The effects of the conditioned medium of estradiol treated mesenchymal stem cells on the structure and function of skeletal muscles in the rat model of type 2 diabetes

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Abstract

Purpose: Diabetes affects various organs, including skeletal muscles, due to high blood sugar levels. In type 2 diabetes, muscle cell damage impairs function, impacting daily activities and quality of life. Currently, there are no drugs available to treat muscle wasting. However, research has demonstrated the potential of stem cells and the conditioned medium they produce in managing or treating complications associated with certain diseases. The aim of this study was to investigate the effect of conditional medium of bone marrow-derived mesenchymal stem cells treated with estradiol on skeletal muscle structure and function in a diabetic rat model based on behavioral, gene and tissue expression changes.

Method: In this study, 40 male Wistar rats were divided into four groups: control, diabetic, diabetic receiving conditioned medium, and diabetic receiving conditioned medium from estradiol-treated cells. The control and diabetic groups received normal saline. Each group received intraperitoneal injections of 100 µl solutions three times during the treatment period (on the 7th, 21st, and 35th days post-diabetes induction). Behavioral tests were conducted 42 days after the initial injection. The right gastrocnemius muscle was collected for gene expression analysis (PI3K, Akt, Atrogin-1), total antioxidant capacity, and malondialdehyde production. The left muscle was preserved for immunohistochemical studies of Bax, Bcl2, and Caspase-3.

Results: The study found that diabetes significantly decreased PI3K/Akt signaling pathway gene levels, and total antioxidant capacity (TAC), while increasing blood sugar levels, malondialdehyde, apoptotic protein expression, Atrogin-1 gene expression, and muscle tissue damage.

Conclusion: Treatment with conditioned medium, with or without estradiol, improved conditions in diabetic rats.

Keywords: diabetes, stem cells, conditioned medium, estradiol, muscle

1. Introduction

Diabetes mellitus is a disease that progresses over time and is characterized by high blood sugar levels. The most common form of diabetes is Type 2 diabetes, which accounts for 85-95% of diabetes cases. While this disease can remain undiagnosed for years, as blood sugar levels rise, specific symptoms like overeating (polyphagia), excessive urination (polyuria), and excessive drinking (polydipsia) may develop.

Microvascular complication such as retinopathy, nephropathy, neuropathy, and myopathy and macrovascular complications such as coronary artery disease, peripheral vascular disease, and cerebrovascular disease are among the long-term effects of type 2 diabetes. One of the significant aspects of type 2 diabetes is insulin resistance in the liver, fat tissue, and skeletal muscle. Type 2 diabetes has been recognized as a cause of atrophy and loss of skeletal muscle fibers in recent years [1]. Skeletal muscle constitutes up to 40% of the human body mass and is the most important glucose absorption system. Insulin action is largely dependent on the skeletal muscle system. Insulin dysfunction can lead to various changes in skeletal muscle, such as functional, structural, and metabolic changes. An atrophic process has been shown to occur in diabetic skeletal muscle, resulting in a decrease in muscle mass and a change in muscle fiber. In addition to the structural changes, there are functional changes that include a decrease in muscle strength and endurance capacity [2]. Additionally, the muscles of certain diabetic rodent models have elevated apoptosis markers such as P53, caspase-3, and Bax/Bcl2 ratio compared to the control muscles [3]. The characteristics of skeletal muscle mass are influenced by physiological and pathological conditions. An increase in muscle mass and fiber size, i.e. muscle growth or hypertrophy, during growth and in response to strength training or anabolic hormonal stimulation (testosterone or adrenergic agonists) and a decrease in muscle mass i.e. muscle atrophy due to aging, starvation, cancer, diabetes, bed rest, loss of neural input (motor neuron disease) or catabolic hormonal stimulation (corticosteroids) occur [4]. The balance between protein synthesis and degradation is crucial for maintaining muscle mass. An increase in protein synthesis or a decrease in protein degradation can lead to an increase in muscle mass; while the decrease in muscle mass can occur as a result of a decrease in protein synthesis or an increase in protein degradation [4]. Muscle growth is positively regulated by the insulin-like growth factor-1 (IGF1)- phosphoinositide-3-kinase-Akt/protein kinase B (Akt/PKB)-mammalian target of rapamycin (mTOR) pathway. In short, IGF1 signaling commences when the IGF1 ligand binds to its receptor, which then attracts IRS1. IRS1 in turn, it activates PI3K, which produces phosphatidyl-inositol 4,5-bisphosphate (PIP2) via phosphorylation of phosphatidyl-inositol 3,4,5, -triphosphate (PIP3), PIP3 activates Akt proteins. The direct activation and phosphorylation of mTOR by Akt, a serine-threonine protein kinase, leads to an enhancement in protein synthesis and cell growth. Akt also prevents the transcription of the main mediators of skeletal muscle atrophy (MuRF1 and MAFbx also called Atrogin) by phosphorylating and preventing the nuclear transfer of FoxO family transcription factors. Increased activation of the IGF1-Akt-mTOR signaling pathway is a major factor in increasing muscle mass [4]. In various diseases, such as sarcopenia, studies have shown that treadmill exercises, resistance exercise, electrical stimulation, and chemical therapy can help rebuild muscles [5]. Cell therapy can be utilized to repair muscles, according to emerging evidence. Different cell populations, such as mesenchymal stem cells derived from bone marrow, have been employed for this purpose [6]. Stem cells, which have the potential for renewal and differentiation, are crucial for different stages of tissue growth and repair [7]. Stem cells are classified into embryonic and adult types depending on their origin. Mature stem cells are the current type of stem cells used in cell therapy [8]. These cells include hematopoietic stem cells (HSC), endothelial stem cells (EPC), mesenchymal stem cells (MSC), and organ-specific stem cells [9].

