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EFFECTS OF DIABETES AND HYPERINSULINAEMIA ON NUMBER OF REGENERATED MUSCLE FIBERS AND THEIR GLYCOGEN CONTENT IN EXTENSOR DIGITORUM LONGUS MUSCLE TRANSPLANTS

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Abstract: Extensor digitorum longus (EDL) muscle was transplanted in control, streptozotocin diabetic, insulin replaced and hyperinsulinaemic rats. Total number of regenerated muscle fibers in four week old control EDL muscle grafts turned out to be 4202±496.88. Three-week old grafts in the diabetic rats had significantly lesser number of regenerated muscle fibers than the respective control value. In insulin replaced rats the number of fibers did not differ significantly from control as well as the diabetic rats. One-week-old EDL muscle grafts in insulin replaced rats had significantly higher glycogenic fibers than the other two categories of the respective transplants. Hyperinsulienaemia caused hyperplasia at 2-week stage. Hyperinsulinaemia promoted the frequency of glycogenic fibers to some extent within the muscle regenerates.

Keywords: EDL muscle regenerates, Insulin and number of regenerated muscle fibers, Insulin and glycogen in regenerated muscle fiber.

INTRODUCTION

Morphological studies of skeletal muscles mainly describe number of muscle fibers and their cross sectional areas for a given muscle. These parameters in many experimental and clinical studies have long been used as major indices to attribute effects of various factors on skeletal muscles (Grimby and Saitin, 1983; Caccia *et al.*, 1984; Ontell, 1986a; Christopher *et al.*, 1988). Many studies on skeletal muscle regeneration have also relied on these attributes for assessing the quality of skeletal muscle regenerates. Ontell (1986b) has summarized that number of myofibers found in long-term grafts is approximately 68% of that found in control muscle. While regarding their diameters, she has explained that many of the regenerating fibers fail to achieve normal size.

Skeletal muscle fibers of the same and different muscles vary in their structural and functional characteristics. Three fibers types are easily distinguished viz red fibers (slow-twitch, oxidative), white fibers (fast-twitch, glycolytic) and intermediate fibers

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(fast-twitch, oxidative, glycolytic). The red fibers have also been referred in literature as type 1, while the others as types 2A (intermediate) and 2B (fast) etc. (Loughlin, 1993). The red muscles, although more slowly they contract are responsible for the sustained activity and are required to maintain body posture. They are continuously fuelled by aerobic glycolysis. White muscles are capable of rapid, powerful action but have relatively limited stores of energy rich compounds. Therefore, white muscles are used for vigorous, intermittent activity. The proportion of type 1 and type 2 fibers varies considerably from one muscle to another in normal individuals. Even the superficial and deep layers of the same muscle have different proportions of type 1 and type 2 fibers. In fact, fiber types distribution within a muscle reflects its particular function (Johnson *et al.*, 1973). These fibers types differ from each other with a number of features ranging from myoglobin content to enzymatic characteristics (Fawcett, 1994). Inasmuch this article is concerned, we need to refer two of the parameters; The white fibers have high glycogen content and large diameters, while the red fibers possesses low glycogen content and small diameters. The intermediate fibers are characterized by medium range of the two features within muscle fibers. Price and Van deVelde (1981) have described that glycogen is distributed throughout the sarcoplasm between myofibrils and beneath the plasma membrane in the form of discrete granules varying from 25 to 40 nm in diameter. And that number of the granules may be dependent on the metabolic demands of the cell, however, glycogen contents are greater in type II fibers.

Extensor digitorum longus (EDL) muscle is a fast contracting muscle. However, a regenerated muscle, like other characteristics, attains different proportion of the fiber types than that of its normal composition. The present investigation was aimed at visualizing the effects of diabetes and exogenously supplied insulin on the total number of regenerated muscle fibers intact EDL muscle grafts under different experimental conditions. Fibers' types in the regenerated EDL muscles based on their glycogen content have also been worked out. Insulin has long been known to promote glycogen storage. It enhances glucose transport across the cell membrane and also affects the activity of glycogen synthetase. Although fast and slow twitch fibers differ from each other in many aspects and recent refined techniques can differentiate them at molecular level. But their identification on the base of glycogen granules is also a reliable method. For instance, Schmalbruch (1979), while studying the membrane systems in different fiber types of triceps surae muscle of cat reported that in electron micrographs of longitudinal and cross sections, fast and slow twitch fibers can be distinguished by the width of the Z-line and the distribution of glycogen granules. Z-lines are narrow in fast and wide in slow twitch fibers. And when the fibers are cross-sectioned at the level of A-band prominent

