Pulsed electromagnetic fields alleviate streptozotocin-induced diabetic muscle atrophy

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Abstract. Diabetic muscle atrophy causes a reduction of skeletal muscle size and strength, which affects normal daily activities. However, pulsed electromagnetic fields (PEMFs) can retard the atrophy of type II fibers (ActRIIB) in denervated muscles. Therefore, the purpose of the present study was to determine whether PEMFs can alleviate streptozotocin (STZ)-induced diabetic muscle atrophy. To do this, 40 Sprague-Dawley (SD) rats were randomly divided into four groups (n=10 per group): The normal control group (NC; nondiabetic rats without treatment); the diabetic mellitus group (DM; STZ-induced rats without treatment); the diabetic insulin-treated group (DT; diabetic rats on insulin treatment, 6-8 U/d twice a day for 6 weeks) as a positive control; and the diabetic PEMFs therapy group (DP; diabetic rats with PEMFs exposure treatment, 15 Hz, 1.46 mT, 30 min/day for 6 weeks). Body weight, muscle strength, muscle mass and serum insulin level were significantly increased in the DP group compared with the DM group. PEMFs also decreased the blood glucose level and altered the activity of metabolic enzymes. PEMFs significantly increased the cross-sectional area of muscle fiber. In addition, PEMFs significantly activated protein kinase B (Akt) and mammalian target of rapamycin (mTOR), and inhibited the activity of myostatin (MSTN), ActRIIB and forkhead box protein O1 (FoxO1) compared with the DM group. Thus indicating that the Akt/mTOR and Akt/FoxO1 signaling pathways may be involved in the promotion of STZ-induced diabetic muscle atrophy by PEMFs. The results of the present study suggested that PEMFs stimulation may alleviate diabetic muscle atrophy in the STZ model, and that this is associated with alterations in multiple signaling pathways in which MSTN may be an integral factor. MSTN-associated signaling pathways may provide therapeutic targets to attenuate severe diabetic muscle wasting.

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by the destruction of pancreatic β cells (1). It affects more than 35 million people worldwide. Reduced muscle mass and myofiber size as well as poor metabolic control in T1DM can result from impaired muscle growth and development (2-4). Diabetic muscle atrophy, a clinical condition, means that the size and strength of skeletal muscles are reduced (5,6), affecting normal daily activities. Protein synthesis or protein degradation is one of the important reasons for diabetic muscle atrophy. Some signaling pathways-such as the activated protein kinase B (Akt)/ rapamycin (mTOR) and Akt/ forkhead box protein O1 (FoxO1) pathways-may be linked to muscle loss in diabetic muscle atrophy (7,8). Myostatin (MSTN), a member of the transforming growth factor β family, is a negative regulator of skeletal muscle growth (9). The negative effect of MSTN may occur via activation of the PI3K/Akt signaling pathway (10).

Growing evidence suggests that pulsed electromagnetic fields (PEMFs) can serve as safe alternatives to drug-based therapies for the treatment of some diseases. In recent years, PEMFs have been widely used in the treatment of osteoporosis, fracture, and other conditions and have produced good therapeutic results. It has been reported, for example, that the atrophy of type II fibers in denervated muscle was retarded by magnetic stimulation (11). However, whether PEMFs can alleviate streptozotocin (STZ)-induced diabetic muscle atrophy has not been investigated.

Accordingly, we examined the effects of PEMFs on STZ-induced diabetic muscle atrophy by evaluating muscle strength, mass, and cross-sectional area of muscle fiber. Furthermore, possible molecular mechanisms were explored through analyses of the gene and protein expression of MSTN, Akt, activin type II receptor (ActRIIB), mTOR, and FoxO1.

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Materials and methods

Animals. This study was conducted with the approval of the ethics committee of Shaanxi Normal University in Shaanxi, China, and was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH; publication no. 85-23, revised 1996).

