Negative Effects of Cx43 during Denervated Skeletal Muscle Atrophy can be Attenuated by Blueberry Extracts via Regulating Akt Pathway

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Research Article

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Abstract

Background: Lack of effective treatment for alleviating the irreversible skeletal muscle atrophy caused by peripheral nerve disfunction has long been a clinical problem. For seeking therapeutic targets, several signaling pathways in muscle cells that activated by denervation have been explored. Important roles of Cx43 in other types of pathologic processes such as have been reported.

Methods: Denervation mice model was created. Hematoxylin–Eosin (HE) and immunofluorescence staining were applied and analyzed. Western blot and quantitative real-time PCR (qPCR) were used. All statistical testing was performed by t test.

Results: Our study investigated the negative role of Cx43 during muscle cells degeneration after denervation. Blueberry extracts was applied orally and its protective effect for denervated skeletal muscle atrophy was found in our study, and we demonstrated that Cx43/Akt pathway in denervated muscle cells was modulated by blueberry extracts.

Conclusion: Negative effects of Cx43 during denervated skeletal muscle atrophy can be attenuated by blueberry extracts via regulating Akt pathway.

Introduction

Denervated skeletal muscle atrophy is characterized as an irreversible process of skeletal muscles mass decrease because of pathological changes in their dominated nerves[1]. It can be one of the most severe sequalae of peripheral nerves diseases such as injury, neuritis, neuroma and so on. Although surgical techniques for nerve repair have been improved a lot now, there is still no satisfying treatment can be applied for alleviating targeted muscle atrophy that already happened.

Family of connexins, proteins that form gap junctions in vertebrates, are recently found to be also important for skeletal muscle cells[2, 3]. Among members of connexins, Cx43 was considered to have something to do with injured skeletal muscle cells and has more negative but key effects on skeletal muscle regeneration. Previous studies reported that Cx43 reduction could protect skeletal muscle tissue from pathological status[4, 5]. Also, in denervation induced muscle atrophy, expression of Cx43 was found in fast muscle cells[6].

For seeking possible therapeutic targets, previous studies have explored several signaling pathways involved in the process of skeletal muscle degeneration induced by denervation. Among numerous signaling pathways, Akt related pathways were noticed by some researchers. Akt, as a classic regulator inside muscle cells, has been proved helpful for skeletal muscle growth[7, 8] and its activation helps to alleviate and prevent muscle atrophy caused by sepsis[9], starvation[10], myocardial infarction[11], chronic kidney disease[12] and so on. In transcriptome sequencing and analysis results from Xin et al.[13], Akt was also down-regulated in denervated skeletal muscle cells. It was attractive that interaction between Cx43

and Akt pathways was reported in recent studies in cardiac muscle[14, 15]. However, there is no study has explored their relationship in denervated skeletal muscle atrophy.

It is widely accepted that blueberry has strong antioxidant function and its protective roles for skeletal muscles is reported recently[16, 17]. Also, the protective roles of blueberry are found to be related to Akt pathway in other pathological models[18, 19]. Here, in our study, we verified the negative effect of Cx43 on denervation induced skeletal muscle atrophy, which can be attenuated by blueberry extracts. And we explored the mechanisms behind protective effect of blueberry extracts, which is achieved via regulating Cx43/Akt pathway.

Methods And Materials

Experimental Animals

Male C57 BL/6 mice aged 6-8 weeks and weighing 22-25 g were purchased from the Vital River Laboratory Animal Technology Co (Zhejiang, China). Mice were housed in standard cages in a room at 23°C and 50% relative humidity on a 12-h:12-h light/dark cycle.

Experimental grouping

The mice were randomly assigned to 6 groups: the mice that received a sham operation (Control group), the denervated mice that were administered with distilled water (Denervation group), the denervated mice received tail intravenous administration with Gap-19 (MedChemExpress, New Jersey, United States) (Gap-19 group) and the denervated mice that were applied with blueberry extracts 200mg/kg and 400mg/kg by intragastric administration (Lanmei Technique Co., Ltd., Zhejiang, China). (Blueberry 200 and 400 groups). Mice in all denervation groups were subjected to unilateral sciatic nerve transection under anesthesia as previously described[20]. Briefly, after deep anesthetization, a 0.5-cm-long portion of the sciatic nerve in the right hind leg of the mouse was resected; the two nerve ends were buried in muscle, and the incision was closed using 4-0 absorbable sutures. After sciatic nerve transection, mice in the Gap-19 group were treated with Gap-19 every two days by tail vein injection.[21] After sciatic nerve transection, mice in the blueberry group were treated daily with blueberry extracts dissolved in 37°distilled water by intragastric administration. Mice in the denervation group received the same amount of distilled water daily.

Wet Weight

At 14 days after denervation, with and without taking blueberry extracts, mice were anesthetized, and the gastrocnemius muscles of both the left and right hind legs were removed, washed with saline, and then weighed. The loss of muscle weight ratio was defined as the weight of the contralateral side minus the muscle weight of the nerve injury side divided by the weight of the contralateral side. The muscle samples were stored in 4% paraformaldehyde at −80°C until use.

