Adenovirus Virus Expressing Myostatin-Somatostatin Fusing Gene Promote the Growth Rate of the Mice

Xinglong Wang (wxlong@nwsuaf.edu.cn)
Northwest Agriculture and Forestry University

Gaoxiao Xu
Nanning University

Ning Zhang
Jinguang Co.,Ltd

Xingfa Chen
DaBeiNong Feed Technology Co.,Ltd

Dongling Yu
Nanning University

Research article

Keywords: myostatin, somatostatin, growth rate, muscle mass

Posted Date: December 17th, 2019

DOI: https://doi.org/10.21203/rs.2.19059/v1

Abstract

Myostatin (MSTN) and somatostatin (SST) are negative regulate factors in the animals. Immunized animals with these proteins induce the related immune response, which could neutralize the normal inhibitory effects of these proteins. Since adenovirus was the mostly wildly used vector for delivering vaccine antigen, here, a recombinant adenovirus expressing MSTN-SST were constructed and mice were inoculated with the recombinant adenovirus. As our predicted, immunization with this adenovirus one time or two times were all induced the specific immune response and resulting in an improve growth rate and muscle mass. And boost immunization improved the effect of the immunization. Accordingly, adenovirus can be used as a vector for deliver myostatin and somatostatin gene, so as to increase animal growth rate and muscle mass of the animals.

1.background

Myostatin (MSTN), a member of transforming growth factor (TGF)-ß superfamily, is also known as growth and differentiation factor 8 (GDF8) [1]. MSTN was found to be capable of modulating the body weight and muscle composition in laboratory and farm animals [2]. Oral feeding recombinant yeast Saccharomyces cerevisiae expressing mammalian MSTN from a plasmid or chromosomal integration gene elicited antigen specific immune responses, and resulted in increased body weight and muscle composition in mice [3, 4]. Down regulation MSTN expression by siRNA or gene knock out can also increase muscle mass [2, 5]. Accordingly, MSTN is an ideal target for regulation animal meat product or human muscle wastage.

Somatostatin (SST) is known to inhibit the release of growth hormone (GH) from the anterior pituitary [6]. Reduction of the concentration of SST in the blood results acceleration of the growth of the animals. Immunization of animals to SST and a result induction of the related antibodies (the anabolic factors) is a means of removing SST's normal inhibitory effects. However, SST is a short peptide hormone with only 14 amino acids,and its half-life in the blood stream is only several minutes. So SST conjugates with various proteins are used for immunization [7]. Here, MSTN were fused to SST, so as to induce the related immune response and acceleration of the growth of animal and enhancement meat product.

Adenovirus vectors are the most commonly employed viral vector for gene therapy and deliver vaccine antigens. It has been used for deliver rabbit hemorrhagic disease virus antigen VP60 [8] and many other virus antigens like, porcine reproductive and respiratory syndrome virus [9], foot-and-mouth disease virus [10]. And the immunization with recombinant adenovirus induced robust immune response and also protection against infection. Here, we used adenovirus to deliver MSTN and SST, and which may induce a strong immune response to regulate the growth rate of the immunized animals.

2. Methods

2.1 Virus and cells

The human type 5 adenovirus expression system (replication-defective) was purchased from TaKaRa (Dalian, China). Recombinant adenoviruse (rAd) and wild type Adenovirus (wtAd) were grown and titered in HEK-293A cells.

All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

2.2 Construction of recombinant adenoviruses (rAd-MSTN-SST)

The open reading frames of MSTN-SST were amplified by PCR using primers listed in Table 1. The PCR amplicons were cloned into a pAd-shuttle-CMV vector. The recombinant adenoviral vectors were generated by homologous recombination of linearized transfer vectors with the pAdEasy-1 in *Escherichia coli* BJ5183 and confirmed by restriction enzyme digestion (New England Biolabs). The recombinant adenoviruses were generated by transfection of 1 μg plasmids (PacI linearized) using 3 μL of Trans Fast TM Transfection Reagent (Promega, Madison, USA), When 90% of the cells showed cytopathic effect, adenoviruses were released by three cycles of rapid freezing and thawing, and stored at −80 ℃ after addition of 10% glycerol.