Healthy male Sprague-Dawley (SD) rats (200±20 g) were obtained from the Laboratory Animal Breeding and Research Center of Xi'an Jiaotong University (Xi'an, China). They were housed in a temperature and humidity controlled room (22±2℃, 60±5% humidity and 12-hour light/dark cycle). After 5 days of acclimation, the rats were randomly divided into a normal control group (NC; n=10) and a T1DM model group (n=50). Experimental T1DM was induced via a peritoneal injection of STZ (Sigma, St. Louis, MO, USA) and 60 mg/kg, 0.1 mol/l sodium citrate buffer, pH=4.5). An equal volume of buffer was injected into the control rats at the same time. The blood glucose levels in tail vein blood samples were measured on the 1st, 3rd, 7th, and 10th days following injection. The rats with blood glucose levels greater or equal to 16.7 mmol/l (300 mg/dl) were considered diabetic. The diabetic rats were then randomly assigned to the DM group (n=10), the diabetic insulin-treated group (DT; n=10) as a positive control, and the diabetic PEMFs therapy group (DP, n=10). The DT group was treated with insulin (6-8 U/d twice a day for 6 weeks; Sigma), and the DP group was exposed to PEMFs (15 Hz, 1.46 mT, 30 min/d for 6 weeks).

PEMFs treatment. The PEMFs exposure system was composed of coils and a pulsed signal generator. There were three identical coils 800 mm in diameter connected in series and placed coaxially 190 mm apart. Each coil was made up of enameled coated copper wire 0.8 mm in diameter. The number of turns on the central coil was 266, and the number of turns on the two outside coils was 500. The pulsed signal apparatus generated an open circuit waveform composed of a pulsed burst (burst width, 5.16 ms; burst wait, 61.4 ms; pulse width, 0.171 ms; pulse wait, 0.171 ms) repeated at 15.08 Hz. The rats in the DP group were put in the center of coils.

Oral glucose tolerance tests. Oral glucose load was administered at 2 g/kg of body weight after overnight fasting. Glucose levels were measured from tail bleeds by cutting off a small part of the tail at 0, 30, 60, 90, and 120 min after glucose administration.

Grip strength. During the final week, forelimb grip strength was measured as maximum tensile force using a rat grip strength meter (YLS-13A; Huaibei Zhenghua Bioinstrumentation Co., Ltd., Anhui, China). Rats were tested 3 times in succession without rest and the results of the 3 tests were averaged for each rat.

Weight and sample preparation. After 6 weeks of treatment, the rats were euthanized with an overdose of diethyl ether and the final body weights were recorded. Blood was collected and centrifuged in order to obtain the serum fractions. Serum was stored at -80℃ for further analysis. After the animals were sacrificed, each quadriceps femoris was harvested and weighed, then immediately stored in liquid nitrogen at -80℃ for reverse transcription-polymerase chain reaction and western blot analysis.

Biochemical analysis. Blood glucose was measured using the eBsensor Blood Glucose Monitor (Visgeneer Inc., Hsinchu, Taiwan). Serum insulin levels were measured using a commercial enzyme linked immunosorbent assay (ELISA; EMT Millipore, Billerica, MA, USA). The activity levels of succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) in the quadriceps femoris were analyzed with standard colorimetric tests using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocols provided by the manufacturer.

Hematoxylin and eosin staining. The quadriceps femoris was cut into 1mmx1mmx1 mm size and then placed in 2.5% glutaraldehyde to fix for 24 h at 4℃. After washing, the specimens suffered the processes of dehydration and transparency, then were embedded into paraffin and cut into 50 nm thickness. Subsequently, the paraffin sections undergo the processes of deparaffinage and dehydration, then were stained with hematoxylin for 8 min. After washing, sections were placed into the eosin counterstain for 5 min. After resinene mount, sections were visualized under the optical microscope (Olympus Corp., Tokyo, Japan).

