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Original Article

Effects of food restriction and/or aerobic exercise on the GLUT4 in type 2 diabetic male rats

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Abstract

Background: The aim of present study was to compare the effects of negative energy balance with food restriction and/or aerobic exercise on the glucose, insulin, and GLUT4 levels in diabetic male rats. **Methods:** Fifty-six 10-week old male Wistar rats were randomly assigned to seven groups: a non-diabetic (ND) group and six diabetic groups. After an infusion of type 2 diabetes, the diabetic groups were given labels as well, namely diabetic control (DC) group, exercise (Ex) group, food restriction with standard diet (FRSD) group, food restriction with low-carbohydrate diet (FRLCD) group, food restriction with standard diet combination in exercise (FRSDE) group, and food restriction with low-carbohydrate diet combination in exercise (FRLCDE) group. Further, to induce caloric restriction (CR), food intake was reduced by 20% and given to food restriction consists of both of (FRSD and FRLCD). Hundred percent food consumption for the Ex group was fixed, but instead, 20% of their energy intake in exercise was calculated, and time of daily exercise was determined. Finally, a combination of reduced food intake (10%) and exercise (10%) was applied in each group FRSDE and FRLCDE for 8 weeks. **Results:** The results showed that type 2 diabetes inductions had reduced glucose, insulin, and GLUT4 gene expression compared to the ND group (P = 0.001). However, there were significant differences in GLUT4 gene expression between groups after 8 weeks of intervention (P = 0.001). A post hoc least significant difference test show that compared to DC group, GLUT4 gene expression level of Ex, FRSDE, and FRLCDE groups was significantly increased 47% (P = 0.004), 60% (P = 0.001), and 65% (P = 0.001), respectively after 8 week of intervention, but it was not significant or with any other diabetic groups (P > 0.05). Moreover, glucose levels were significantly higher in the FRLCDE, FRLCD, FRSD, FRSDE, Ex groups compared with the DC group in the same period (P = 0.0.01). **Conclusions:** It was concluded that FRSD and FRLCD combination in regular exercise was elevated of GLUT4 gene expression in type 2 diabetes. These results may help to develop new methods for the treatment of obesity and type 2 diabetes mellitus.

Keywords: *GLUT4 gene expression, low-carbohydrate diet, standard diet, type 2 diabetes*

Introduction

Type 2 diabetes is a disease that is preventable but not curable and more than 280 million people worldwide suffer from this disease. Insulin resistance is the most important disorder and a precursor state of type 2 diabetes mellitus that occurs even before the improvement of hyperglycemia.[1,2] Hyperglycemia and high intramuscular glucose levels play a major role in the onset of insulin resistance.[3] The major causes of insulin resistance are owing to defects in insulin signaling, changes in insulin target genes, metabolic problems, and contrast in other hormones.[4-6] Changes in gene expression and transportation of GLUT4 are directly related to insulin resistance, especially in skeletal muscle.[7] Most studies report that type 2 diabetes reduces gene expression and transportation of GLUT4 in skeletal muscle cells.[8] Studies have shown that the reduction of calorie intake and low-carbohydrate diet are the common methods to reduce exogenous carbohydrate availability (glycemia) and the prevention and/or improvement of type 2 diabetes.[9] There is some evidence that low-carbohydrate diets reduce fasting plasma insulin and glucose concentrations in overweight and obese individuals with insulin resistance and in patients with type 2 diabetes mellitus.[10] Exercise and diet are two certain ways to reduce calorie intake as well as improve insulin resistance and glucose homeostasis in patients with type 2 diabetes.[11]