2.3 IFA

IFA was used to identify the expression of MSTN-SST. Briefly, HEK293 A cells were infected with rAd-MSTN-SST or wtAd at a multiplicity of infection (MOI) of 5. After 24 h incubation, the cells were washed, fixed with 4% paraformaldehyde (30 min at 25 °C) and incubated with the mouse-anti-MSTN-SST polyclonal antibodies (diluted 1:800) for 1 h at 37℃. Cells were stained with goat anti-mouse FITC-Conjugated Antibody (Abcam, ab6785, diluted 1:800) for 1 h at 37 °C. The cells were then washed with PBS, and the expression of MSTN-SST was visualized using a fluorescence microscope (OLYMPUS IX73).

2.3 Western blot

The 293A cells infected with rAd-MSTN-SST were collected at 18, 24 and 36 h post infection. The cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Pall Corporation). The 293A cells infected with wtAd were used as a negative control.

The membrane was incubated overnight in blocking solution (10% fat-free milk in PBS, PBS-M) at room temperature and incubated with anti MSTN-SST polyclonal mouse antiserum for 2 h. The membrane was subsequently reacted for 1 h with Goat anti-Mouse IgG conjugated with horseradish peroxidase (AS003) at a dilution of 1/2000 in PBS-M. Detection was performed using chemiluminescence lumi-nol reagents (Super Signal West PicoTrial Kit, Pierce). By gray scan and compared with the known concentration marker bands, the concentration of proteins were calculated according the obtained gray value of the bands.

β-actin was used as a referee and the primary antibody againstβ-actin was purchased from BOSTER Co., Ltd.. (Wuhan, BM0627).

2.6 Immunization

Thirty 4 week old male Chinese Kunming mice (male) were purchased from Chengdu Dashuo experimental animal co. LTD, and were randomly divided into 3 groups of 10. The mice were with very similar weight. The first group received wtAd (at day 0) and served as a control (Group A). The second group was given rAd-MSTN-SST at day 0 by intramuscular injection (Group B). And the third group were given rAd-MSTN-SST at day 0 and boosted at day 14 (Group C). Mice were kept in cages system with automatic ventilation system. Five mice were kept in one cage and they were free choice feeding.

The mice were weighted every week and blooding at day 14 and 28. The mice were gave euthanasia by using CO2 inhalation (CO2 were infused at 10%, 30%, or 100% volume per minute displacement rates) followed by cervical dislocation. To observed the muscles, the skin of the mice. The muscle fibers (biceps femoris muscle) were observed by making tissue slides.

2.7. Histopathology test

The collected biceps femoris muscle were fixed and embedded in paraffin wax and cut into 4 to 5 μm slices. For micro structure observation, the slices were stained with haematoxylin and eosin. The densities of muscle fibers in one view were analyzed using software Image J.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Guangzhou Jet Bio-Filtration Co., Ltd., China) were coated with 0.2 μg (each) /well of mixed purified MSTN-SST (expressed in Escherichia coli and stored by our laboratory). Then, the plates were reacted with sera from mice immunized with corresponding proteins. Commercial peroxidase-conjugated rabbit anti-mice IgG (Sigma) was used as the secondary antibody. Following incubation with TMB (tetra-methylbenzidine) substrate (Tiangen Biotech (Beijing) Co., ltd., China), the reaction was stopped with 0.5 M sulphuric acid and the optical density (OD) of each well was measured at a wavelength of 450 nm.

2.10. Statistical analysis

The body weight of mice was expressed as mean ± standard deviation (SD) and evaluated with ANOVA. The body weight of mice was, and the differences between control groups and immunized groups were analyzed by a two-tailed independent Student's t-test. A difference was considered to be significant if $P < 0.05$ was obtained.

