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Original Research

The Effect of MitoTEMPO on Rat Diaphragm Muscle Contraction Parameters in an Experimental Diabetes Model Induced with Streptozotocin

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ABSTRACT

Objective: Diabetes Mellitus (DM) not only causes hyperglycemia but also leads to clinical challenges involving respiratory functional impairments. The contraction of the diaphragm reduces pleural pressure, thereby contributing significantly to the process of breathing. This study examines the functional impairments in diaphragm muscle isometric contraction parameters due to increased reactive oxygen species (ROS) associated with DM, as well as the effects of MitoTEMPO, a mitochondria-specific antioxidant, on these impairments.

Methods: Wistar Albino male rats at 12-14 weeks of age were randomly divided into three groups: the control group (CON, n=6), the diabetes group (DM, n=6), and the diabetes + MitoTEMPO (MT, n=6) group. A single dose of 50 mg/kg streptozotocin (STZ) was administered to the rats in the DM and MT groups. When the rats in the MT group reached a blood glucose level of 300 mg/dl, they were administered MitoTEMPO at a dose of 0.7 mg/kg/day for 28 days. Isometric contraction recordings were obtained from diaphragm muscle preparations isolated from the experimental animals at the end of the 28-day period.

Results: Although the effectiveness of mitochondria-specific antioxidants in reducing blood glucose levels in DM is debated in the literature, results for the MT group were interestingly indicative of a statistically significant decrease in blood glucose levels following MitoTEMPO administration at the end of the fourth week. Furthermore, MitoTEMPO exhibited therapeutic effects on diaphragm muscle contraction parameters impaired by DM.

Conclusion: The findings suggest that in DM patients, MitoTEMPO could be utilized for blood glucose control and might also be effective in the treatment of DM-induced diaphragm muscle mechanical dysfunction.

Keywords: Diabetes Mellitus, Diaphragm muscle, Isometric contraction, MitoTEMPO

INTRODUCTION

Hyperglycemia caused by Diabetes Mellitus (DM), which is known as a group of metabolic disorders, is a result of impaired insulin secretion from the pancreatic β-cells, insulin sensitivity, or a combination of both [1]. Chronic hyperglycemia in DM leads to damage and dysfunction in various tissues and organs. On the other hand, in recent years, there has been a significant increase in the prevalence of DM, and World Health Organization (WHO)

statistics indicate that this upward trend is likely to continue [2].

Strong evidences support that oxidative stress plays important roles in the pathogenesis of damage and dysfunction caused by DM in many tissues and organs [3]. Reactive oxygen species (ROS) are recognized as the predominant origin of oxidative stress for cells. The increased amount of ROS may disrupt the physiology of intracellular signal transduction mechanisms, stimulate inflammatory responses, and lead cells to apoptosis [4]. As a result, diseases that increase ROS production, such as DM, can cause distant tissue and organ damage through these free radicals entering the bloodstream.

Although NADPH-oxidase, xanthine oxidase, immune reactions, arachidonic acid metabolism mechanisms contribute to ROS production, it is mostly produced in the electron transport chain localized to the inner membrane of the mitochondria [5]. The expected results from traditional applications, such as vitamin E and vitamin C, against mitochondria-induced oxidative stress cannot be achieved in clinical practice [6]. According to this information, researchers have focused on the idea of targeting antioxidants to mitochondria in recent years, thereby inactivating ROS at the production site before they enter the bloodstream. The mostly used molecule when targeting mitochondria is triphenylphosphonium (TPP), which is a lipophilic cation [7]. Another molecule bounds to TPP for drag into the intracellular matrix. It is more negative than the extracellular matrix, creating electrical force thanks to the cationic property of TPP. Then it continues to drag into the mitochondrial matrix, which is also more negative than the intracellular matrix, by the same mechanism. TPP can easily pass the membrane layers it encounters during this movement, thanks to its lipophilic feature [8].

Main Points;

- This study investigates the relationship between respiratory function impairments observed in patients with DM and the parameters of diaphragm muscle contraction, as well as the effects of MitoTEMPO, a mitochondria-specific antioxidant, on these functional impairments. Through this and similar studies, the effectiveness of mitochondria-specific antioxidants against secondary diseases observed in DM patients can be determined, potentially leading to higher quality of life standards for DM patients in the future.