glycogen granules that belong to the longitudinal rows are found between the thick filaments of fast twitch fibers, whereas in slow twitch fibers glycogen granules are scarce. It should be noted that these details have been worked out at electron microscope using ultra thin sections. In comparison, cross sections of tissues prepared for light microscopic analysis are two thick to be restricted or specified to the level of A band etc. Thus here, at light microscopic level, fast twitch fibers would appear characteristically with higher glycogen contents, while slow twitch fibers with scarce glycogen deposition in cross sections. Degree/intensity of staining with Periodic acid-Schiff (PAS) reaction can throw light on types of muscle fibers in a cross section. Sjostrom *et al.* (1982) have described the identification of type 1 and type 2 fibers of skeletal muscle, selectively depleted of glycogen by sustained sub maximal muscular exercise at light and electron microscopic levels by examining thin and ultra thin sections treated particularly for visualization of glycogen. They concluded that type 1 fibers were, in general, some what smaller in size than type 2 fibers. Moreover, PAS stained sections from the trained (exhausted) individuals had numerous glycogen depleted fibers, which were exclusively of type I. Some of such fibers were practically completely depleted, whilst others had been reported having low glycogen content, which was assessed by the degree of staining. Based on above cited explanation cross-sections of various experimental EDL muscle regenerates in this study have been worked out as comprising of fast and slow twitch fibers, as assessed by their intensity of PAS stainings. This work is part of studies pertaining to the effect hyperinsulinaemia on the regeneration of muscle fibers in freely transplanted EDL muscles in rats. Average cross-sectional areas of regenerated muscle fibers of such grafts have been reported earlier (Qazi and Mufti, 2003). This report describes total number of the regenerated muscle fibers and their frequencies with respect to varying glycogen contents in control, diabetic, insulin replaced and supplemented EDL muscle orthotransplants.

MATERIALS AND METHODS

Animals

Seventy-two adult male rats were used in this study. They were kept in standard animal room facilities with roughly 12 hours dark/light cycle, fed commercially prepared food and given a constant supply of drinking water.

Diabetic rats

Twenty of the rats reported here were made diabetic by a single intraperitoneal injection of streptozotocin (2mg/100g b.wt.). The muscle transplantation surgeries were

completed within 2-weeks starting from day the animals received the streptozotocin. The control animals received similarly an injection of 0.2ml of sodium citrate buffer (pH=4.01). The rats were anesthetized with ether and extensor digitorum longus (EDL) muscle of each leg was orthotopically transplanted, in control as well as in experimental animals. The muscle was taken out and then grafted back in its original bed in proper orientation, by suturing both proximal and distal tendons with respective stumps with 6-0 silk. After suturing the fascia by 6-0 silk the two ends of the skin were sutured with 4-0 silk. The operated animals were given 0.06% terramycin in drinking water for 3-4 days postoperative.

Experimental design

Experiments pertaining to the effect of induced diabetes comprised of three groups. Control rats have been designated as C-STP. The streptozotocin induced diabetic rats were divided into two groups. The group, STP received no treatment following muscle transplantation. While, the animals designated as STP-lN-IIwere injected daily with ID of regular insulin/100 g b.w. Following the transplantation of EDL muscles, the rats serving the experiments meant to see the effects of hyperinsulinaemia were categorized into three groups. The control rats (C-SL) were injected with 0.9% saline in an amount of 0.lml/ l00g b.w./day intra-muscullarly, starting from the day following the muscle grafting. In the second group (IN-I) the animals received a daily intra-muscular injection of regular insulin in an amount of 0.5 U/100 b.w. starting from the day after the transplantation. Rats in the third set (1N-1I) were injected with a higher dose of 1U of the insulin in the same manner. The insulin was diluted in sterilized physiological saline prior to administration in such a way that 0.lml of the solution contained either 0.5 or 1 unit of the insulin.

