

## Original Article

# The role of PPAR- $\gamma$ and NFKB genes expression in muscle to improve hyperglycemia in STZ-induced diabetic rat following magnesium sulfate administration

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**Abstract:** The present study was designed to investigate the possible role of magnesium ( $Mg^{2+}$ ) on activation of the peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) and inhibition of nuclear factor-KB (NFKB p65) in muscle to increase glucose transporter 4 (GLUT4) gene expression. Fifty rats were divided into five groups, namely 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 with streptozotocin (STZ) injection. 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 every week. After 16 weeks, intraperitoneal glucose tolerance test (IPGTT) was done and then animals were decapitated, blood samples were taken to determine the plasma levels of  $Mg^{2+}$  and gastrocnemius muscle legs were isolated for both PPAR- $\gamma$  and NFKB (p65) genes and proteins expression. Administration of  $MgSO_4$  improved IPGTT, lowered blood glucose levels and increased PPAR- $\gamma$  gene and protein expression. Diabetes increased NFKB gene and protein expression. Although  $Mg^{2+}$  therapy could not decrease NFKB (p65) gene expression, the protein decreased by  $Mg^{2+}$  therapy. Insulin decreased NFKB (p65) gene and protein expression, without any effect on PPAR- $\gamma$  gene and protein expression. According to our findings it seems that suppressing NFKB (p65) protein synthesis and increases in PPAR- $\gamma$  gene and protein expression could help  $Mg^{2+}$  administration to decrease blood glucose levels. But decreasing in NFKB (p65) gene and protein expression help insulin to decrease blood glucose level.

**Keywords:** Diabetes, insulin, magnesium, PPAR- $\gamma$ , NFKB (p65)

## Introduction

There is evidence suggesting that peroxisome proliferator-activated receptors (PPARs) may have a role in chronic diseases like diabetes, obesity and atherosclerosis [1]. There are three types of PPARs including PPAR- $\alpha$ , PPAR- $\beta/\delta$  and PPAR- $\gamma$  [2]. These nuclear receptors are expressed in adipose, muscle and liver tissues [3]. But each PPAR isoform has exclusive roles in vivo. PPAR- $\alpha$  is a major activator of fatty acid oxidation pathways and is the target of the hypolipidemic fibrate drugs [4]. However, in the key metabolic tissues such as skeletal muscle, liver and heart PPAR- $\delta/\beta$  has a crucial role in

fatty acid oxidation. PPAR- $\gamma$  is the most highly expressed in white adipose tissue and muscle [5] and its agonists cause to improve glycemic control, decrease plasma insulin levels, and increase insulin sensitivity [6, 7]. PPAR- $\gamma$  ligands improve insulin sensitivity by promoting fatty acid storage in fat tissues and expression of GLUT4 (a major glucose transporter in skeletal muscle) mRNA [8]. Some studies observed that PPAR- $\gamma$  agonist receptor therapy could enhance muscle glucose metabolism via activation of insulin cell signaling. Other studies demonstrated that PPAR- $\gamma$  increases insulin-stimulated glucose uptake in insulin target cells via GLUT4 [8, 9]. Several studies have shown that

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the PPAR- $\gamma$  agonist can inhibit macrophage activation and inflammatory secretion such as nuclear factor-KB (NFKB) [10-12]. But its utility is limited due to undesired side-effects of PPAR- $\gamma$  agonist. It has been recently implicated that NFKB may serve as an important agent in adipose tissue for developing insulin resistance and diabetes [12]. Prominently, magnesium seems to play an essential role in regulating the PPAR-mediated signaling pathways as a key cofactor in the protein phosphorylation. Thus, controlling magnesium level in the body is essential for defense against some diseases [13].

In our previous studies [14-16] we introduced magnesium as a new anti-diabetic agent that develops insulin sensitivity and reduces plasma glucose and blood pressure in diabetic animal model and type II diabetic patients. Our previous findings indicated that  $Mg^{2+}$  deficiency occurs after diabetes induction and oral magnesium sulfate ( $MgSO_4$ ) administration reduces blood sugar through increased GLUT4 gene expression, not insulin secretion [17]. Because the mechanism of this action is not clear, the present study was designed to investigate the possible effect of  $Mg^{2+}$  on activation of PPAR- $\gamma$  receptor and inhibition of NFKB (p65) in skeletal muscle for increasing GLUT4 gene expression and translocation.