Western blot analysis. The removed quadriceps femoris muscles were homogenized in ice-cold lysis buffer. Protein concentrations were quantified with the BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein concentrations were determined and equal amounts of sample were uploaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. After electrophoresis and separation, samples were transferred onto nitrocellulose membranes. The immunoblots were incubated with primary antibodies overnight at 4℃, followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. Blots were visualized with ECL-plus reagent, and the results were quantified with Lab Image Version 2.7.1. The primary antibodies used were as follows: the expression of MSTN [EPR4567(2), ab124721], ActRIIB (EPR10739, ab180185) from Abcam (Cambridge, UK); AKT (Rabbit Ab 9272S); phospho-AKT (S473 9271S); mTOR (Rabbit 2972S), phospho-mTOR (S2448, 2971P), FoxO1 (C29H4, Rabbit 2880P), and phospho-FoxO1 (S256, 9461P) from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Statistical analysis. Statistical analysis was performed using SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance was employed to evaluate the differences between the three groups. Once a significant difference was detected, Tukey's multiple comparisons test was used to determine the significance between any two groups. $P<0.05$ was considered to indicate a statistically significant difference. The results are expressed as the mean ± standard deviation.

Results

Changes of blood glucose and oral glucose tolerance tests before and after model establishment. Blood glucose levels
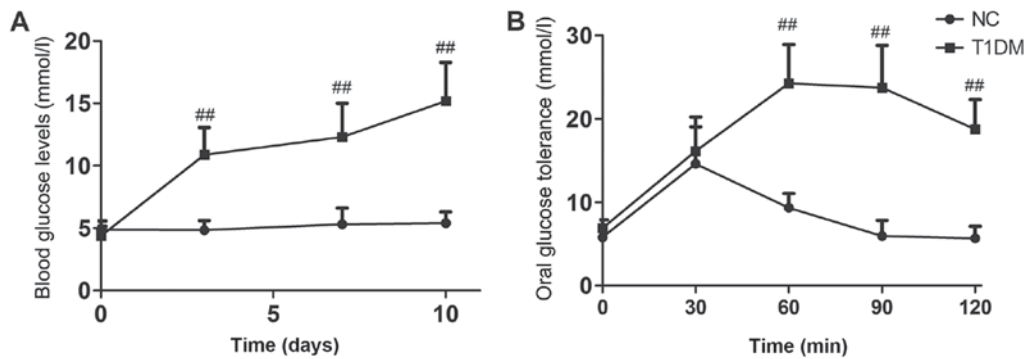
Figure 1. Comparison of (A) blood glucose levels and (B) oral glucose tolerance in the NC and T1DM groups prior to and following model establishment. Data are expressed as mean ± standard deviation (n=8/group). ##P<0.01 vs. NC. T1DM, type 1 diabetes mellitus; NC, normal control.
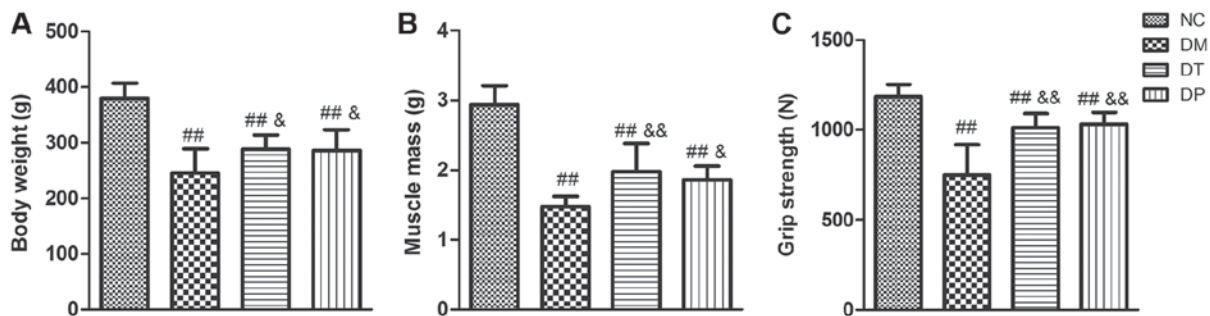

Figure 2. Effects of pulsed electromagnetic fields on (A) serum body weight, (B) muscle weight and (C) muscle strength. Data are expressed as the mean ± standard deviation (n=8/group). ##P<0.01 vs. NC; &P<0.05 and &&P<0.01 vs. DM. NC, normal control; DM, diabetic mellitus group; DT, diabetic insulin-treated group; DP, diabetic pulsed electromagnetic fields-therapy group.

in diabetic mice remained extremely high throughout the experiment (P<0.01, Fig. 1A). Oral glucose tolerance tests were performed after 6 weeks of treatment. Rats in the DB group showed impaired glucose tolerance compared with those in the NC group (P<0.01, Fig. 1B). The T1DM model was successfully established.