Hematoxylin–Eosin (HE)

Gastrocnemius muscle samples from mice were fixed in 4% paraformaldehyde and embedded in paraffin. The samples were cut at a thickness of 5 μm and the sections were stained with hematoxylin–eosin (HE) (Beyotime, Shanghai, China) to evaluate histopathologic changes.

Immunofluorescence

To reveal Akt and Cx43 proteins in denervated muscle, double immunostaining was conducted. Paraformaldehyde-fixed denervated muscle were incubated with the primary antibody, followed by the secondary antibody, and subsequently mounted with DAPI. The primary antibodies included rabbit anti-Akt (1: 100, cat.no. AF1789 Beyotime Institute of Biotechnology Co., Ltd.) and rabbit anti-Cx43 (1:100, cat. no. 52559 Cell Signalling, Danvers, MA, USA).

Western Blot Analysis

Frozen gastrocnemius muscle samples were homogenized in radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Roche Applied Science). Lysates were centrifuged for 20 min at 12,000 × g (4◦C) and the protein level in the supernatant was quantified with a bicinchoninic acid assay kit (Beyotime). Proteins were separated by SDS–PAGE (Beyotime) and transferred to a polyvinylidene difluoride membrane (Beyotime) that was blocked with 5% non-fat dry milk in Tris-buffered saline at room temperature, followed by incubation with primary antibodies: rabbit anti-Akt (1: 1000, cat.no. AF1789 Beyotime Institute of Biotechnology Co., Ltd.), rabbit anti-Cx43 (1:5000, cat.no. C6219 Sigma) and rabbit anti-p-Cx43 (1:1000, cat.no. PA5-104820, Invitrogen). After three washes, the membrane was incubated with appropriate secondary antibody (Abcam) at room temperature for 1 h. Enhanced chemiluminescence detection reagent and X-ray film were used for protein visualization.

Quantitative Real-Time PCR (qPCR)

The RNeasy kit (Qiagen, Valencia, CA, United States) was used to extract total RNA from gastrocnemius muscle. cDNA was synthesized using a first-strand cDNA synthesis kit with oligo dT primers (Invitrogen, Carlsbad, CA, United States) and used for quantitative real-time PCR (qPCR) (MJ Research, Waltham, MA, United States). The thermal cycling conditions were as follows: 94◦C for 5 min; 35 cycles at 94◦C for 30 s, 55◦C for 45 s, and 72◦C for 1 min; and 72◦C for 5 min. Relative expression level of the target gene was calculated using the cycle threshold (Ct) value. Akt and Cx43 expression levels were normalized to β-actin levels. We used the Primer3 to design all primer sequences.

Statistical analysis of data

All data are presented as means ± SD. All statistical testing was performed by t test. All statistical analyses were conducted with a SPSS Software Version 17.0 (SPSS Inc., Chicago, IL,United States). P-value less than 0.05 was considered statistically significant.

Results

Denervation leads to increased expression and activated function of Cx43 in atrophied skeletal muscle

Unilateral sciatic nerve was transected on mice in denervated groups. Wet weight ratio decreased significantly at 14 days post-operation (Figure 1.A). Ipsilateral gastrocnemius also showed obvious atrophy at 14 days post-operation in gross and histology (Figure 1.A, B). All these changes at 14 days were more obvious than those at 7 days (Supplementary Figure 1.A-C), so materials at 14 days post-operation were used for the further study. Compared with control group, denervation induced increased Cx43-positive expression in skeletal muscle cells in both transcription (Figure 3. E) and translation (Figure 1.C, D) level. These results showed numbers of Cx43 raise after denervation. To explore if their function was activated, p-Cx43/Cx43 was analyzed by Western Blot and found that p-Cx43/Cx43 elevated continuously post-denervation till 14 days (Figure 1.E).

Gap-19 inhibited the function of Cx43 and attenuated denervated muscle atrophy

To determine the effects of Cx43 on skeletal muscle cells after denervation, we applied Gap-19 by tail intravenous administration, a selective inhibitor of Cx43, in mice of Gap-19 group. Gap-19 reduced Cx43 expression in denervated skeletal gastrocnemius muscle in both transcription (Figure 3. F) and translation (Figure 2.B.C) level and inhibited Cx43 activation (Figure 2. C). In histology (Figure 2.D) and gross level (Figure 2.A), Gap-19 attenuated skeletal muscle atrophy at 14 days after denervation. Gap-19 showed no obvious effects on skeletal muscle without denervation (Figure 2.E). Combined with previous results, we can conclude that activation of Cx43 promoted denervation induced skeletal muscle atrophy.