Morphomelric and histochemical analysis of the EDL muscle regenerates

The muscle grafts were removed out of the animals of each experiment at 1, 2, 3 and 4 weeks post-transplantations. The rats were then killed by an overdose of the anesthetic. A portion of each muscle graft comprising of widest girth was trimmed and kept in Benin's fixative for at least 6-hours. The fixed tissues were then processed routinely for paraffin embedding and sectioning at 8 um with the help of a Cambridge rotary microtome. The sections were stained with periodic acid-Schiff (PAS) reaction (Loughlin, 1993). They were studied under microscope at various magnifications. All muscles fascicles of each cross-section were sketched out at low magnification. Then number of regenerated muscle fibers was then counted in each of the fascicle. Thus total number of regenerated muscle fibers/cross-section was worked out for each muscle graft.

While counting, the fibers were categorized as darkly stained, pink and faint based upon their relative intensity of the stain retention.

Muscle fibers' PAS staining degrees have been used by various workers to discriminate between types of the fibers (Schmalbruch, 1979; Sjostrom *et al.,* 1982). In this regard Swatland (1975) had earlier termed muscle fibers with PAS stained intermyofibrillar glycogen as PAS positive. He termed the fibers without a detectable reaction or with only a very faint intermyofibillar reaction as PAS-negative. The author included the fibers with intermediate intensity PAS reactions in the PAS positive category. Based upon the intensity of PAS staining, the fibers were categorized into three groups, in the present study. They were counted as strongly positive (it), positive (+) and negative (-) to be translated as glycogenic, intermediate and non-glycogenic. From representative cross-section of each animal of an experimental group number of each category of muscle fibers were counted and calibrated as %age of total number of the regenerated muscle fibers. Data of each animal of a group were pooled to measure average and standard error of means. Control and different experimental groups of a given regeneration stage were statistically compared by employing one-way analysis of variance (Campbell, 1989).

Photographs of representative sections were taken with the help of a camera fitted microscope.

RESULTS AND DISCUSSION

Sterptozotocin-induced diabetic (STP) and insulin replaced (STP-1NH) rats

Total number of regenerated muscle fibers in the three types of the muscle transplants at 1-week stage ranged from 3152 to 4247 and the differences were non significant (Table 1). However, 3-week old STP muscle regenerates harboured significantly reduced total number of regenerated muscle as compared to the values for the controls (Table 1). At first week of grafting the control EDL muscle regenerates contained about 99% non- glycogenic fibers, while the transplants in STP rats indicated about 4% mild glycolytic fibers. The difference was statistically significant. In case of STP-1N-II animals the muscle grafts attained about 32% mild glycolytic fibers, significantly higher than the corresponding values both for the control and STP transplants (Table 1). In the control transplants the glycogenic muscle fibers mostly pertained to the central cores of necrotic muscle fibers. While, in STP grafts mainly regenerated muscle fibers showed the glycogen content. More or less same situation appeared in STP-1N-I1 animals. In two-week old BDL muscle grafts in diabetic rats only

few percent of the regenerated muscle fibers could retain the glycogen (Fig. 1A) and the differences of the number of the mild glycogenic fibers between C-STP and STP were non-significant. All the regenerated muscle fibers in the three categories of the EDL muscle transplants appeared devoid of glycogen content at 3rd week stage. By the completion of one month, the EDL muscle transplants showed nonsignificant differences in terms of total number of regenerated muscle fibers and their glycogen content (Table 1). In one-month old STP-IN-II grafts all the fibers were non-glycogenic (Table-1). Some of the grafts did show weekly glycogenic regenerated muscle fibers (Fig. 1 B, C).

Quiroz-Rothe and Rivero (2004), while offering new prospects for muscle fiber typing in porcine experimental studies, described that slow fibers had the highest SDH activity (oxidative capacity), the lowest GPDH activity (glycolytic metabolism) and glycogen content, the smallest cross-sectional areas (CSA) and the greatest capillary and nuclear densities. These workers also reported that reverse pattern was true for pure type IIB fibers, whilst hybrid fibers had mean values intermediate in between their respective pure phcnotypes. These morphological parameters appear valuable for muscle fiber typing. In the present work only frequencies of slow, fast and intermediate fibers as assessed by their glycogen contents have been reported. Detailed morphological analyses are required for establishing correlation of the fibers' CSA, their nuclear and capillary densities etc with their histochemical profiles. Inasmuch present results are concerned, it is concluded that the induced diabetes caused hypoplasia in regenerating EDL muscle grafts. Glucogenesis became evident at first week stage. Exogenous insulin caused a significantly higher proportion of glycogenic regenerated muscle fiber in diabetic rats. At this stage, possibly due to failure of regeneration of tendenous connections of the freely grafted EDL muscle, differences in frequencies of glycolytic fibers in different categories of the transplants became evident. While, in the later stages, regenerated muscle fibers in different experimental grafts might had been exhausted more or less equally in terms of their glycogenolysis at time of sampling. If this explanation is plausible then like others deficits of regenerated muscle fibers, the process of glucogenesis has also been regenerated with lower efficiency than its level, encountered in a normal intact muscle.