PEMFs increased body weight, muscle weight and muscle strength. After 6 weeks of PEMFs treatment, the body weight, quadriceps wet weight, and grip strength of the rats were measured (as shown in Fig. 2). Compared with the NC group, the body weight, quadriceps wet weight, and grip strength of the DM group were significantly decreased (P<0.01, P<0.01, and P<0.01, respectively). Although the body weight, muscle weight, and grip strength in the DT and DF groups were significantly lower than those in the NC group (P<0.01, P<0.01, and P<0.01, respectively), insulin treatment statistically significantly affected the loss of body weight, muscle weight, and grip strength (P<0.01, P<0.01, and P<0.01, respectively) in this group compared with the DM group. Furthermore, the body weight, muscle weight, and grip strength in the DF group were significantly increased (P<0.05, P<0.05, and P<0.01, respectively) compared with the DM group.

PEMFs decreased blood glucose level and increased insulin level. Before the animals were sacrificed, the blood glucose levels were detected. The blood glucose levels of the DM group were significantly higher than those of the NC group (P<0.01); insulin and PEMFs treatment caused a decrease

in blood glucose levels compared with the DM group (P<0.01) (Fig. 3A). Moreover, insulin levels were significantly decreased in the DM group (P<0.01). Insulin and PEMFs treatment caused a significant decrease in blood glucose (P<0.01 and P<0.01, respectively) and a significant increase in serum insulin compared with the DM group (P<0.01 and P<0.05, respectively) (Fig. 3B).

PEMFs altered the activities of metabolic enzymes. Fig. 4 shows the effect of PEMFs on activity levels of key muscle metabolism enzymes in the several experimental groups. In the diabetic rats, activity levels of SDH (Fig. 4A) and MDH (Fig. 4B) were significantly decreased (P<0.01 and P<0.01, respectively) as compared with the normal, nondiabetic rats. Insulin treatment statistically significantly increased the SDH and MDH levels (P<0.05 and P<0.05, respectively). PEMFs treatment resulted in significantly higher SDH and MDH activity levels (P<0.05 and P<0.05, respectively) as compared with the untreated diabetic rats.

Effect of PEMFs on cross-sectional area of muscle fiber. As shown in Fig. 5, the cross-sectional area of muscle fiber in DM group was significantly decreased (P<0.01) as compared with the normal. The cross-sectional area of muscle fiber in DT and DP groups were significantly increased (P<0.01 and P<0.05, respectively) as compared with the DM group.