Results

3.1. Construction recombinant viruses

Shuttle vectors containing MSTN-SST gene under the control of the CMV early promoter were constructed and verified by sequencing (data not shown). By recombination with adenovirus backbone vector pAdEasy-1 in BJ5183 cells, recombinant adenoviral plasmids, pAd-MSTN-SST was obtained. Then the recombinant plasmids were linearized with endonuclease Pac I and transfected into HEK293A to generate recombinant adenoviruses, rAd-MSTN-SST. After about 10 days incubation, those recombinant adenoviruses were successfully packaged with characteristic cytopathic effect in transfected cells. While mock-transfected cells (control samples) retained their singularity (data not shown).

The obtained recombinant adenoviruses, rAd-MSTN-SST was purified three times with plaque-purified method and titered in HEK293A cells. The titer of this recombinant adenoviruses were 3.6×10^{8} vp ml^{-1}. The expressions of the proteins MSTN-SST were confirmed by IFA and WB.

The expression of MSTN-SST was examined by IFA. Green fluorescence indicating the expression of MSTN-SST was observed in rAd-MSTN-SST infected cells but not in wtAd infected cells (Fig. 1A). As show in Fig 1B, the protein bands corresponding to MSTN-SST were observed in Line 1, 2 and 3, in which the proteins derived from cells infected with rAd-MSTN-SST at 18, 24, and 36 h post infection were separated. While the related band was not observed in Line 4, in which the proteins from cells infected with wtAd were added.

3.2 Humoral immune responses against MST-SST following vaccination

The sera were collected at 7, 14, 21 and 28 days post immunsation and were detected by ELISA with the recombinant proteins (E.coli expressed, 0.2 μg in one well) as reported [11].

The antibody against MSTN-SST was detected as early as 7 days post inoculation. And the antibody titer reached a peak at day 21 with only one time immunization as that shown in group B (Fig 2). The antibody titer was still increasing even at end of the experiments in group C, which were given two times immunization. The immunized groups gained significant high level of antibody titer since 7 days post inoculation and till to the end of the experiment *($P\leq0.05$*). The immunized two groups showed significant difference since day 28 (*$P\leq0.05$*) (Fig 2).

3.3 Body weight gain

The experiment procedure were lasted a total of 4 weeks before the mice were sacrificed. The body weights of each animal were recorded each week. When the experiment started all mice were about the same size with little difference. At the end of experiment, however, they weighted from 34 to 42.3 g and the average sizes varied among groups. In Group A, the average increase of body weight was 14.18± 0.36 g, whereas it was 16.70 ± 0.252 g in Group B, suggesting that vaccination against MSTN-SST modulated the body weight of animals. Interestingly, the increases of body weight in Groups C were significantly higher than that of Group B, suggesting that boosting immunization was effective and further confirm the role of MSTN-SST on regulation growth rate of the mice (Fig 3).

3.4 Muscle morphology observation

To show the affection of the immunization on the growth of the mice muscle, post-mortem examinations were performed on these animals. As show in Fig 4, the muscles of the mice immunized are more defined and the increased sizes could also be easily observed. The size of Longissimus dorsi (LD) and Biceps femoris (BF) were measured and show in Fig 5. The average size of LD and BF in control group was 0.358±0.002 and 0.239±0.005. Immunization increased these to 0.463±0.004 and 0.314±0.002 in group B and 0.494±0.003 and 0.367±0.005 in group C

3.5 Histology examination

Histology examination was used to show the micro-level differences among the different groups. As show in Fig 6, the muscle fibers of BF from each group were observed under microscope. The thicken muscle fibers in group B, C were observed when compared the muscle collected from group A. In addition, the densities of muscle fibers were significantly increased as the numbers of the fibers were significantly increased in one view. According to statistical results of 10 views, the proportions of muscle fibers were increased from 63.43±3.28 to 72.18±4.28 and 83.72±1.67, respectively. And the differences were significant among each group.

