonal antibody showed improvements in physical performance and force generation, and resulted in a

decrease of serum CK to near wild-type levels[23]. The researchers interpreted that inhibition of myostatin had rendered most dystrophin-deficient muscles resistant to necrosis during normal daily activity[23, 38].

The presence of anti-myostatin antibodies can be a definite evidence of myostatin blockade. In this study, the level of anti-myostatin antibodies was significantly increased in the treated group compared with the control group. The mean increase was about 5.5 folds. Additionally, the antibody titer was well correlated with the decrease of serum CK. The delivery of myostatin antigen via *Lactobacillus casei*-pgsA was proved to be effective with this result. Generally, this type of oral vaccination first induces mucosal immunity. The extracellularly accessible antigens expressed on the surface of bacteria are better recognized by the immune system than those that are intracellular[39]. The production of circulatory IgG antibody as well as mucosal IgA antibody was proved in a previous study with a similar design[27, 40].

The typical dystrophic features of mdx mice were size variation of myocytes, centrally located nuclei, infiltration of inflammatory cells, and diffuse fibrosis. In the treated group, these dystrophic features were improved; more homogenous distribution of muscle cellular size, and a decrease of fibrosis and

inflammation were noted. However, the centrally located nuclei, indicating regeneration of myofibers, still existed. The most striking finding was hypertrophy of myocytes in the treated group. The cell surface area was significantly increased in the treated group compared with the control mdx mice (P=0.001). The hypertrophy of myocytes is a typical finding and the major mechanism of myostatin blockade in the postnatal period[21, 22]. This was also demonstrated in a study with monoclonal antibodies against myostatin[23].

The underlying cause for the improvement of muscle pathology and gross function by inhibition of myostatin has been well explained. The primary effect of myostatin inhibition is hyperplasia and hypertrophy of myocytes. These may be the answer for weight gain and increase of muscle power, but it may not be a proper explanation in cases with DMD. Dystrophin functions as a mechanical linkage to transmit force across the surface membrane of the fiber between the internal contractile apparatus and the overlying basement membrane[41]. The hypertrophic muscle fibers are resistant to degeneration in anti-myostatin treated mdx mice contravenes the observation that small fibers survive better than large fibers in dystrophin-deficient muscles [38, 42]. According to a previous study, blocking of myostatin may permit hypertrophic signals to drive muscle growth above the point

where it is compromised by normal workloads, resulting in shifting of dystrophic muscles above the damaging levels of stress[38]. A similar phenomenon has been shown in the positive effect of IGF-1-induced hypertrophy in mdx mice[18].

The measurement of serum myostatin level can be a direct evidence of the action of myostatin oral vaccine. There have only been few studies which measured myostatin level, because myostatin exists at a very low level in mice and even lower in humans at 8.0±0.3ng/ml[43]. According to these studies, measurement of myostatin was possible with monoclonal antibody. At present, there is no commercially available myostatin monoclonal antibody thus the development of hybridoma generating anti-myostatin monoclonal antibody should be considered to measure myostatin level and to compare the effects of myostatin blockade with myostatin oral vaccine.

In conclusion, myostatin oral vaccine which was made via presentation of the myostatin antigenic moiety conjoined to the pgsA vector to the surface of *Lactobacillus casei* successfully blocked myostatin with circulatory antibodies. Myostatin blockade resulted in the functional and histologic improvements of dystrophic features of mdx mice. This can be applied to human muscular dystrophy after further verification of efficacy, safety, and optimal dose of myostatin oral

vaccine in large animal models such as mdx beagles.

Table 1. Comparison of physical measurements between the control and the treated mdx groups (mean ± SEM).

