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ORIGINAL ARTICLE

Quantitative Morphometric Study of the Skeletal Muscles of Normal and Streptozotocin-Diabetic Rats

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ABSTRACT

Objectives Quantitative morphometry under light microscope was applied to analyze changes in the number and the diameters of skeletal muscle fibers and their myonuclei in the extensor digitorum longus and rectus femoris muscles of normal and streptozotocin-diabetic rats.

Animals Twelve adult male albino Fischer rats each weighing 300 g were used in the study.

Interventions Streptozotocin (STZ)-diabetes was induced by a single intravenous injection of STZ (75 mg/kg body weight) via the tail vein. Six normal and 6 STZ-diabetic rats were sacrificed; samples of the extensor digitorum longus and rectus femoris muscles were taken, fixed in modified Bouin's fluid and processed for paraffin sectioning. The muscle samples were properly oriented during paraffin embedding for cross and longitudinal sectioning. Sections from each block were cut, processed and stained with hematoxylin and eosin. Randomly selected samples from normal and STZ-diabetic rats were analyzed using a 100x objective lens of a light microscope.

Main outcome measures The diameters of the fibers and the length of the myonuclei were determined from the longitudinal sections while the diameters and number of fibers and myonuclei were measured from the cross sections. A length measuring 10x reticule fitted to the microscope eye piece was used for the direct measurement of the fibers and myonuclei profiles. Morphometric measurement from each rat was determined and the data were pooled for the control and diabetic groups of rats. All data presented are means with standard error and were statistically analyzed using the Student's t-test.

Results Histological examination of the stained sections from diabetic rats revealed the presence of areas of inflammation and necrosis in the myofibers of both muscles. The estimated mean diameter of the muscle fibers in the STZ-diabetic rats was reduced by 36% and 31% respectively in the longitudinal and cross sections of the extensor digitorum longus. Similarly, the diameter of the fibers of the rectus femoris in the longitudinal and the cross sections were reduced by 44% and 31%, respectively. On the other hand, a corresponding increase in the number of fibers per unit area was recorded in both muscles of the STZ-diabetic rats which amounted to 13% and 16%, respectively as compared to those of normal rats. Analysis of the myonuclei in normal and diabetic rats revealed a slight decrease in their length and diameter which amounted to 4% and 6%, respectively for the extensor digitorum longus and to 4% and 18%, respectively for the

rectus femoris. The estimated numerical density of myonuclei per unit area was 10% lower in both muscles of the diabetic rats.

Conclusions Skeletal muscular atrophy is a well-documented complication in long-standing diabetes and has been attributed to the direct effect of low serum insulin on the motor end plates and on the synthesis of contractile proteins. The present morpho-metric study illustrates a reduction in the diameter of the myofibers of the extensor digitorum longus and rectus femoris muscles of STZ-diabetic rats. A slight but significant decrease in the length and diameters of the myonuclei between the diabetic and the normal rats was recorded. The results also indicate more evident morphologic changes in the myofibers of the hindlimb muscle.

INTRODUCTION

The concept of insulin action on the skeletal muscles is of great significance in glucose homeostasis, especially in the treatment of type II diabetes. Normally, about 75% of the total body glucose uptake stimulated by insulin is mediated by the skeletal muscles [1, 2]. Insulin action on skeletal muscle fibers takes place via specific membrane receptors coupled to tyrosin kinase activity which eventually leads to glucose uptake by activated GLUT 4 transporters [2]. A large number of reports on the physiology of skeletal muscle fibers in human type I diabetes and in experimentally induced streptozotocin- (STZ) diabetes have revealed a synaptic delay at the motor end plate with a decrease in the contractility of the fibers [3, 4, 5, 6]. Histochemical and morphologic studies with light and electron microscopes have illustrated degeneration and necrosis of the myofibers with atrophy of type 2A and 2B myofibers in the hindlimb muscles of STZ-diabetic mice and rats [7, 8], and myofibril derangement and mitochondrial swelling in the hindlimb muscles of spontaneous diabetic WBN/Kob rats [9]. Recent studies on human type II diabetes have illustrated atrophy in the distal muscles of the lower limbs [10]. Several cases of acute skeletal muscular infarction in type II diabetics which are attributed to microangiopathic and coagulopathic causes induced by prolonged lack of insulin have been reported [11, 12, 13]. Reviewing the literature, it seems that few quantitative analytic studies on diabetic skeletal muscles have been conducted. Therefore, the aim of this study was to comprehensively analyze the morphometric changes in the number and size of myofibers and their myonuclei in the extensor digitorum longus and rectus femoris muscles of STZ-induced diabetic rats. A comparison in the morphometric data between forelimb and hindlimb muscles was also carried out in order to point out any regional differences in the effect of insulin depletion.