EDL muscle transplants in hyperinsulinaemic rats

Total number of regenerated muscle fibers in 1-week 1N-II muscle transplants significantly reduced as compared to the corresponding control value (Table 2). Two-week old IN-II muscle regenerates attained significantly higher total number of regenerated muscle fibers from both the C-SL and IN-I regenerates. Three-week old IN-II regenerates still contained higher, but non-significantly, total number of the fibers as

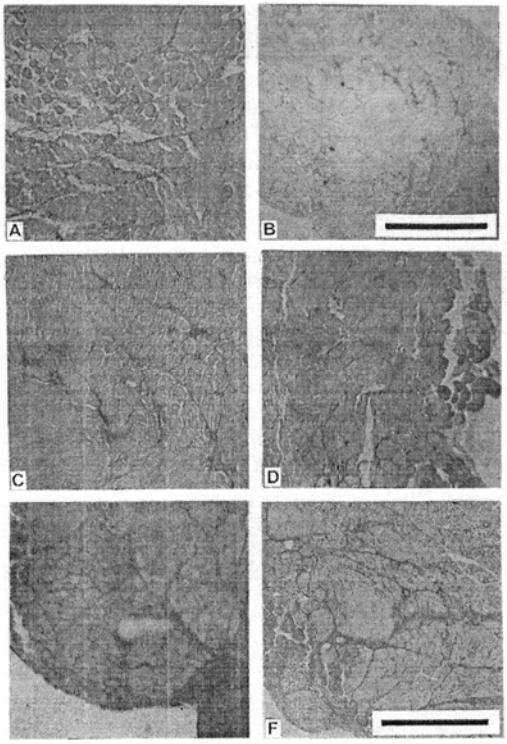

Fig. l: A. Portion of cross-section of 2-week old EDL muscle regenerate from an STP rat. Note some mildly glycolytic regenerated muscle fibers (light pink) distributed randomly with in grafts. **B.** A cross-section of a 4-week old EDL muscle graft from a STP-1N-11 rat showing central mass of week glycolytic regenerated muscle fibers. Note the dark staining of connective tissue (scarce amount) indicative of its glyeoprotein rich nature. Bar = 500 pm **C.** A higher magnification of a potion of cross-section shown in previous photograph. **D.** A portion of cross section of 1- week old EDL muscle graft from an insulin supplemented (1N-1) rat. Some highly glycogenic fibers (darkly stained) are visible. **E.** A portion of a cross- section of 2-week old EDL muscle grafts from an 1N-1 rat. Some highly glycogenic muscle fibers are visible at peripheral location. Note also intensely stained tendon and associated connective tissue of the regenerate. **F.** A portion of cross-section of 1-week old EDL muscle regenerate from an IN-11 rat. Randomly distributed mild glycolytic regenerated muscle fibers and darkly stained inter-fasicular connective tissue are visible. Bar = 200 μm.

Note: All photographs represent PAS-stained cross-sections and are of same magnifications, except the **B**.

Table 1: Number of regenerated muscle fibers and their % distribution according to PAS reaction in the EDL muscle transplants in control (C-STP) streptozotocin diabetic (STP) and insulin replaced (STP-IN-M) rats.

