Changes in Muscle Biomarkers Following Swimming Exercise in Streptozotocin-induced Rats*

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ABSTRACT

Youngsik Kim · Storm Reid · Eunkee Park · Yehchan Ahn · Byeonghwan Jeon. Changes In Muscle Biomarkers Following Swimming Exercise in Streptozotocin-induced Rats. *The Journal of Kinesiology*, 2017, 19(3): 1-13. **[PURPOSE]** The purpose of this study was to assess the effect of swimming exercise in diabetic atrophy by monitoring changes in biomarkers associated with musculoskeletal growth and abnormalities. **[METHODS]** Ninety-two male Sprague-Dawley rats were randomly divided into four groups: control plus exercise group (CO+Ex), control and no exercise group (CO+No Ex), diabetes plus exercise group (DM+Ex) and diabetes and no exercise group (DM+No Ex). Swimming exercise was implemented for 60 min/day, 5 day/week for a total of eight weeks. Muscle strength and atrophy biomarkers as well as muscle morphology were compared at the first, second, fourth and eighth week of experimentation. **[RESULTS]** ACE levels were significantly higher in the DM groups ($p<.05$), however swimming exercise did not significantly affect ACE levels. Irisin levels were significantly higher in the DM and DM+Ex groups compared to the CON, in week one ($p<.05$), and greater in the DM+Ex group at week two compared to CON+Ex ($p<.05$), but swimming did not significantly affect irisin levels over the eight-week period in DM rats. In terms of muscle damage, Myl3 expression was unchanged in the CON groups, but significantly higher in the DM+Ex group compared to the CON+Ex group ($p<.05$). AST was found to be in higher levels in the DM+No Ex group, compared to CON and CON+Ex ($p<.05$); and DM+Ex group, compared to the CON+Ex group at weeks one, four and eight ($p<.05$). Tibialis anterior muscle fibers were thicker in the DM+Ex group compared to DM+No Ex, but only at week four ($p<.05$). Otherwise, no other notable differences were recorded in muscle morphology. **[CONCLUSIONS]** These results suggest that swimming exercise alone may not be sufficient to induce muscle strength and/or protective effects against muscle atrophy associated with diabetes mellitus. However, this mode of exercise may be considered safe by not compounding the deteriorative effects of this disease.

Key words : atrophy, AST, ACE, irisin, myl3

주요어 : 근위축, AST, ACE, irisin, myl3

Introduction

Diabetes mellitus (DM) is a disease that has a high rate of mortality due to its complications, and is reported to be a rising global epidemic (Robertson et al., 2010; Zimmet et al., 2014). The diabetic condition characteristically manifests in the form of insulin resistance and its associated complications; one of them being skeletal muscle atrophy. Muscle atrophy is the gradual loss of muscle tissue caused

by the environment and several diseases including malnutrition, immobilization, cachexia, and denervation (Evans, 2010; Hepple, 2012). Skeletal muscle abnormalities associated with defective insulin receptor signaling (Sloniger et al., 2005; Wei et al., 2006; Lee et al., 2015), skeletal muscle mitochondrial dysfunction (Kelly et al., 2002; Russell et al., 2014), decreased oxidative fiber number, and atrophy (Perry et al., 2016) have all been implicated in the DM condition.

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Involvement of the renin-angiotensin-aldosterone system (RAAS) in muscular degradation that takes place in catabolic conditions, such as DM, has been reported in the literature. RAAS is a key systemic regulator of blood pressure, salt and fluid homeostasis, and cardiac function. However, excess activation of RAAS, typically via increased production of angiotensin II (Ang II), can induce skeletal muscle abnormalities. The infusion of Ang II resulted in cachexia and decreased IGF-1 circulation - possibly by inhibiting the autocrine IGF-1 system (Brink et al., 2001), via the Akt/mTOR/p70S6K pathway (Song et al., 2005). Wei et al. (2006) demonstrated that Ang II markedly enhanced NADPH oxidase activity, subsequently increasing ROS generation and impairing insulin signaling in skeletal muscle. Moreover, Kadoguchi et al. (2015) corroborated these results, suggesting a pivotal link between mitochondrial dysfunction and a decrease in oxidative fiber number, leading to muscle atrophy. Several studies on DM have shown that by blocking the synthesis of Ang II with angiotensin-converting enzyme (ACE) inhibitors, muscle dysfunction may be prevented (Cameron, Cotter & Robertson., 1992), mitochondrial dysfunction in skeletal muscle ameliorated and exercise capacity improved (Takada et al., 2013).