METHODS

Twelve adult male albino Fischer-344 rats (about 300 g in body weight) were used in the study. A group of 6 rats received citrate buffer as a drug vehicle and were maintained on normal food pellets and water while another group of 6 rats were made diabetic by a single intravenous injection of STZ (75 mg/kg body weight) in 0.05 M citrate buffer (pH 4.5) via the tail vein. The STZ-injected rats were kept in separate cages and maintained on the same food pellets. The diabetic status in the STZ-injected animals was confirmed from the elevated blood glucose levels and markedly positive glucosuria. Body weight, blood glucose and glucosuria in the normal and diabetic rats were regularly recorded until sacrifice. Four weeks after the onset of the experiment, normal and STZ-diabetic rats were sacrificed under nembutal anesthesia after recording their body weight, fasting blood sugar level and glucosuria. Blood samples for serum insulin assay were taken, the fore and hind limbs of each rat were dissected and samples from the central part of the extensor digitorum longus and rectus femoris muscles were cut, cleaned of fibrous tissue and fixed in modified Bouin's fluid (picric acid:formaldehyde 3:1) for 24 h at room temperature with continuous shaking. The tissue samples dehydrated in graded ethanol series were embedded in paraffin with

proper orientation for cross and longitudinal sectioning.

For the morphometric analysis, an unbiased sampling procedure was applied. Four tissue blocks for cross and longitudinal sectioning per animal were randomly selected, and serial sections of 4 µm thickness were cut and mounted on egg albumin-coated slides. All sections were processed for routine histological examination, stained with hematoxylin-eosin, and examined with a light microscope (Olympus, Tokyo, Japan). One cross and one longitudinal section per block were selected, and three microscopical fields per section were randomly selected for measuring selected parameters. The mean diameter of the muscle fibers and the length of the myonuclei per field were measured from longitudinal sections while the mean number of the muscle fibers, the diameter of the muscle fibers, the number of myonuclei and the diameter of the myonuclei per field were recorded from the cross sections. The diameters of the muscle fibers and myonuclei from the cross sections were calculated from their major and minor profile semi-axes according to the formula of Abercrombie [14]. All quantitative measurements were carried out with a100x objective lens using a calibrated length reticule.

The serum insulin level from each rat was measured by means of the microparticle enzyme immunoassay (MEIA) method using AxSYM insulin assay apparatus (Abbott Laboratories, Abbott Park, IL, USA).

ETHICS

The rats were bred at Jordan University Animal House following the principles of laboratory animal care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals (1996)" prepared by the National Academy of Sciences.

STATISTICS

Data from each animal were estimated separately (n=4 blocks), and the mean data were pooled for the entire group of normal and STZ-diabetic rats (n=6 animals in each

Table 1. The estimates of body weight, fasting blood sugar level, glucosuria and serum insulin in the normal and the STZ-diabetic rats.