Discussion

Adenovirus vectors are the most commonly employed viral vector for gene therapy and deliver vaccine antigens. Adenovirus vectors used as vaccines are mostly replication-defective with certain essential viral genes deleted and replaced by a foreign gene expression cassette [12, 13]. Here, the MSTN-SST fusing gene were inserted in Ad5 genome, which were used to expression the protein MSTN-SST. The immunization with the recombinant adenovirus induce the related antibody response against recombinant protein MSTN-SST, resulting accelerated the body weight of the mice immunized and also the muscle mass of the mice muscle. In a previously research, MSTN were delivered though heat-killed whole recombinant yeast Saccharomyces cerevisiae expressing mammalian MSTN from a plasmid, and which elicited antigen specific cell and humor response in mice [4]. The immunization increased body weight and muscle composition in mice. Soon after, another reports demonstrated that heat-inactivated MSTN-recombinant yeast could promote the growth of the rabbits and significantly increase the development of muscle [14]. And then, saccharomyces cerevisiae harboring MSTN in genome were constructed and used to deliver MSTN gene to mice by oral immunization [3]. Similar results happened to the immunized mice were observed. In addition, monoclonal anti-MSTN antibody injected into the yolk significantly increased body weight (4.2%) and muscle mass (5.5%) [15]. Though the oral immunization with Saccharomyces cerevisiae or directly given monoclonal antibody could induce immune response against MSTN and acceleration of growth of the animal, the immune operation is not easy to carryout in field. Immunization by muscle injection is most used in clinical production, because it is easy to operation and quantification. Here, adenovirus expression MSTN-SST was given by intramuscular injection and all the immunized mice gained similar antibody level.

Animals immunized with SST have an increased average daily weight gains of 10–20%, and appetite reduced by 9% and an 11% increase in the efficiency of food utilization [7]. Wherein improved absorption of food components and a slower passage of food through the gastrointestinal tract with sluggish peristalsis is observed. Animals immunized with SST, and also their offspring, have correct proportions, and the distribution of the weight of the animals between the muscles, bones and fat is the same as in the control [1, 7]. Another research found that immunization of gestated goats results in an increased in the weight of newly-born by 10% and an increase in milk yield [7]. Here, according to our results, immunization with MSTN-SST improved daily weight gains of the mice and also the gross weight of the mice. However, we did not statistic the food consumption and the mice were free feed intake.

Conclusion

In summary, we constructed an adenovirus, which expressed fusing proteins MSTN-SST. Immunized mice with this adenovirus enhance daily weight gain of the mice and also improved the muscle mass of them. This recombinant adenovirus can be used to increase animal meat production, and may also can be used for treating muscle atrophy.

Abbreviations

MSTN
Myostatin
SST
somatostatin
ELISA
Enzyme-linked immunosorbent assay
IFA
indirect immunofluorescence assay
TGF-ß
transforming growth factor ß
GDF8
growth and differentiation factor 8
GH
growth hormone
rAd
recombinant adenoviruses
MOI
multiplicity of infection
PBS
Phosphate Buffer solution
IgG

immunoglobulin G

Declarations

Ethics approval and consent to participate

All experimental procedures involving animals were reviewed and approved by the Ethics Committee at Northwest A&F University. Guidelines from the independent Animal Care and Use Committee in Shaanxi Province, China, were strictly adhered to.

Consent for publication

The authors all agree to publish these data.

Competing interests

The authors declare no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China (No. 31672581), The Scientific Research and Technology Development Major Project of Nanning（20192004-1）, The Scientific Research and Technological Development Program of Wuming District of Nanning City (20180101), The Basic Ability Promotion Project for Young and Middle-Aged Teachers in Guangxi Universities （2019KY0941）, The Guangxi Innovation-Driven Development Project（Gui Ke AA18118021-7）and The Research project of Nanning University (2018XJ36).

Authors' contributions

GX and NZ conducted the experiment. XC and DY did the data analysis and XW prepared the manuscript.

Acknowledgements

We would like to show our thanks to Xiang Biao who gave us much help for making the histology examination.