Blueberry extracts attenuated denervated muscle atrophy and downregulated Cx43 expression

Mice in blueberry groups were treated daily with blueberry extracts dissolved in 37°distilled water by intragastric administration from the day of operation. Figure 3.A and Figure 3.B showed the sizes of ipsilateral gastrocnemius in Blueberry 200 (Figure 3. A left) group and Blueberry 400 (Figure 3.B left) group were obviously improved versus denervation group (Figure 3. A, B right) and wet weight ratio (Figure 3.C) also showed that denervation induced skeletal muscle atrophy could be partly reversed by both doses of blueberry extracts. Also, in histology (Figure 3, D), skeletal muscle tissue showed larger stained area of myocytes and narrower intercellular space in both blueberry groups. What's more, higher doses of blueberry extracts showed stronger protective effect. Next, we further explored whether Cx43 is involved in the protective role of blueberry extracts against denervated muscle atrophy. Cx43 expression and p-Cx43/Cx43 were upregulated in denervated skeletal muscle, while blueberry extracts downregulated the expression of Cx43 in both transcription (Figure 3. F) and translation (Figure 3.E) level and p-Cx43/Cx43 (Figure 3.E) also decreased in blueberry 200 group. However, the ratio of p-Cx43/Cx43 did not decrease significantly in blueberry 400 group.

Cx43/Akt pathway was involved in the effect of blueberry extracts on denervated muscle atrophy

Then we explored whether Akt participated in the protective effect of blueberry and if Cx43 interacted with Akt. HE staining (Figure 4.A) and immunofluorescence (Figure 4. B) showed Gap-19 upregulated Akt expression in denervated skeletal muscle tissue. To explore whether Akt was involved in the protective effects of blueberry extracts on denervated skeletal muscle, we used Western Blot analysis and found that upregulated Akt expression in blueberry groups versus denervation group (Figure 4.C). Also, relative gene expression of Akt was downregulated in denervation group while Gap-19 and blueberry extracts could reverse this change and even upregulated Akt expression versus control in transcription level (Figure 4.E). These results demonstrated that Cx43/ Akt interaction was involved in the effect of blueberry extracts on denervated muscle atrophy.

Discussion

Overall, our study indicated that blueberry extracts protects against denervation induced skeletal muscle atrophy. In addition, Cx43 was activated by denervation and aggravated the skeletal muscle atrophy, while blueberry extracts could downregulate the number and function of Cx43, which could be the target of the protective effects of blueberry extracts. Blueberry extracts and the Cx43 selective inhibitor Gap-19 reversed Akt expression inhibited by denervation in skeletal muscle cells. Therefore, this study provides proof of concept that blueberry extracts attenuates denervated skeletal muscle atrophy via regulating Cx43/Akt pathway.

Skeletal muscle denervation can be caused by acute nerve injury, chronic neuritis and many other pathological conditions. After denervation, dominated skeletal muscle started irreversible atrophy, which resulted in loss of muscle mass and function [22]. Clinically, patients suffered from reduced functional status and quality of life and, even, reduced survival caused by denervated muscle atrophy. Sciatic nerve transection can be one of the most commonly used methods to create the denervated muscle atrophy animal models. Our study operated unilateral sciatic nerve transection on C57BL/6 mice and observed related changes of ipsilateral gastrocnemius. Obvious atrophy was observed in both gross and histology.

Connexins were family of proteins that forming or connexons at cell membrane, which mediate molecules exchange between the cells[23]. And their extracellular part, called hemichannels, form gap junction channels between neighboring cells[24]. Previous research found that Cx43, a member of connexins family, critically contributes to the progression of skeletal muscle atrophy caused by denervation[6, 25, 26]. And ion channels imbalance maybe mechanism behind its harmful roles on skeletal muscle atrophy. In this study, we used Cx43 selective inhibitor Gap-19 to attenuate denervated skeletal muscle atrophy successfully, which furtherly proved that augmented Cx43 expression induced by denervation contributed to skeletal muscle atrophy. Beyond just changes in quantity of Cx43, qualitive changes such as phosphorylation could better reflect functional activation of Cx43[27]. Here, we found that p-Cx43/Cx43 increased significantly after denervation, and Gap-19 could also reduce this ratio. According to these results, we could conclude that denervation induced enhanced Cx43 quantity and quality that furtherly resulted skeletal muscle atrophy.

Blueberry is one of the most beneficial fruits that world-wide popular for its health effects. A recent clinical trial found that blueberry extracts could enhance muscular strength, power, and endurance[28]. Similarly, Sato et al.[16] found that *Montgomerym [Mont]*, a kind of blueberry cultivar, protects skeletal muscle loss induced by sex steroid deficiency. Study from Valeria et al.[29] demonstrated that raw blueberries attenuated dopaminergic denervation and partially reversed motor disorders on rats. Inspired by previous discoveries, in this study, we tried blueberry extracts on mice undergone sciatic nerve transection and found its obvious protective effects on gastrocnemius. Combined with results before, we explored the if Cx43 was involved in potential mechanism by which blueberry extracts protected against denervation induced skeletal muscle atrophy. We found that blueberry could partially reverse the increase in Cx43 and p-Cx43/Cx43 caused by denervation, which indicated that blueberry attenuated denervation induced skeletal muscle atrophy maybe by inhibiting the function of Cx43. Noticed that p-Cx43/Cx43 did not decreased significantly in blueberry 400 group, this may be due to the amount of total Cx43 had been reduced nearly 80% in blueberry 400 group, even the amount of p-Cx43 alone had been reduced significantly, the ratio decline was not remarkable.