Effect of PEMFs on MSTN/Akt/mTOR/FoxO signaling pathways. To investigate the molecular mechanism of the effect of PEMFs
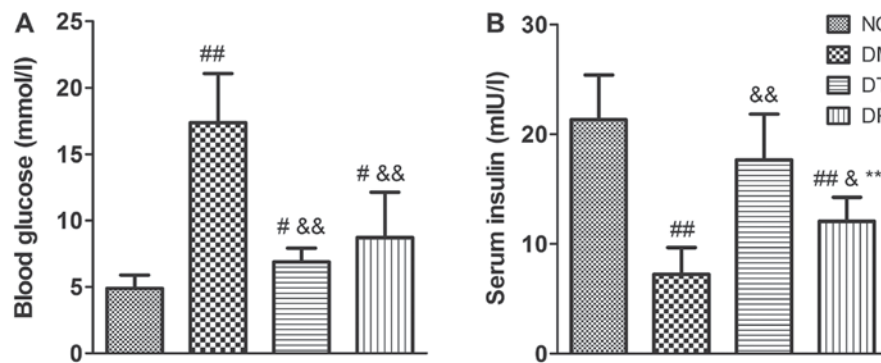
Figure 3. Effects of pulsed electromagnetic fields on (A) blood glucose level and (B) increased insulin level. Data are expressed as the mean ± standard deviation (n=8/group). #P<0.05 and ##P<0.01 vs. NC; &P<0.05 and &&P<0.01 vs. DM; **P<0.01 vs. DT. NC, normal control; DM, diabetic mellitus group; DT, diabetic insulin-treated group; DP, diabetic pulsed electromagnetic fields-therapy group.
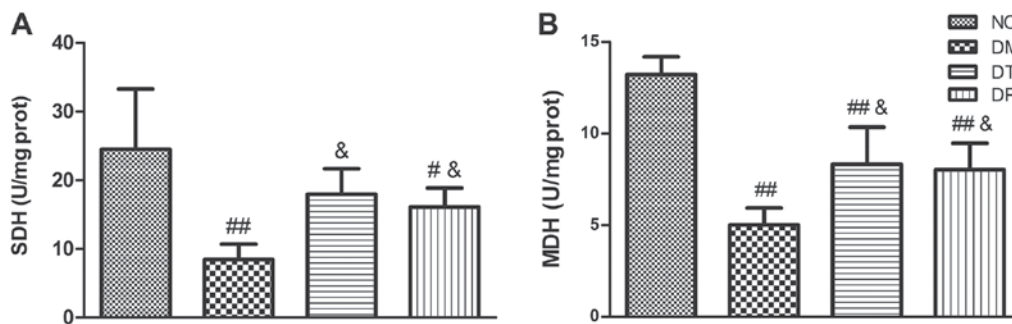

Figure 4. Effects of pulsed electromagnetic fields on the levels of (A) SDH and (B) MDH. Data are expressed as the mean ± standard deviation (n=8/group). #P<0.05 and ##P<0.01 vs. NC; &P<0.05 vs. DM. SDH, succinate dehydrogenase; MDH, malate dehydrogenase; NC, normal control; DM, diabetic mellitus group; DT, diabetic insulin-treated group; DP, diabetic pulsed electromagnetic fields-therapy group.

on reversal of muscle atrophy in diabetic rats, we explored the MSTN, Akt/mTOR, and FoxO3 signaling pathways, which are closely related to muscle protein synthesis and subject to degradation by Western blotting. The results showed that STZ markedly decreased p-Akt (P<0.01) (Fig. 6D), mTOR (P<0.01) (Fig. 6E), and p-mTOR (P<0.01) (Fig. 6F) while it increased MSTN (P<0.01) (Fig. 6A), ActRIIB (P<0.01) (Fig. 6B), FoxO1 (P<0.01) (Fig. 6G), and p-FoxO1 (P<0.01) (Fig. 6H) as compared with the nondiabetic rats. Insulin and PEMF increased p-Akt (P<0.01 and P<0.05, respectively) (Fig. 6D), mTOR (P<0.01 and P<0.01, respectively) (Fig. 6E), p-mTOR (P<.01 and P<0.01, respectively) (Fig. 6F) whereas it reduced MSTN (P<0.01) (Fig. 6A), ActRIIB (P<0.01 and P<0.01, respectively) (Fig. 6B), FoxO1 (P<0.01 and P<0.05, respectively) (Fig. 6G), and p-FoxO1 (P<0.01 and P<0.01, respectively) (Fig. 6H) as compared with the DM group. Akt expression did not change significantly among the groups (Fig. 6C). These results suggest that PEMFs can not only increase muscle protein synthesis by activating Akt/mTOR but also inhibit protein degradation by inactivating MSTN and FoxO1 protein.

Discussion

Because of the absence of insulin in T1DM, blood glucose levels rise dramatically when glucose cannot be taken up into the major insulin-sensitive tissue-skeletal muscle.

Individuals with T1DM are at a high risk of muscle atrophy. This means that many of these individuals' lives are lost, despite insulin therapies (12). PEMFs are dynamic and able to penetrate all the way through the body, thus having many effects. It has been proved that PEMFs can promote the proliferation and differentiation of C2C12 myoblasts (13,14) and facilitate tendon healing (15). Therefore, we sought to investigate the effects and potential mechanisms of PEMFs on STZ-induced diabetic muscle atrophy. Our findings indicate that PEMFs alleviate diabetic myopathy by increasing protein synthesis and decreasing protein degradation in MSTN-associated signaling pathways.