### Materials and methods

#### Animals

Animals were treated in accordance with standard guidelines [18]. The experimental protocol was approved by the Ethical Committee for Animal Care of Hormozgan University of Medical Science (Ethic number HUMS.REC.1395.88). Male Wistar rats, weighing 180-250 g were selected and maintained at a constant temperature of  $22\pm 2^\circ C$  with a fixed 12:12-h light-dark cycle. Nutritionally balanced pellets and water were freely available. The animals were divided into five groups (10 rats in each group) that include 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 with a single I.P injection of 60 mg/kg STZ and 10 days after diabetes

induction, fasting blood glucose level was measured and the presence of diabetes was confirmed by blood glucose levels of above 250 mg/dl. Animals with diabetes lasted for 16 weeks were defined as 'chronic diabetic'. At 10 days after diabetes induction  $Mg^{2+}$ -CD and  $Mg^{2+}$ -NDC groups received 10 g/l of  $MgSO_4$  added to drinking water [14, 15, 17], for 16 weeks duration and water consumption was measured.  $Mg^{2+}$ -treated chronic diabetic and  $Mg^{2+}$ -treated non-diabetic control groups have significant lower water consumption compared to chronic diabetic group ( $43\pm 3$ ,  $41\pm 2$  and  $201\pm 2.1$  ml/24 h for  $Mg^{2+}$ -treated chronic diabetic,  $Mg^{2+}$ -treated non-diabetic control and chronic diabetic respectively). So the mean of exact dose of daily consumption of  $MgSO_4$  was 0.43 g/24 h.

The Ins-CD group received 2.5 U/kg of insulin (1/3 regular and 2/3 NPH) to keep the level of blood glucose in Ins-CD group around  $134\pm 4$  mg/dl [17], 10 days after I.P induced diabetes for 16 weeks. Animals were monitored for blood glucose concentrations and body weight every week at 9 AM over the experiment. Blood glucose was measured with an Ascensia ELITE XL glucometer and Ascensia Elite blood glucose test strips while body weight of all rats recorded using a digital weighing scale.

#### Intraperitoneal glucose tolerance test (IPGTT)

For the purpose of conducting IPGTT, animals in all groups were fasted overnight for 15 h, and were given 1.5 g/kg glucose via I.P injection. Blood sample was taken from the tail vein at 0, 10, 20, 30, 60, 90 and 120 min after glucose administration [14, 19].

#### Plasma magnesium measurement assay

Animals were anesthetized with ketamine HCl (50 mg/kg, I.P), before decapitating. Blood samples were taken from the neck vascular trunk in order to determine the plasma levels of magnesium by a Micro plate reader (Biotek, USA) and appropriate kits (Pars Azmun, Iran).

#### PPAR- $\gamma$ and NFKB (p65) genes and proteins expression assay

Muscle was isolated under anesthesia for both PPAR- $\gamma$  and NFKB (p65) genes and proteins expression via Real time PCR and immunohistochemistry methods, respectively.

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**Table 1.** List of primer sequence used in Real Time-PCR

Gene	Primer (5'→3')	Amplicon Size (bp)	TM
β-actin	F: CAC ACC CGC CAC CAG TTCG R: ACC CAT TCC CAC CAT CAC ACC	165	60/5
PPAR-γ	F: CTG TTC GCC AAG GTG CTC CA R: GCT CAT ATC TGT CTC CGT CTT CTT	102	68/4
NFKB (p65)	F: TTA CGG GAG ATG TGA AGA TG R: ATG ATG GCT AAG TGT AGG AC	94	59/9

### 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 at 37°C for 30 min and then 1 μl EDTA was added. Then it was incubated at 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 were visualized under a transilluminator. The yield was quantified spectro-photometrically at 260 and 280 nm by Nano Drop 1000 (Thermo, USA). The extracted RNA had a ratio of 1.8 to 2.

