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Effect of oral magnesium sulfate administration on blood glucose hemostasis via inhibition of gluconeogenesis and FOXO1 gene expression in liver and muscle in diabetic rats

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ABSTRACT

The present study was designed to investigate the possible role of Mg^{2+} in suppression of phosphoenolpyruvate carboxy kinase (PEPCK) enzyme via inhibition of FOXO1gene expression in liver and we also examined whether Mg contributes to decrease blood glucose in muscle via inhibiting FOXO1 gene and protein expression. Fifty rats in five groups of experiment were considered as; non-diabetic control (NDC), Mg^{2+}-treated non-diabetic control (Mg^{2+}-NDC), chronic diabetic (CD), Mg^{2+}-treated chronic diabetic (Mg^{2+}-CD), and insulin-treated chronic diabetic (Ins-CD). Streptozotocin (STZ) was used for diabetes induction. The Mg^{2+}-CD and Mg^{2+}-NDC groups received 10 g/l of magnesium sulfate ($MgSO_4$) added to drinking water, and Ins-CD group received 2.5 U/kg of insulin. The blood glucose level and body weight were measured weekly. After 16 weeks, intraperitoneal glucose tolerance test (IPGTT) was done and blood samples were taken to determine the plasma levels of Mg and gastrocnemius muscle legs, and liver were isolated for both Forkhead transcription factor (FOXO1) and PEPCK enzyme genes and proteins expression. Administration of $MgSO_4$ improved IPGTT, lowered blood glucose levels and decreased FOXO1 and PEPCK genes and proteins expression in muscle and liver, while insulin just could decrease FOXO1 gene and protein expression in the muscle. These findings illustrated that $MgSO_4$ improved hyperglycemia via inhibition of FOXO1 gene and protein level in the muscle and liver, and it also decreased blood glucose level by prohibition of gluconeogenesis pathway in the liver. However, long time administration of insulin did not have any effect on liver.

1. Introduction

There are different pathways to regulate the blood sugar including the control of the key enzyme (phosphoenol pyruvate carboxykinase enzyme, PEPCK) activity of the gluconeogenesis pathway in the liver. Increasing the activity of this enzyme is one of the responsible factors to increase blood glucose in diabetic patients [1]. It is reported that PEPCK expressions is down-regulated by insulin [2]. The other way to improve glucose hemostasis is stimulating glucose transporter 4(GLUT4), gene expression and translocation from intracellular pool to the plasma membrane [3]. Insulin stimulates glucose uptake in adipose and muscle tissues by eliciting GLUT4 gene expression [4].

In addition, Forkhead transcription factor (FOXO1) plays an important role in mediating insulin action and regulating target gene expression [5]. Some findings [6] indicate that FOXO1 is a negative regulator of insulin sensitivity in liver, adipocytes and pancreatic β-cells. FOXO1contains highly conserved Akt phosphorylation sites (Thr-24, Ser-253, and Ser-316) and its activity is regulated by Akt-mediated phosphorylation of these sites. Phosphorylated FOXO1 is excluded from the nucleus, thereby decreasing its transcriptional activity (7). FOXO1 also stimulates the expression of phosphoenolpyruvate carboxy kinase (PEPCK) and glucose 6-phosphatase (G6P-ase) via direct binding of insulin response element (IRE) mapped in the promoters of these genes [7]. So over activity of the FOXO1 promotes diabetic hyperglycemia, dyslipidemia, and acute-phase response [5]. Recent studies demonstrated that insulin can suppress FOXO1 activity [8,9], and there have

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been documented by us and others that magnesium (Mg^{2+}) deficiency occurs during diabetes induction [10] and Mg deficiency increases the activity of PEPCK and G6P-ase enzymes [11], however; the mechanism of this phenomenon is not completely understood. Our previous findings support the hypothesis that magnesium could decrease blood glucose via an increase in GLUT4 mRNA expression not by increasing in insulin secretion [12]. So the present study was designed to investigate the possible role of Mg^{2+} in suppression of PEPCK enzyme via inhibition of FOXO1gene expression in liver. It is also examined whether Mg^{2+} contributes to the activation of GLUT4 gene expression and translocation in muscle via inhibiting FOXO1.