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Table

Table1 Primers used for amplification MST-SST gene

Name	Sequence(5'-3')	RE Site
MST-SSTs	agatctatgcaaaaactg	*Bgl* II
MST-SSTa	tgagcacccacagcgatctac	
MST-SSTa2	gatatcctaacaggatgtgaaagtcttccagaagaaattcttgcagccagctgagcacccacagcgatc	*Eco*R V

RE: restriction endonuclease

Figures

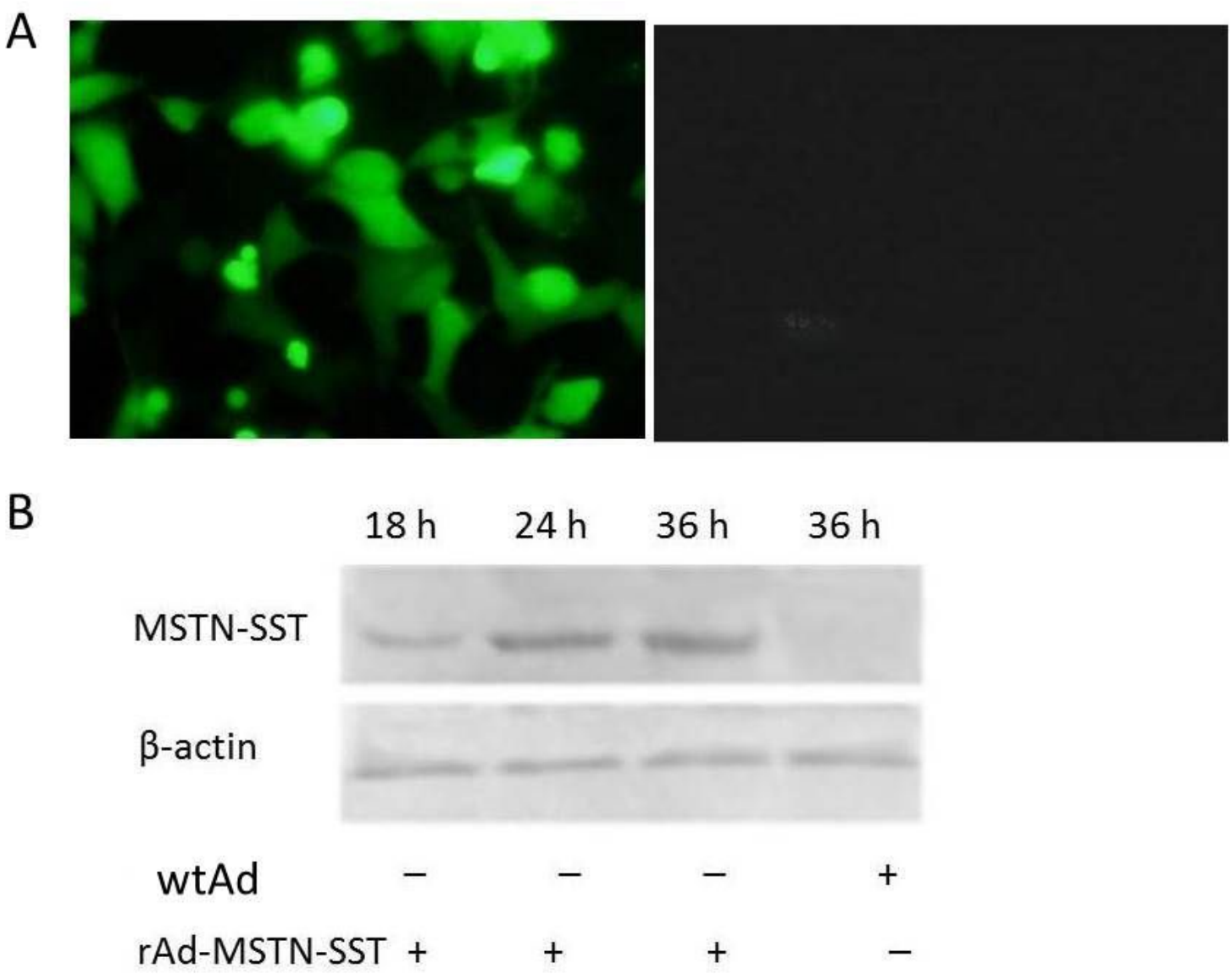

Figure 1

Detection MSTN-SST expression by IFA (A) and WB (B). (A) The cells were fixed and stained with mouse antibody against MSTN-SST. Positive signals were showed with goat anti mouse FITC-conjugated antibody. (B) Target bands of MSTN-SST were detected in cells infected with rAd-MSTN-SST.