Measurement	Control mdx group	Treated mdx group	*P* value
Body weight change (g)	6.33 ± 0.50	10.25 ± 0.51	0.001
Weight of gastrocnemius (g)	0.230 ± 0.004	0.227 ± 0.010	1.000
Weight of soleus (g)	0.015 ± 0.003	0.051 ± 0.020	0.03
Serum creatine kinase (IU/L)	37,010 ± 11,636	5,308 ± 584	0.001
Serum anti-myostatin Ab	0.13 ± 0.01	0.71 ± 0.05	0.001
Rota-rod test (second)	46.9 ± 36.2	271.2 ± 63.4	0.003

Ab: antibody

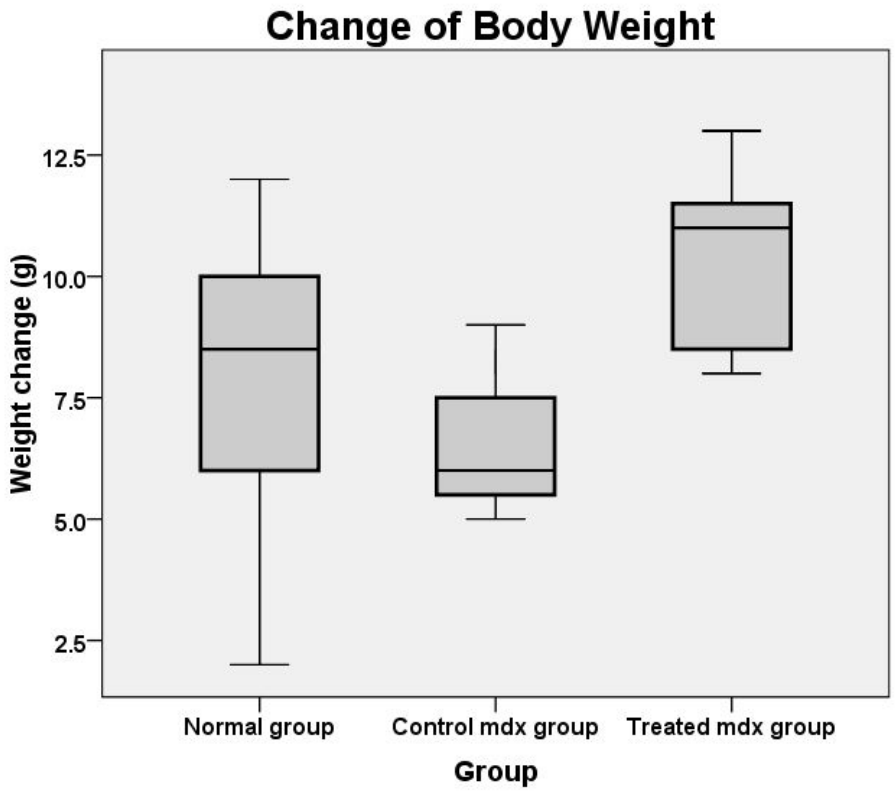

Fig. 1. Change of body weight during ten weeks of myostatin oral vaccine treatment.

The change of body weight in the treated mdx group is significantly higher

than that in the control mdx group (P= 0.001), and it is also higher than the body weight increase in the normal mice group (P= 0.04).

