

Effect of Erythropoietin on Metabolic and Contractile Functions of Soleus Muscle in Type I Diabetic Rats

Gehan A. Shaker, Reem M. Emam, Refka K. Messiha, and Hanaa A. Abd-El-moneim

Department of Medical Physiology/ Faculty of Medicine/Mansoura University, Mansoura, Egypt

Abstract

Diabetes mellitus has been linked with specific morphological and metabolic abnormalities in skeletal muscle in a fiber specific manner. Erythropoietin (EPO) is a glycoprotein hormone that regulates the development of erythrocytes through binding to a high affinity receptor expressed in erythroid progenitor cells. EPO receptor expression in non hematopoietic tissue, including skeletal muscle progenitor cells, raises the possibility of a role for EPO beyond erythropoiesis. So the aim of the present study was to evaluate the effect of EPO on skeletal muscle changes as a complication of type 1 diabetes mellitus in STZ- rat experimental model. **Methods:** 40 male Sprague Dawely rats were divided into 5 groups : control group , diabetic group (STZ-induced , 50 mg/kg I.P.), insulin treated diabetic ; (received 0.75 IU/ 100g body weight daily, S.C; for 4 weeks) , EPO treated diabetic; (received EPIAO® S.C , 200 IU/Kg, 3 times weekly, day after day for 4 weeks),and insulin and EPO treated diabetic groups. At the end of the experiments, fasting blood glucose, insulin levels, lipid profile, contractile changes in soleus muscle and glucose transporter 4 (GLUT4) expression in soleus muscle were evaluated. **Results:** All biochemical parameters were improved in the group treated with insulin or EPO with greater improvement in insulin treated group. The greatest improvement was in the group treated with combined insulin and EPO. Contractile function of soleus muscle in diabetic group showed significant decrease in muscle tension either before or after fatigue, significant decrease in time taken to reach complete fatigue, significant increase in time taken to reach peak and in time taken to relax to 50% when compared with normal group. All parameters were improved in insulin treated and EPO treated groups, with greater improvement in insulin treated group. The greatest improvement was in combined insulin and EPO treated group. The reduced **GLUT 4 expression** in diabetic soleus muscle was significantly increased in insulin treated group as compared to EPO treated group, however combined EPO and insulin treated group showed greater increase in GLUT4 expression. **Conclusion:** The present results showed that, EPO injection improved hyperglycemia, hypoinsulinemia, hyperlipidemia, and skeletal muscle changes observed in STZ-induced diabetes in rats. Therefore, EPO could be beneficial in managing diabetic disorders and the application of EPO in treatment of diabetes can be considered.

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Keywords

- Erythropoietin (EPO)
- GLUT4
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- Rats

Corresponding author: Dr. Gehan A. Shaker, Physiology department, Faculty of Medicine, Mansoura University. Tel: 01066020009. Email: dr.gehanshaker911@gmail.com

Introduction

Diabetes mellitus has been linked with specific morphological and metabolic abnormalities in skeletal muscle in a fiber specific manner. The reduction in absolute force production in T1DM may be explained by a fiber type-specific atrophy, as slow-twitch or type I fibers exhibit minimal loss or a slight gain in fiber area, while fast-twitch fibers, especially the fatigable glycolytic (type IIB) fibers, exhibit the most severe atrophy (1).

Hyperglycemia has a negative effect on skeletal muscle as it leads to proteins glycation and further oxidation reactions that leads to advanced glycation end products (AGE) which have been implicated in the pathophysiology of the aging process, and are clearly established as contributing to the other T1DM-related complications (2). Fast-twitch/type II myosin fibers are the most affected by AGE (3). Previous studies demonstrated that fast-twitch/type II myosin fibers commonly exhibit atrophy and loss of contractile function in various models of T1DM (4). Hyperglycemia possibly affects skeletal muscle through activation of the polyol pathway, leading to tissue damage by reducing cellular defenses against oxidative stress (5). DM is also associated with rapid down regulation of GLUT4 which is the major insulin-responsive glucose transporter, and is widely responsible for insulin-stimulated glucose transport into muscle and adipose tissues (6).