Animals	Body weight at onset of experiment (g)	Body weight at sacrifice (g)	FBS at onset of experiment (mg/dL)	FBS at sacrifice (mg/dL)	Glucosuria at onset of experiment[a]	Glucosuria at sacrifice[a]	Serum insulin (µU/mL)
Normal rats							
Rat 1	290	302	65	78	–ve	–ve	21.4
Rat 2	300	310	58	62	–ve	–ve	38.1
Rat 3	285	290	72	68	–ve	–ve	17.6
Rat 4	288	296	85	70	–ve	–ve	19.0
Rat 5	302	306	68	58	–ve	–ve	20.5
Rat 6	298	310	62	80	–ve	–ve	18.4
Mean±SEM	**293.8±2.9**	**302.3±3.3**	**68.3±3.9**	**69.3±3.5**			**22.5±3.2**
STZ-diabetic rats							
Rat 1	290	265	78	420	–ve	++++ve	4.1
Rat 2	310	284	55	380	–ve	++++ve	0.9
Rat 3	294	270	65	480	–ve	++++ve	3.9
Rat 4	305	260	85	350	–ve	++++ve	5.5
Rat 5	285	250	58	280	–ve	++++ve	3.5
Rat 6	295	262	64	310	–ve	++++ve	6.5
Mean±SEM	**296.5±3.8**	**265.2±4.6**	**67.5±4.8**	**370.0±29.9**	**-**	**-**	**4.1±0.8**
P value	0.589	<0.001	0.895	<0.001	-	-	<0.001

FBS: fasting blood sugar
SEM: standard error of mean
STZ: streptozotocin
[a] -ve: less than 50 mg/dL; ++++ve: greater than 1,000 mg/dL

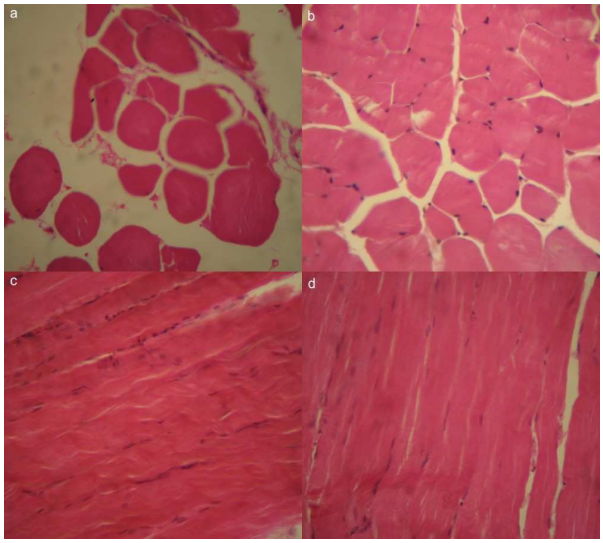

Figure 1. H&E stained sections of the extensor digitorum longus muscle. **a.** Cross section of a normal rat. **b.** Cross section of a STZ-diabetic rat. **c.** Longitudinal section of a normal rat. **d.** Longitudinal section of STZ-diabetic rat. (Magnification x400).

group). The data are presented as means with standard error of the mean (SEM), and they were analyzed with the Student's t-test. Data were analyzed by means of the SPSS Version 12 for Windows. Two-tailed P values less than 0.05 were considered statistically significant.

RESULTS

The STZ-diabetic rats showed a significant decrease in their body weight with marked hyperglycemia and positive glucosuria. The serum insulin level was remarkably reduced in streptozotozin-diabetic rats. Table 1 illustrates the values of body weight, fasting blood sugar, glucosuria and serum insulin levels for each rat in the control and the diabetic groups.