### cDNA synthesis and quantitative real-time PCR

The total RNA (2 μg) was reversely transcribed to cDNA using PrimeScript™ RT Reagent Kit for RT-PCR (Cat. No.RR037A, 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 Syber Green (Takara, Japan) according to the manufacturer's instructions. All reactions were carried out in a terminal volume of 20 μL containing 1 μL of cDNA sample, 1 μL of the primers, 10 μL Syber Green, 0/4 μL ROX and 7/6 μL of sterile double-distilled water for all β-actin, PPAR-γ or NFKB (p65). The thermal cycling status for β-actin was an initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for each primer for 15 s. For PPAR-γ the thermal cycling status was an initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 s and each primer was annealed for 30 s at 65°C. Also for NFKB (p65) the thermal cycling status was an initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation at

95°C for 5 s and the 15 s annealing at 58°C for each primer. All the Standards and the samples were analyzed in duplicates. Melting curve analysis was used to characterize the specifications of each PCR product.

Expression levels of PPAR-γ and NFKB (p65) 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 for real-time PCR was used (Table 1).

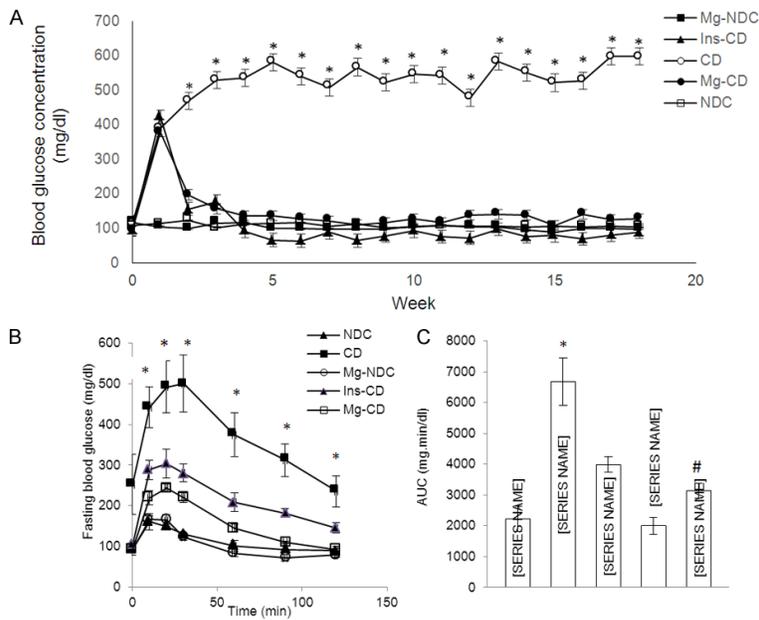
### Immunohistochemistry

Five-micrometer sections of formalin-fixed paraffin-embedded tissue were cut and prepared for IHC staining. Before immunostaining the specimens were processed through xylene and a graded alcohol series and treated with 3% hydrogen peroxide to eliminate the endogenous peroxidase activity and blocked with 5% normal goat serum. These slides were then treated with mouse anti-rat PPAR-γ or NFKB Ab at 10 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA). After an overnight incubation, horse anti-mouse IgG-biotin secondary antibody (Vector Labs, Burlingame, CA) 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.