Erythropoietin (EPO), a hypoxia-responsive glycoprotein hormone produced mainly by the kidney, is the primary regulator for erythroid differentiation. It acts by binding to a high affinity

receptor on the surface of erythroid progenitor cells to promote survival, proliferation, and differentiation. However, EPO receptor expression extends beyond the hematopoietic progenitor cells that include the cardiovascular system, neural progenitors and neurons, and skeletal muscle progenitor cells (7 & 8). Mice lacking EpoR die in utero from severe anemia and exhibit many developmental defects in heart and brain, including decreased progenitor cell proliferation and increased apoptosis (9).

Previous studies have revealed that EPO exerts multiple protective effects, including anti-oxidative (10& 11), anti-inflammatory (12& 13) and anti-apoptotic effects (14). It has been suggested that EPO could have a positive effect on skeletal muscle regeneration, growth, oxidative capacity and angiogenesis(15). In clinics, EPO is widely used in diabetic patients receiving hemodialysis, and this application established the need for critical exploration of the interplay between EPO and glucose homeostasis (16).

Based on this background, the present study was designed to explore whether EPO therapy can reduce metabolic and contractile changes in soleus muscle in STZ-induced Type 1 DM rat model.

Material and Methods

Experimental animals:

Fourty Sprague Dawely male rats, weighting 150-200g were included. The study was conducted at the Medical Experimental Research Center (MERC), Mansoura faculty of medicine. The study protocol was approved by the local ethics committee for animal experiments, Mansoura faculty of medicine. The animals were kept at a

standard animal care environment and fed with standard animal food.

Chemicals:

Erythropoietin (EPIAO)®: is a recombinant human erythropoietin, manufactured by Shenyang sunshine pharmaceutical co.,Ltd. It is available in a vial 1.0 ml colorless transparent solution, with pH 6.9±0.5., and 2000IU/vial. It should be stored at 2-8 °C and avoid direct sunshine .

Streptozotocin(STZ) : was purchased from Sigma (St. Louis, MO, USA) ; 1 gm powder, Stored at -20c, away from dry air and moisture, dissolved in cold citrate buffer PH 4.5 (0.9gm citric acid and 1.47 gm sodium citrate in 100 ml saline), **Insulin mixtard 30:**(Novo Nordisk, Denmark) 100 IU/ml Suspension for injection in a vial. containg 10 ml is equivalent to 1000 IU.

Experimental groups:

Animals were divided into **5 groups, (n=8 in each group):**

Group I: control group, were injected with 0.75 ml normal saline subcutaneous (S.C) once daily for 4 weeks. **Group II: Diabetic group:** Rats were injected with 0.75 ml normal saline S.C once daily for 4 weeks. **Group III:Diabetic insulin treated Group:** (D+insulin) : Rats were injected S.C with mixtard insulin 30 , 0.75 IU/ 100g body weight (17) once daily for 4 weeks. Injection was started 48 hours after induction of diabetes. **GroupIV : Diabetic EPO group:** (D+ EPO): Diabetic rats were injected with EPO in a a dose of (200 I.U/Kg body weight S.C, 3 times weekly , day after day) (18) for 4 weeks. Injection was started 48 hours after induction of diabetes. **Group V : Diabetic Insulin-EPO group:** (D+ insulin +EPO) :Rats

were treated with insulin and EPO with the same doses in groups III and IV.

Experimental Model: (Type 1 DM)

For induction of type 1 diabetes: Overnight fasted rats were intraperitonealy (IP) injected with with streptozotocin (STZ; 50 mg/kg , single injection) dissolved in 0.1 mmol/L citrate buffer (19). One week later, blood samples were used to determine fasting blood glucose (FBG) and insulin levels. Hyperglycemia and hypoinsulinemia were used to identify the success of the model. Animals with FBG >300 mg /dl were included in the study (20). After STZ injection, rats were allowed to drink 10% glucose solution to prevent hypoglycemia (21). FBG was measured weekly during the whole experimental period with an Accu- check Go strip test using glucometer.