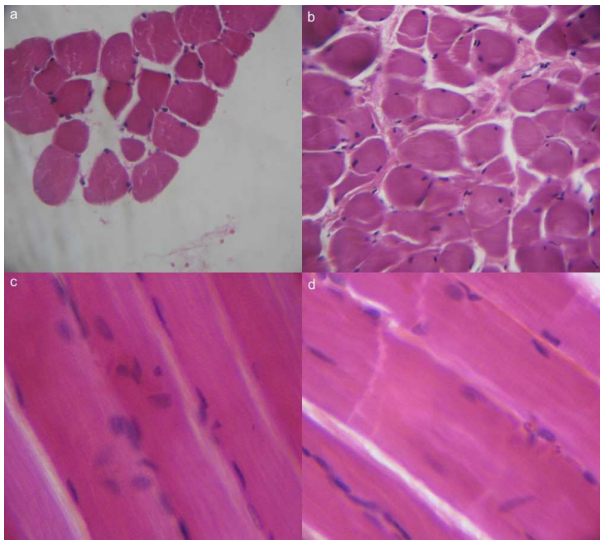

Figure 2. H&E stained sections of the rectus femoris muscle. **a.** Cross section of a normal rat. **b.** Cross section of a STZ-diabetic rat. **c.** Longitudinal section of a normal rat. **d.** Longitudinal section of STZ-diabetic rat. (Magnification x400).

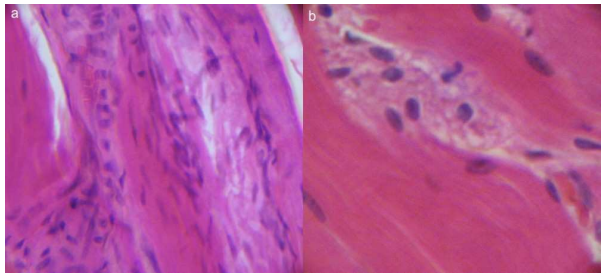

Figure 3. a. H&E stained section of the extensor digitorum longus muscle in a STZ-diabetic rat showing an area of inflammatory cell infiltration among the normal myofibers. **b.** H&E stained section of the rectus femoris muscle in an STZ-diabetic rat showing necrosis of the myofibers. (Magnification x400).

Histological examination of hematoxylin-eosin stained sections from the extensor digitorum longus and rectus femoris of normal and diabetic rats showed adequately preserved myofibers with clear striation and peripheral myonuclei (Figures 1a-d and 2a-d). Areas of inflammation infiltrating the fibers of both muscles were seen in the STZ-diabetic rats (Figure 3a). These zones showed an increase in the number of blood capillaries with many inflammatory cells invading the intercellular spaces. Occasionally, small areas of necrosis and lyses were seen among normal looking fibers (Figure 3b).

Table 2 illustrates the morphometric data of parameters measured from all fields analyzed in the longitudinal and cross sections of the extensor digitorum longus in one of the normal rats.

Table 3 illustrates the mean data obtained from the longitudinal and cross sections of the extensor digitorum longus muscle in the normal and STZ-diabetic rats. The diameters of the muscle fibers estimated from the longitudinal sections was 36% lower in diabetic rats (20.6±1.3 µm *versus* 32.4±0.2 µm, P<0.001). Similarly, the diameter of the muscle fibers estimated from the cross

Table 2. The morphometric data of the parameters measured in the cross and longitudinal sections of the extensor digitorum longus in one of the normal rats.