Existing treatments are not able to treat type 2 diabetes [10]. The use of mesenchymal stem cells (MSCs) has been shown to have therapeutic effects on blood sugar control [11]. The duration of effect and useful life of MSCs is short, however [12]. The performance of these cells in vitro and in vivo can be improved by pretreatment with various agents (hypoxia, estradiol, chemical drugs), genetic modification, and optimized MSC culture conditions [13]. Studies have demonstrated that estradiol has an impact on various physiological processes in mammals, including cell proliferation, apoptosis, differentiation, and metabolism of treated stem cells [14]. The expression of estradiol receptors on stem cells may be a role for

estradiol in regulating some functions in these cells [15]. Recent research shows that the main mechanism of the therapeutic effects of mesenchymal stem cells (MSCs) is the secretion of paracrine factors by these cells. The supernatant from the culture medium of these cells, known as mesenchymal stem cell conditioned medium (MSC-CM), contains paracrine factors and is extensively researched for various restorative and therapeutic applications [16]. It has been reported that MSC-CM conditioned medium can enhance recovery from stroke and promote cell migration to wound sites, aiding in the repair process for diabetic patients [17]. When mesenchymal stem cells (MSCs) are treated with estradiol, their migration to the damaged pancreas in a diabetic mouse model is enhanced [18]. However, the effects of the conditioned medium of stem cells treated with estradiol on any of the diabetic complication have not been studied before. The aim of this study was to investigate some molecular and tissue mechanisms involved in improving muscle wasting in diabetic rat model treated with conditioned medium of bone marrow-derived mesenchymal stem cells (BMSC-CM) and BMSC-CM treated with estradiol.

2. Materials & method

2.1. Animals and grouping: In this study, 40 male Wistar rats, each weighing between 180-220 gr, were used. These rats were sourced from the laboratory animal breeding and maintenance center at the Faculty of Science, Urmia University. Throughout the study period, the rats were provided with a standard rodent diet and had unlimited access to water. They were housed in cages under standard laboratory conditions, which included a 12-hour light/dark cycle, 38% ±2 humidity, and a temperature of 22 ± 2 degrees Celsius. The rats were randomly divided into four groups of ten: control, diabetic (STZ/NA), diabetic receiving conditioned medium (STZ/NA + CM), and diabetic receiving conditioned medium from estradiol-treated cells (STZ/NA + ECM). The control and diabetic groups received a normal saline solution. All solution, with a volume of 100 microliters, were administered intraperitoneally (i.p.) three times during the treatment period, specifically on the 7^{th}, 21th, and 35^{th} days post diabetes induction. After 42 days of injections, behavioral tests were conducted, and subsequently, the animals were euthanized.