Collection of blood and tissue samples

At the end of experimental protocol, Rats were anesthesitised with thiopental (I.P, 120 mg/kg). Blood was collected from the heart. , left to coagulate and centrifuged at 3000 rpm for 30 minutes .Separated serum was stored at -20° C for biochemical analysis. **Tissue samples:** Soleus muscle on one side was separated and broken down into small cubes,weighting 30mg. Cubes was stored immediately into cryotubes (RNase-free), then stored at liquid nitrogen at - 196° C to prevent RNA destruction to be ready for PCR technique. Soleus muscle on the other side was hold by a thread attached to its distal tendon to the force transducer of the biopac apparatus and immersed in krebs solution(120 mM NaCl, 25 mM

NaHco₃, 1.2mM NaH₂PO₄ , 1.2 mM MgSO₄, 5.0 mM KCl, 2.5mM calcium gluconate, 11.5 mM glucose) at 30° C. It was continuously bubbled with 5% CO₂ , 95% O₂, and the pH was kept at 7.4. Specimen can be kept in -20° C to preserve . Serum – coagulation at room temperature 10-20 mins, then centrifugation 20 min at the speed of 2000-3000 r.p.m.

Biochemical assessment:

-Fasting blood glucose was measured by the glucose oxidase method . Lipid profile: Triglycerides, cholesterol, HDL, LDL, were determined. All kits were manufactured by Spinreact company.

-Insulin level was determined by using rat insulin ELISA kit , manufactured in Sun red biological technology company , was purchased from biogene company (REF).

Measurement of GLUT 4 expression:

PCR was carried out in clinical pathology department , Mansoura university. kits was purchased from Thermo scientific company, lot 00269687. It was stored at -20 °C.

Primers used: According to gene bank , primers sequences were:

Gene	(5'-3') forward primer	(5'-3') reverse primer	Base pairs	Annealing temperature °C	Cycle (S)
GLUT 4 (SLC2a 4)	GAAACG CAAGTT GGAAAG A	CTACTA AGAGCA CCGAGA CC	225	55	30
β-actin	GCCAAC CGTGAA AAGATG	CCAGGA TAGAGC CACCAAT	681	57	30

These primers gene were manufactured in BIOSEARCH TECHNOLOGIES company. DNA molecular weight marker type 50 bp DNA ladder was applied to identify the size of the PCR products. The ladder was purchased from **Thermo scientific** , Lot 00145906, concentration 0.1 µg/ µl (22).

Contractile changes in the soleus muscle:

BIOPAC was used determining the optimal force range for the experiment (force transducer 100 for soleus muscle).

Statistical analysis:

Data were analysed by SPSS software ver. 17 (IBM, US). The findings were expressed as the mean ± SD. Statistical analyses were undertaken using independent One-way ANOVA with post-hoc tukey test. The t-test was used for two sample assuming unequal variances, which confirmed the statistically significant difference (p < 0.05). A P-value <0.05 was accepted statistically significant.

Results

As shown in table (1), insulin treatment produced significant decrease in FBG (P=0.0001). Also EPO produced significant decrease in FBG (P=0.0001) and non significant increase in insulin (P=0.099). Combined treatment with insulin and EPO significantly decreased FBG (P=0.0001), and non significant increase in fasting insulin (P=0.225) when compared with diabetic group

Also in table (2) ,Insulin treatment produced significant decrease in cholesterol (P=0.0001), triglycerides (P=0.0001), LDL (P=0.0001), and significant increase in HDL (P= 0.049) when compared to diabetic group. Also, EPO treatment

produced significant decrease in cholesterol (P=0.0001), triglycerides (P=0.0001), LDL (P=0.0001), but non significant increase in HDL (P= 0.099) when compared to diabetic group. Combined treatment with insulin and EPO produced significant decrease in cholesterol (P=0.0001), triglycerides (P=0.0001), LDL (P=0.0001), and significant increase in HDL (P=0.0001) when compared to diabetic group.

Contractile functions:

Data was obtained by analysis of the recorded curves .In table (3), diabetic animals showed significant decrease in muscle tension either before or after fatigue (P=0.0001), significant decrease in time taken to reach complete fatigue (P=0.0001) that means rapid fatigue. However time taken to reach peak and time taken to relax 50% were significantly increased (P=0.0001) as compared to control.

Diabetic rats treated with insulin , showed significant increase in muscle tension before fatigue (P=0.0001) and insignificant increase in tension after fatigue (P=0.933). Significant increase in time taken to reach complete fatigue was also observed (P=0.0001) , Also significant decrease in time to reach peak and time to reach 50% relaxation was observed (P=0.0001) as compared to diabetic non treated group.