Figure 2

Antibody response against MSTN-SST after immunization. The sera were collected at 7, 14, 21 and 28 days post inoculation. The date collected from group A, B, and C were shown as A, B and C in figure. Data are shown as Mean±SD. Significant difference ($p \leq 0.05$) were marked with different letter.

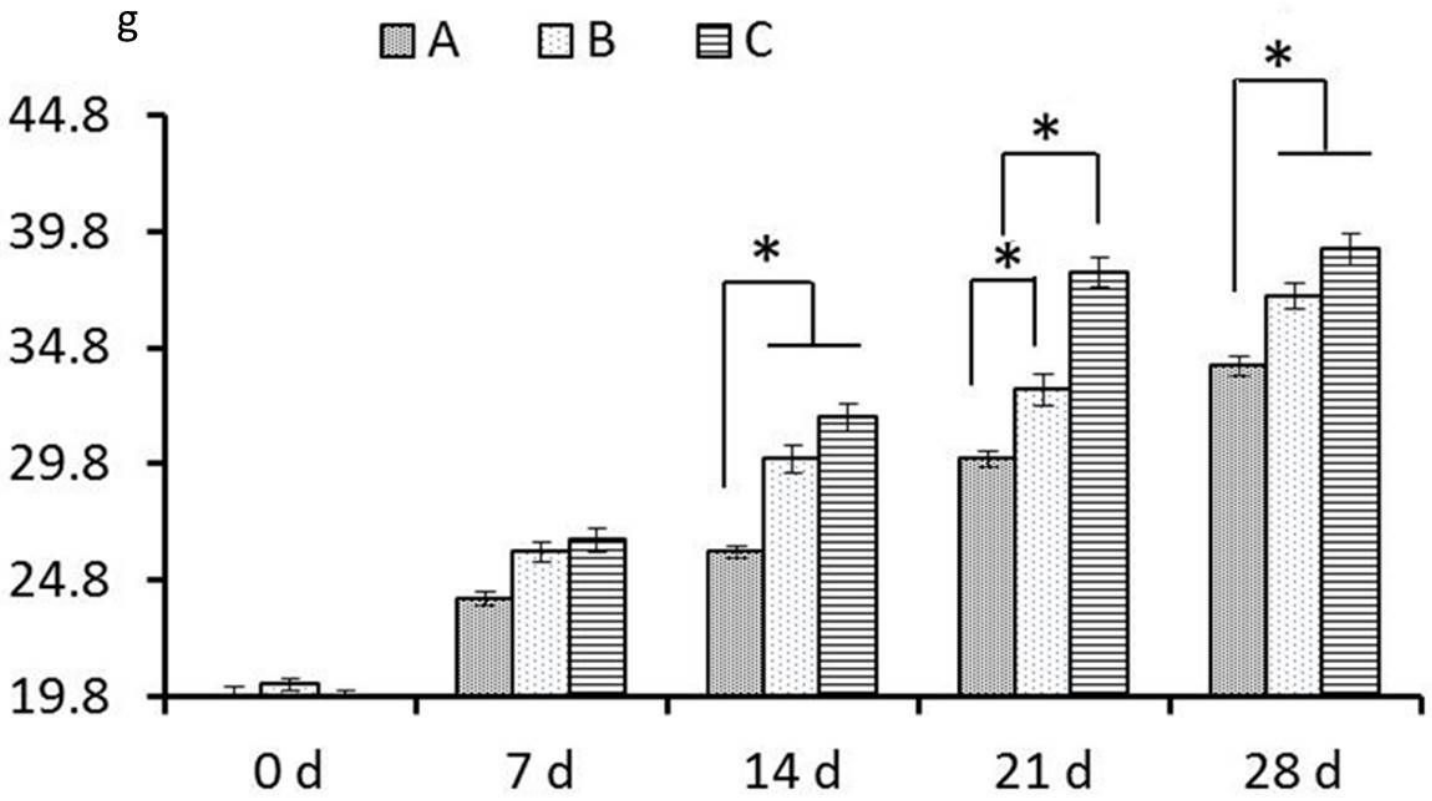

Figure 3

Body weight gains of the mice during the experiment. The mice were weighed every week after immunization. The mean body weight of mice in each group were compared. The date collected from group A, B, and C were shown as A, B and C in figure.* was used to show the significant difference. Data are shown as Mean±SD