	Longitudinal sections		Cross sections			
	Diameter of muscle fiber (µm)	**Longitudinal length of muscle fiber (µm)**	**Diameter of muscle fiber (µm)**	**Number of muscle fiber per field**	**Diameter of myonuclei (µm)**	**Number of myonuclei per field**
Section 1						
Field 1	33.4±2.7	15.3±0.6	45.8±2.4	10	4.53±0.80	18
Field 2	39.7±2.9	15.1±0.6	47.1±2.7	14	4.02±0.23	19
Field 3	37.5±1.9	14.9±0.7	42.5±2.3	16	4.10±0.18	25
Mean±SEM	**36.9±1.5**	**15.1±0.1**	**45.1±1.1**	**13.3±1.4**	**4.22±0.13**	**20.7±1.8**
Section 2						
Field 1	29.3±2.7	14.8±0.8	41.4±2.1	14	4.20±0.40	17
Field 2	32.7±2.8	15.6±1.06	41.2±2.6	12	5.23±0.35	22
Field 3	32.3±1.9	15.9±1.1	34.4±1.8	10	3.95±0.34	26
Mean±SEM	**31.4±0.9**	**15.4±0.3**	**39.0±1.9**	**12.0±0.9**	**4.46±0.32**	**21.7±2.1**
Section 3						
Field 1	30.6±1.8	13.8±1.5	38.6±1.8	14	4.42±0.60	16
Field 2	28.6±2.2	14.4±1.4	40.4±2.3	12	3.84±0.24	20
Field 3	32.4±1.8	14.8±0.8	42.6±1.8	12	4.90±0.16	24
Mean±SEM	**30.5±0.9**	**14.3±0.2**	**40.5±0.9**	**12.7±0.5**	**4.38±0.25**	**20.0±1.9**
Section 4						
Field 1	34.2±1.6	14.2±0.6	42.6±1.4	12	5.23±0.60	19
Field 2	32.6±1.3	13.8±1.2	40.8±1.6	10	4.82±0.42	21
Field 3	30.8±1.9	14.6±0.8	40.4±1.2	12	4.21±0.34	22
Mean±SEM	**32.5±0.8**	**14.2±0.2**	**41.3±0.6**	**11.3±0.5**	**4.80±0.24**	**20.7±0.7**

Table 3. The mean morphometric data of the parameters measured from the cross and longitudinal sections of the extensor digitorum longus muscle in the normal and the STZ-diabetic rats.

	Longitudinal sections		Cross sections			
	Diameter of muscle fiber (µm)	**Longitudinal length of muscle fiber (µm)**	**Diameter of muscle fiber (µm)**	**Number of muscle fiber per field**	**Diameter of myonuclei (µm)**	**Number of myonuclei per field**
Normal rats						
Rat 1	32.8±1.2	14.8±0.3	41.5±1.1	12.3±0.4	4.5±0.1	20.8±0.3
Rat 2	32.6±1.3	14.2±0.1	37.3±0.8	11.8±0.3	4.3±0.2	20.0±0.4
Rat 3	32.5±0.8	14.5±0.5	41.1±0.3	13.2±0.3	4.3±0.04	17.6±1.9
Rat 4	32.6±0.5	14.7±0.5	43.1±0.5	13.6±0.3	4.4±0.1	19.0±1.4
Rat 5	32.7±0.2	14.8±0.1	44.8±0.9	12.7±0.3	4.3±0.1	17.0±0.8
Rat 6	31.4±1.3	14.8±0.3	44.0±0.6	12.4±0.4	4.3±0.1	16.1±1.4
Mean±SEM	**32.4±0.2**	**14.6±0.1**	**42.0±1.1**	**12.7±0.3**	**4.4±0.03**	**18.4±0.7**
STZ-diabetic rats						
Rat 1	24.8±0.8	14.4±0.5	29.1±1.2	15.4±1.1	4.1±0.1	17.0±0.5
Rat 2	24.3±1.5	15.0±0.7	28.4±0.7	13.7±0.3	4.7±0.1	16.3±0.5
Rat 3	22.3±1.2	13.6±0.2	29.8±1.2	14.2±0.4	4.5±0.1	16.1±1.8
Rat 4	16.9±0.7	14.0±0.5	28.2±0.8	13.0±0.5	3.8±0.1	14.2±0.3
Rat 5	18.0±0.4	14.7±0.3	29.4±0.6	16.2±1.0	3.9±0.1	19.5±1.3
Rat 6	17.5±0.4	14.8±0.4	28.8±1.1	15.3±0.8	4.8±0.1	16.0±1.1
Mean±SEM	**20.6±1.5**	**14.4±0.2**	**29.0±0.2**	**14.6±0.5**	**4.3±0.2**	**16.5±0.7**
Difference (%)	36%	4%	31%	13%	6%	10%
P value	<0.001	0.384	<0.001	0.005	0.783	0.094