Experiment	Time Post-Grafting			
	1-Week	2-Week	3-Week	4-Week
C-STP	**3593.7 ± 552.34**[a]	**3231 ± 269.38**	**3561 ± 522.191**	**4204 ± 496.88**
	98.80 ± 0.598[b]	96.89 ± 1.566	100	96.97 ± 1.195
	(-)	(-)	(-)	(-)
	0 ± 0	0 ± 0	0 ± 0	0.026 ± 0.0265
	(++)	(++)	(++)	(++)
	1.18 ± 0.592	5.36 ± 0.859	0 ± 0	2.62 ± 1.053
	(+)	(+)	(+)	(+)
STP	**4247 ± 655.38** 95.66	**3698.33 ± 619.18**	**2660*** ± 68.723**	**3908 ± 1 17.36**
	± 0.924	96.38 ± 2.155	100	86.72 ± 9.77
	(-)	(-)	(-)	(-)
	0 ± 0	0.040 ± 0.4046	0 ± 0	0 ± 0
	(++)	(++)	(++)	(++)
	4.327* ± 0.9242	3.37 ± 2.116	0 ± 0	3.88 ± 1.150
	(+)	(+)	(+)	(+)
STP-IN-II	**3152.3 ± 397.058**	**3352.3 ± 309.225**	**2948 ± 276.32**	**2964 ± 493.35**
	68.88***--- ± 0.884	100	99.54 ± 0.2266	97.49 ± 1.375
	(-)	(-)	(-)	(-)
	0 ± 0	0 ± 0	0 ± 0	2.41 ± 1.43
	(++)	(++)	(++)	(++)
	31.74***--- ± 0.885	0* ± 0	0.410 ± 0.2049	0.084 ± 0.0837
	(+)	(+)	(+)	(+)

Values are means of three replicates +S.E.M a= Total number of regenerated muscles fibers of EDL muscle grafts. b = % of the fibers expressing their glycogenic nature. Number of (+) signs within parentheses corresponds to the intensity of PAS reaction. (-) = fibers appeared faint and considered PAS-negative. Values with asterisk(s) are significantly different from respective controls. Significant difference between STP-20 and STP-20-IN-II is represented by * = p≤0.5.***, --- p < 0.01 (single factor analysis of variance).

compared to the corresponding C-SL and IN-I regenerates. The 4-week old control muscle regenerates had non-significantly higher number of regenerated muscle fibers than IN-I and IN-II grafts. All the three categories of one-month old muscle grafts showed decreases in total regenerated muscle fibers as compared to the values for corresponding 3-week grafts. This may reflect the spontaneous degeneration of the

regenerated muscle fibers. In week old control (C-IN) EDL muscle grafts about 20% fibers were mildly glycogenic (Table 2). These were, however, represented mainly by peripheral original surviving muscle fibers and central core of necrotic fibers. In the insulin supplemented rats 2% and 3.26% of the regenerated fibers were highly glycogenic in IN-I and IN-II grafts, respectively. Similarly 1.87% and 8.44% were mildly glycogenic in one-week old IN-I and IN-II, EDL muscle transplants. These regenerated glycogenic fibers were found scattered within the insulin-supplemented grafts (Fig.1 D-F). Two-week old C-SL muscle grafts contained only 1.64% mild glycogenic regenerated muscle fibers. However, the IN-I and IN-II muscle regenerates showed 4.14% and 4.5% highly glycogenic regenerated muscle fibers, respectively in addition to some mild glycogenic fibers (Table 2). The glycogenic fibers were randomly distributed within the cross sections of the transplants. However, some of the regenerates had frequency of glycogenic fibers located peripherally (Fig.1E). At the end of three-week period 100% of the regenerated fibers in all three types of the grafts were non-glycogenic (Table 2). One-month old IN-I EDL muscle regenerates possessed 4.04% and 4.62% of highly and mildly glycogenic regenerated muscle fibers, respectively as compared to 100% non-glycogenic fiber in the control regenerates. While the IN-II EDL muscle regenerates showed only 1.72% mild glycogenic fibers (Table 2). The glycogenic fibers were randomly distributed within the cross sections of the regenerates. It is known that the hormone insulin decreases plasma glucose concentration by increasing the uptake rate of the metabolites across the cell membrane of skeletal and cardiac muscles, adipose tissue and mammary gland. The hormone also facilitates the formation of glycogen from glucose, by bringing more rapid accumulation of glucose and also by increasing activity of glycogen synthetase in muscle. In addition to increasing the glucogenesis, the hormone decreases glycogenolysis in muscle. Infact, under conditions of increased glucose uptake into muscle, the insulin-induced increase in glycogen synthesis is quantitatively more important than the increase in glucose oxidation. With increasing levels of insulin, the increased glucose entry into muscle is preferentially shunted toward glucose storage (Bentley, 1982; Porterfield, 2001; Fisher and Khan, 2002). Fligher frequencies of glycolytic regenerated muscle fibers is the insulin supplemented grafts clearly indicate that the surplus amount of the hormone have been successful in promoting the process of glucogenesis in the regenerated muscle fibers. These observations are suggestive to increase the Table 2: Number of regenerated muscle fibers and their % distribution according to PAS reaction in the EDL muscle transplants in control. (C-SL) and insulin supplemented (IN-I and IN-II) rats. observational period of skeletal muscle regenerates till their glycogenetic potential stablizes. Holmang *et al.* (1993), while reporting the

Table 2: Number of regenerated muscle fibers and their % distribution according to PAS reaction in the EDL muscle transplants in control (C-SL) and insulin supplemented (IN-I and IN-II) rats.