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It has been suggested that calorie restriction (CR) for 6 weeks reduces 53% of insulin resistance in rats that were fed a high-fat diet.[12] Similarly, aerobic exercise reduces insulin resistance by the improvement of gene expression and transportation of GLUT4 in skeletal muscle cells.[13] Shicheng Cao *et al*. (2012) showed that long-term exercise training increases the phosphorylation and GLUT4 gene expression in rats with type 2 diabetes.[14] In addition, Sung-Tae Park *et al*. (2011) have shown that streptozotocin (STZ) injection reduces the amount of GLUT4 gene expression in the skeletal muscles of healthy rats, but exercise can improve GLUT4 gene expression in diabetic rats.[15] The diet includes decreasing total energy intake or decreasing one or more components of food (especially macronutrients).[16] A low-fat diet (LFD) with high carbohydrates, combined with regular exercise, is traditionally recommended for type 2 diabetics. However, such a lifestyle is not suitable for patients with type 2 diabetes because high level of carbohydrates in the diet increases plasma glucose and insulin secretion, thereby increasing the risk of cardiovascular disease, hypertension, dyslipidemia, and diabetes.[17] Recent studies have created a change in our insight of the low-carbohydrate diet and its interaction with type 2 diabetes.[17] In this context, studies have shown an extreme correlation between low-carbohydrate diets and improvement of insulin sensitivity in type 2 diabetes.[18] Studies have also shown that low-carbohydrate diets reduce hyperglycemia in patients with type 2 diabetes.[19,20] However, few studies have examined the effect of low-carbohydrate diets on GLUT4 in the skeletal muscles of type 2 diabetic rats. To prevent or improve the health condition of type 2 diabetes, promoting the quality of life, reducing economic costs, and mortality in patients, so the mechanism of a low-carbohydrate diet alone and in combination with aerobic exercise on GLUT4 in the muscle of patients with type 2 diabetes, are vital. In fact, finding ways to reduce the risk for obesity-related disorders, including type 2 diabetes is important. Such approaches can include lifestyle interventions by diet and exercise. The aim of this study was, therefore, to test whether the addition of aerobic exercise to a food restriction (FR) and food restriction with low-carbohydrate (FRLC) diets leads to greater improvements in glucose, insulin, and GLUT4 gene expression levels compared to FRLC diet alone in diabetic male rats. In light of recent evidence, we hypothesize that the novel combination of a FRS and FRLC diets and aerobic exercise represents a promising lifestyle strategy for the treatment of type 2 diabetes.

Methods

Fifty-six 10-week old male Wistar rats (body weight 229 ± 19.5 g) were kept in separate polycarbonate cages (groups of four) at room temperature (23 ± 2°C) with 12 light/12 dark cycles. The animals were randomly separated to seven groups: a non-diabetic group (ND; n = 8) and six diabetic groups. Following an infusion of type 2 diabetes, type 2 diabetic groups were accidently separated into six groups. These are the diabetic control group (DC; n = 8), exercise group (Ex; n = 8), food restriction with standard diet group (FRSD; n = 8), food restriction with low-carbohydrate diet group (FRLCD; n = 8), food restriction with standard diet combination in exercise group (FRSDE; n = 8), and food restriction with low-carbohydrate diet combination in exercise (FRLCDE; n = 8).

Infusion type 2 diabetes

Type 2 diabetes was infused in Wistar male rats that have fasted overnight, by injecting 60 mg/kg STZ (Sigma Aldrich, USA) 15 min after an injection of 110 mg/kg of nicotinamide (Sigma Aldrich, USA).[19] STZ and nicotinamide were dissolved in citrate buffer (pH 4.5) and saline.[21,22] The ND control group was injected with citrate buffer.[20] After 1 week, blood was taken from the rats' tail veins for diagnosis of diabetes. Levels of fasting plasma glucose above 126 mg/dl were considered as evidence of type 2 diabetes.[23]

Animals and diets

According to the Association of Official Analytical Chemists (AOAC) and AIN-93G formulas for the growth of rats,[24] rat chow diets were mixed. Two kinds of diets, namely the standard diet (SD) and the low-carbohydrate diet (LCD) were used in this study. Where the LCD was concerned, the amount of starch was decreased to 24% of the original formula. Meanwhile, the amounts of other nutrients were chosen similar to the SD to ensure that there are sufficient nutrients for the rats to grow normally, [Table 1].[25] Food consumption by each group was measured for 1 week, and daily consumption of food was determined for all groups. Further, to induce CR, 20% of food was reduced consists of both of (FRSD and FRLCD). Hundred percent of food consumption for the Ex group was fixed, but instead, 20% of their energy intake in exercise was

Table 1: Composition of rat chow diet (diet divided into standard and low-carbohydrate food and given to rats for total protocol)

Components	Standard diet		Low carbohydrate diet	
	g/kg Diet	% Energy	g/kg Diet	% Energy
Casein	200	20.2	333.3	33.8
Vitamin mix	10	-	16.6	-
Mineral mix	35	-	58.3	-
Sucrose	50	5	83.3	8.4
Corn starch	585	58.9	308.3	31.2
Fiber	50	-	83.3	-
Lipid	70	15.9	116.6	26.6
Total amount (g)	1000	100	1000	100
Total calorie/kg diet (Kcal)	3970		3950	

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Table 2: Energy intake and expenditure through exercise and/or caloric restriction per each day