EPO treatment also produced significant increase in muscle tension before fatigue (P=0. .027) and insignificant increase in tension after fatigue (P=0. .927). Significant increase in time taken to reach

complete fatigue was also observed (P=0.007). significant decrease in time to reach peak and time to reach 50% relaxation was observed(P=0.0001) as compared to diabetic non treated group.

Combined treatment of diabetic rats with insulin and EPO by the same doses produced more significant increase in muscle tension before fatigue (P=0.0001) and insignificant increase in tension after fatigue (P=0.067). significant increase in time taken to reach complete fatigue (P=0.0001) and Significant decrease in time to reach peak and time to reach 50% relaxation was observed (P=0.0001) when compared to diabetic non treated group. From the tables, we can find that ,the greater improvements of diabetic changes was obtained in combined insulin and EPO treated group.

PCR (area ratio) (GLUT4 expression ratio):

Data was obtained by analysis of PCR bands by **image j** program. As shown in fig.(1), (2), (3); The decreased GLUT4 expression In STZ-diabetic model is significantly reversed (P=0.0001) with insulin treatment. Also EPO treatment by the same previous doses significantly increased GLUT4 (P=0.0001). Combined treatment of diabetic rats with insulin and EPO produced more significant increase in GLUT4 expression when compared to diabetic non treated group (P=0.0001).

Table (1) Effect of insulin , EPO , and combined treatment on the level of fasting blood glucose (FBG) , and fasting insulin in type 1 diabetic rats:

Groups N=8	control (G:I)	(D.M) (G:II)	D.M+I (G:III)	D.M +EPO (G:IV)	D.M+I+EPO (G:V)
fasting blood glucose (mg/dl)	92.77±10.23	386.48±14.41*	237.87±13.47*#	278.87±10.62*#\$	178.62±17.64*#S#
fasting insulin (mIU/L)	11.39±4.587	7.22±1.720*	8.65±2.673	7.216±1.216*	10.071±1.643

Results are expressed as mean ± SD. Significance means p <0.05.

(*) significant as compared to control. (#) significant as compared to D.M.

(\$) significant as compared to D.M+I. (S) significant as compared to D.M +EPO

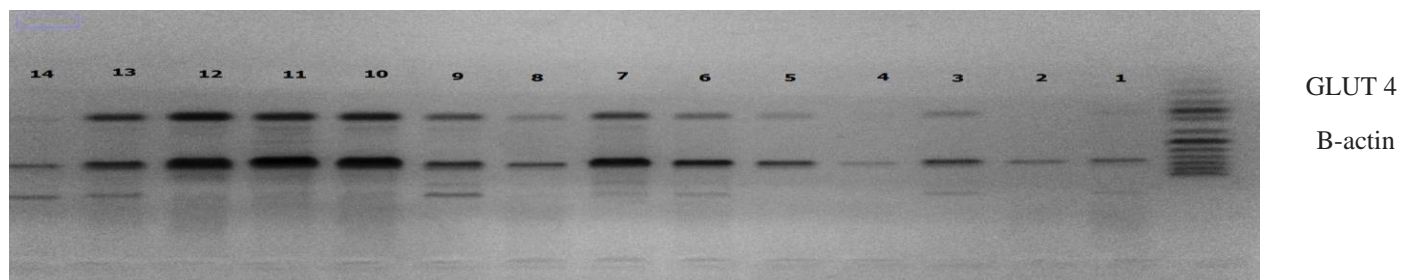
Table (2) Effect of insulin , EPO , and combined treatment on the level of cholesterol, triglycerides, LDL, and HDL in type 1 diabetic rats:

Groups N=8	control (G:I)	diabetic (D.M) (G:II)	D.M+I (G:III)	D.M +EPO (G:IV)	D.M+I+EPO (G:V)
Cholesterol (mg/dl)	148.18±14.5 8	372.43±17.01 *	250.25±8.18 *#	312.00±15.44* # \$	198.93±12.65*#S# S#
TGS (mg/dl)	97.81±13.44	270.50±15.52 *	207.00±8.34*#	237.75±9.808*#S	176.93±12.31*#S#
LDL (mg/dl)	85.95±14.12	277.13±35.68 *	171.65±8.029 *#	231.33±14.97*#S	119.45±12.33*#S# S#
HDL (mg/dl)	43.75±4.803	29.87±4.793*	35.00±3.295*#	29.8750±2.35*#S	42.50±3.33*#S#

Results are expressed as mean ± SD. Significance means p <0.05.