Other biomarkers that may indicate atrophy of skeletal muscle in the DM state include, Myl3 (myosin essential light chain 3); released into the circulation as a result of slow twitch muscle damage - which is specifically decreased in the DM population. Exercised-induced irisin has also become a growing area of interest. It has been positively related to muscle mass, strength and metabolism and negatively correlated to fasting blood glucose (Chen et al., 2016), and shown to directly modulate muscle metabolism through AMPK kinase phosphorylation (Huh et al., 2014).

Though the mechanisms explaining these

abnormalities remain equivocal, exercise interventions to combat the loss of muscle are constantly being refined in order to provide effective and practical methods of managing DM, particularly diabetic atrophy. Swimming offers an alternative to weight bearing exercise, especially for those who struggle with weight-bearing exercise (Hart et al., 2001) - including elderly, arthritic, osteoporotic and diabetic populations. Aerobic exercise, including swimming, has been shown to induce structural and functional adaptations to muscle (Broderick et al., 2005; Lee et al., 2012; Junior et al., 2013) possibly mediated through the activation of signaling pathways regulating apoptosis and protein degradation. Swimming has also been put forward as an effective mode of exercise for the diabetic population in terms of lowering cardiovascular risk and overall mortality in type 1 and 2 diabetes patients (Colberg et al., 2016). To create an experimental condition of diabetes in animal subjects, the current study utilized the widely implemented administration of streptozotocin (STZ) to induce DM, and muscle atrophy as a result. STZ causes pancreas swelling, degeneration in Langerhans islet beta cells and changes normal metabolism in diabetic rats (Akbarzadeh et al., 2007).

The purpose of this study, therefore, was to focus on the effect of swimming exercise in diabetic atrophy, by monitoring changes in biomarkers associated with musculoskeletal growth and abnormalities.

Methods

Animal experiments

Ninety-two male Sprague-Dawley rats (Hyochang Science, Daegu, Korea) were used in this study. All groups of animals were purchased at the age of seven weeks and acclimated for one week before the

experiment. The temperature in the room in which the rats were kept was maintained at 22–23°C with a humidity of 50–55 % and a 12-h light-dark cycle. A diet of commercial rodent pellets and water were supplied ad libitum. After the first week, the body weight of rats was between 280–310g (Data was not shown). The animals were randomly divided into four groups: control plus exercise group (CO+Ex), control and no exercise group (CO+No Ex), diabetes plus exercise group (DM+Ex) and diabetes and no exercise group (DM+No Ex). Every week, animals in each group were sacrificed to collect whole blood and to dissect the tibialis anterior (TA) muscle. TA muscle was chosen to compare muscle weight and morphology among groups. The animal care and experimental procedures were approved by the Animal Ethics Committee of Kosin Gospel Hospital, Busan, Korea.

Swimming exercise

After the injection of streptozotocin (STZ), swim exercise was conducted in the exercise group. Swim exercise occurred in large containers filled with lukewarm water (30–34°C). The container condition was composed with deep depth and rats could not touch the bottom or hang on the sides and were supervised the entire swim time. The training phase consisted of swimming 60 min/day, 5 day/week for a total of 8 weeks in the rats of eighth week sacrificed rats.

Streptozotocin-induced diabetic rats

Diabetes was induced by STZ. The STZ was dissolved in 0.1 M citrate buffer (pH 4.5) before the injection. The buffer and streptozotocin solution were made fresh for every injection. The solution was administrated by intraperitoneal injection (55 mg/kg B.W.). After 24 h, glucose levels were measured

using a glucose checker (Accu-Chek® Performa; Roche, Basel, Swiss). A glucose level higher than 300 mg/dL was considered successful for inducing diabetes (Wohaieb & Godin, 1987; Masiello et al., 1998; American Diabetes Association, 2006).