2.2. Hyperglycemia induction: Diabetes was induced following the Torres-Piedra protocol and the Massier-Lo et al [19]. guidelines. After a 12-hour fasting period, each rat received an intraperitoneal injection of 100 mg/kg nicotinamide dissolved in a 0.9% sodium chloride solution. Fifteen minutes later, an intraperitoneal injection of 65 mg/kg streptozotocin was administered. To prevent hypoglycemia-induced death, a 5% glucose solution was provided in the cages of the diabetic groups for 24 hours post injection. Diabetes was confirmed seven days after the STZ/NA injection by measuring blood glucose levels from the caudal vein using a glucometer. Rats with blood glucose levels exceeding 150 mg/dL were classified as diabetic. Throughout the treatment period, the weight and fasting blood glucose levels of the rats were monitored weekly.

2.3. Conditioned media preparation:

According to the standard protocol, for bone marrow extraction, two Wistar male rats were used at the age of 8 weeks. Briefly, flushed out marrow was washed and centrifuged in PBS and cells were plated in 75-cm^2 tissue-culture flasks at the concentration of 0.4 × 106 cell/cm^2 in DEMEM (Dulbecco's modified eagle medium) [20]. 72 hours after the initial culture began, the cells were examined with the invert microscope, and the non-sticky cells were discarded, and the culture of the sticky cells continued. To achieve proper density, replacement of the culture medium was done every three or four days. Adherent cells were separated from the bottom of the flask using trypsin containing 2% EDTA and were transferred to the next flasks with a ratio of 3:1 using trypan blue staining and the direction of passage. After reaching a density of 70%, the cells of the third passage were cultured for 24 hours in the presence of a final concentration of

100 µM of 17-beta-estradiol (this is the same stage of treatment of cells in the culture medium) [15]. After washing the cells, they were incubated for another 24 hours in the culture medium without FBS to prepare the conditioned medium. The conditioned medium (which is the culture medium of the treated cells) was collected inside the flask then centrifuged for 30 minutes at a speed of 12000 rpm to remove the cell debris. The conditioned medium obtained from each mouse was filtered through a 0.2 micrometer filter and stored -80°C [21].

2.4. Real time PCR

To measure the expression of Akt, PI3K and Atrogin-1 genes, the primer sequences of (Table 1) were used. These sequences were designed and constructed with the help of NCBI software program.

Table 1. Characteristics of primers used for target and control genes

Gene	Primer sequence	Genomic Fragment
Atrogin- 1	Forward: CCATCAGGAGAAGTGGATCTATGTT	215
	Reverse: ATGACGTGAAACCCCCTTCG	
PI3K	Forward: GGTCAGAACGGGAGTGATGT	224
	Reverse: TGAGCACAATGAAGCAGACC	
Akt	Forward: AAGAAGGTGGTGAAGCAGGCATC	247
	Reverse: CGAAGGTGGAAGAGTGGGAGTTG	
β-actin	Forward: GTGGAAGAGTGGGTGGTG	315
	Reverse: CAGACCACAATGAAGA TA	

2.5. Immunohistochemistry

Immunohistochemistry (IHC) is a technique in histology used to detect specific protein markers. The main steps of this technique are as follows: Use of primary antibody: Primary antibody specifically binds to the target antigen. There are two types of antibodies:

Polyclonal: reacts with multiple antigens and may react with non-target antigens.

Monoclonal: reacts with only one antigen and has more specific staining but less sensitivity. Sample preparation: Samples are autoclaved in retrieval solution for 3 minutes to allow proteins and antigens to rise. Then they are placed in the retrieval solution and in the autoclave for 10 minutes to denature the proteins. Inactivation of endogenous peroxidase: tissues were exposed to 3% hydrogen peroxide for 5 min and then rinsed with PBS buffer. Addition of primary antibody: primary antibodies (Bax, Bcl2, and caspase-3) are added dropwise to tissue sections and incubated for 15 min at 4°C. then the slides were washed with cold PBS for 5 minutes. Addition of secondary antibody: secondary antibody is added and incubated for 30 minutes at room temperature. Then the slides are washed with PBS for 5 minutes. Staining with DAB chromogen: DAB chromogen is added to the slides and incubated for 5 minutes. The slides are then stained

with hematoxylin for 2-3 minutes, washed with PBS or sterile water, and covered with a cover slip. These steps help to accurately detect protein markers in tissue samples.