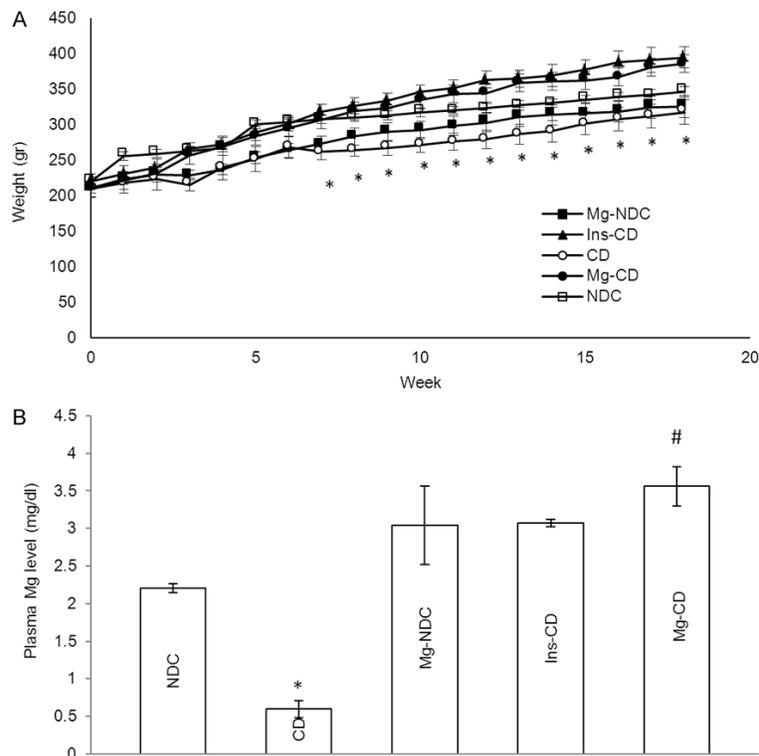
### Statistical analysis

Data was expressed as mean ± S.E.M and between group differences were evaluated by one-way and two-way ANOVA with the Tukey post-hoc test. The relative changes of gene expression were calculated by  $2^{-\Delta\Delta Ct}$  formula, where  $\Delta Ct = C_t (PPAR-\gamma \text{ or } NFKB) - C_t (\beta\text{-actin})$  and  $C_t$  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, Microsoft Excel and Prism-5 software and in all instances  $P < 0.05$

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**Figure 1.** Comparison of fed blood glucose (A), intraperitoneal glucose tolerance test (IPGTT) (B) and the area under the glycemic 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 ( $Mg^{2+}$ -CD) groups (10 rats in each group, data are expressed as mean  $\pm$  SEM). \*Significant difference between CD and other groups ( $P < 0.00001$ ). #Significant difference between  $Mg^{2+}$ -CD and other groups ( $P < 0.0001$ ).



**Figure 2.** Comparison of body weight (A), plasma magnesium levels (B) in non-diabetic control (NDC), chronic diabetic (CD),  $Mg^{2+}$ -treated non dia-

betic 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  $\pm$  SEM). \*Significant different between CD and other groups ( $P < 0.001$ ). #Significant difference between  $Mg^{2+}$ -CD and Ins-CD ( $P < 0.001$ ).

was considered as an acceptable statistical significance.