Measurement of growth factors and inflammatory factors

The concentrations of key hormones involved in the synthesis of skeletal muscle protein and inflammation within tissues were measured in blood circulation, as follows. These factors play a critical role in communication between tissues, in muscle atrophy and the inflammation process, in DM.

Irisin, an exercised-induced myokine said to play an important role in metabolic regulation in disease states, such as diabetes, was reported in the present study. Measurement of irisin in rat serum samples was performed using a commercial EK-067-29 irisin recombinant enzyme immunoassay (ELISA) kit with a measurement interval of 0.1–1000 ng/mL (Phoenix Pharmaceuticals, Inc.; Burlingame, CA, USA) according to the manufacturer's instructions. Absorbance from each sample was measured using a microplate reader (EMax Precision Microplate Reader; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm.

In order to evaluate muscle damage to slow-twitch skeletal muscle, serum Myl3 concentration was measured. To measure Myl3 concentration, we collected blood samples from the abdominal vein with a syringe. The collected blood was stored in plain tubes. When the whole blood was fully clotted, the clots were removed by centrifuging at 3,000 rpm for 15 min. These serum samples were diluted to the proper concentration before the ELISA experiment. ELISAs were conducted using commercial kits [Myl3: Abnova

Myl3 (mouse, rat) ELISA kit, Seoul, Korea]. The specific method was according to manufacturer's protocol. The intensity was measured using a microplate reader (EMax Precision Microplate Reader; Molecular Devices, Sunnyvale, CA, USA).

ACE mediates the cleavage of the synthetic substrate (FAPGG=N-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-glycyl-L-glycine) into an amino acid derivate and a dipeptide. The kinetic of this cleavage reaction is measured by recording the decrease in absorbance at 340 nm using a commercially available kit from BÜHLMANN Laboratories (Basel, Switzerland).

AST (Aspartate aminotransferase, or glutamate oxalacetate transaminase) belongs to the transaminases, which are enzymes that catalyze the transfer of α -amino groups from aspartate to the α -keto group of ketoglutaric acid to generate oxalacetic acid - important contributors to the citric acid cycle. A standardized procedure provided by the manufacturer (COBAS from Roche Diagnostics, Indianapolis, USA) was used for AST. AST measurement included optimization of substrate concentrations, employment of Tris buffers, pre-incubation of a combined buffer and serum to allow side reactions with NADH to occur, substrate start, and optional pyridoxal phosphate activation.

Histological analysis

To confirm morphological changes associated with atrophy, TA muscle was fixed with 10% neutral buffered formalin. These tissues underwent paraffin embedding and were sliced into thin sections using a microtome in order to generate serial sections of tissues. The serial sections were then fixed on glass slides and stained with hematoxylin and eosin. The images were examined and captured by a digital camera (Digital Sight DS-U1; Nikon, Japan) attached to a microscope (Eclipse 80i, Nikon, Japan).

Statistics

Statistical analysis was performed using the paired t-test to compare values in the same group. Comparisons between groups were performed with one way ANOVA for the independent. The level of statistical significance was set at $p < .05$. All statistical analyses were performed with SPSS 19.0 (IBM Corporation, Armonk, NY, USA).

Results

Body weight, blood glucose levels, and muscle weight

The results following an 8-week swimming exercise program on muscle biomarkers in streptozotocin-induced diabetic rats confirmed that the presence of diabetes resulted in weight loss and muscle atrophy <Table 1 and 2, respectively>. Exercise intervention, however, did not significantly alter body weight or muscle weight in the experimental groups. Blood glucose levels were also significantly higher in the DM groups compared to CON, as expected <Table 3>. However blood glucose levels were not significantly different between Ex and No Ex rats.

Table 1. Animal body weight (g)

Group	Exercise	1st	2nd	4th	8th
Control	No Ex	312.0 (17.0)a	359.7 (10.4)a	426.5 (22.4)a	518.7 (9.8)a
	Ex	298.5 (14.5)a	388.6 (13.3)a	426.4 (9.6)a	454.1 (33.0)a
Diabetes	No Ex	252.5 (22.6)b	264.5 (9.6)b	241.7 (76.7)b	167.9 (13.7)b
	Ex	231.6 (12.5)b	241.9 (25.8)b	249.0 (27.2)b	216.1 (51.3)b

Data presented as mean (S.D.). a,b: indicates a significant difference among groups in the same week. Statistical significance was determined using an ANOVA (post hoc test is Tukey test, $p < .05$).