2. Materials and methods

2.1. Animals

Animals were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The experimental protocol was approved by the Ethical Committee for Animal Care of Hormozgan University of Medical Sciences (Ethic number HUMS.REC.1395.105.). Male Wistar rats, weighing 180–250 g were obtained from Hormozgan University of Medical Sciences breeding center (Bandar Abbas, Iran) and maintained at a constant temperature of 22 ± 2 °C with a fixed 12:12-h light-dark cycle. Nutritionally balanced pellets and water were freely available. The animals were divided into five groups (n = 10 rats in each group): non-diabetic control (NDC); Mg^{2+}-treated non-diabetic control (Mg^{2+}-NDC); chronic diabetic (CD); Mg^{2+}-treated chronic diabetic (Mg^{2+}-CD); and insulin-treated chronic diabetic (Ins-CD).

Diabetes was induced by a single IP injection of 60 mg/kg streptozotocin dissolved in isotonic normal (STZ, Sigma Aldrich, Hamburg, Germany) and the same volume of normal saline was injected to the animals in the NDC group. At 10 days post STZ injection, fasting blood glucose level was determined and the presence of diabetes was confirmed by blood glucose levels of above 250 mg/dl. Animals in which the diabetes lasted for 16 weeks were defined as chronic diabetic. Ten days after diabetes induction, the Mg^{2+}-CD and Mg^{2+}-NDC groups received 10 g/l of $MgSO_4$ (Sigma Aldrich, Hamburg, Germany) added to their drinking water for 16 weeks. Water consumption was measured. Mg^{2+}-treated chronic diabetic and Mg^{2+}-treated non-diabetic control groups appeared to have significantly lower water consumption compared to chronic diabetic group (42 ± 2, 40 ± 2 and 203 ± 3.1 ml/24 h for Mg^{2+}-treated chronic diabetic, Mg^{2+}-treated non-diabetic control and chronic diabetic respectively). So the exact dose of $MgSO_4$ that rats were consuming was 0.42 g/24 h.

The Ins-CD group received 2.5 U/kg of insulin (1/3 regular and 2/3 NPH, from Aksir Company Iran) for 16 weeks to keep the blood glucose around 100 ± 2 mg/dl [13]. Animals were monitored for blood glucose concentrations and body weight every week at 9 A M over the course of the experiment. Blood glucose was measured with an Ascensia ELITE XL glucometer and Ascensia Elite blood glucose test strips and body weight of all experimental was recorded using a digital weighing scale.

2.2. Intraperitoneal glucose tolerance test (IPGTT)

For IPGTT, the animals in all groups were subjected to 15 h overnight fastening, and were given 1.5 g of glucose (Sigma Aldrich, Hamburg, Germany) per kilogram of body weight via IP injection. Blood was drawn from the tail vein at 0, 10, 20, 30, 60, 90 and 120 min after glucose administration [14].

2.3. Biochemical assay

Animals were anesthetized with ketamine HCl (50 mg/kg, IP, from Rotexmedica Trittau, Germany), then the animals were decapitated. Blood samples were taken from the neck vascular trunk in order to determine the plasma levels of Mg by Micro plate reader (Biotek, USA) and appropriate kits (Zistshimi, Tehran, Iran).

Liver and muscle were isolated for FOXO1 gene and protein expression via Real-time PCR and immunohistochemistry methods respectively. The gene expression of PEPCK enzyme and their proteins in liver were also measured by Real-time PCR and ELISA (mybiosource,USA), respectively.

2.4. RNA extraction

The total RNA was extracted from muscle tissue using RNeasy Fibrous Tissue mini kit (Cat No.74704 QIAGEN, Germany) according to the manufacturer's protocol. Briefly, RNA was treated with 1 µl DNase-I in 37 °C for 30 min and then 1 µl 50 mM EDTA was added. Then it was incubated in 65 °C for 10 min for DNase inactivation. The extracted RNA was quantified by running on 1.5% agarose gel electrophoresis. The 18S and 28S RNA bands should be visualized under a transillumiator. The yield was quantified spectro-photometrically at 260 and 280 ηm by NanoDrop 1000 (Thermo, USA). The obtained proportion of 1.8 to 2.0 from indicated the quality of extracted RNA.