2.6. Measurement of total antioxidant capacity (TAC) of muscle

To perform this test, samples must be lysed first. According to the work schedule available in Arsam Farazist, first 50-100 mg of the desired sample was weighed. Then 10 times the weight of the sample buffer in the kit was added and the samples were lysed with a glass homogenizer. Centrifugation was performed for 10 minutes at 10000 rpm and the supernatant was separated and used as the required sample for testing. Next, the standard solution was poured into two wells of the plate and the sample was poured into the next wells. Two repetitions were done for each sample and finally the average of these repetitions was calculated. 250 microliters of the working solution in the kit were added to all the wells and the optical absorbance of the samples was read after 5 minutes at a wavelength of 593 nm.

2.7. Measuring the level of malondialdehyde (MDA) in muscle

Solution No. 1: 1.5040 g of and 2.1790 g of was poured into a 100 ml flask and brought to volume and the pH was adjusted to 7.4.

Solution number 2: 10 grams of tricarboxylic acid (TCA) was poured into a 100 ml flask and distilled water was added to it. Then 0.67 grams of thiobarbituric acid (TBA) was added to it and 28 ml of HCl was added and brought to volume.

The desired sample was weighed and for each gram of tissue, 5 ml of No. 1 solution was poured on the sample and pounded in a mortar. Tissue homogenization should be done on ice. After homogenizing the tissues, the test tubes were placed in a centrifuge for 10 minutes at 3000 rpm, then the supernatant was separated and poured 2 separate microtubes for biochemical tests and placed in a freezer at minus 20 degrees Celsius. 24 hours later, the homogenized tissues were taken out of the freezer and centrifuged at 3000 rpm for 5 minutes and these samples were used for the intended experiments.

2.8. Statistical analysis

For statistical analysis, one-way ANOVA and Tukey tests were used for continuous quantitative data, and Kruskal-Walli's test was used for discontinuous data using SPSS software. Before performing the statistical tests, the kolmograph-Smirnov or Shapiro-Wilk method was used to check the normal distribution of the data amount of data. A confidence level of 95% was considered to check the significance level of the data.

3. Results

3.1. CM and ECM led to increased muscle mass by increasing the signaling pathway of PI3K/Akt

According to (Figure 1.A), the rate of expression of the PI3K gene in the gastrocnemius muscle was significantly reduced at the end of seven weeks of treatment in the diabetic group compared to healthy rats. Treatment of diabetic rats with simple condition medium caused a significant increase in the expression of the PI3K gene in the muscle of the rats studied and reached the control group level, so that there was no significant difference between healthy and diabetic rats treated with a conditional medium. Treatment of diabetic rats with conditioned medium treated with estradiol significantly increased the PI3K gene expression.

According to (Figure 1.B), the level of Akt gene expression in the gastrocnemius muscle decreased significantly at the end of seven weeks of treatment in the diabetic group compared to healthy rats.

Treatment of diabetic rats with simple conditioned medium caused a significant increase in Akt gene expression in the muscle of the studied rats. Treatment of diabetic rats with conditioned medium treated with estradiol significantly increased Akt gene expression. According to (Figure, 1C), the amount of Atrogine-1 gene expression in the gastrocnemius muscle increased significantly at the end of seven weeks of treatment in the diabetic group compared to healthy rats. Treatment of diabetic rats with conditioned medium treated with estradiol significantly reduced the expression of Atrogine-1 gene and brought it to the level of the control group, so no significant difference was observed between healthy and diabetic rats treated with estradiol.