## Results

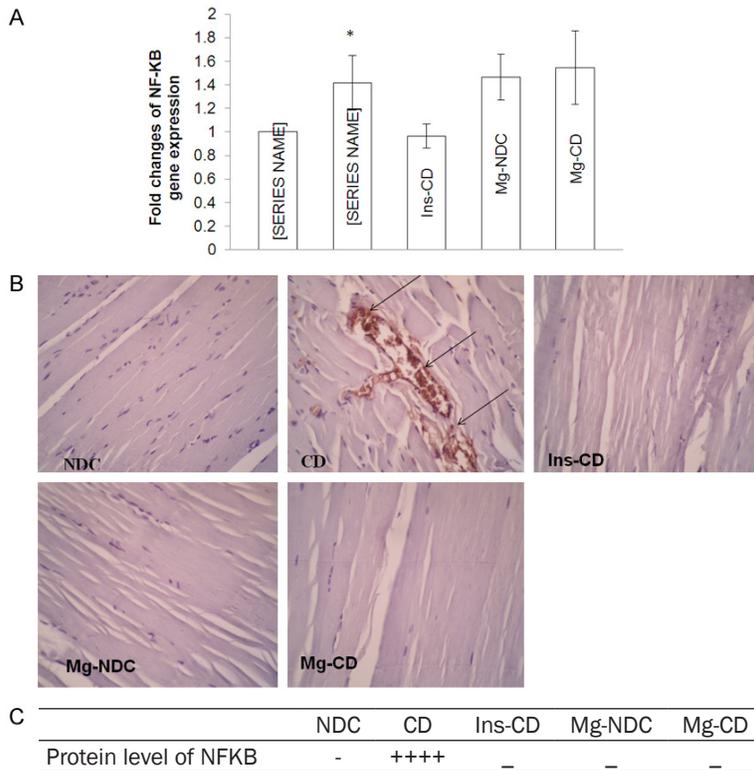
### Changes in plasma glucose

Changes in feeding plasma glucose were measured in all groups (Figure 1A). Diabetes induction caused an increase in plasma glucose concentration ( $389.16 \pm 24.43$  mg/dl) in one week and blood glucose continued to be elevated ( $597 \pm 1/91$  mg/dl) 16 weeks after diabetes induction. Administration of  $MgSO_4$  or insulin for 16 weeks (from day 10) led to a decrease of plasma glucose concentrations in  $Mg^{2+}$ -CD and Ins-CD groups ( $127/5 \pm 1.25$  mg/dl,  $90 \pm 16.74$  mg/dl), respectively.

### Effects of $MgSO_4$ on intraperitoneal glucose tolerance test (IPGTT)

Before administration of  $MgSO_4$  and STZ, the IPGTT patterns were similar for all groups and there was no significant difference between the groups when the glycemic response was expressed as the area under the curve (AUC) (data not shown). However, after 16 weeks, the CD group displayed severe glucose intolerance (Figure 1B), which was significantly improved in  $Mg^{2+}$ -CD group (Figure 1C, AUC: NDC vs CD vs  $Mg^{2+}$ -CD vs Ins-CD =  $2228.33 \pm 408.4$  vs

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**Figure 3.** Comparison of the fold changes of NFKB gene expression (A), immunohistochemistry for NFKB (B and C) and protein level of NFKB in chronic diabetic (CD), Mg<sup>2+</sup>-treated non diabetic control (Mg<sup>2+</sup>-NDC), insulin-treated chronic diabetic (Ins-CD) and Mg<sup>2+</sup>-treated chronic diabetic (Mg<sup>2+</sup>-CD) groups (10 rats in each group, data are expressed as mean  $\pm$  SEM). \*Significant different between CD and Mg<sup>2+</sup>-CD or Ins-CD rats ( $P < 0.01$ ).

6674.8 $\pm$ 765.1 vs 3137.83 $\pm$ 235.4 vs 3990 $\pm$ 246.3  $P < 0.0001$ ).

### Changes in the body weight and plasma magnesium levels

The body weight of CD animals was decreased insignificantly in comparison with NDC animals; increased body weight was significantly observed after administration of Mg<sup>2+</sup> and insulin compared to CD animals (**Figure 2A**).

To find the concentration as well as the duration of oral-administered Mg<sup>2+</sup> in plasma, changes in plasma magnesium (Mg<sup>2+</sup>) level were measured in all groups. The plasma magnesium level in CD (0.59 $\pm$ 0.11) significantly ( $P < 0.001$ ) decreased compared to the NDC group (2.2 $\pm$ 0.06). Administration of Mg<sup>2+</sup> and insulin for 16 weeks (from day 10) caused an increase plasma magnesium concentrations in Mg<sup>2+</sup>-CD and Ins-CD groups (Mg<sup>2+</sup>-CD = 3.56 $\pm$ 0.26, Ins-CD = 3.07 $\pm$ 0.04) (**Figure 2B**).

### Changes in PPAR- $\gamma$ and NFKB (p65) gene expression

The mean level of Ct obtained from Real-time PCR in five groups of examined rats as well as the effect of MgSO<sub>4</sub> and insulin in NFKB (p65) and PPAR- $\gamma$  expression have been shown in **Figures 3A, 4A**, respectively. In magnesium treated diabetic rats, the PPAR- $\gamma$  gene expression exhibited almost 1.5 fold increase ( $P < 0.0001$ ) in comparison with chronic diabetic rats (CD). But MgSO<sub>4</sub> administration in diabetic rats could not reduce NFKB (p65) gene expression in comparison to CD group.

Although Insulin could not significantly up-regulate PPAR- $\gamma$ , reduction in NFKB (p65) gene expression was observed in Ins-CD group in comparison with chronic diabetic (CD) animals.