The diaphragm is a skeletal muscle that plays a crucial role in respiration by contracting to decrease pleural pressure, enabling inspiration. The contraction and relaxation activities of skeletal muscles are determined by the ion concentrations on both sides of the cell membrane [9]. Increased ROS levels can lead to lipid peroxidation, potentially disrupting the physiological balance of the cell membrane potential [10]. Therefore, protecting respiratory functions against the increased amount of ROS in the tissues of patients with DM is vital for quality of life.

The aim of this study is to determine the dysfunctions that the amount of ROS, which is likely to increase with DM, may cause on the contraction-relaxation activity of the diaphragm muscle. In addition, it is another aim to determine whether possible dysfunctions can be treated with MitoTEMPO, a mitochondria-specific antioxidant.

MATERIALS AND METHODS

Experimental Animals and Groups

Wistar Albino male rats (12-14 weeks old) weighing 200-300 grams were used in the experiments. Rats were housed with a maximum of 5 animals per cage, on 12h/12h light/dark cycles, and with no restrictions on their access to feed and water. Experimental animals were randomly divided into three groups: control group (CON, n=6), diabetes group (DM, n=6) and diabetes + MitoTEMPO (MT, n=6) group. A single dose (50 mg/kg) intraperitoneal (i.p.) streptozotocin (STZ) (dissolved in 0.1 M sodium citrate, pH 4.5) was administered to the DM and MT groups, while the same dose of citrate vehicle injections were administered to the CON group. One week after the STZ injection, blood glucose measurements greater than 300 mg/dl were accepted as diabetic [11]. Starting one week after the STZ application, 0.7 mg/kg MitoTEMPO (cat. no: SML0737, Sigma-Aldrich, Darmstadt, Germany) was applied to the MT group [12] and vehicle gavage was applied to the other groups at the same dose for 4 weeks. The MT dose was determined in our previous studies as the value that maintains the oxidative stress index at the level of the control group [12,13]. At the beginning and subsequently for 4 weeks, the blood glucose (mg/dl) and body weight (g) values of the experimental animals were measured and recorded weekly. All procedures of this study were approved by the Animal Experiments Local Ethics Committee of Necmettin Erbakan University with the approval no 18-007.

Diaphragm Muscle Isolation and Isometric Contractility Recordings

Diaphragm tissue was quickly isolated from rats decapitated using a guillotine, and placed in a petri dish with a fresh modified solution of Krebs (composed of mM: 119 NaCl, 4.8 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 20 $NaHCO_3$ and 10 glucose, pH 7.4, and gassed with a mixture of 95% O_2 and 5% CO_2). The muscle strips prepared by trimming 20x5mm were placed in an isolated tissue bath with the costal side up, connected to a force transducer (FT03 Force Displacement Transducer, Grass Instruments) and the other side connected to a micromanipulator. The isolated tissue bath was continuously perfused with the modified Krebs solution and the temperature was kept constant at 37 ^oC. Diaphragm muscle preparations suspended in this way were placed in a 30-minute equilibration period under a tension of 2 grams. After the equilibration period, a rectangular field stimulation of 1 ms duration was given via a stimulator (S48, Grass Instruments). To determine the maximal contraction response, the voltage value was gradually increased and fixed at 12 Volts. Directly applying a field stimulus to the muscle tissue allowed for obtaining contraction data without the effects of neuromuscular junction and neural transmission disorders. Basic contraction recordings were obtained with a 1 Hz stimulus frequency. From these recordings, contraction force, maximum speed of force development ($+dF/dt_{max}$), and maximum speed of force decrease ($-dF/dt_{max}$) parameters were evaluated. Additionally, for the assessment of frequency-dependent contraction force, stimuli at 0.2, 0.5, 1, 2, 3, 4, and 5 Hz were applied [14]. Furthermore, for the responses to the post-rest potentiation protocol, pre-rest intervals of 10, 20, 30, 40, 50, 60, 70, and 80 seconds were implemented before each 100-second recording [15]. Isometric contraction data were simultaneously recorded using a data acquisition unit (MP45, Biopac) via software (BSL Pro 3.7.5, Biopac) for further analysis. Finally, the muscle masses were weighed, and the contraction data were normalized by dividing the wet muscle mass [16]. The presented data on isometric contraction were created by averaging the 10 data obtained from the recording of each rat.