Akt, also known as protein kinase B, plays critical roles in many cell activities. Recent research also found that Akt signaling was involved in skeletal muscle cell responses to denervation[30, 31]. Strong interaction between Akt signaling and Cx43 was found in many other diseases models[14, 19, 32, 33], and previous evidences indicated they shared mutually inhibiting relationship. In our study, we found Gap-19 enhanced Akt expression, which meant that Cx43 interacted with Akt signaling during denervation skeletal muscle atrophy. Also, we investigated if this kind of interaction was involved in the effects of blueberry on denervated skeletal muscles. After treatment of blueberry extracts, Akt expression was enhanced. Therefore, Cx43/Akt interaction played an important role in blueberry extracts-mediated protection against denervated muscle atrophy. (Figure 5.)

There are still some limitations in our study including. First, the effects of chemical compositions of blueberry extracts remain unclear, which are needed to be analyzed and confirmed to facilitate reproducibility and for further medicine research. Secondly, we only determined the effect of blueberry extracts at 14 days after denervation in mice, which does not enough for a fine-tuning of the molecular events during the process, and the effect of blueberry extracts and its regulation on Cx43/Akt interaction at other time points is still unknown. What's more, follow-up experiments are still required to determine how Cx43 and Akt signaling interacted during denervation and after treatment with blueberry extracts to more detailed level. Beside these limitations, our study could be a good evidence for potential medicinal value of blueberry extracts on denervated skeletal muscle atrophy and provided a initial attempt on exploring the mechanisms behind its protective effect. Clinical application could be expected after more follow-up research is carried out.

Conclusion

Our study demonstrates that blueberry extracts protects against denervation induced skeletal muscle atrophy by regulating Cx43 expression and activation and interacting with the AKT signaling pathway. These results suggest that blueberry extracts may be an effective treatment for denervated skeletal muscle atrohpy.

Declarations

Code availability: Not applicable

Competing interests: The authors declare no competing interests.

Ethics approval: This study was performed in full compliance with the ethics in force in our institution.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent for publication: The authors affirm that patients provided informed consent regarding publishing their data and photographs.

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Data Availability Statement: Some or all data, models, or code generated or used during the study are available in a repository or online in accordance with funder data retention policies (Provide full citations that include URLs or DOIs.).