Total RNA from liver tissue was extracted using Iraizol kit, (RNA Biotech.IRAN) following the manufacturer's instruction.

2.5. cDNA synthesis and quantitative real-time PCR

The total RNA (2 µg) was reversely transcribed to cDNA using PrimeScript™ RT Reagent kit Fast cDNA Synthesis Kit ready for Real Time (Cat. No.RR037 A, Takara, Japan) by applying random hexamer primer following the manufacturer's protocol. Quantitative RT-PCR was conducted on a Real-time PCR (Corbett, Rotor-Gene 6000, Australia) and SYBR Green (Takara, Japan) according to the manufacturer's instructions. All reactions were carried out in a terminal volume of 20 µl containing1µL of cDNA sample, 1 µl of the primers, 10 µL SYBR Green, 0/4 µL ROX and 7/6 µL of sterile double-distilled water for all β-actin, FOXO1 or PEPCK. The thermal cycling status for β-actin was an initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 68 °C for each primer for 30 s. For FOXO1: the thermal cycling status was an initial denaturation at 95 °C for 3 min followed by 40 cycle of denaturation at 95 °C for 10 s, annealing at 62 °C for each primer for 30 s. Also for PEPCK the thermal cycling status was an initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and the annealing at 65 °C for each primer for 30 s. All the standards and the samples measurements were repeated twice. Each PCR product was characterized by melting curve analysis.

Expression levels of FOXO1and PEPCK were normalized by β-actin expression as the house-keeping gene and calculated through the $2^{-\Delta\Delta CT}$ method. The sequence of specific primer sets for each gene, amplicon sizes and annealing temperature of the primers real- time PCR time was used (Table 1).

2.6. Immunohistochemistry

Five-micrometer sections of formalin-fixed paraffin-embedded tissue were cut and prepared for IHC staining. Processed through xylene

Table 1
Show Primers designed for amplification of, *β-actin*, FOXO1 and PEPCK.

Gene	primer (5′→3′)	Amplicon Size (bp)	TM
β-actin	F: CAC ACC CGC CAC CAG TTCG	165	60/5
	R: ACC CAT TCC CAC CAT CAC ACC		
FOXO1	F:GAT AAG GGC GAC AGC AAC AG	125	60/5
	R:TGA GCA TCC ACC AAG AAC TT		56/4
PEPCK	F: CTC ACC TCT GGC CAA GAT TGG TA	195	64/7
	R:GTT GCA GGC CCA GTT GTT GA		60/5