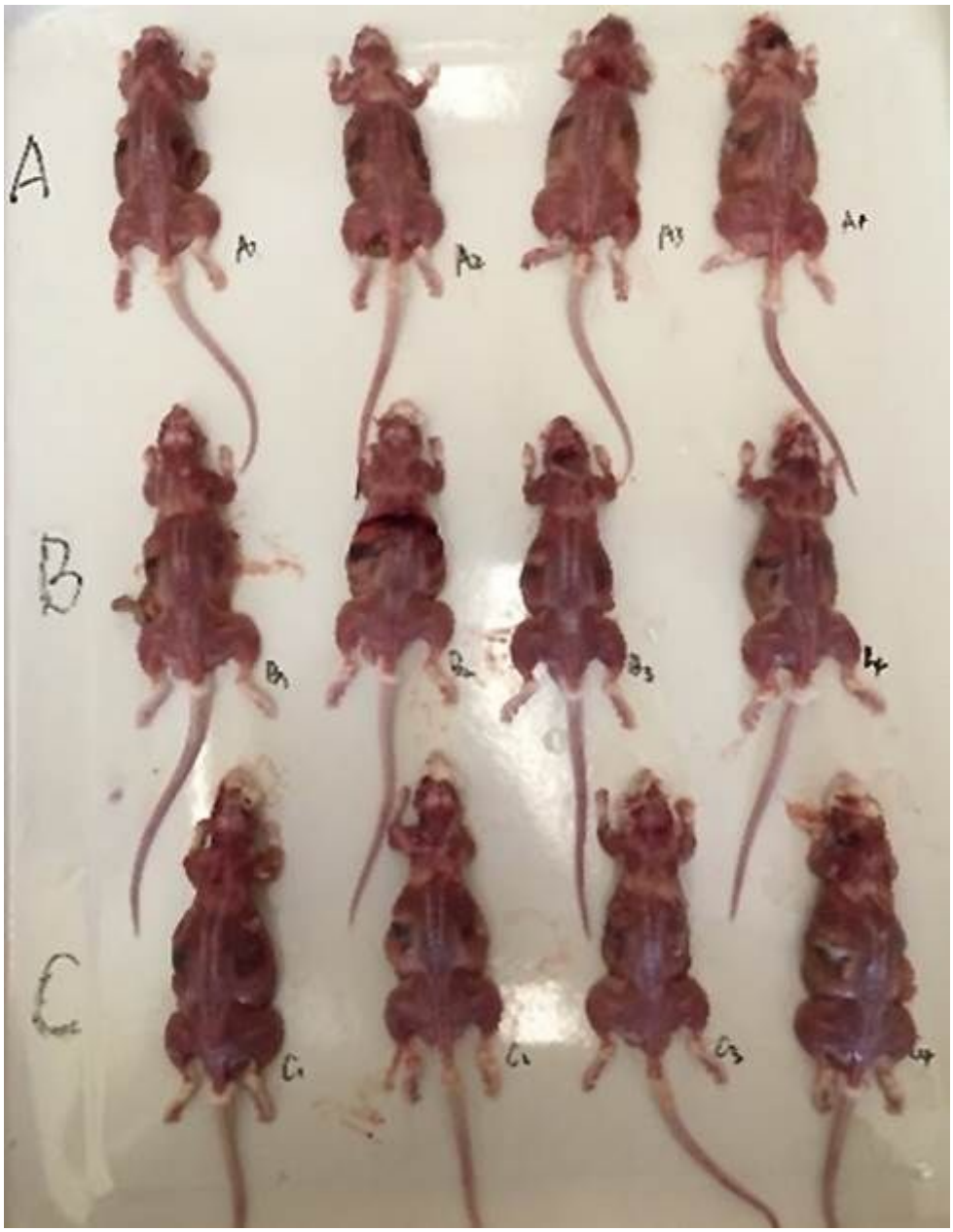

Figure 4

Display muscle shape of the mice by removing the skin. Random selected four mice from each group were used to show the muscle shape. The skin of the mice were removed after given euthanasia. The date collected from group A, B, and C were shown as A, B and C in figure. The differences of the muscle between the groups can be visually seen.

Figure 5

The size of Longissimus dorsi (LD) and Biceps femoris (BF) of mice from different group. LD and BF from each mice were collected and weighted. Immunization significant increases the size of these two