Data Analysis and Statistics

All data were presented as mean ± standard error of the mean (SEM), and the normal distribution of data was tested with Kolmogorov-Smirnov. The significance of the rats' body weight and blood glucose data compared to the previous measurements was analyzed using a nonparametric paired t-test. For all other data, a statistical analysis was performed using One-way ANOVA, followed by the Tukey post-hoc test.

RESULTS

Both the blood glucose and body weight parameters were statistically evaluated for inter-group comparison at the same measurement time as well as for the intra-group changes over the weeks. In the DM group, following the STZ injection, statistically significant differences were observed in blood glucose values in all measurements compared to both the initial value and the CON group. In an interesting manner, the 2nd-week blood glucose value in the MT group was found within the range of values for both the DM and CON groups. Furthermore, in the 4th-week measurement, the MT group did not show statistical significance compared to the initial and CON group values but differed significantly from the DM group value. Regarding the body weight parameter, while no statistically significant differences were found between groups in all measurements, the differences detected in comparison to the initial value are as indicated in the table. No other statistical significance was observed between weeks for both blood glucose and body weight values within the same group, except for the significance marked in the table compared to the initial measurements (Table 1).

According to the contraction recordings taken at a basic stimulus frequency of 1 Hz, the contraction force did not show significant differences between the experimental groups (Figure 1-A). While the $+dF/dt_{max}$ parameter from the same recordings did not show differences between the groups (Figure 1-B), the $-dF/dt_{max}$ parameter increased in the DM group compared to the CON group, whereas the MT group remained at the CON level (Figure 1-C). The maximum tetanic force value obtained at a 100 Hz stimulation frequency increased in the DM group, whereas the MT group remained at the same level as the CON group (Figure 1-D).

It was observed that there was no statistically significant change in the contraction force across groups with varying stimulus frequencies (Figure 2-A). Similarly, the contraction force values obtained through the post-rest potentiation protocol did not show significant differences between the groups (Figure 2-B).

Table 1. Blood glucose and body weight measurements of the rats.

Blood glucose (mg/dl)					
	Initial	**1st week**	**2nd week**	**3rd week**	**4th week**
CON	109.83±3.66	101.83±3.72	99.67±6.78	100.17±5.03	100.67±1.65
DM	107.50±5.77	330.83±16.43[a,*]	345.50±28.23[a,*]	413.33±41.70[a,*]	390.33±27.22[a,*]
MT	103.67±2.79	313.00±12.32[a,*]	235.83±18.56[a,b,*]	218.17±42.57[b,*]	161.83±23.97[b]
p	0.5966	<0.0001	<0.0001	<0.0001	<0.0001
Body weight (g)					
	Initial	**1st week**	**2nd week**	**3rd week**	**4th week**
CON	240.83±6.99	273.67±23.95	289.33±25.32	301.83±25.68	316.67±20.33*
DM	249.50±18.33	282.33±25.33*	287.83±27.32*	297.83±28.53*	280.00±32.44
MT	216.67±8.20	266.17±20.88*	270.67±20.56*	282.50±19.41*	286.33±21.05*
p	0.1808	0.8887	0.8385	0.8462	0.5592

[a] p<0.05 vs CON, [b] p<0.05 vs DM, * p<0.05 vs initial measurement.

Abbreviations: control group (CON, n=6), diabetes group (DM, n=6) and diabetes + MitoTEMPO (MT, n=6). Between-group comparisons were conducted using One-way ANOVA with Tukey post-hoc test, while within-group measurements across weeks were compared using nonparametric paired t-tests. p<0.05 was considered statistically significant. Values are given mean ± SEM.