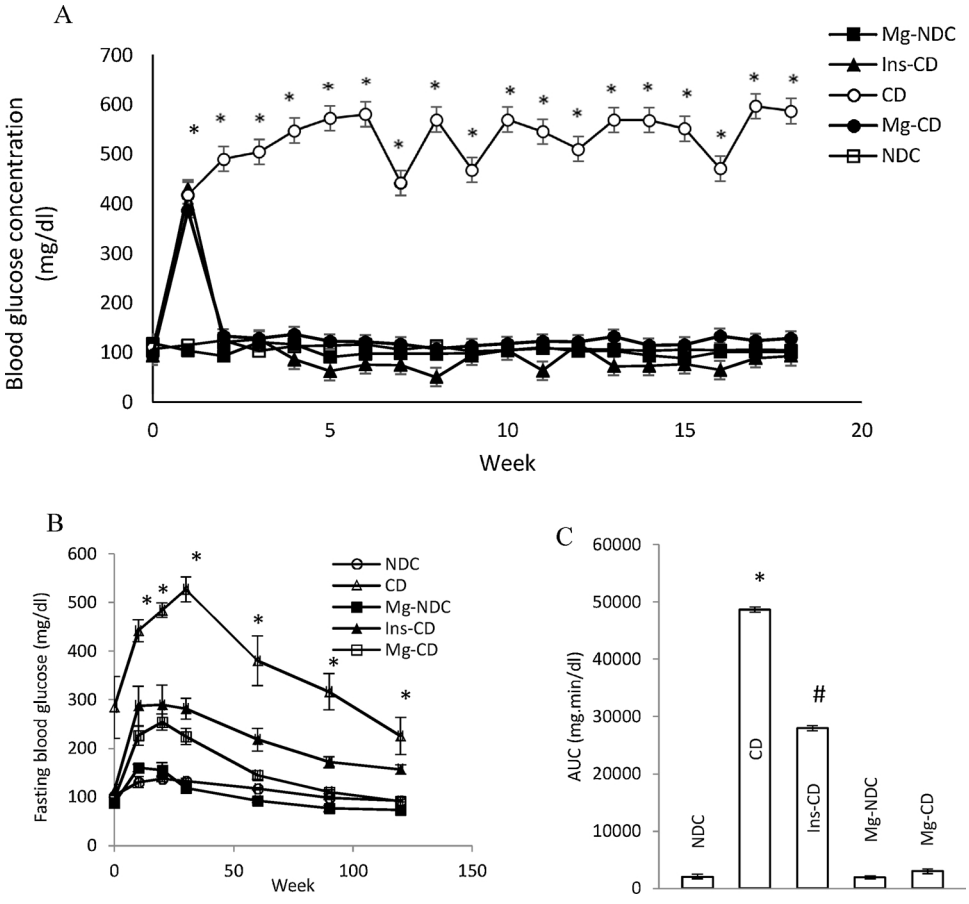


Fig. 1. Comparison of fed blood glucose (A), intraperitoneal glucose tolerance test (IPGTT) (B) and the area under the glycaemic curve (AUC) (C) in non-diabetic control (NDC), chronic diabetic (CD), Mg^{2+}-treated non diabetic control (Mg^{2+}-NDC), insulin -treated chronic diabetic (Ins-CD) and Mg^{2+}-treated chronic diabetic groups (10 rats in each group. data are expressed as mean ± SEM).
*Significant difference between CD animals and other groups ($P < 0.0001$).
Significant difference between Ins-CD animals and Mg^{2+}-CD and Mg^{2+}-NDC groups ($P < 0.001$).

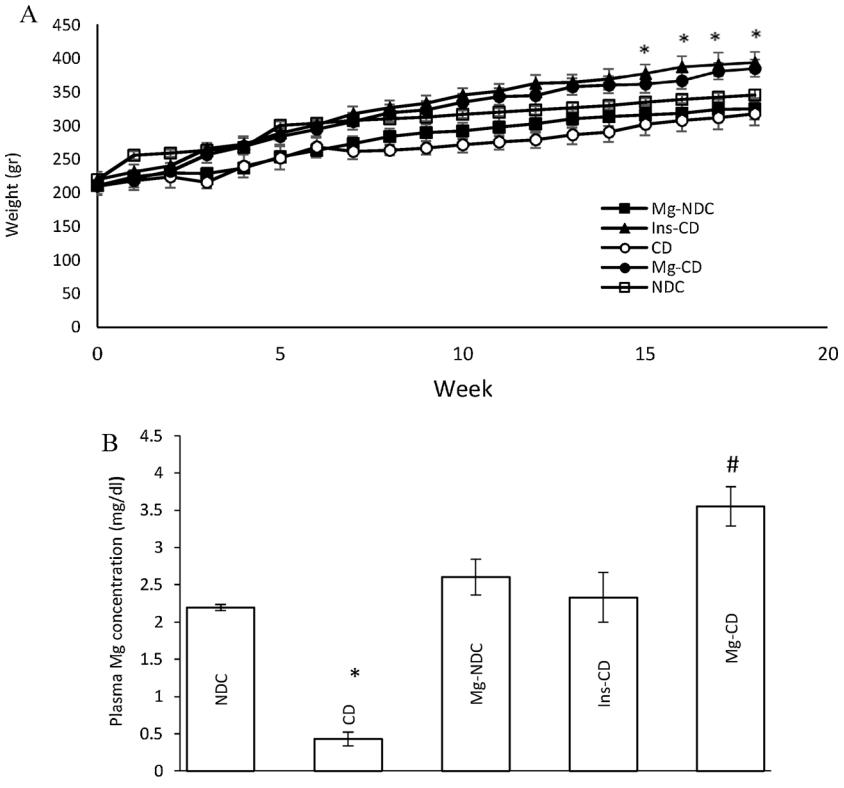
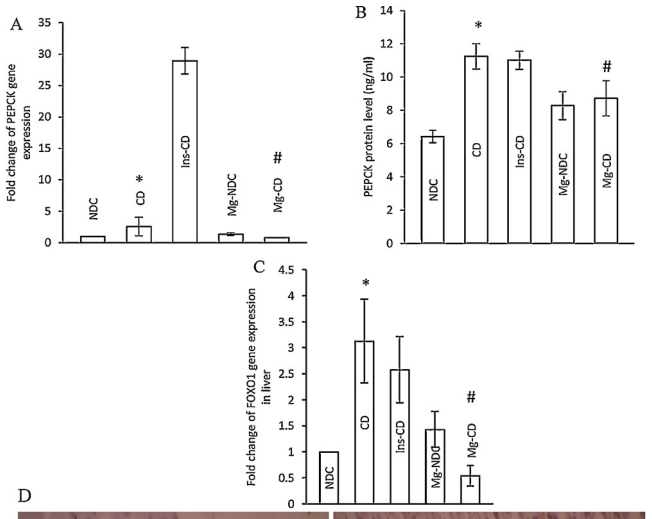