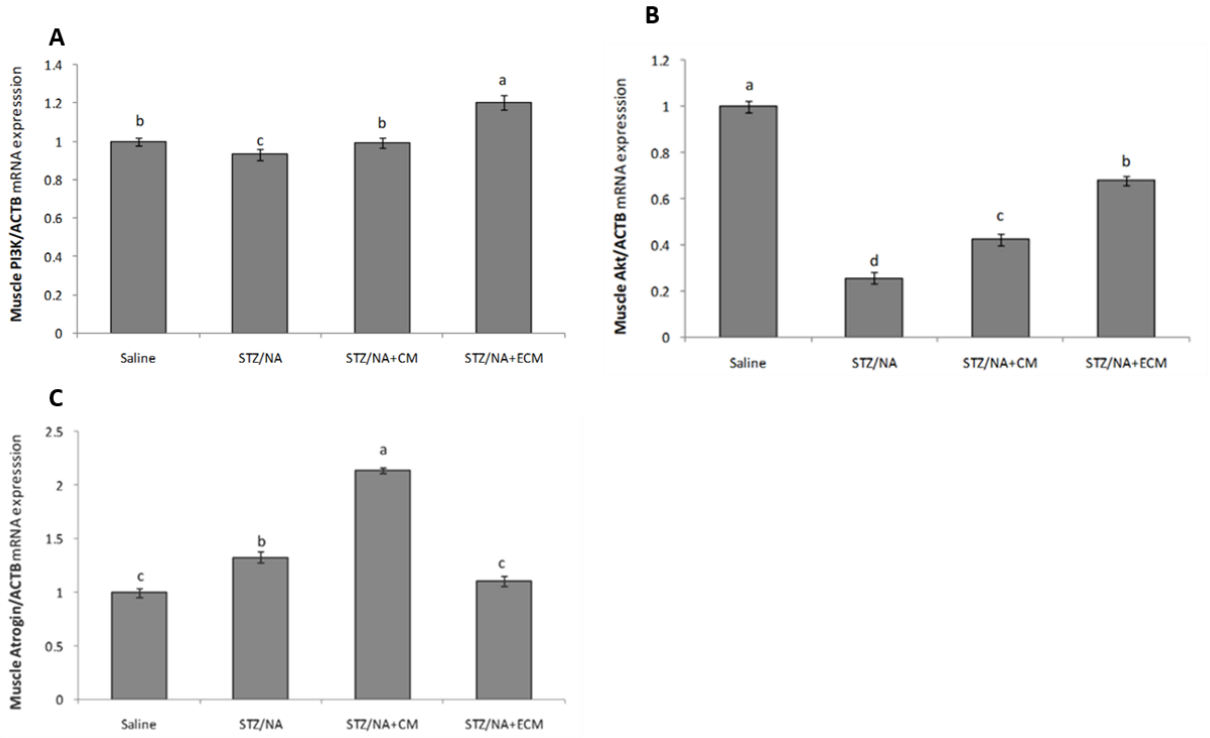

Figure 1. (A) PI3K gene expression level in gastrocnemius muscle at the end of seven weeks of treatment. Data were shown as mean ± standard deviation. Different letters indicate significant differences at the P<0.05 level. (B) Akt gene expression level in gastrocnemius muscle at the end of seven weeks of treatment. Data were shown as mean ± standard deviation. Different letters indicate significant differences at the P<0.05 level. (C) Atrogine-1 gene expression level in gastrocnemius muscle at the end of seven weeks of treatment. Data were shown as mean ± standard deviation. Different letters indicate significant differences at the P<0.05 level.

3.2. CM and ECM reduced oxidative stress in the gastrocnemius muscle of diabetic rats

As shown in (Figure,2A), the total antioxidant capacity in the muscle tissue of diabetic rats decreased significantly at the end of the treatment period. Treatment of diabetic rats with simple conditioned medium and treated with estradiol significantly increased this index compared to the diabetic group. According to (Figure, 2B), the amount of malondialdehyde production in the diabetic group was significantly higher than

that of healthy rats. Treatment of diabetic rats with simple conditioned medium or estradiol resulted in a significant decrease of this index compared to rats in the diabetic group.