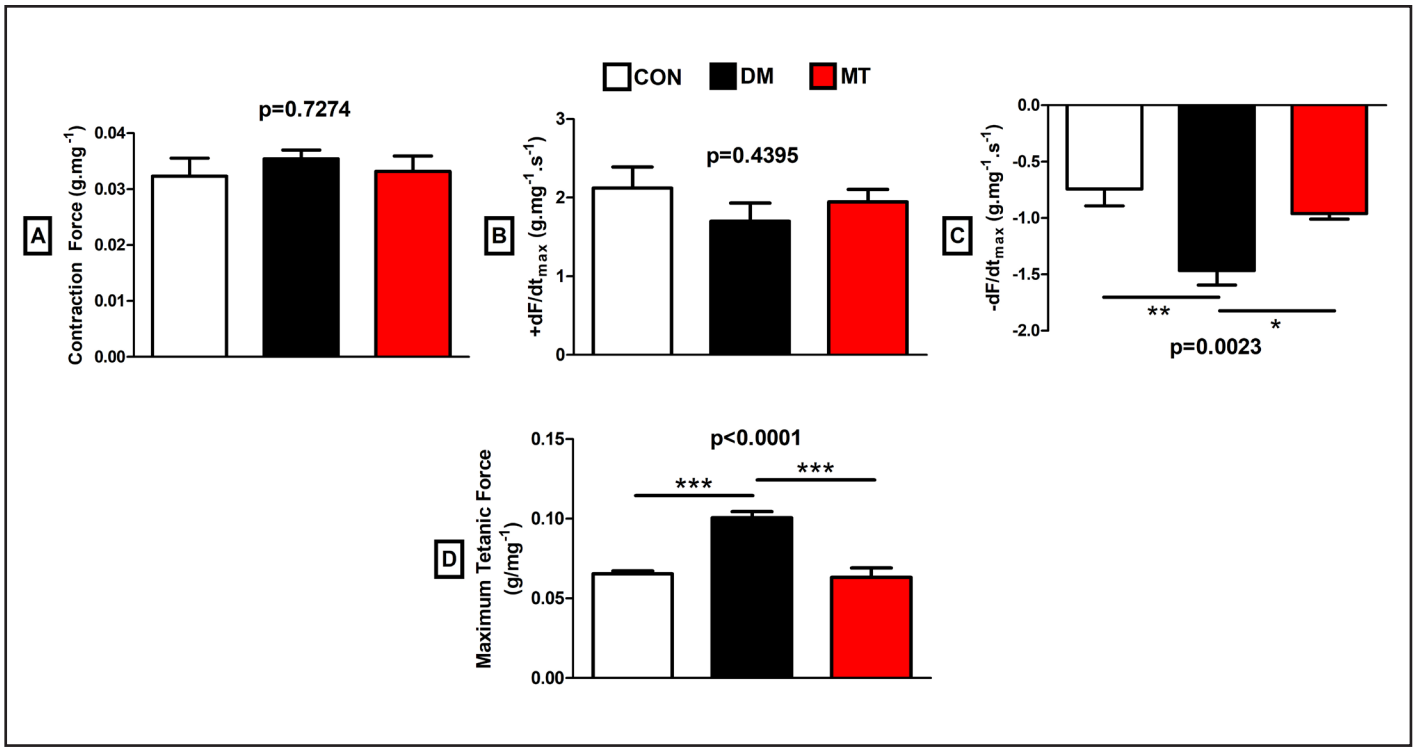

Figure 1. A, B, C- 1 Hz basic contractility parameters, D- maximum tetanic force. Abbreviations: control group (CON, n=6), diabetes group (DM, n=6) and diabetes + MitoTEMPO (MT, n=6). Between-group comparisons were conducted using one-way ANOVA with Tukey post-hoc test. Values are given as mean ± SEM.* p<0.05, ** p<0.01, *** p<0.0001.