type of muscle. The date collected from group A, B, and C were shown as A, B and C in figure. Data are shown as Mean±SD. Significant difference ($p \leq 0.05$) were marked with *

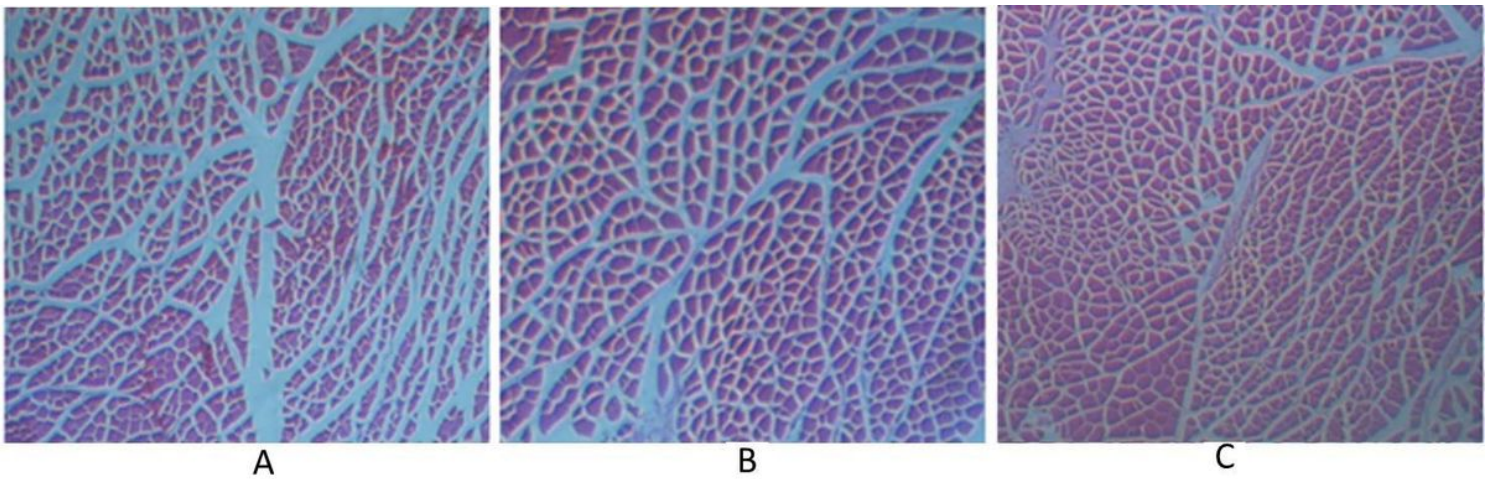

Figure 6

Display the change of muscle fibers shape by histological examination. BF collected from each group were fixed and transected to make slices. The slice from group A, B, and C were shown as A, B and C in figure. The muscle fibers were observed under microscope. The increased muscle fiber densities were observed in immunized groups.

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