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Figures

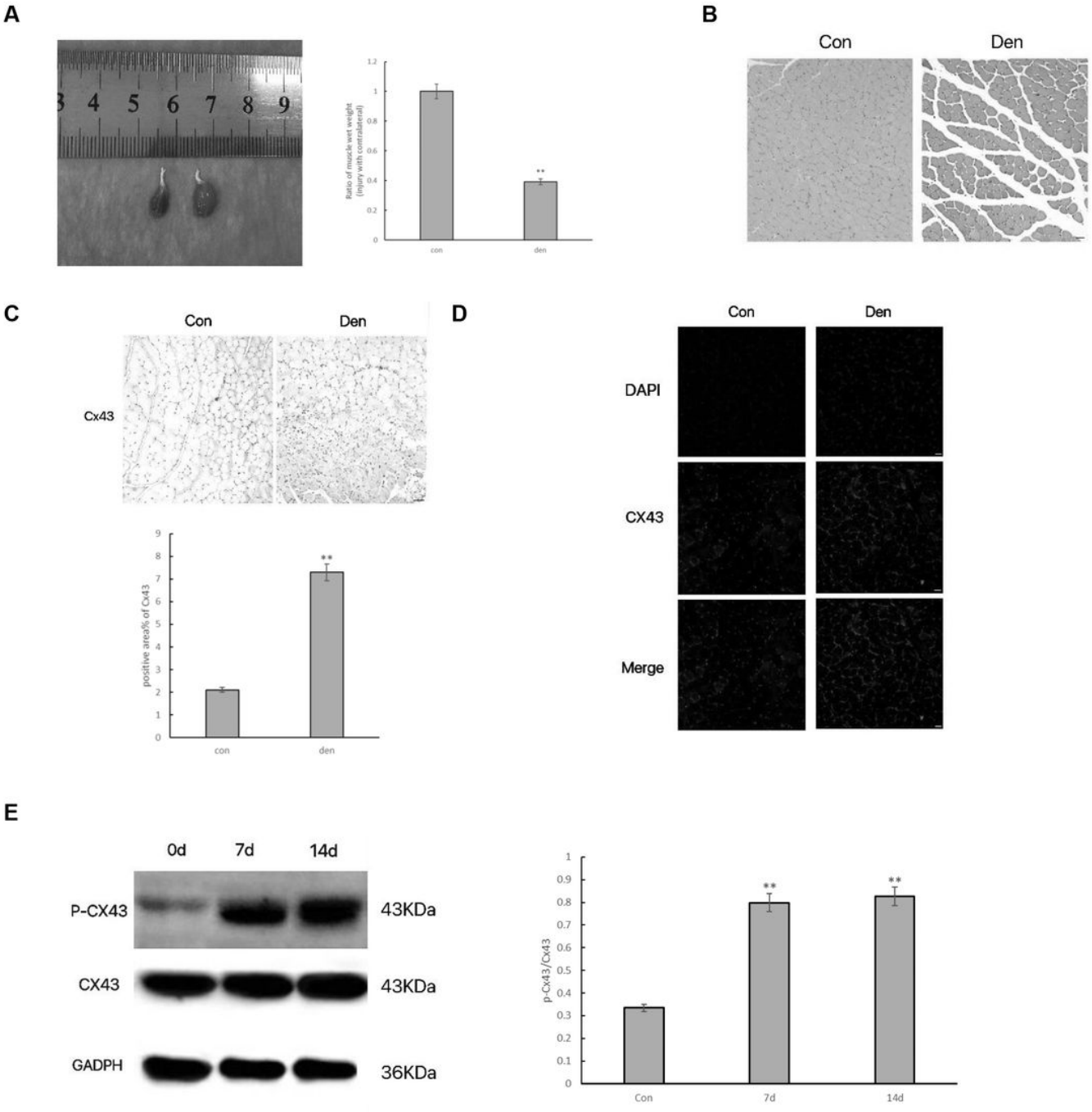

Figure 1

Denervation leads to increased expression and activated function of C x 43 in atrophied skeletal muscle. A) The decreased wet weight ratio of gastrocnemius at 14 days post denervation. B) Denervation le d to

skeletal muscle atrophy. HE staining showing the irregular arrangement o f myocytes in 14 days of denervated muscle and a smaller stained area compared to normal (non denervated) gastrocnemius muscle. C , D) Denervation leads increased expression of C x 43. Compared to control group muscle, C x 43 positive expression was increased i n the denervated muscle . E) Denervation le d to activation (phosphorylation) of C x 43. Elevated ratio of P C x 43/C x 43 from 0 to 14 days post denervation was determined by western blot. mice. Scale bar, 50 μm. * p < 0.01 versus Control.