Increase in blood glucose, appetite, urination, and thirst during the experimental period confirmed the induction of T1DM. The insufficient insulin therapy could cause the development of muscle function in individuals with T1DM. Furthermore, glycemic control is directly related to muscle metabolism and could be an important determinant of muscle force and power in T1DM (16). In accordance with earlier studies (17-19), our experimental rats with T1DM were characterized by increased blood glucose, appetite, urination, and thirst and by decreased blood insulin, weight mass, muscle mass, and grip strength compared with the NC group rats. Furthermore, compared with DM, PEMFs significantly increased strength and mass of quadriceps muscle, and cross-sectional area of quadriceps muscle fiber. These results indicate that PEMFs can improve the muscle atrophy induced by STZ.

It is well known that SDH and MDH are marker enzymes in the metabolism of muscle (20,21). Decreased SDH and MDH activity in patients with diabetes has been reported (22-25).
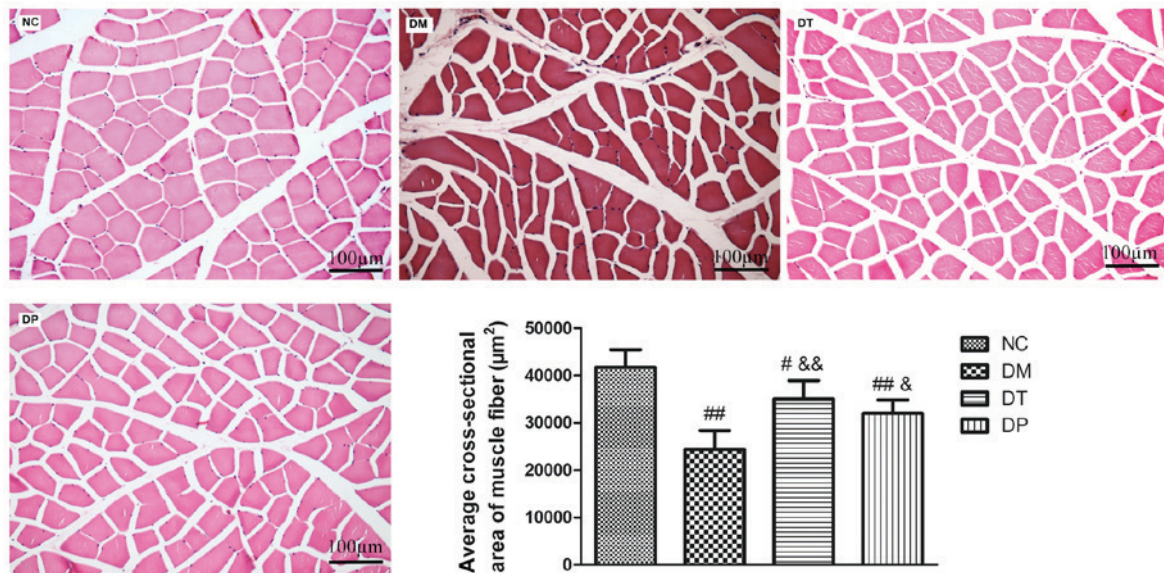