(*) significant as compared to control. (#) significant as compared to diabetic (D.M).

(\$) significant as compared to D.M+I. (S) significant as compared to D.M +EPO

**Figure (1):** PCR bands: 1,2: D.M +EPO , 3-11:D.M +I +EPO , 12-14: D.M

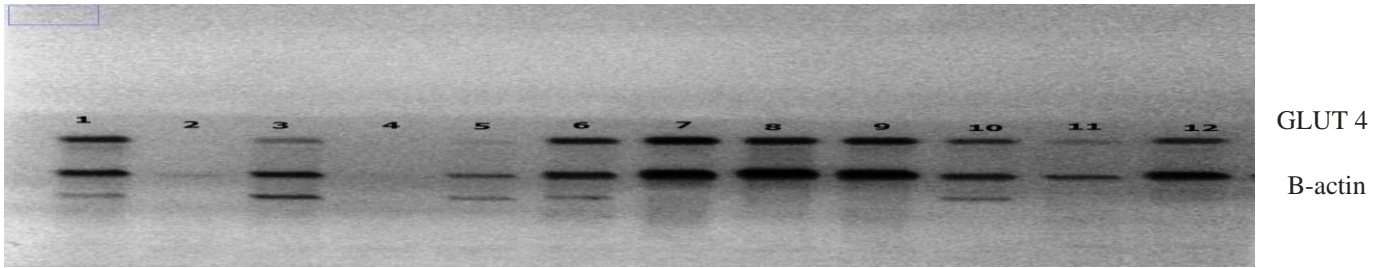


Figure (2): PCR bands 1-7: D.M (control) ,8-12:D.M +I+EPO

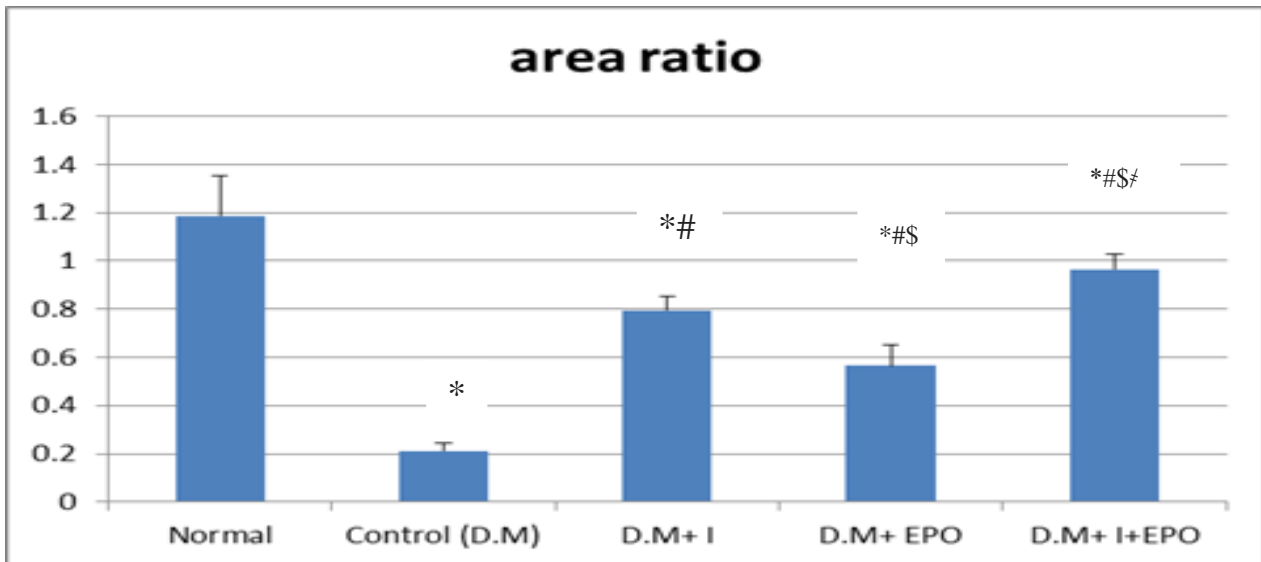


Figure (3): GLUT4 expression ratio (area ratio) in normal, diabetic, insulin treated, EPO treated, and combined treated groups (*) significant as compared to normal. (#) significant as compared to control (D.M). (\$) significant as compared to D.M+I. (¢) significant as compared to D.M +EPO.