Fig. 2. Comparison of body weight (A), Plasma magnesium level (B) in non-diabetic control (NDC), chronic diabetic (CD), Mg^{2+} -treated non diabetic control (Mg^{2+}-NDC), insulin-treated chronic diabetic (Ins-CD) and Mg^{2+}-treated chronic diabetic (Mg^{2+}-CD) groups (10 rats in each group, data are expressed as mean ± S EM).
*Significant different between CD and other groups ($P < 0.0001$).
#Significant different between Mg^{2+}-CD and Ins-CD and Mg^{2+}-NDC groups ($P < 0.001$).



E

	NDC	CD	Ins-CD	Mg^{2+}-NDC	Mg^{2+}-CD
Protein level of FOXO1 in liver	+	+++	++	++	+

Fig. 3. Comparison of phosphoenol pyruvate carboxykinase (PEPCK) gene expression in liver(A), Protein level of PEPCK in liver (B), Forkhead transcription factor (FOXO1) gene expression in liver (C) and immunohistochemistry for FOXO1 in liver (D and E) in non- diabetic control (NDC), chronic diabetic (CD), Mg^{2+} -treated non diabetic control (Mg^{2+}-NDC), insulin-treated chronic diabetic (Ins-CD) and Mg^{2+}-treated chronic diabetic (Mg^{2+}-CD) groups (10 rats in each group, data are expressed as mean ± S EM).
*Significant different between CD and other groups ($P < 0.001$(.
Significant different between Mg^{2+}-CD and Ins-CD and Mg^{2+}-NDC groups ($P < 0.001$).

and a graded alcohol series and treated with 3% hydrogen peroxide to eliminate the endogenous peroxidase activity prior to immune-staining and blocked with 5% normal goat serum. Slides were then treated with mouse anti- rat FOXO1 m Ab at 10 µg / ml (Santa Cruz Biotechnology, Santa Cruz, CA). After an overnight incubation, horse anti-mouse IgG-biotin (Vector Labs, Burlingame, CA) secondary antibody was added at a 1 : 200 dilution, followed by streptavidin – HRP (Jackson Labs, West Grove, PA) at 1 : 1000. Slides were developed with AEC Reagent (Zymed Laboratories, San Francisco, CA) and counterstained with hematoxylin (Sigma Co, St. Louis, MO). Cells were viewed and photographed under bright field.