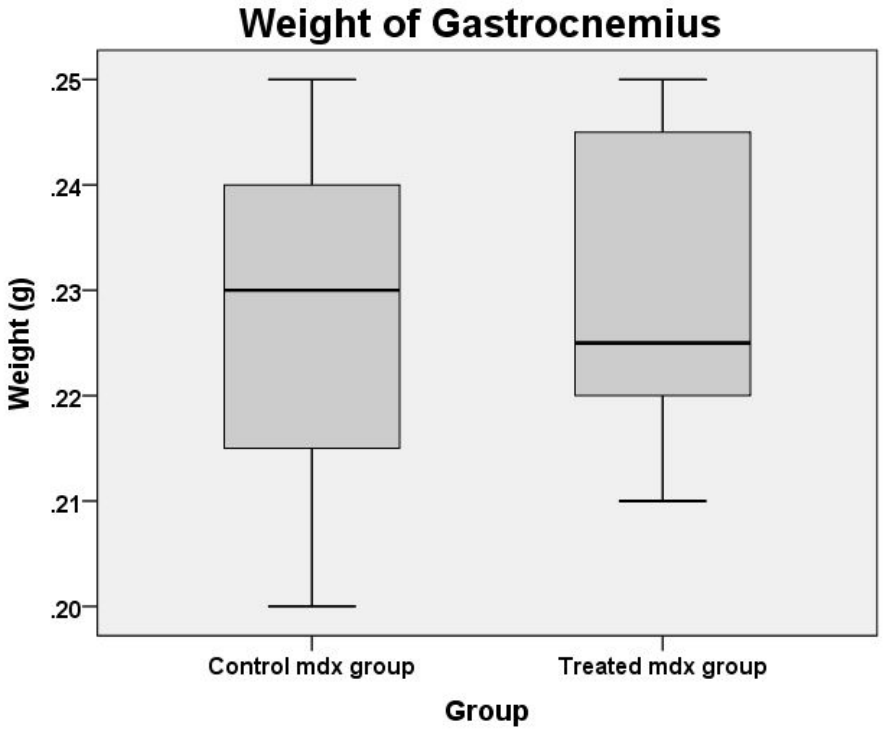

Fig. 2. Comparison of the weight of gastrocnemius between the control and the treated mdx groups.

The weight of gastrocnemius in the treated mdx group was not significantly different from that in the control mdx group (P= 1.00).

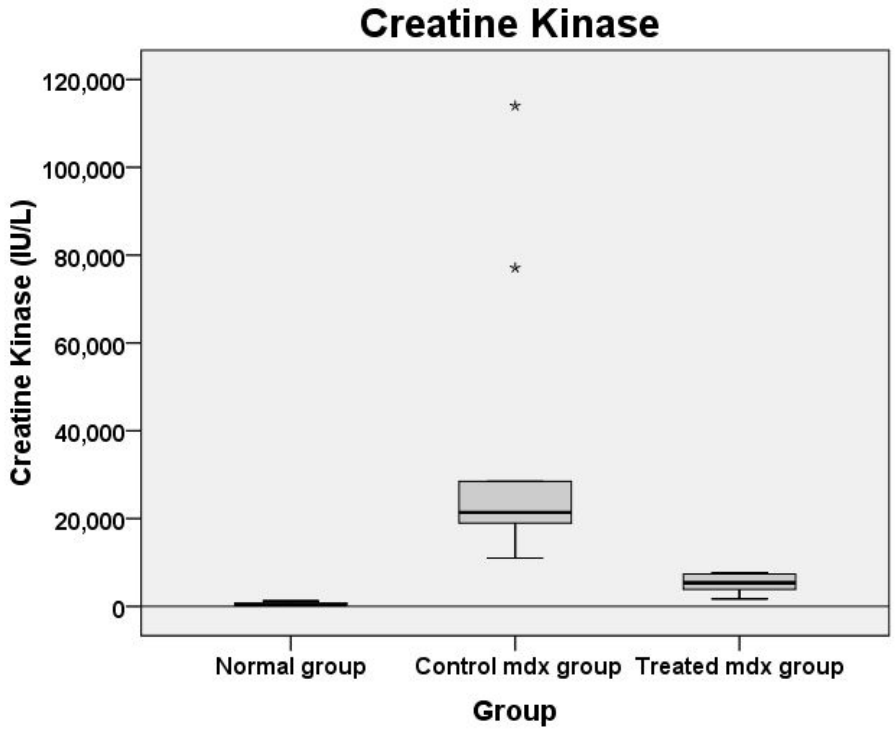

Fig. 3. Comparison of serum creatine kinase.

The serum creatine kinase of the treated mdx group was significantly lower than that of the control mdx group (P= 0.001). The mean value of creatine kinase in the normal mice group was 587.6±426.8 (IU/L), and the mean

value in the treated mdx group was 5,308 ± 584 (IU/L), which is about ten folds higher than the normal mice group.