Table (3) shows the effect of insulin , EPO , and combined treatment on contractile properties of soleus muscle:

Groups N=4	control (G:I)	(D.M) (G:II)	D.M+I (G:III)	D.M +EPO (G:IV)	D.M+I+EPO (G:V)
Tension before fatigue (gm)	1.787±0.070	0.439±0.019*	0.771±0.020*#	0.573±0.033*# \$	1.182±0.093*#S¢
Tension after fatigue(gm)	0.390±0.041	0.192±0.014*	0.215±0.0138*	0.216±0.014*	0.280±0.0821*
Complete fatigue (sec)	401.25±23.9 3	137.50±6.45*	216.25±17.01* #	188.75±8.53*#	285.00±24.15*#S¢
Time to peak(sec)	0.018±0.003 5	0.160±0.0182 *	0.071±0.003*#	0.088±0.004* #	0.047±0.0047*#S¢
Half relaxation time (sec)	0.050±0.008 1	0.392±0.0403 *	0.142±0.025*#	0.205±0.012*# \$	0.090±0.0020*# \$¢

Results are expressed as mean ± SD. Significance means p <0.05. (*) significant as compared to normal. (#) significant as compared to control (D.M). (\$) significant as compared to D.M+I. (¢) significant as compared to D.M +EPO

Discussion

EPO may modify several health factors that could modify the onset of many diseases. Previous researches have shown that EPO injection may reduce hyperglycemia in diabetic rats through activation of EPO receptors (23). On the other hand, diabetes-related changes in skeletal muscles is a less studied complication of poorly controlled diabetes, in spite that, it is a common condition that is characterized by decrease in muscle mass, weakness, and an overall reduced physical capacity (24). Therefore, the aim of The present study was to examine the possible role of EPO hormone solely or in combination with insulin in improving diabetes-induced derangement in metabolic and contractile changes in soleus skeletal muscle in Type 1 D.M rat model.

The study demonstrated that EPO treatment (200 U/kg, three times a week for 4 weeks) was effective in reducing FBG and serum insulin, in STZ-induced experimental diabetic rats. These results was in agreement with previous study that explain the results by the EPO effect in normalizing the state and the quantity of mitochondria and golgi apparatus, and increased the number of insulin secreting granules in the pancreatic β -cells (25). Another explanation may be due to antioxidant-like action of EPO (26) which may be due to upregulating hemoglobin oxidase-1 (27), and increasing RBCs number in the circulation, thus reducing cellular oxidative stress (28).

Concerning lipid metabolism , EPO causes significant decrease in serum triglyceride, cholesterol, LDL, with significant increase in HDL

.These results was in accordance with previous study(29). The significant decrease of triglyceride level may be related to an improved response to insulin, since increased insulin resistance was known to be associated with diminished lipoprotein lipase activity, while the triglyceride production remains normal and thus results in hypertriglyceridemia (30). The increased serum HDL levels may be attributed to improvement in tissue oxygenation which increases activity of ATP-binding cassette transporter (ABCA1) or other enzymes involved in maturation of HDL and increase its levels (31).

Regarding contractile function , contraction of soleus muscle (Type 1 or slow twitch or red muscle) in diabetic rats , as observed in the study , showed significant decrease in isometric contraction (muscle tension) as compared to control before and after fatigue . This was previously illustrated by (32&33). Normal skeletal muscles are able to produce maximum force of contraction of higher magnitude than diabetic muscle due to better fuel supply and its utilization along with low oxidative stress and lactate levels (34).

Diabetic muscle showed more rapid fatigue than control one and than groups treated with insulin or EPO or both , this was cleared in this study by measuring time taken by the muscle to complete fatigue . (35), illustrated that repeated stimulation of diabetic muscle leads to reduced glycogen stores and increase lactate level , exhausted their ATP supply significantly greater than normal group.