Experiment	Time Post-Grafting			
	1-Week	2-Week	3-Week	4-Week
C-SL	**2734.3 ± 374.42**[a]	**2145.67 ± 139.69**	**3565 ± 638.99**	**2927.67 ± 175.75**
	79.93 ± 11.13[b]	98.36 ± 1.642	100	100
	(-)	(-)	(-)	(-)
	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	(++)	(++)	(++)	(++)
	20.06 ± 11.13	1.64 ± 1.642	0 ± 0	0 ± 0
	(+)	(+)	(+)	(+)
IN-I	**2313.67 ± 3566.51**	**1.994 ± 264.34**	**3049 ± 551.88**	**2471 ± 266.67**
	95.31 ± 2.653	95.31 ± 2.653	100	91.33 ± 4.082
	(-)	(-)	(-)	(-)
	2.813 ± 1.965	4.136 ± 2.0797	0 ± 0	4.04 ± 2.630
	(++)	(++)	(++)	(++)
	1.867 ± 0.945	1.867* ± 0.945	0 ± 0	4.62 ± 1.867
	(+)	(+)	(+)	(+)
IN-II	**1927.00* ± 222.82**	**2666---*** ± 82.3005**	**3823 ± 656.79**	**2463.67 ± 220.66**
	88.27 ± 4.783	92.85 ± 4.223	100	98.28 ± 1.672
	(-)	(-)	(-)	(-)
	3.263 ± 2.832	4.476 ± 2.354	0 ± 0	0 ± 0
	(++)	(++)	(++)	(++)
	8.44 ± 1.964	2.579 ± 1.788	0 ± 0	1.723 ± 1.685
	(+)	(+)	(+)	(+)

Values are means of three replicates +S.E.M [a]= Total number of regenerated muscles fibers of EDL muscle grafts. b = % of the fibers expressing their glycogenic nature. Number of (+) signs within parentheses corresponds to the intensity of PAS reaction. (-) = fibers appeared faint and considered PAS-negative. Values with asterisk(s) are significantly different from respective controls. Significant difference between IN-I and IN-II, represented by; * = $p < 0.5$; *** = $p < 0.01$ (single factor analysis of variance).

effects of insulin on rat muscle fiber composition have described that hyperinsulinaemia changed muscle fiber composition and elevated 2- deoxyglucose uptake toward more fast twitch, type II, fibers, whereas the proportion of slow twitch, type I, fibers diminished. These workers also noticed elevations in capillary density both per unit muscle surface area as well as per muscle fiber. They suggested that muscle fiber composition alterations might be a consequence rather than a cause of hyperinsulinaemia and that capillarization

rather than fiber composition is of importance for insulin sensitivity in muscle. These hyperinsulinaemic effects on muscle fiber composition and capillarization are the responses of intact muscle. In the present study failure of pictorial effects of hyperinsulinaemia on the composition of muscle fibers and persistence of slow twitch fibers in the EDL muscle regenerates indicates that various factors involved /required in the mechanism of insulin actions, such as insulin receptors, possibly had not yet been fully regenerated within the regenerated muscle fibers. Further diabetic condition as well as higher amount of exogenously supplied insulin, especially for prolong period exert negative effects on the development of regenerating muscle fibers. This notion has earlier been suggested in these and allied experimental models (Qazi and Mufti, 1990; Qazi and Raiz, 2005). EDL muscle regenerates in hyperinsulinaemic rats had prominent, strongly PAS-positive extra fiber areas. It is known the glycocalyx is rich in glycoproteins (Johnson, 1991). Vivid deposition of glycocalyx in such zones, as assessed by PAS reaction, possibly does not require the cascade of metabolites needed for a regenerating (ed) muscle fiber to accumulate glycogen.

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