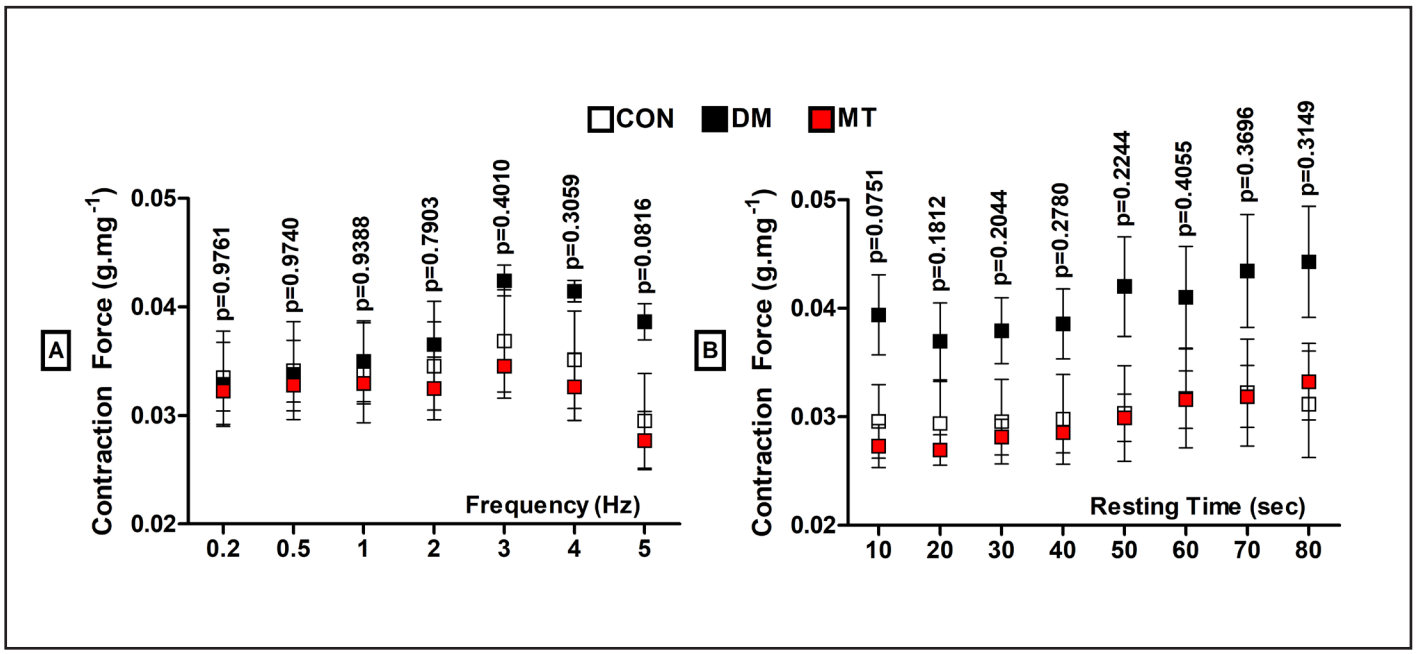

Figure 2. A- frequency dependent contraction force, B- postrest-potentiation protocol contraction force. Abbreviations: control group (CON, n=6), diabetes group (DM, n=6) and diabetes + MitoTEMPO (MT, n=6). Between-group comparisons were conducted using one-way ANOVA with Tukey post-hoc test. $p<0.05$ was considered statistically significant. Values are given as mean ± SEM.

DISCUSSION

DM has been considered an important comorbidity that affects the natural course of various respiratory system diseases. One of the significant causes of damage occurring in various biological structures in diabetic patients is the increased production of ROS due to hyperglycemia [17]. In line with the undisputed findings in the literature, we had previously identified the regulation of oxidative stress parameters following the application of MitoTEMPO in our studies [13,18]. Kabitz et al. have suggested that the impairment in diaphragm muscle functions resulting from DM is largely responsible for respiratory function impairment [19]. Clinical studies show that diaphragmatic breathing exercises prevent oxidative damage and regulate glycemic parameters in patients with type 2 diabetes [20]. Conversely, impairment in diaphragm muscle functions in these patients could become a source of various pathophysiologies due to oxidative damage and impaired glycemic parameters. In this study, we examined the contraction parameters of the diaphragm muscle preparation isolated from diabetic rats and the effects of MitoTEMPO on these parameters.

Mason et al. proposed that, contrary to our findings, the application of mitochondria-specific antioxidants to DM patients did not significantly alter glycemic parameters [21]. On the other hand, Jeong et al. found that in the glucose tolerance test, conducted by intraperitoneal glucose injection on the experimental animals, the high-fat diet group exhibited significantly higher blood glucose levels compared to the control group. However, the blood glucose levels of the group treated with MitoTEMPO were like those of the control group, in line with our findings [22]. Virgana et al. found in their DM model created with STZ that the group treated with mitochondria-specific antioxidants had a significantly lower fasting blood glucose level compared to the diabetic group [23]. In their study, Xiao et al. found a significant decrease in blood glucose levels in the mitochondria-specific antioxidant group compared to the DM group starting from the 12th week [24] (Table 1). The decrease in blood glucose levels may be associated with the improvement in β-cell functions, as indicated in the studies by Plecitá-Hlavatá et al., highlighting the potential involvement of mitochondria-specific antioxidants [25]. Based on this information, we believe that the role of MitoTEMPO in regulating blood glucose levels in DM patients' needs to be confirmed by further research. The body weights of the experimental animals did not show any significant differences between the groups in all measurements. However, while there was a significant weight gain in the CON and MT groups compared to the initial measurement at the end of the 4th week, there was no statistically significant weight gain