Groups	Measured required food (g)	Type of food	Calculated required energy (kcal)	Food intake (g)	Energy intake (kcal)	Exercise energy expenditure (kcal)	Negative energy (kcal)	% of negative energy balanced
FRSD	10	Standard	39.70	8	31.76	0	7.94	–20%
FRLCD	10.5	Low carbohydrate	41.47	8.4	33.18	0	8.29	–20%
Ex	8	Standard	31.76	8	25.40	6.352	6.35	–20%
FRSDE	11	Standard	43.67	9.9	39.30	4.367	8.73	–20%
FRLCDE	9.5	Low carbohydrate	37.52	8.55	33.77	3.75	7.50	–20%
ND	10	Standard	39.7	10	39.70	0	0	0
DC	10.5	Standard	41.68	10.5	41.68	0	0	0

FRSD=Food restriction with standard diet, FRLCD=Food restriction with low-carbohydrate diet, Ex=Exercise, FRSDE=Food restriction with standard diet combination in exercise, FRLCDE=Food restriction with low-carbohydrate diet combination in exercise, ND=Non-diabetic, DC=Diabetic control

calculated, and the time of daily exercise was determined.[26] The intensity of exercise was fixed (28 m/min),[25] but the time of exercise was variable to control exact negative energy balance (similar to 20% of their energy intake). Finally, a combination of reduced food intake (10%) and exercise (10%) was applied in two groups, namely FRSDE and FRLCDE. The time duration of daily exercise of the rats was determined according to their energy expenditure in exercise. Exercise training involved daily running in 2 different sessions with 15 min' rest between each session, for 8 weeks.[26]

Exercise training program

To adapt the rats at first week, Ex, FRSDE, and FRLCDE groups were exercised on a rat motor-driven treadmill at speed of 10 m/min for 15 min and gradually increased up to 28 m/min for 60–70 min during the adaptation stage. The exercise started between 7 A.M and 11 A.M in an almost dark place.[26] To shorten adaptation phase, it was tried to choose runner rats. A slight electric shock was used for encouragements of rats. After adaptation, the rats ran at treadmill speed of 28 m/min (70–75% VO_2 max ~7.78 ml. 100 g. min^{-1})[27] and duration of (Ex, 71 min), (FRSDE, 46 min), and (FRLCDE, 39 min). The duration was equal to 20% of Ex group and 10% of FRSDE and FRLCDE groups baseline energy requirements. The FRSDE and FRLCDE groups remained in the cage without exercise.

Exercise calorie expenditure

Resting oxygen consumption during exercise was assessed through Shepherd and Gollnick equations from running time, intensity, and total body weight listed in Table 2.[27] Resting oxygen uptake was calculated by multiplying body weight times 2.42 ml O_2/100 g per min. Calorie expenditure was achieved by subtracting resting O_2 consumption from exercise O_2 uptake and multiplying by 4.86 Kcal/l O_2 (assuming an respiratory quotient (RQ) of 0.85).[27]

Exercise calorie expenditure = Exercise oxygen (7.77 ml O_2/100 g per min – Resting oxygen (2.42 ml O_2/100 g per min) × 4.86 Kcal/l O_2 (assuming an RQ of 0.85).[27]

Therefore, in accordance with the cited equation, exercise sessions designed corresponding 20% or 10% of daily calorie intake (DCI) through equations demonstrated below:

VO_2 = % of DCI/4.86

Exercise duration (min) = VO_2/(exercise O_2 uptake ml O_2/100 g per min - resting O_2 uptake ml O_2/100 g per min) × body weight.[27]

Measurement of serum parameters

At the end of the experimental protocol, blood samples from the rats, which have fasted overnight, were gathered from their hearts. Glucose was measured by Accu-Chek Active (from Iran), and Serum insulin was measured with an insulin ELISA kit (Kristal De Biotik from China).

Real-time polymerase chain reaction

Total RNA was extracted from the rats' soleus muscles. To prepare the total RNA, the muscles were homogenized in trizol solution. RNA was then isolated according to the manufacturer's instructions. The extracted RNA was dissolved in 500 ml of diethyl pyrocarbonate (DEPC) water and stored at –70°C. We used optical density (OD) to characterize the quality and density of the extracted RNA. The OD was measured at a specified wavelength of 260 nm. The amount of 1 mg of isolated RNA from each sample was used to make cDNA by M-MLV inverse transcriptase (TAKAR, Japan). For real-time PCR reaction, we used a commercial kit (SYBR® Green PCR Master Mix, Applied Biosystems, USA). GLUT4 and GAPDH genes (as housekeeping genes) were multiplied by using (SYBR Green PCR Master Mix ABI, USA) and ABI step one plus device. The intended thermal cycle of reaction includes a step to activate polymerase in 95°C in 10 min followed by 40 cycles (95°C in 15 s and 60°C in 1 min). After the PCR was done and to study the primers, a temperature range from 55 to 99 was used to derive the melting curve. The following primers were used: REV: CAGCGAGGCAAGCTAGA and FOR: GGGCTGTGAGTGAGTGCTTTC. The cycle of threshold (CT) of the reactions was extracted by real-time-PCR

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software. We also used the 2-ΔΔCt method to quantify the GLUT4 mRNA expression.