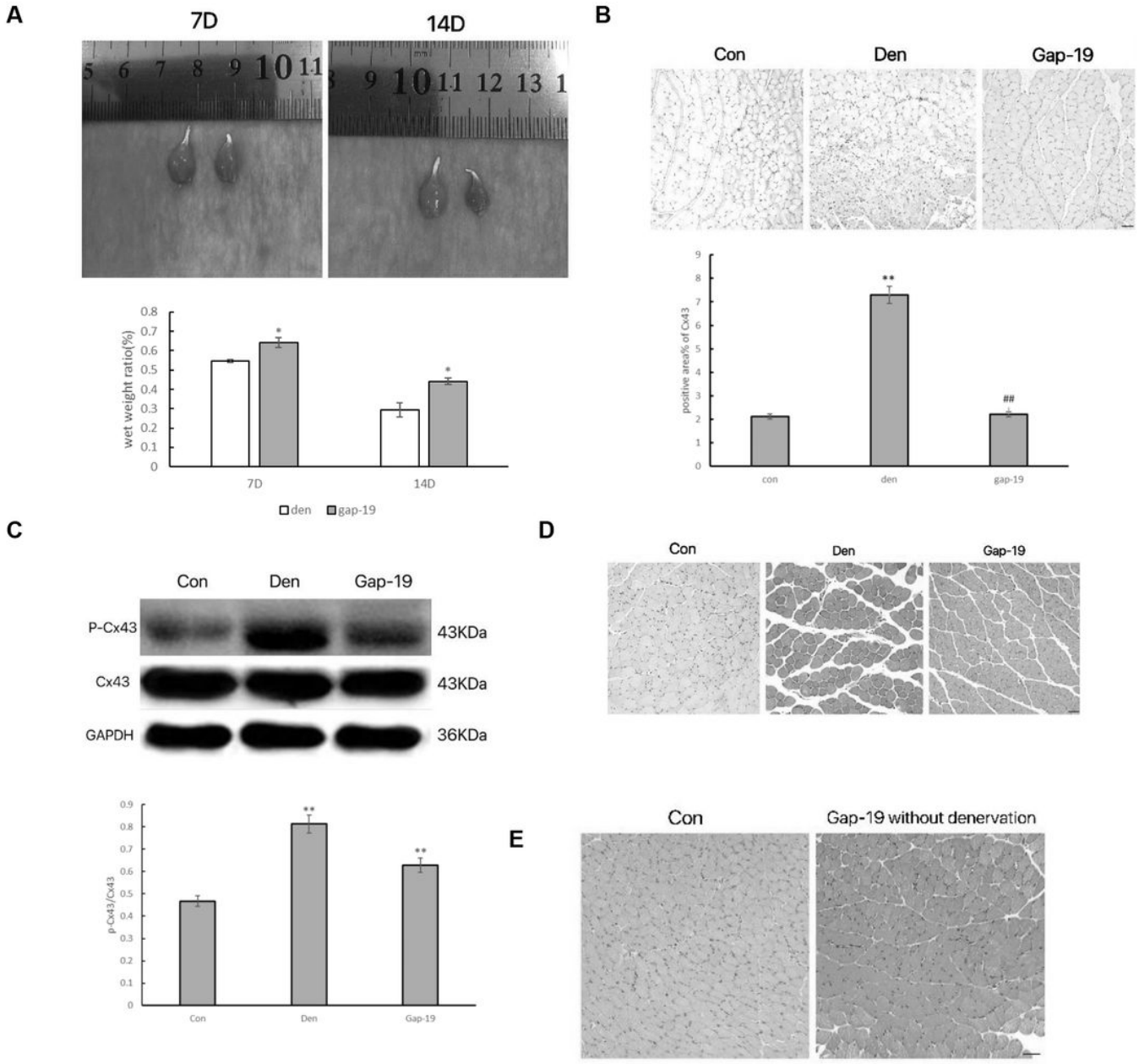

Figure 2

C x 43 blocker Gap 19 showed protective effect in denervated muscle atrophy.

A) The increased wet weight ratio of gastrocnemius in Gap 19 (at 7 and 14 days post denervation versus denervation (left) group. B) Reduced expression of Cx43 in Gap 19 group versus denervation group. C)

Gap 19 reduced the activation of C x 43. Decreased ratio of P C x 43/C x 43 in Gap 19 group versus denervation group was determined by western blot. D HE staining showed muscle atrophy was reduced by Gap 19. E) HE staining showed no significant difference between control group and control group with gap 19 injection. mice. Scale bar, 50 μm. p < 0.01 versus con p < 0.0 1 versus den. *p < 0.0 5 versus den.