in the DM group (Table 1). Lin et al. did not find a significant difference in the body weights of the experimental animals belonging to the control, DM, and MitoTEMPO groups in a study similar to this study's findings [26]. In contrast, Xing et al. found that the body weights of diabetic mice were significantly higher compared to the control group, and they also observed that MitoTEMPO treatment had no effect on weight gain [27]. It appears that there is a lack of a study elucidating the mechanisms of weight gain or loss in experimental animals following the administration of MitoTEMPO.

Brotto et al. did not find a significant difference between the groups in diaphragm muscle contraction force on the 4th day, similar to ours; however, unlike ours, they observed a significant decrease in the DM group compared to the control in the data at the 4th week [28]. On the other hand, De Jong et al. did not find a significant difference in the isometric contraction parameters of the diaphragm muscle in diabetic rats compared to the control group, parallel to our findings [29]. RodríGuez-Reyes et al. also found that the normalized contraction force value of the skeletal muscle did not change in the DM group, in line with our findings [30] (Figure 1-A). Lamberts et al. indicated diastolic function impairments in cardiac muscle contraction parameters of diabetic patients, parallel to the diaphragm muscle contraction parameters in our study [31]. In another study, the diaphragm muscle contraction parameters of diabetic fatty rats did not show a difference between the groups in $+dF/dt_{max}$, which is in line with this study. However, unlike ours, $-dF/dt_{max}$ did not show a significant difference in the diabetic fatty group [29] (Figure 1-B,C). Peixoto et al. found the maximum tetanic force of the diaphragm muscle isolated from diabetic rats to be high, similar to our findings. They suggested that this might be due to increased resistance to fatigue [32]. This could be associated with the increased ATP levels in diabetes [33-35]. The injection of MitoTEMPO resulted in the treatment of oxidative damage, thereby maintaining the data of this group at the level of the CON group (Figure 1-D).

Laitano et al. did not observe any significant difference between the groups in terms of frequency-dependent contraction force of the diaphragm muscle in their study on a chronic heart failure model, which is in line with our findings [36]. Eshima et al. demonstrated an increase in blood glucose levels in mice in a high-fat diet model and, parallel to our findings, did not detect any significant difference in the frequency-dependent contraction parameters of skeletal muscle in these animals [37] (Figure 2-A). In our data, where the post-rest potentiation protocol was applied to test the sarcoplasmic reticulum Ca^{++} reuptake mechanism without altering the extracellular Ca^{++} influx, no differences were observed between the groups [38] (Figure 2-B). Eshima et al., in contrast to our findings, reported the disruption of Ca^{++} homeostasis in diabetes [39]. Perhaps this situation could be a phenomenon involving different diabetic durations.

Limitations

This study was conducted only in male rats, aged 12-14 weeks, without evaluating gender- and age-dependent parameters. For instance, a difference in the rate of occurrence of cardiac diseases in diabetic patients has been identified in relation to age and gender [40]. Furthermore, only the mechanical activity of the diaphragm muscle was identified for respiratory dysfunctions related to DM. Another limitation of our study is the lack of investigation into the molecular mechanisms associated with the regulatory effects of MitoTEMPO treatment on blood glucose and diaphragm muscle mechanical activity. Among these mechanisms, oxidative stress parameters, intracellular Ca^{++} concentration as a contraction-relaxation regulator, insulin levels, etc., can be mentioned.

CONCLUSIONS

In conclusion, it is essential for DM patients to maintain the physiological working capacity of the diaphragm muscle for healthy respiratory functions. Our findings demonstrated that MitoTEMPO treatment regulates blood glucose levels in diabetic rats, thereby correcting the impairment in the maximum tetanic force and $-dF/dt_{max}$ values of the diaphragm muscle. Thus, MitoTEMPO can be used as an alternative therapeutic agent in patients with DM.

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