SEM: standard error of the mean
STZ: streptozotocin

sections was 31% lower in STZ-diabetic rats (29.0±0.2 µm *versus* 42.0±1.1 µm, P<0.001) with a 13% increase in their numerical density (14.6±0.5 *versus* 12.7±0.3, P=0.005). The lengths and diameters of the myonuclei in the diabetic rats were slightly reduced in both muscle fibers which amounted to 4% (14.4±0.2 µm *versus* 14.6±0.1 µm, P=0.384) and 6% (4.3±0.2 µm *versus* 4.4±0.03 µm, P=0.783), respectively. A 10% decrease in the numerical density of the myonuclei from the cross sections of the diabetic rats was also recorded (16.5±0.7 *versus* 18.4±0.7, P=0.094).

Table 4 records the data obtained from the longitudinal and the cross sections of the rectus femoris muscle in both normal and STZ-diabetic rats. The mean diameter of the fibers in the longitudinal sections was 44% lower in diabetic rats (28.4±1.1 µm *versus* 50.7±3.2 µm, P<0.001). In the cross sections, the reduction in the diameter of the muscle fibers of the diabetic rats was 31% (47.3±0.6 µm *versus* 68.1±1.2 µm, P<0.001), and their numerical density increased by 16% (12.4±0.3 *versus* 10.4±0.2, P<0.001) as compared to the data of the normal rats. The analysis of the myonuclei in the diabetic rats revealed a 4% reduction in their length (17.9±0.2 µm *versus* 18.7±0.3 µm, P=0.093) and an 18% reduction in their diameter (4.0±0.1 µm *versus* 4.9±0.1 µm, P<0.001). The numerical density of the myonuclei from the cross sections was 10% lower in the diabetic rectus femoris (17.6±0.03 *versus* 18.4±0.54, P=0.257).

DISCUSSION

Muscular atrophy and necrosis are well known complications of longstanding human diabetes, and commonly affect the lower limb muscles [11, 12]. This has been attributed to microangiopathies and abnormal coagulation in the skeletal muscle blood vessels caused by persistent hyperglycemia. Most of these cases have been diagnosed from clinical features and radiological imaging procedures, such as MRI, computed tomography, sonography and gallium scintigraphy [12]. Although, 95% of the cases of human diabetic muscular

Table 4. The mean morphometric data of the parameters measured from the cross and longitudinal sections of the rectus femoris muscle in the normal and the STZ-diabetic rats.

	Longitudinal sections		**Cross sections**			
	Diameter of muscle fiber (µm)	**Longitudinal length of muscle fiber (µm)**	**Diameter of muscle fiber (µm)**	**Number of muscle fiber per field**	**Diameter of myonuclei (µm)**	**Number of myonuclei per field**
Normal rats						
Rat 1	41.6±1.6	18.2±0.5	69.8±4.1	10.0±0.4	4.8±0.1	16.4±0.2
Rat 2	45.3±0.5	20.2±0.8	70.6±3.9	11.0±0.1	4.7±0.1	17.6±0.6
Rat 3	52.0±1.8	19.0±1.1	63.2±0.2	10.4±0.3	4.9±0.1	17.6±0.6
Rat 4	63.6±4.6	18.6±0.2	67.5±1.9	10.5±0.3	4.8±0.1	20.0±1.4
Rat 5	54.4±1.1	18.0±0.2	70.6±1.5	10.3±0.2	5.0±0.1	20.0±0.5
Rat 6	47.0±2.3	18.2±0.2	67.0±1.7	10.0±0.3	5.1±0.2	18.6±0.5
Mean±SEM	**50.7±3.2**	**18.7±0.3**	**68.1±1.2**	**10.4±0.2**	**4.9±0.1**	**18.4±0.6**
STZ-diabetic rats						
Rat 1	31.0±1.2	17.5±0.3	46.4±0.6	12.5±0.3	4.2±0.1	18.6±0.6
Rat 2	31.8±0.6	17.2±0.2	47.0±1.6	13.7±0.3	4.1±0.1	16.8±0.4
Rat 3	29.1±0.7	18.5±0.1	48.8±1.3	12.6±0.5	4.0±0.1	17.5±1.3
Rat 4	26.0±0.6	18.7±0.2	49.4±1.2	11.9±0.5	4.1±0.1	16.7±0.3
Rat 5	26.2±0.6	17.6±0.3	46.6±0.5	12.0±1.0	3.6±0.2	18.5±0.7
Rat 6	26.4±1.4	18.1±0.3	45.8±1.8	11.8±0.2	4.1±0.1	17.2±0.4
Mean±SEM	**28.4±1.1**	**17.9±0.2**	**47.3±0.6**	**12.4±0.3**	**4.0±0.1**	**17.6±0.3**
Difference (%)	44%	4%	31%	16%	18%	10%
P value	<0.001	0.093	<0.001	<0.001	<0.001	0.257