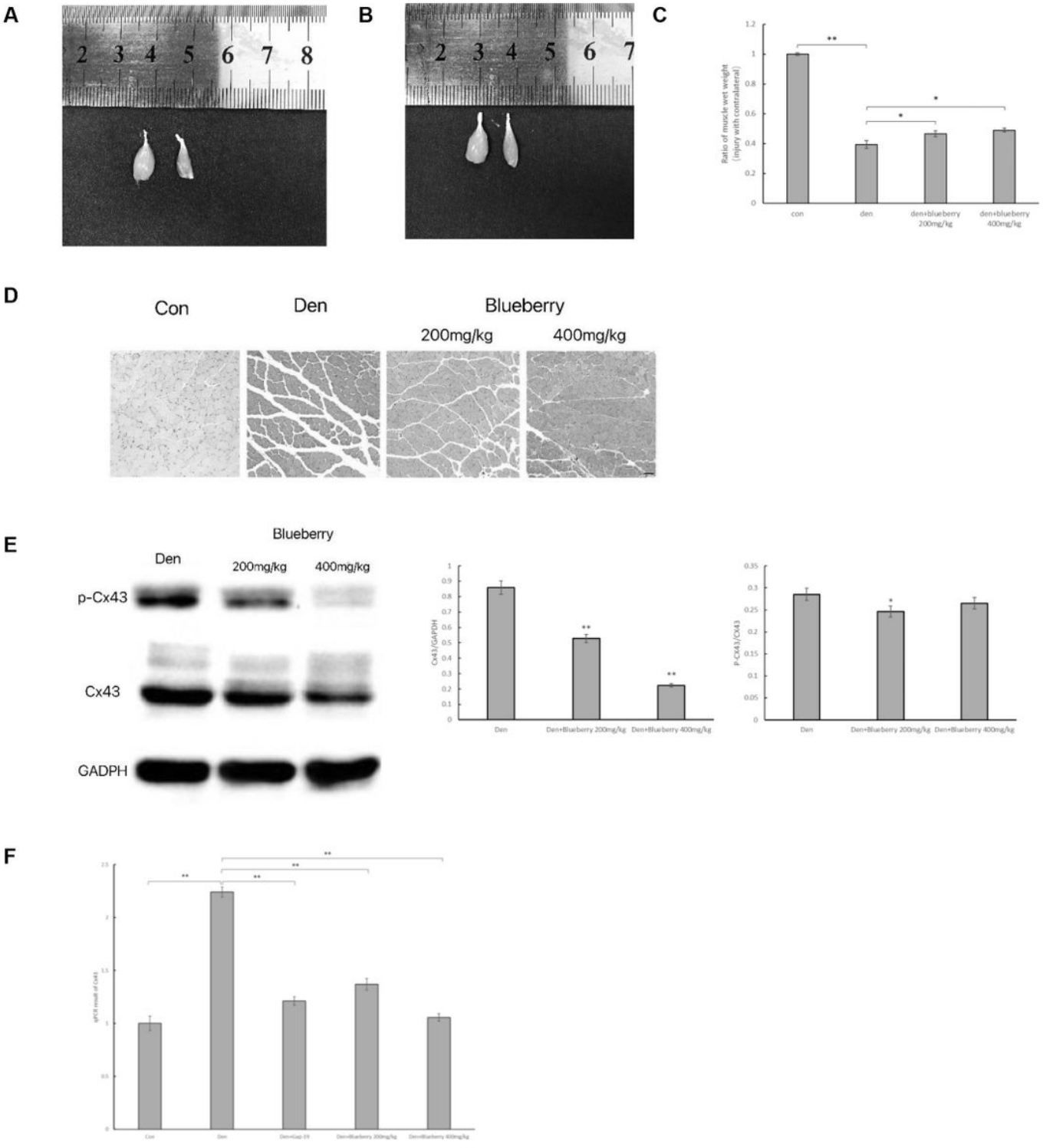

Figure 3

Blueberry extracts protected denervated muscle atrophy by reducing the function of C x 43.

A, B, C) The increased wet weight ratio of gastrocnemius in blueberry 200 (B) and 400 (C) groups at 14 days post denervation. D) Blueberry extracts protected denervated skeletal muscle atrophy. HE staining showing larger stained area of myocytes in blueberry 200 and 400 groups at 14 days of denervated muscle compared to denervated gast rocnemius muscle. E) Blueberry extracts reduced expression of C x 43. Decreased C x 43 expression in blueberry 200 and 400 groups at 14 days post denervation was determined by western blot. The ratio of p Cx43/Cx43 decreased in blueberry 200 group but not significant in blueberry 400 group. F) R elative gene expression of C x 43 was reduced by blueberry extracts. mice. Scale bar, 50 μm. *p < 0.0 5 p < 0.0 1 versus Den.

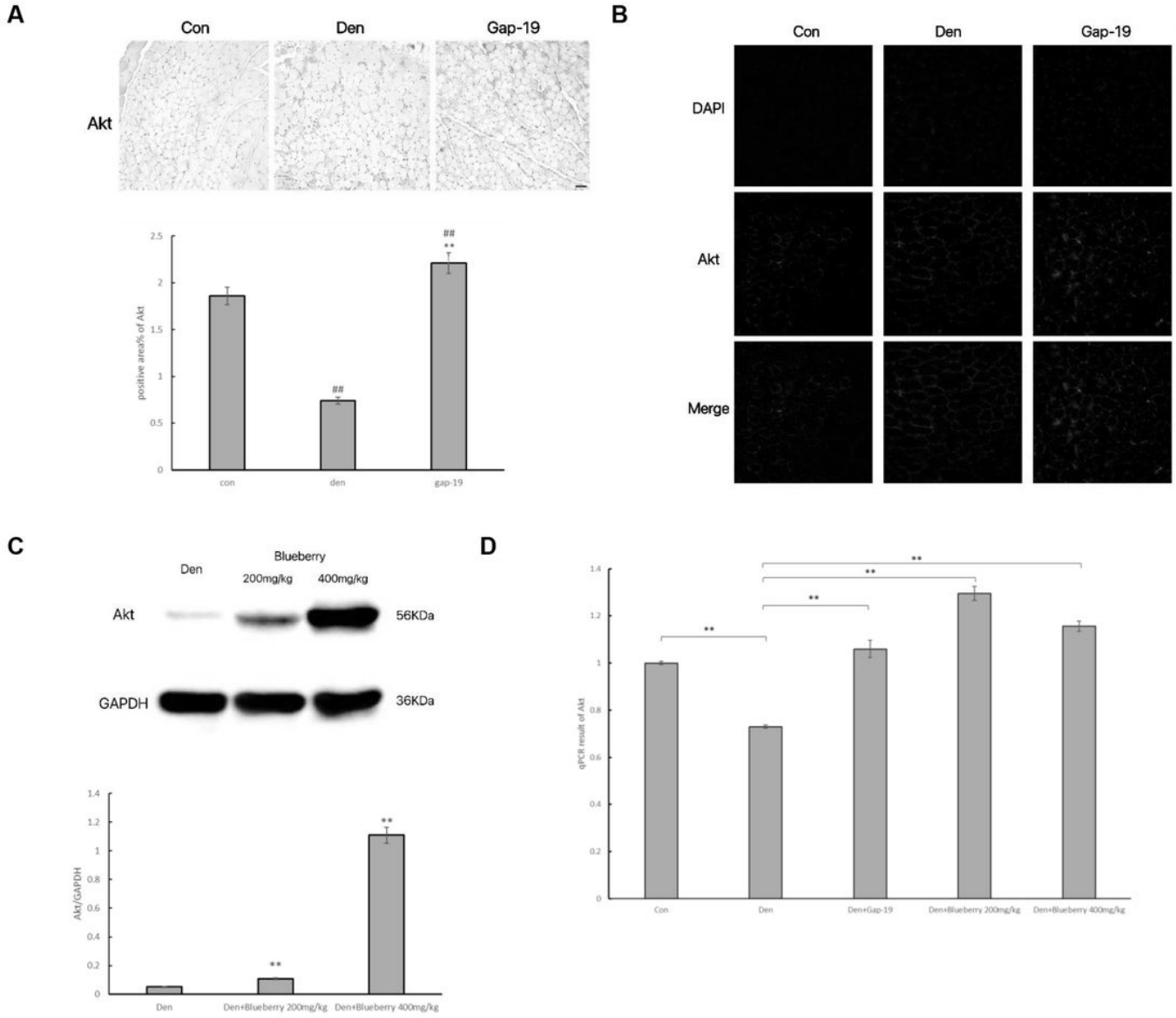

Figure 4

Blueberry extracts delayed denervated skeletal muscle atrophy via C x 43 A kt pathway. A , B) C x 43 interacted with A kt during the process of denervation induced skeletal muscle atrophy. HE staining and