Statistical analyses

All analyses were performed using SPSS for Windows (version 19.0, SPSS Inc, Chicago, IL, USA). A Shapiro-Wilk test was applied to determine the normality of distribution of measures, which were found to be normally distributed. A one-way analysis of variance (ANOVA) was performed to determine the differences in a parameter among the groups. Significant differences were identified using the least significant difference (LSD) *post hoc* test. All data were expressed as mean ± SD, and significance was set at the alpha level $P \leq 0.05$.

Results

Glucose level

There were significant differences in glucose levels between groups after 8 weeks of intervention ($P = 0.002$). However, type 2 diabetes clearly increased the glucose serum compared to the ND group ($P = 0.001$). The *post hoc* LSD test analysis revealed that compared to DC group, glucose level of FRSD group was significantly decreased 14.8% after 8 week protocol ($P = 0.002$); In addition, 15.9% significant decrease was observed in FRLCD group in comparison to DC group ($P = 0.001$). Ex, FRSDE, and FRLCD groups fasting glucose level showed 13.6%, 16.5%, and 19% decrease comparing to DC, respectively ($P < 0.01$) [Table 3].

Insulin level

Insulin fasting concentration did not altered significantly in any diabetic groups after 8 week intervention ($P > 0.05$). However, the results showed that 2 diabetic male rats had a significant decrease in insulin concentration compared to ND group ($P = 0.001$) [Table 3].

GLUT4 gene expression

There were significant differences in GLUT4 gene expression between groups after 8 weeks intervention ($P = 0.001$). However, the results showed that type 2 diabetes inductions had reduced GLUT4 gene expression compared to the ND group ($P = 0.001$). The *post hoc* LSD test analysis revealed that compared to DC group, GLUT4 gene expression level of Ex group was significantly increased 47% after 8 week protocol ($P = 0.004$); In addition, 60% significant increase was observed in FRSDE group in comparison to DC ($P = 0.001$). In FRLCDE group, GLUT4 gene expression level showed 65% significant increase compared to DC ($P = 0.001$), but it was not significant or with any other diabetic groups [Table 3].

Discussion

Our study examined the effect of negative energy balance with food restriction only or combination with aerobic exercise on the GLUT4 in diabetic male rats. Diet restrictions, SD and LCD, Ex, or a combination of exercise and CR caused a significant reduction in glucose level compared to the DC group. CR increases glucose metabolism in rats.[28] Evidence suggests that this is owing to large part to increased muscle glucose utilization.[29] Studies have shown increased insulin sensitivity after activation of 5' adenosine monophosphate-activated protein kinase (AMPK) in the skeletal muscle of rats during CR.[30] CR activates a cellular energy regulator (AMPK), a cellular manager that activates energy production when calorie and glucose levels are low. AMPK makes cells more energy-efficient by facilitating glucose transport across cell membranes, which can reduce glucose levels in rats.[31] Furthermore, the RLCE group had the most significant effect on glucose reduction. These results are consistent with the result of a study by Filaire *et al*.[32] who reported that a combination of exercise and CR considerably reduced the density of blood glucose compared to diabetic groups.[33] Evidence from many studies, such as the Nurses' Health Study[34] and Health Professional Addition Study[35] has shown that carbohydrate intake reduction has a positive correlation with improvement in type 2 diabetes. Reducing the intake of high-carbohydrate foods is one of the most important methods of improving hyperglycemia in type 2 diabetic patients.[36] Many studies have shown that an LCD markedly lowers serum glucose and serum insulin levels after meals.[37,38] Samaha *et al*., have shown that there is a major decrease in fasting serum glucose levels in LCD diabetic groups compared to the LFD groups.[12] Many mechanisms can improve glucose uptake during and after exercise, notably those, involving the enhancement of muscle blood flow, insulin resistance (IR) turnover, insulin binding to its receptor (IR), and glucose is transported by stimulating GLUT4 translocation to the muscle cell surface.[39,40] Skeletal muscle reduces blood glucose during exercise, which is independent of insulin signaling pathways in patients with type 2 diabetes.[41] The results show that the RLCE group had the most important effect on blood glucose reduction that might be attributed to the combination of dietary carbohydrate reduction and increased glucose utilization by muscles as a result of