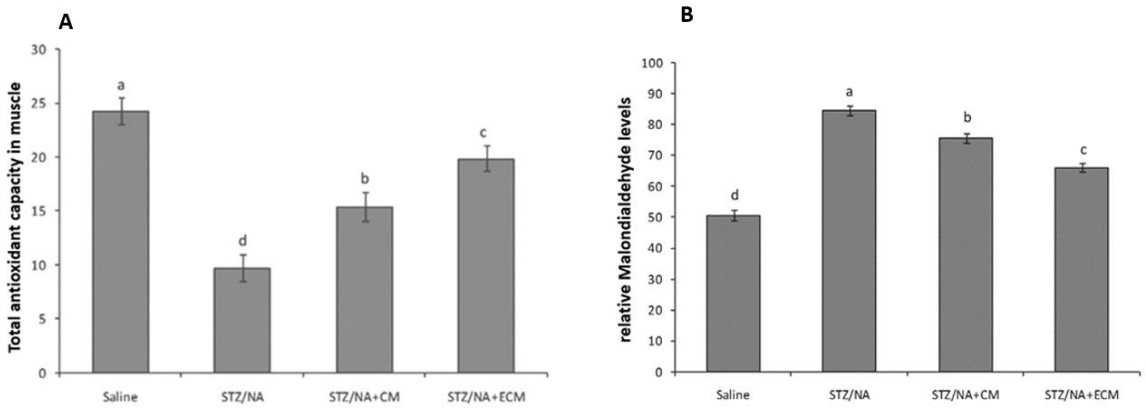

Figure 2. (A) Total antioxidant capacity changes in the gastrocnemius muscle at the end of seven weeks of treatment. Data were shown as mean ± standard deviation. Different letters indicate significant differences at the $P<0.05$ level. (B) Changes in the amount of malondialdehyde in the gastrocnemius muscle at the end of seven weeks of treatment. The data were shown as mean ± standard deviation. Different letters indicate significant differences at the $P<0.05$ level.

3.3. CM and ECM reduced apoptosis in the gastrocnemius muscle of diabetic rats

(Figure 3) shows the changes in the expression of apoptotic proteins Bax, BCL-2 and caspase-3, which were stained by immunohistochemical method and specific antibody. As seen in the figure, the number of cells expressing Bcl-2 as an anti-apoptotic protein is higher in the group receiving saline compared to other groups. Nevertheless, the treatment of diabetic rats with simple conditioned media and estradiol increased the number of cells expressing Bcl-2. The expression of Bax protein in diabetic muscle cells was higher than that of healthy rat. Treatment with simple conditioned medium or estradiol was able to reduce the expression of this protein in the muscles and the last two groups was lower compared to the diabetic group. A similar situation existed for caspase-3 protein.

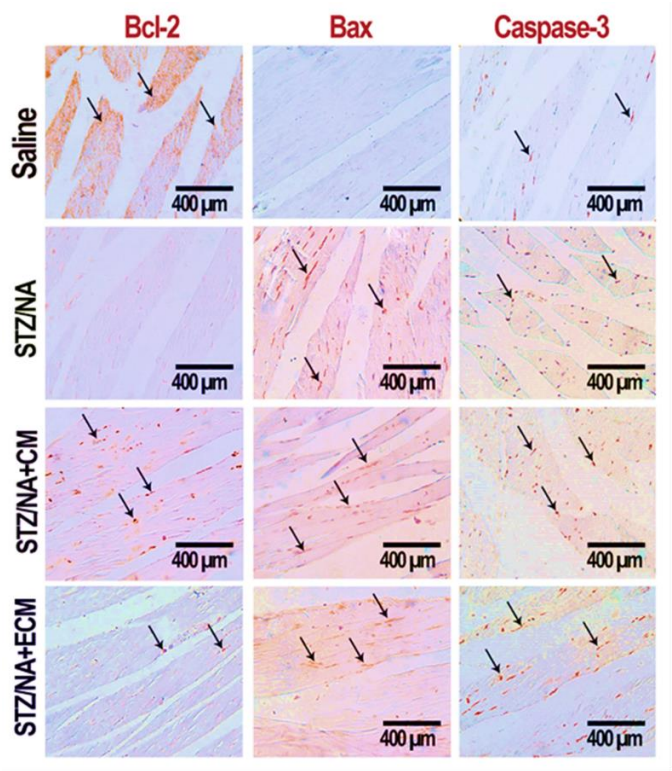

Figure 3. Changes in the expression of apoptotic proteins (Bcl-2, Bax, Caspase-3) in gastrocnemius muscle following diabetes (STZ/NA) and treatment of diabetic rats with simple conditioned medium (STZ/NA+CM) and estradiol conditioned medium (STZ/ NA+ECM).

3.4. CM and ECM can regulate blood glucose

Table 2 shows fasting blood sugar levels at the beginning and end of the experiment. According to this table, the blood sugar level in the diabetic groups at the end of the treatment period was significantly higher than the initial blood sugar in the same rats. Treatment of diabetic rats with simple conditioned medium or estradiol caused a significant decrease in blood sugar of treated rats compared to diabetic rats. However, there is still a significant difference between the initial blood sugar level and the final blood sugar level in the diabetic groups receiving the conditioned medium.