2.7. Statistical analysis

Data is expressed as mean ± S.E.M. Differences among groups was evaluated by one-way and two-way ANOVA with the Tukey post-hoc test. The relative changes of gene expression were calculated by the $2^{\wedge -\Delta\Delta Ct}$ formula, where $\Delta Ct = C_t$ (FOXO1 or PEPCK)–C_t (β-actin) and Ct demonstrates threshold cycle number. In addition, Spearman and Mann-Witney U-tests were used to investigate the expression pattern of target gene. Statistical analysis was carried out using SPSS-21 software. $P < 0.05$ was selected for acceptance of statistical significance.

3. Results

Before the intervention, there were no differences in blood glucose, IPGTT, body weight and plasma Mg level among the animals in each group.

3.1. Changes in blood glucose

Changes in feeding blood glucose were measured in all groups (Fig. 1A). Diabetes induction caused blood glucose concentration to increase (418.33 ± 12.88 mg/dl) and blood glucose continued to be elevated (587 ± 9.57 mg/dl) 16 weeks after diabetes induction. Administration of $MgSO_4$ or insulin for 16 weeks (from day 10) caused the blood glucose concentrations in the Mg-CD and Ins-CD groups decreased (128.25 ± 0.62 mg/dl, 93 ± 15.99 mg/dl) respectively.

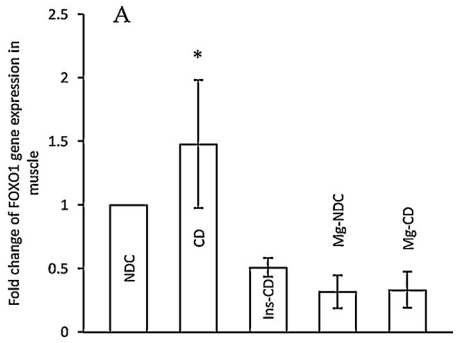
3.2. Effect of MgSO4 on intraperitoneal glucose tolerance test (IPGTT)

Before starting $MgSO_4$ and STZ administration, the IPGTT patterns for all groups were similar, and there were no significant differences between the groups when the glycemic response was expressed as the area under the curve (AUC) (data was not shown). However, after 16 weeks, the CD group displayed severe glucose intolerance (Fig. 1B), which was significantly improved in the Mg^{2+}-CD group to a degree that was judged effective (Fig. 1C, AUC: NDC vs CD vs Mg^{2+}-CD vs Ins-CD: 2799 ± 433.5 mg.min/dl vs 6150.4 ± 455.1 mg.min/dl vs 2990.8 ± 432.5 mg.min/dl vs 4097.25 ± 324.5 mg.min/dl, $P < 0.0001$).

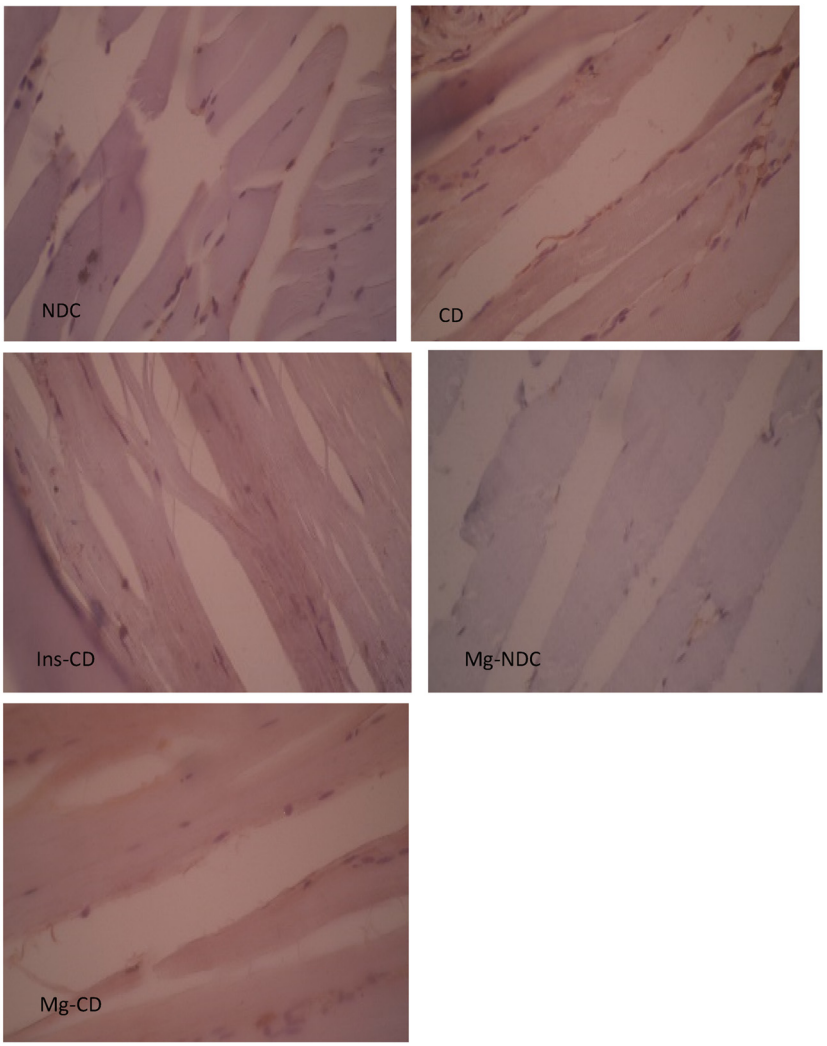
3.3. Changes in the body weight and plasma magnesium levels