Figure 1. Changes in muscle growth and damage biomarkers: angiotensin converting enzyme (ACE) (A), Irisin (B), MyI3 (encoding slow twitch alkali myosin light chain) (C), and AST (aspartate aminotransferase) (D). All measurements were determined at 1, 2, 4 and 8 week intervals. Each point indicates the mean and S.D. a,b,c: indicates significant difference among groups in the same week. The statistical significance was determined using an ANOVA (post hoc test is Tukey test, $p < .05$).

Table 2. Animal tibialis anterior muscle weight (g)

Group	Exercise	1st	2nd	4th	8th
Control	No Ex	0.88 (0.04)a	0.96 (0.08)a	1.16 (0.03)a	1.28 (0.11)a
	Ex	0.79 (0.08)a	0.91 (0.08)a	0.95 (0.11)a	1.29 (0.14)a
Diabetes	No Ex	0.66 (0.12)b	0.68 (0.07)b	0.58 (0.20)b	0.39 (0.08)b
	Ex	0.62 (0.09)b	0.55 (0.14)b	0.45 (0.16)b	0.46 (0.12)b

Data presented as mean (S.D.). a,b: indicates a significant difference among groups in the same week. Statistical significance was determined using an ANOVA (post hoc test is Tukey test, $p < .05$).

Table 3. Animal blood glucose level (mg/dl)

Group	Exercise	1st	2nd	4th	8th
Control	No Ex	111.0 (10.8)a	143.2 (14.3)a	108.0 (9.4)a	108.8 (8.7)a
	Ex	106.0 (6.3)a	122.0 (26.4)a	123.0 (10.9)a	97.3 (10.9)a
Diabetes	No Ex	443.2 (55.0)b	486.8 (55.0)b	474.7 (72.3)b	462.3 (51.2)b
	Ex	439.0 (67.7)b	417.3 (37.3)b	489.6 (70.4)b	491.0 (34.0)b

Data presented as mean (S.D.). a,b: indicates a significant difference among groups in the same week. Statistical significance was determined using an ANOVA (post hoc test is Tukey test, $p < .05$).

Figure 2. Serial sections of tibialis anterior muscle stained with hematoxylin and eosin taken at week one, two, four and eight in CON, CON+Ex, DM and DM+Ex rats. Note significantly thicker muscle fibers in DM+Ex group compared to DM+No Ex at week four.

Table 4. Normalized Irisin level for muscle weight (ng/ml/g)

Group	Exercise	1st	2nd	4th	8th
Control	No Ex	28.6 (5.3)a	25.3 (5.5)a	19.8 (2.9)a	16.9 (1.4)a
	Ex	25.7 (4.6)a	24.6 (2.8)a	28.0 (4.5)a	22.5 (2.8)a
Diabetes	No Ex	47.8 (10.2)b	35.5 (2.1)b	34.1 (7.5)a	53.7 (14.6)b
	Ex	57.5 (13.4)b	60.5 (9.7)c	71.4 (19.4)b	71.8 (15.3)b

Data presented as mean (S.D.). a,b: indicates a significant difference among groups in the same week. Statistical significance was determined using an ANOVA (post hoc test is Tukey test, $p < .05$).

Muscle growth and damage factors

ACE levels were significantly higher in the DM

groups ($p < .05$), however swimming exercise did not significantly affect ACE levels following the 8-week experimental period <Figure 1A>. Irisin levels in the CON group were enhanced with the introduction of exercise, as shown by the gradual increase of plasma irisin with each week <Figure 1B> in the CON+Ex group. Irisin levels were significantly higher in the DM and DM+Ex groups compared to the CON, in week 1 ($p < .05$), and greater in the DM+Ex group at week 2, compared to CON+Ex ($p < .05$). However, exercise itself did not modulate change in plasma irisin over the course of the 8 week experimental period in rats with DM. When normalizing irisin data for muscle weight, the DM+Ex group showed an enhanced effect of exercise compared to DM+No

Ex <Table 4> significant at week two and four ($p<.05$).