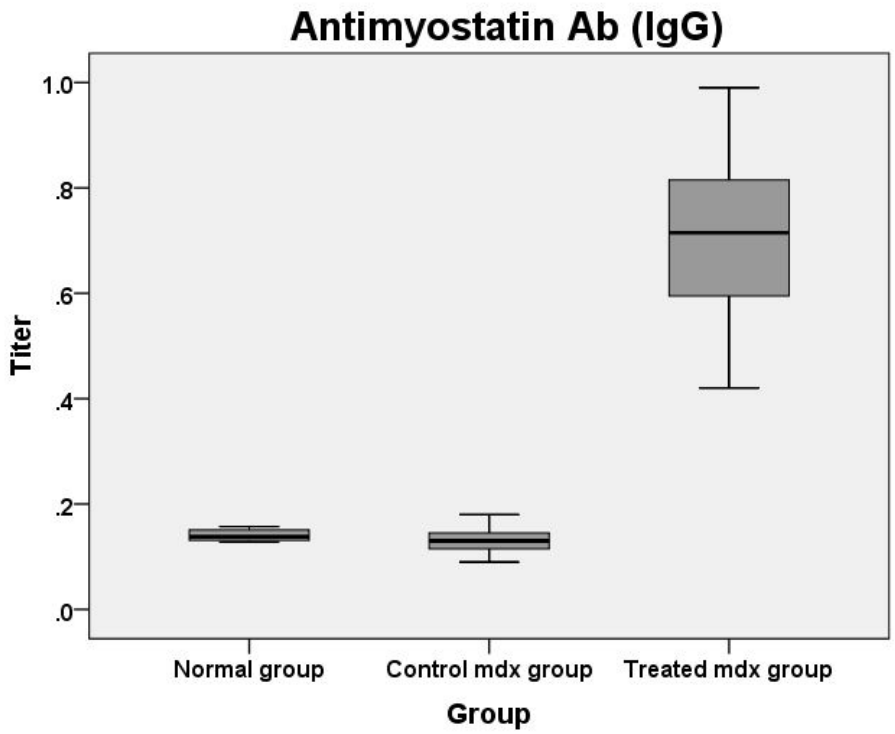

Fig. 4. Serum anti-myostatin antibody (IgG) titers (OD=450nm).

The anti-myostatin antibody titers were measured with ELISA method. The anti-myostatin IgG antibody titer is not significantly different between the normal group and the control mdx group (*P*= 1.00). The treated mdx group shows 5.5 folds increase in the anti-myostatin IgG antibody titer (*P*= 0.001).

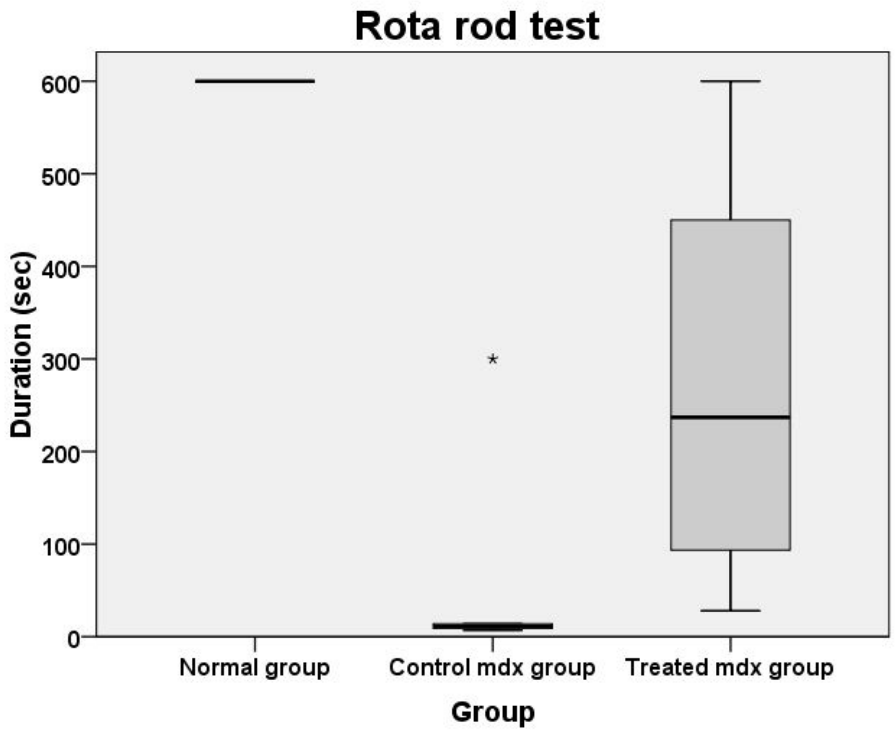

Fig. 5. The results of Rota-rod test (20rpm)

All mice in the normal mice group stayed over 600 seconds in Rota rod test with 20 rpm. The treated mdx group stayed significantly longer (5.8 folds) than the control mdx group (*P*= 0.003).