The body weight of CD animals was insignificantly decreased when compared to the levels in NDC animals; increased body weight was significantly observed after administration of Mg^{2+} and insulin when compared to the levels in CD animals (Fig. 2A).

To find the concentration as well as duration of oral-administered Mg in plasma, changes in plasma Mg^{2+}level were measured in all groups. The plasma Mg level in CD (0.42 ± 0.09 mg/dl) significantly ($P < 0.001$) decreased compared to the NDC group (2.19 ± 0.04 mg/dl). Administration of Mg^{2+} and insulin for 16 weeks (from day 10) caused plasma Mg^{2+} concentrations in the Mg^{2+}-CD and Ins-CD groups to increase (Mg^{2+}- CD = 3.55 ± 0.26 mg/dl, Ins-CD = 2.33 ± 0.34 mg/dl), but there is a significant differences



B



C

	NDC	CD	Ins-CD	Mg^{2+}-NDC	Mg^{2+}-CD
Protein level of FOXO1 in muscle	++	+++	+	+	+

Fig. 4. Comparison Forkhead transcription factor (FOXO1) gene expression in muscle (A)and immunohistochemistry for FOXO1 in muscle (B and C) in non-diabetic control (NDC), chronic diabetic (CD), Mg^{2+} -treated non diabetic control (Mg^{2+}-NDC), insulin-treated chronic diabetic (Ins-CD) and Mg^{2+}-treated chronic diabetic (Mg^{2+}-CD) groups (10 rats in each group, data are expressed as mean ± S EM).
*Significant different between CD and other groups ($P < 0.001$).

between Mg^{2+}-CD and Ins-CD groups ($P < 0.001$) (Fig. 2B).

3.4. Changes in FOXO1 and PEPCK gene expression

The average Ct obtained from Real-time PCR of five groups of examined rats as well as the effect of $MgSO_4$ and insulin in expression of PEPCK and FOXO1 in liver and muscle have been showed through Fig. 3A, C and Fig. 4A respectively. Transcription of FOXO1 in muscle decreased ($p < 001$) in comparison to chronic diabetic rats (CD) due to Mg^{2+} treatment. But insulin administration in diabetic rats could not reduce FOXO1 gene expression in liver in compare to CD group. Mg therapy could significantly down-regulate PEPCK gene expression in liver in comparison with chronic diabetes (CD) group, but this effect was not observed in insulin-treated diabetic animals compared with chronic diabetes and Mg^{2+}-CD groups.

3.5. Changes in FOXO1and PEPCK proteins levels

Immunohistochemistry was done for all groups to observe FOXO1 protein level expression in muscle and liver and results are shown in Figs. (4 B, C, and 3 D and E respectively). According to our findings FOXO1 protein level in muscle increased in CD group, however this result was not observed in Mg^{2+} or insulin treated groups. FOXO1 protein level in liver in Mg^{2+} treated group decreased as compare to CD animals, but this finding was not observed in Ins-CD group compared to CD and Mg^{2+}-CD animals. Our results showed that the protein level of PEPCK in liver significantly decreased in Mg^{2+}-CD groups compared with CD and Ins-CD groups.