In regards to muscle damage, Myl3 expression was unchanged in the CON group as a result of exercise. However, Myl3 expression was significantly higher in the DM+Ex group compared to the CON+Ex group ($p<.05$) <Figure 1C>. Muscle damage was thus observed to be greater in DM sufferers in the presence of exercise than in a normal cohort. AST, an enzyme elevated in the blood as a result of muscle damage, was found to be in higher levels in the DM+No Ex group, compared to CON and CON+Ex ($p<.05$); and DM+Ex group, compared to the CON+Ex group at weeks 1, 4 and 8 ($p<.05$) <Figure 1D>.

Morphological assessment of skeletal muscle showed that the DM+Ex had thicker muscle fibers than the DM+No Ex group, but only in the fourth week of the present study <Figure 2>.

Discussion

The results from the current study demonstrate that swimming exercise had no significant effect on body weight, muscle weight and/or blood glucose levels. Tissue morphology didn't indicate a significant effect of swimming on TA muscle fiber thickness. Muscle damage biomarker, Myl3, levels were also unchanged by swimming exercise intervention, despite being higher in the DM+Ex group. Nonetheless, swimming exercise was shown to prevent a deteriorative response in ACE, irisin and AST levels. Diabetes mellitus is a complex pathology that can lead to several other complications (Emerging Risk Factors Collaboration, 2010). DM is an independent risk factor for the development of skeletal muscle atrophy and linked to numerous diseases (Evans, 2010). It is therefore

important to ascertain the mechanisms involved in this disease and develop safe interventions, such as the use of exercise, to help manage diabetes-related muscle atrophy.

The renin angiotensin aldosterone system (RAAS) plays a key role in the regulation of blood pressure in the body (Atlas, 2007). With specific reference to skeletal muscle function, the ACE enzyme, responsible for converting angiotensinogen to angiotensin, is involved in promoting the degradation of protein in muscle (Brink et al., 2001). Furthermore, several studies have implemented the use of ACE inhibitors as medication in muscle atrophy (Semprun-Prieto, 2011; Sukhanov 2011). In the present study, the diabetes groups had higher levels of ACE than the control groups, but the swimming exercise intervention did not significantly affect ACE levels after the 8 weeks. The higher levels of ACE in the DM+Ex group may be related with the loss in muscle weight shown in the current study. Ang II, the resulting hormone of ACE-stimulated conversion of Ang I, is associated with anorexigenic and catabolic effects on skeletal muscle, including an inhibitory effect on the autocrine IGF-1 system (Brink et al., 2001). In addition, Tidgren et al. (1991) demonstrated a positive correlation between exercise intensity and renal renin secretion rates, subsequently leading to increased plasma renin activity; implying that swimming exercise was a safe intervention, not causing additional deteriorative effects on diabetes - marked by an increase in ACE. Interestingly, leisure-time physical activity has been inversely related to mortality in type 1 diabetes, particularly, the total amount of activity (Waden et al., 2015). This continues to raise the challenge of prescribing optimal exercise programs for the diabetic population, with adequate, but safe exercise frequency and intensity. Previous studies have implicated ACE, and the increase in plasma Ang II,

in the decrease in skeletal muscle protein kinase B (Akt) phosphorylation, a key component of protein synthesis (Wei et al., 2006; Ohta et al., 2011; Fukushima et al., 2014) and diabetic neuropathy in type 1 (Lewis et al., 1993) and type 2 (Amann et al., 2003; Lindhardt et al., 2016) DM. However, a clear mechanism is yet to be elucidated, and intervention applied, linking aerobic exercise, the RAAS system and diabetic atrophy.