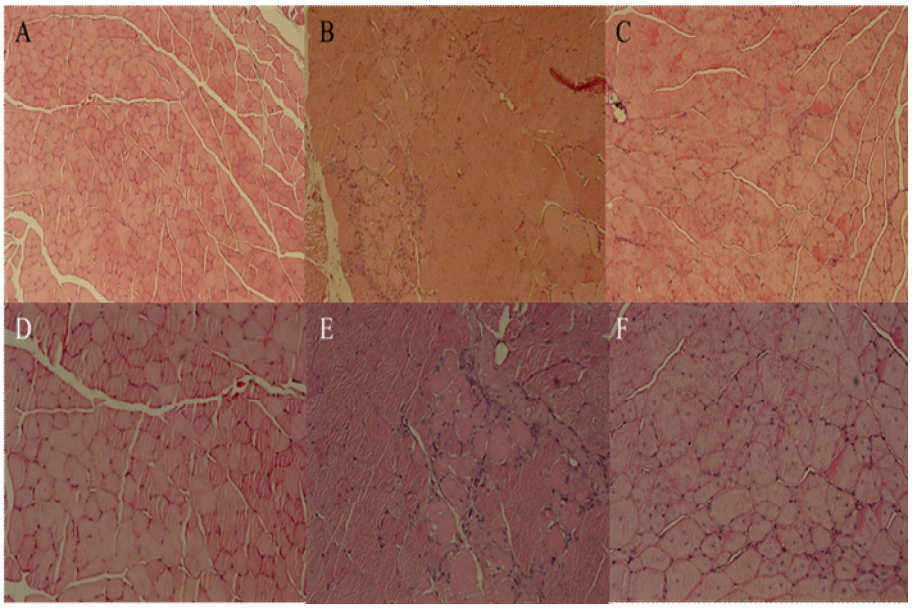

Fig. 6. Histologic improvement with myostatin oral vaccine (gastrocnemius, H&E stain, A/B/C: X100, D/E/F: X400).

A, D: Normal muscle specimen shows polygonal appearance with multiple peripheral nuclei.

B, E: The muscle specimen of the control mdx group shows remarkable size variation, fibrosis, central nuclei and infiltration of inflammatory cells.

C, F: The histology of the treated mdx group reveals less severe degree of

size variation comparing with the control mdx group. There are hypertrophy of myocytes, reduction of fibrosis, and minimal infiltration of inflammatory cells.