4. Discussion

Diabetes is recurrently linked with both extracellular and intracellular Mg shortages [15]. Insulin and glucose are main supervisors of Mg metabolism. Intracellular Mg plays an important role in changeable insulin action, insulin-mediated-glucose-uptake and insulin sensitivity [15]. Our previous study showed that oral Mg^{2+} administration for 8 weeks played a crucial role in glucose hemostasis through attenuated GLUT4 gene expression [3] while the mechanism of this phenomenon was not clear. So, the present study was designed to investigate to examine whether Mg^{2+} contributes to the activation of GLUT4 gene expression and translocation in muscle via the inhibition of FOXO1 gene and protein expression. We also investigated the possibility role of Mg^{2+} on suppression of PEPCK enzyme via inhibition of FOXO1gene expression in liver to prevent of hyperglycemia.

We observed that Mg reduction after diabetes induction and 16 weeks of Mg^{2+} or insulin therapy could reach plasma Mg level to the acceptable value. This result demonstrated that insulin accumulation Mg^{2+} in plasma and probably part of insulin action on its target cell mediated by Mg. Takaya et al showed that Mg has an important role in insulin action and insulin stimulates magnesium uptake in insulin-sensitive tissues and Mg is required for both proper glucose utilization and insulin signaling [11].

However in the CD and NDC groups receiving Mg^{2+}, weight gain was not observed during the study. However; in diabetic groups treated with insulin or Mg^{2+}, after 16 weeks, weight was significantly increased compared to other groups due to the maintenance of fat storage in the body. Based on the current results, it seems that Mg^{2+} administration is not suitable for NDC group, because it interrupts the electrolyte balance.

Extreme hepatic sugar manufacture is a donating factor to fasting hyperglycemia in diabetes. Insulin destroys hepatic glucose creation via preventing the expression of two important gluconeogenic enzymes [16]. Studies confirmed that practical inhibition of FOXO1is related to decreased hepatic gluconeogenic activity and improved fasting blood glucose in diabetic animals [17]. Type 2 diabetes mellitus, obesity and insulin deficiency are accompanying diminished regulation of GLUT4 gene expression and increased blood glucose level [16]. On the other hand insulin unplugs FOXO1 through the PI3K/Akt pathway. FOXO1 factors, in turn, activate upstream signaling factors that control both insulin sensitivity and glucose metabolism [18,19]. Our findings in this study indicated that Mg^{2+} administration in diabetic animals in contrast with insulin, suppressed FOXO1 gene expression in liver. We reported that Mg^{2+} inhibits the expression of gluconeogenic genes such as PEPCK. Mg^{2+} decreased the mRNA levels of this gluconeogenic enzyme, whereas this enzyme was significantly higher in Mg^{2+}-NDC group in comparison to Mg^{2+}-CD and Ins-CD groups; however FOXO1 gene was suppressed in this group. So in the liver the mRNAs for PEPCK remains high and gluconeogenesis continues to prevent of hypoglycemia in non-diabetic rats by Mg^{2+} administration.

Researcher showed that under normal conditions, dietary glucose stimulates insulin secretion from the pancreas [20]. The insulin prevents of FOXO1 phosphorylation which in turn down regulates genes required for gluconeogenesis, most prominently PEPCK [20]. The result is a decrease in hepatic glucose output, which helps to keep blood glucose low [21,22]. But the results of the present study showed that FOXO1 gene expression was not suppressed by insulin in the liver, whereas mRNA for PEPCK in liver was blocked by insulin and gluconeogenesis pathway was stopped by the other mechanism.

4.1. Conclusion

Overall, the results of this study showed that $Mg^{2+}$is more effective than insulin in controlling blood glucose and prevent of gluconeogenesis pathway in liver while Mg^{2+} has both positive liver and the muscle effects.

Conflict of interests

The authors declare that they have no competing interests.

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