Muscle fatigue was measured as a decrease in maximum force of contraction due to the

repeated contractions at tetanic frequencies. Significant fatigue (early fatigue) was observed in the study in diabetic muscle. This was explained by (32), who illustrated pronounced fatigue in soleus diabetic muscle when compared to normal, and explained these by alternation in Ca^{++} homeostasis and compromised muscle oxygenation following muscle contractions. (36) reported that, soleus muscle of STZ diabetic rats showed decline in the level of sarcoplasmic reticulum Ca^{2+} -ATPase protein levels. So, resolution of the oxygen dysregulation, and the role of Ca^{+2} in those signaling processes will constitute an important goal in combating diabetes-induced skeletal muscle dysfunction. As represented by animal model, the peripheral circulation is profoundly dysfunctional in diabetes (37), increasing the possibility that acute and chronic diabetes-induced muscular deficits at least by impaired oxygen transport. Contractile function is sensitive to increased or decreased blood oxygen content and muscle oxygen supply (38). hypoxia exacerbates intracellular disorders in the form of increase free ADP, decrease intracellular phosphocreatine(37).

The study also revealed that, time taken by the muscle to reach peak and time taken to relax 50% was significantly increased in diabetic compared to control group. Time to peak depends on the velocity of Ca^{+2} release from sarcoplasmic reticulum, but half relaxation time depends upon the rapidity of Ca^{+2} pumping from the sarcoplasm to the sarcoplasmic reticulum (39). Calcium handling characteristics are altered in the STZ-diabetic muscle, so longer rise and half-relaxation times of single muscle twitches occur (1).

Insulin treatment produced significant improvement in muscle tension, half relaxation time and time to peak when compared to diabetic group. This is due to the important role of insulin in control glucose uptake, activate the pathway for protein synthesis and by inhibiting degradation of skeletal muscle protein (40). EPO treatment also, showed significant increase in muscle tension when compared to diabetic group, which illustrated in a previous study done by (41), who explained this significant improvement in contractile function of soleus muscle when compared to diabetic, due to the role of EPO in protecting myoblasts against hypoxia-induced apoptosis. Another study illustrated that EPO supplementation alone or coupled with enhanced EPO-R expression promoted rat myoblast survival (42). It has been suggested that, local EPO treatment increases rat soleus muscle regeneration and strength following mechanically induced injury, a response associated with increased proliferating satellite cells numbers (40). A study in skeletal muscle from EPO-deficient mice showed increases in genes related to muscle hypoxia, proteolysis, cell death and apoptosis as well as reductions in genes involved in glycolysis and mitochondrial function. These findings support the hypothesis that EPO has a protective effect in skeletal muscle. (43). **Nachbauer et al.,2012** claimed that prolonged EPO administration increases muscle capillary density and contribute to improve the motor function.

A decrease in insulin-mediated glucose uptake caused by a lower levels of GLUT4 expression has been documented in diabetic skeletal muscle, a major site for glucose disposal (45). The present study revealed a significant increase in GLUT4

expression in the insulin treated group. The effect of insulin on GLUT4 translocation is mediated through intracellular signaling pathway through tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) associated with phosphatidylinositol 3-kinase (PI3K), phosphorylation of AKT/PKB and TBC14D/AS160 which is a down stream target of AKT in the distal insulin signaling pathway (46). GLUT 4 expression was also improved in EPO treated group in the study. Despite the significant role of GLUT4 in glucose metabolism, the molecular mechanisms underlying the transcriptional regulation of GLUT4 are poorly understood (47). One of the marked signals associated with EPORs for glucose homeostasis is AMPK (48). EPO treatment might increase the phosphorylation of AMPK in muscles. It has been established that activation of AMPK may enhance GLUT4 levels (49).

The greater improvement was obtained when EPO combined with insulin, this may be due to synergistic effect of both drugs on all parameters, or due to increase blood supply and O₂ to the tissue by the effect of EPO that enhance the action of insulin. But the precise mechanism still need further researche.

conclusion

From the previous results, it is possible to conclude that, EPO may be beneficial in managing diabetic disorders particularly skeletal muscle dysfunction. EPO injection reduce hyperglycemia and hyperlipidemia in diabetic rats and upregulate GLUT 4 expression in skeletal muscle, with a better effect when it is used in combination with insulin. EPO receptor will be a good target for the

development of antihyperglycemic agent(s) in the future.

Recommendations: EPO could offer a great promise as a therapeutic strategy in addition to insulin in patients with type 1 diabetes to improve skeletal muscle functions and myopathy.

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