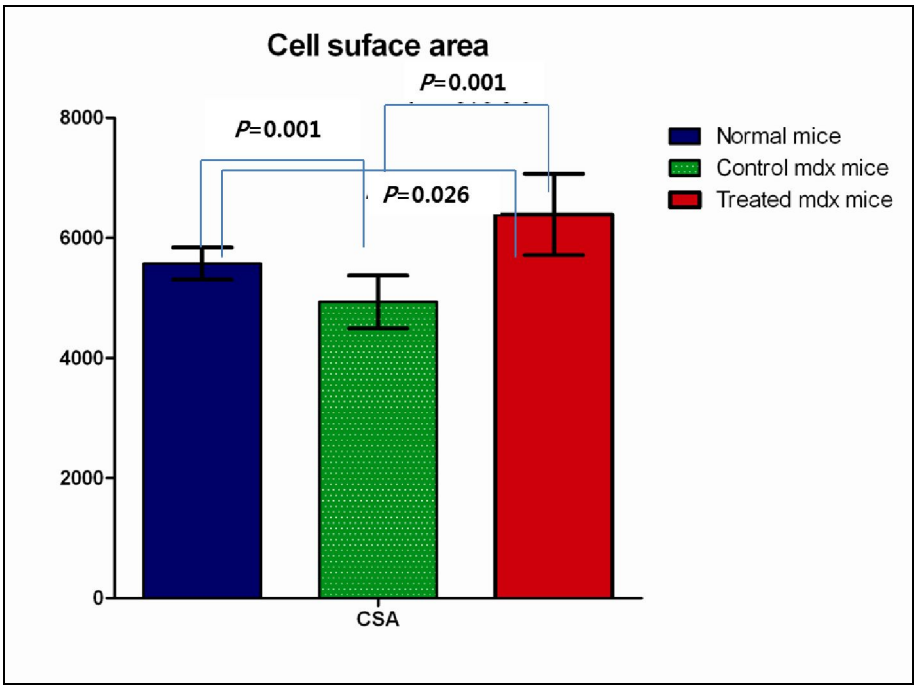

Fig. 7. Hypertrophy of myocyte with myostatin oral vaccine.

The mean cell surface area of the control mdx mouse group is decreased than the normal mice group, and that of the treated mdx mouse group is significantly higher than that of the normal mice group.

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국문 초록

연구 배경: 듀센 근디스트로피는 X염색체와 연관하여 유전되는 가장 흔한 근디스트로피로 아직까지 이에 대한 근본적인 치료방법은 없다. 이에 대한 대체치료로서 마이오스타틴(myostatin)에 대한 단클론항체가 개발되어 동물실험에서 그 효과를 입증하였으나, 성인의 근디스트로피 환자를 대상으로 한 임상시험에서는 효과적인 결과를 제시하지 못하였다. 최근 마이오스타틴의 항원성 부위를 *Lactobacillus casei*에 연결한 마이오스타틴 경구용 백신이 개발되어 마이오스타틴을 효과적으로 억제하여 근디스트로피의 치료에 이용될 것으로 기대되고 있다.

연구 목적: 저자는 마이오스타틴 경구용 백신을 듀센 근디스트로피 쥐 모델에 투여하여 혈청 항체가 생성되는 지 관찰하고 이에 의해 근디스트로피의 기능적 및 조직학적 호전이 발생하는 지 확인하고자 하였다.

연구 대상 및 방법: 생후 6주된 남성 mdx 마우스 (C57BL/10ScSn-Dmd$^{mdx/J}$)를 무작위로 대조군과 실험군으로 나누고 각각 3%의 *Lactobacillus casei*에 벡터가 연결된 *Lactobacillus casei* with pgsA 혹은 *Lactobacillus casei*에 벡터 및 마이오스타틴 항원이 연결된 *Lactobacillus casei* with pgsA-myostatin이 포함된 사료를 10주간 투여하였다. 2주 간격으로 체중과 혈청 크레아틴키나아제(creatine kinase)를 측정하였고 실험 종료 후 로타-로드(Rota-rod)검사를 시행하였다. Mdx 마우스를 희생한 후 혈청과 근육을 분리하여 단위 근육의 무게, 혈청 크레아틴키나아제, 항마이오스타틴 항체가를 측정하였다. 수집된 근육을 이용하여 조직학적 분석을 실시하였다.

연구 결과: **실험군에서 실험기간 동안 유의한 몸무게의 증가를 보였다** (*P*=0.001). **실험군의 혈청 크레아틴 카이네이즈는 대조군의 약** 14%**정도로 감소하였으며** (*P*=0.001), **혈청 항마이오스타틴 항체는 대조군보다** 5.5**배 증가하였다** (*P*=0.001). **로타-로드검사에서는 실험군이 대조군에 비해 약** 5.8**배 긴 시간 동안 머무는 것이 관찰되었다** (*P*=0.003). **조직학적 분석에서 실험군의 경우 세포크기의 다양성이 감소하고**, **섬유화가 줄어들었으며**, **근세포의 비대가 관찰되었다**. **실험기간 동안 마이오스타틴 경구용 백신과 연관된 부작용은 관찰되지 않았다**.

결론: *Lactobacillus casei* **표면에 벡터인** pgsA**를 통해 마이오스타틴 항원성부위가 연결된 마이오스타틴 경구용 백신은 혈청 항체를 생성하여 효과적으로 마이오스타틴을 억제하였으며**, **이를 통해 근디스트로피가 기능적 및 조직학적으로 개선되었다**.

주요어: **듀센 근디스트로피**, mdx **마우스**, **마이오스타틴**, **백신**

학번: 2005-31206