Irisin is a signaling protein that is secreted into the blood from skeletal muscle; purportedly, to mediate the beneficial effects of exercise on metabolism (Moreno-Navarrete, 2013; Pekkala, 2013) and convert subcutaneous white fat into brown fat, enhancing metabolic uncoupling, thus increasing caloric expenditure (Boström et al., 2012). The function of irisin in humans is not well understood at present, but it is attracting investigation for its relationship with insulin resistance and metabolic syndrome, and role as a putative exercise-induced myokine. Subjects with DM demonstrated higher levels of irisin throughout our study. This is in accordance with levels found in type 1 diabetes subjects (Espes, et al., 2015) and may be a result of early-onset diabetes diminishing the response of skeletal muscle and adipose tissue to insulin, thus affecting the expression of FNDC5 (fibronectin type III domain-containing protein 5). The present study does not report a significant effect of exercise on irisin levels in DM subjects, until results are adjusted for muscle weight; of which, the DM+Ex group showed an enhanced effect of exercise compared to DM+No Ex - significant at week two and four. The same phenomena has been demonstrated in previous studies (Anastasilakis et al., 2014; Espes et al., 2015). This adds to the controversial data arising from research into the regulatory role of exercise on serum irisin levels. Circulating irisin levels have been shown to be significantly lower in obese and type 2 diabetic

patients (Moreno-Navarrete, 2013; Liu et al., 2013; Liu et al., 2014). However, in vitro these results were not replicated in palmitate and glucose lowered Fndc5 mRNA treated muscle cells, indicating a regulatory effect of diabetes-related factors on circulating irisin and its precursor FNDC5 (Kurdiova et al., 2014). In support of this premise, several complications of DM, including hyperglycaemia and triglyceridaemia, were negatively associated with circulating irisin (Kurdiova et al., 2014). Furthermore, Choi et al. (2013) demonstrated that 2 h plasma glucose was an independent variable influencing serum irisin levels. Thus, considering the gradual decrease in muscle weight in the DM+Ex group, it may be put forward that - in the presence of metabolic disease - adipose tissue, plasma glucose levels and other DM associated complications are more closely related to irisin levels.

Myl3 is released into the circulation following damage to cardiac and slow twitch muscle (Schiaffino et al., 2015). According to recent reports, the expression of Myl3 was induced by electrical stimulation in paralysis patients (Adams, 2011). The current study demonstrated that the prescribed exercise did not affect Myl3 expression in the control group, but Myl3 levels were significantly higher in the DM+Ex group. These results provide a strong indication of muscle damage present in the DM condition, but not that swimming exercise significantly improves these biomarkers.

Other results that confirm the negative impact of DM include blood glucose levels which were significantly higher in both diabetes groups, however a modulating effect of exercise on blood glucose was not observed. High concentration of blood glucose is considered an independent risk factor for muscle loss, in people with (Toledo et al., 2007) and without diabetes (Barzilay et al., 2009). In addition, hyperglycaemia and insulin resistance have also been associated with slower walking speeds (Kuo et al.,

2009). Toledo et al, demonstrated that moderately intense exercise in addition to weight loss significantly improved fasting and 2-h post-challenge plasma glucose values, but the relatively unchanged body weight in the present study may indicate that exercise in conjunction with diet is necessary to induce significant improvements in blood glucose and muscle atrophy.

In support of swimming exercise having a regulatory role in DM, AST levels in the current study increased in DM+No Ex group as a result of DM and the complications associated, after the 8 weeks. AST - increased by liver, heart and muscle problems (Vozarova, 2002; Giannini, 2003; Nathwani, 2005) - is elevated in the presence of DM (Giboney, 2005). The DM+Ex group did not, however, demonstrate the same degree of degeneration, but exercise had more of a protective effect on liver condition. Vigorous exercise has been condoned for the management of non-alcoholic fatty liver disease (Kistler et al., 2011), which is associated with DM. This further highlights the importance of exercise intensity in prescribing the optimal dose of exercise, while considering the contraindications of high intensity exercise in severe DM.

Conclusions

The results from the current study indicate that swimming exercise alone may not suffice to induce prophylactic effects on the DM condition, particularly atrophy related. There was no profitable effect on body weight, muscle weight and/or blood glucose levels by swimming exercise. Tissue histology also highlighted the lack of observable change in muscle tissue following swimming exercise. Some promise was shown regarding the health preserving impact of swimming exercise on

DM, with ACE, irisin and AST levels not demonstrating deteriorative responses. The complications associated with DM need further investigation in terms of their inhibitory effects on exercise-induced maintenance and/or improvement of the DM atrophic condition.

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