Figure 5. Effect of pulsed electromagnetic fields on cross-sectional area of muscle fiber. Data are expressed the mean ± standard deviation (n=8/group). Each sample represents ten different fields of view, images were taken at magnification x200 under a light microscope (scale bars, 100 μm). [#]P<0.05 and [##]P<0.01 vs. NC; [&]P<0.05 and [&&]P<0.01 vs. DM. NC, normal control; DM, diabetic mellitus group; DT, diabetic insulin-treated group; DP, diabetic pulsed electromagnetic fields-therapy group.
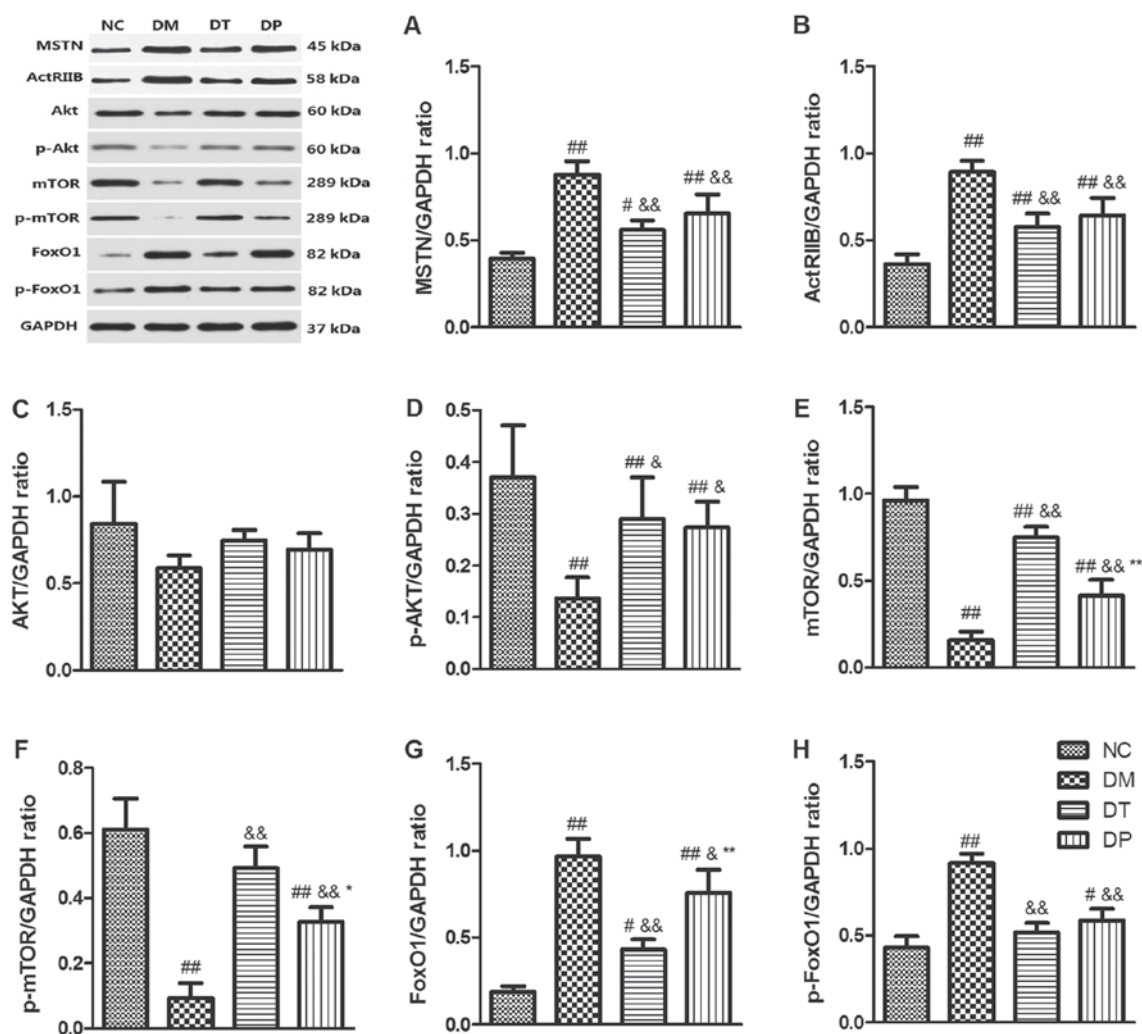

Figure 6. Effects of pulsed electromagnetic fields on the protein expressions of (A) MSTN, (B) ActRIIB, (C) Akt, (D) p-Akt, (E) mTOR, (F) p-mTOR, (G) FoxO1 and (H) p-FoxO1 in quadriceps. Data are expressed as the mean ± standard deviation. [#]P<0.05 and [##]P<0.01 vs. NC; [&]P<0.05 and [&&]P<0.01 vs. DM; [*]P<0.05 and [**]P<0.01 vs. DT. NC, normal control; DM, diabetic mellitus group; DT, diabetic insulin-treated group; DP, diabetic pulsed electromagnetic fields-therapy group; MSTN, myostatin; ActRIIB, activin type II receptor; Akt, protein kinase B; mTOR, mammalian target of rapamycin; p-, phosphorylated; FoxO1, forkhead box protein O1.