Table 3: Effects of 8 weeks negative energy balance on glucose, insulin plasma levels, and GLUT4 gene expression

Group	ND	DC	FRSD	FRLCD	Ex	FRSDE	FRLCDE
Glucose (mg/dl)	104±12.26	286±36.70#	240.125±16.96*	236.7±29.07*	244.37±27.5*	235.07±20.06*	227.87±28.27*
Insulin (υU/mL)	2.3±0.29	1.53±0.27#	1.48±0.30	1.35±0.25	1.47±0.31	1.45±0.28	1.287±0.16
GLUT4 (mRNA)	1	0.52±0.13#	0.61±0.12	0.63±0.13	0.71±0.11*	0.76±0.13*	0.8±0.14*

Data have been expressed as mean±SD. #Level of significant difference is $P<0.05$ compared to ND group. **Post hoc* LSD revealed a significant difference between the groups compared to DC group ($P<0.01$)

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exercise. We certainly cannot suggest that this reduction in blood glucose is caused by the combined effect of exercise and FRLCDE, as identification of this process requires a more extensive investigation. Rats with type 2 diabetes showed a significant decrease in insulin levels compared to ND rats. The injection of STZ destroys the beta-cells significantly by reducing the amount of insulin production.[42] It is now well established that type 2 diabetes mellitus (T2DM) develops when beta-cells are unable to supply the amount of insulin needed to maintain normal glucose levels.[43] However, fasting insulin density did not change considerably in any of the diabetic groups after the 8-week protocol. These results are contrary to the findings of a previous study conducted by Khowailed *et al*.,[44] who have reported that CR alone or in combination with exercise significantly decreases insulin levels. The results showed that reduction of type 2 diabetes induction reduced GLUT4 gene expression more significantly than the ND group. Food restriction alone cannot lead to a major increase in GLUT4, although Ex, FRSDE, and FRLCDE groups had significantly higher GLUT4 gene expressions than to the DC group. FR and exercise stimulate the GLUT4 by different signaling pathways, whereby GLUT4 is translocated to the cell membranes and transverse tubules, where it mediates the transport of glucose into the muscle cells.[45-47] Exercise training markedly increases insulin's ability to stimulate glucose uptake in skeletal muscles and neutralize insulin resistance, a generic feature of type 2 diabetes. Although it seems that certain dimensions of the molecular relationship between exercise and insulin sensitivity improvement remain unknown, a large body of literature suggests that aerobic exercise improves glucose transport into muscle cells. The number of glucose transporters associated with the plasma membrane and transverse tubules increase sharply directly after exercise[48] with a concomitant increase in glucose uptake. As already mentioned, a substantial portion of glucose ingested after exercise is taken up by skeletal muscles to replenish glycogen stores.[49] Therefore, the recently suggested functional association between muscle glycogen and GLUT4 is particularly interesting.[50] These authors hypothesize that glycogen reduction would result in a larger available pool of free GLUT4 vesicles. This hypothesis is supported by the observation that rat over-expressing GLUT1, which have dramatically increased muscle glycogen levels, are insulin resistant.[51,52] Exercise training improves the phosphorylation and expression of the protein kinase cascade in the activated protein kinase AMPK signaling pathway, which can increase GLUT4 content, and subsequently increase glucose uptake in skeletal muscles. Furthermore, exercise training increases GLUT4 gene expression in type 2 diabetic patients.[53,54] The results of the present study showed that induction of type 2 diabetes decreased GLUT4 gene expression in the skeletal muscles.[47] However, exercise training alone or with CR could improve GLUT4 gene expressions in type 2 diabetic patients. El-Tablawy and Khaleel have shown that exercise training increases GLUT4 protein expression in insulin deficiency and insulin resistance on the rats' skeletal muscles.[15,55]

Conclusions

Results indicated that adding exercise to the FR programs has any effect on the insulin levels than to the DC group, but it increased the blood glucose level and GLUT4 gene expression of diabetic groups. Exercise may be considered a treatment option with positive effects on obesity and metabolic changes in type 2 diabetes mellitus. However, the exact mechanism in which a combination of exercise with FRSD and FRLCD affects GLUT4 needs further research. The results of our study show that a LCD combined with aerobic exercise is a viable plan for type 2 diabetes patients. However, these results may help to develop new approaches for the treatment of obesity and type 2 diabetes mellitus.

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Conflicts of interest

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