SEM: standard error of the mean
STZ: streptozotocin

infarction were confirmed by biopsies, physicians usually prefer noninvasive methods and spare muscle biopsy for atypical and uncertain cases of musclular infarction. Based on this information and from a review of the literature, our study was aimed at exploring the morphological changes in the skeletal muscles of the fore and hind limbs of STZ-diabetic rats using quantitative morphometry under a light microscope. Large, randomly selected samples from the extensor digitorum longus and rectus femoris muscles were analyzed using both longitudinal and cross sections. The size and number of the myofibers and the myonuclei were quantified in both muscles of the normal and the STZ-diabetic rats. The results indicate a reduction in the diameter of the fibers with a corresponding increase in their numerical density per unit of reference area. Similarly a slight decrease in myonuclei length and diameter was also recorded in STZ-diabetic rats. These findings were more obvious in the rectus femoris muscle.

Very few quantitative morphometric studies on the skeletal muscles of STZ-diabetic animals have been published. Hegarty and Rosholt [15], Fahim *et al*. [5], Klueber and Feczko [7], and Ozaki *et al*. [9], in their studies on different muscles of STZ-diabetic mice and rats, have reported a decrease in the number and diameters of the muscle fibers. Studies on human type II diabetes have illustrated impaired skeletal muscle strength with a decreased muscle area, and infarctions [4, 11]. Studies on different muscles from STZ-diabetic rats have shown a decrease in protein synthesis and an increase in ribosomal degradation associated with changes in the circulating and muscle amino acids [16, 17]. Chaudhary *et al*. [18], from their study on the fore and hindlimb muscles of STZ-diabetic rats, have demonstrated a decrease in the weight and protein content of fast conducting muscles. They concluded that diabetic atrophic changes in skeletal muscles vary according to fiber composition and function. Similarly, Kawaguchi *et al*. [19], from their work on skeletal and cardiac muscles in human diabetes and in STZ-diabetic rats, observed small myocytes due to decreased F-actin production.

In the course of this study, patches of inflammatory reaction were occasionally seen infiltrating the myofibers of the STZ-diabetic rats. Torres *et al*. [20], in their study on non-complicated type II diabetic patients, recorded an increase in nitric oxide production and elevated levels of CD163, CD154 and TNF-alpha in samples of quadriceps femoris muscles. This was associated with macrophage infiltration and concluded that an inflammatory process occurs in the skeletal muscles of diabetic patients. Similar inflammatory changes in the skeletal muscles have also been reported in STZ-diabetic rats [21].

The process of skeletal muscle atrophy caused by insulin depletion is rather complicated and seems to involve several overlapping mechanisms including metabolic derangement, blood vessel changes, motor end plate degeneration and impairment of myocyte protein synthesis. In conclusion, our study clearly illustrates a decrease in the size of the myofibers in the fore and hind limb muscles of STZ-diabetic rats, there being a more prominent effect in the hindlimb muscles. Further studies on diabetic muscular atrophy and peripheral neuropathy are required to elucidate and understand the role of insulin as a causative agent.

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Abbreviations FBS: fasting blood sugar; STZ: streptozotocin

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