In the current study, SDH and MDH activity was decreased in the muscles of diabetic rats. These results are in line with previous studies. The observed increase in the activity of SDH and MDH in the quadriceps femoris muscles of the diabetic rats was significantly enhanced by PEMFs therapy, indicating that PEMFs contributed to increasing the metabolic capacity of skeletal muscle by alleviating STZ-induced diabetic muscle atrophy.

MSTN is a potent negative regulator of skeletal muscle mass as demonstrated by the hypermuscularity caused by its inactivation (26). MSTN is expressed in skeletal muscle predominantly, and the muscle mass increases significantly if its gene is disrupted (9). It has been demonstrated that MSTN can activate the TGFβ2/activin type II receptor (ActRIIB) and then activate the downstream signaling pathway (27,28). Animals with STZ-induced T1DM that were treated with follistatin (an inhibitor of MSTN) demonstrated improvement in the regenerative capacity of skeletal muscle (29), and elevations in MSTN expression have been observed in STZ-induced T1DM (7,30). In accordance with earlier studies, our results also pointed to a significant increase in MSTN expression in the animals with STZ-induced T1DM. PEMFs significantly inhibited mRNA and protein expression of MSTN and ActRIIB compared with DM. These results indicate that inhibition of MSTN may play a role in PEMFs promotion of STZ-induced diabetic muscle atrophy.

Muscle mass depends on a homeostatic balance between protein synthesis and degradation. It is believed that the Akt-mTOR pathway is the principal signaling protein cascade regulating protein synthesis (31,32). Akt is a key regulator of several signaling pathways associated with skeletal muscle homeostasis, and its major direct target downstream is the mammalian target of mTOR kinase (33). The mTOR blocker can inhibit muscle hypertrophy (34). Furthermore, inhibition of the Akt/mTOR pathway can lead to muscle atrophy (35). Rodriguez *et al* (36) have reported that MSTN negatively regulates the activity of the Akt pathway, which promotes protein synthesis.

Moreover, muscle-specific FoxO1 overexpression in mice has been linked to muscle atrophy (37), and Akt can control the activation of FoxO transcription factors (38). In the present study, STZ enhanced the activity of Akt and mTOR and inhibited FoxO1 activity and then induced skeletal muscle hypertrophy (7,39). Some findings suggest that PEMFs exposure might function in a manner analogous to soluble growth factors by activating a unique set of signaling pathways, including the Akt/mTOR pathway (40). We found that PEMFs significantly activated Akt and mTOR and inhibited the activity of MSTN, ActRIIB, and FoxO1 compared with DM. This means that both the Akt/mTOR and Akt/FoxO1 signaling pathways may be involved in the alleviation of STZ-induced diabetic muscle atrophy by PEMFs.

In terms of broadness, future studies should investigate the mechanistic links between the proteins identified in our study and muscle atrophy in various diabetic models and investigate methods of inhibiting or knocking down elements of the MSTN pathway. In terms of depth, changes in vascular lesions and nerve evoked potentials as a more in-depth discussion of electromagnetic field effects on diabetic muscle atrophy, which is the focus of our next study. We will be ready to cooperate with electrophysiology laboratories to research this issue.

Our results show that PEMFs stimulation can alleviate diabetic muscle atrophy in an STZ model in association with the alteration of multiple signaling pathways, wherein MSTN may be an important factor. MSTN-associated signaling pathways may provide therapeutic targets for the attenuation of severe diabetic muscle wasting.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LT and SA conceived the study. JY, BY, XF, LS and YK performed the experiments, and collected and analyzed the data. JY and LS prepared the manuscript. XF revised the manuscript and all authors edited the manuscript. All authors contributed to the writing of the manuscript.

Ethics approval and consent to participate

The present study was conducted with the approval of the Ethics Committee of Shaanxi Normal University (Shaanxi, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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