immunofluorescence showed augmented A kt positive expression in Gap 19 group at 14 days post denervation. C,) Blueberry extracts enhanced expression of A kt . Elevated express ion of A kt in blueberry 200 and 400 groups at 14 days post denervation was determined by western blot. D) R elative gene expression of A kt was increased by blueberry extracts. mice. Scale bar, 50 μm. *p < 0.0 5 **p < 0.01 versus Den ##p < 0.01 versus C on

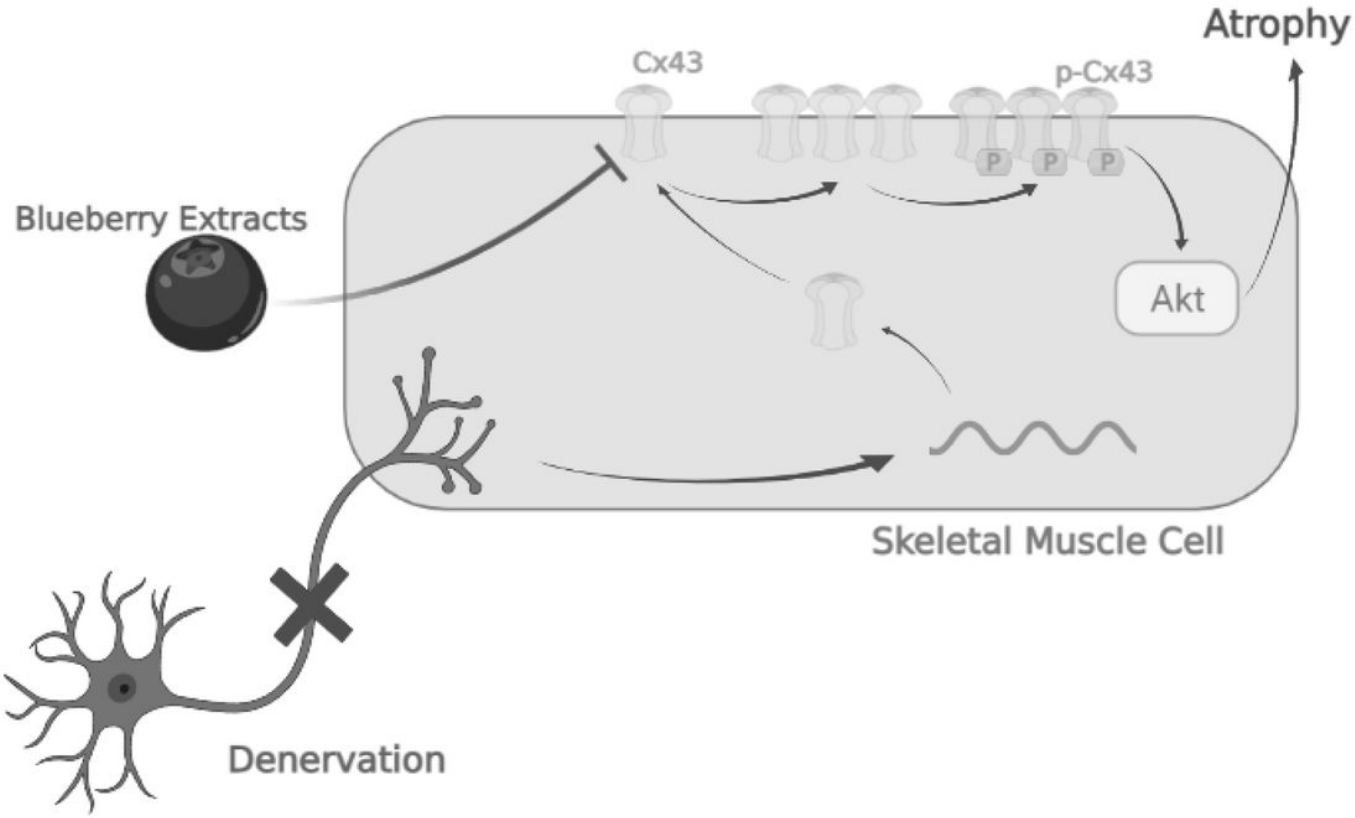

Figure 5

A scheme for blueberry extracts attenuates denervated skeletal muscle atrophy via regulating C x 43 /Akt pathway