Groups	fasting blood sugar at the beginning of the test (mg/dl)	fasting blood sugar at the end of the test (mg/dl)
saline	±33/92 4/3[a]	±87/97 4/1[a]

STZ/NA	±84/96 7/5[a]	±54/320 18/7[c]
STZ/NA+CM	±06/92 6/2[a]	±16/233 14/6[d]
STZ/NA+ECM	±12/102 7/4[a]	±71/160 9/9[e]

Table 2. Data are shown as mean ± standard deviation. P<0.05 was considered significant level. Different letters indicate significant differences between groups.

4. Discussion

In the present study, in order to investigate the mechanism of conditioned medium in the recovery of muscle strength in diabetic rats, the PI3K/Akt signaling pathway was studied by evaluating the expression of PI3K and Akt genes. In the present study, the expression of both PI3K and Akt genes in the skeletal muscle of diabetic rats decreased compared to healthy animals, while the administration of conditioned medium increased the expression of these genes compared to untreated diabetic conditions. This signaling pathway plays an essential role in a wide range of biological functions, including metabolism, macromolecular synthesis, cell growth, proliferation, and survival [22]. The PI3K/Akt signaling pathway is a central axis in cell signaling, responding to various extracellular stimuli such as insulin, insulin-like growth factor, epidermal growth factor, and fibroblast growth factor [23]. Enhanced activation of this pathway is a key factor in promoting muscle mass growth. When the balance shifts towards protein degradation in conditions like cancer, diabetes, kidney failure, and aging, it leads to muscle atrophy. This condition is marked by a decrease in muscle mass, a reduction in muscle fiber cross-section, muscle volume, and muscle protein content. Atrogin1 and MuRF1 play crucial roles in regulating muscle atrophy. Their expression in muscle is influenced by the activity of FOXO transcription factors, which are regulated by the Akt pathway. In atrophic muscle, reduced Akt activation permits the phosphorylation and nuclear translocation of FOXO, leading to elevated protein degradation through increased expression of Atrogin1 and MuRF1[24]. In this study, the expression of the Atrogin1 gene, an indicator of muscle atrophy, was examined in the gastrocnemius muscle of the rats. The findings revealed that diabetic rats exhibited increased expression of this gene. However, treating diabetic rats with either simple conditioned medium or estradiol reduced the gene's expression. Recent studies have shown that the loss of muscle mass is not only a cause of type 2 diabetes, but can also be a complication of this disease. Oxidative stress, caused by the abundance of reactive oxygen species (ROS), is considered a critical factor for the pathogenesis of diabetes. Uncontrolled production of ROS can damage proteins, lipids and nucleic acids and lead to cellular dysfunction including loss of energy metabolism and changes in signaling. These reactive species are important signaling molecules necessary for muscle function. However, excessive production of ROS and reduction of antioxidant defense have a negative effect on muscle performance, because it disrupts muscle growth and strength and changes metabolic capacity. Malondialdehyde (MDA) is often used as an indicator of oxidative damage [25]. Manganese superoxide dismutase (MnSOD) is a key mitochondrial antioxidant that mitigates mitochondrial ROS by transforming superoxide into hydrogen peroxide, which is subsequently converted to water by catalase [26].

In the present study, diabetes increased the expression of pro-apoptotic proteins Bax and caspase-3 and decreased the expression of anti-apoptotic protein Bcl-2. While the treatment with conditioned mediums moderated these conditions and decreased the expression of proapoptotic proteins and increased the

expression of antiapoptotic proteins. Apoptosis, a meticulously controlled process, enables multicellular organisms to sustain tissue and cellular equilibrium. In certain physiological scenarios, including chronic heart failure, motor neuron diseases, spinal cord injuries, muscular dystrophy, and muscle atrophy due to immobility, the rate of apoptosis rises in impaired skeletal muscles [27].

The findings of this study can provide a basis for the design of preclinical studies in the direction of using the conditioned media obtained from mesenchymal stem cell culture in the treatment or control of diabetes.

5. Data availability

The input files and analysis scripts are available on request from the corresponding author.

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