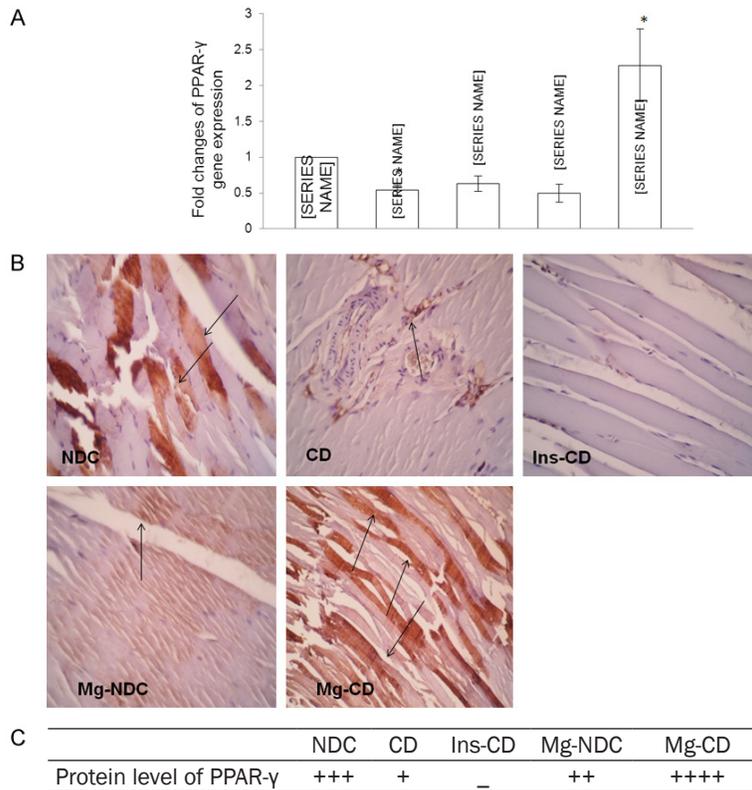
### Changes in PPAR- $\gamma$ and NFKB (p65) protein level

Immunohistochemistry was done for all groups to observe NFKB (p65) and PPAR- $\gamma$  protein levels and results were shown in **Figures 3B, 3C** and **4B, 4C** respectively. According to our findings, NFKB (p65) protein level increased in CD group, however this result was not observed in the other groups. Interestingly, although 16 weeks of Mg<sup>2+</sup> therapy in Mg<sup>2+</sup>-CD and Mg<sup>2+</sup>-NDC groups could not suppress NFKB mRNA gene expression, it seems that Mg<sup>2+</sup> inhibited NFKB (p65) protein production in those groups. PPAR- $\gamma$  protein level increased by Mg<sup>2+</sup> therapy in diabetic rats, but this effect was not observed through insulin administration.

### Discussion

It has been previously shown [14-17, 20] that eight weeks use of oral magnesium in STZ-induced diabetic rats could decrease plasma glucose level and it can be mediated by increasing GLUT4 gene expression in skeletal muscle independent of insulin secretion. This study

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**Figure 4.** Comparison of the fold changes of PPAR- $\gamma$  gene expression (A), immunohistochemistry for PPAR- $\gamma$  (B and C) and protein level of PPAR- $\gamma$  in non-diabetic control (NDC), chronic diabetic (CD), Mg<sup>2+</sup>-treated non diabetic control (Mg<sup>2+</sup>-NDC), insulin-treated chronic diabetic (Ins-CD) and Mg<sup>2+</sup>-treated chronic diabetic (Mg<sup>2+</sup>-CD) groups (10 rats in each group). \*Significant different between Mg<sup>2+</sup>-CD and other groups (P<0.0001).

has tried to examine the possible role of *PPAR- $\gamma$*  and *NFKB (p65)* gene expression in increased *GLUT4* gene expression and improve glucose hemostasis following oral magnesium sulfate administration in skeletal muscle of STZ-induced diabetic rats.

The results of our study showed that magnesium therapy not only reduce blood glucose level, but also improves insulin resistance, although long term use of insulin reduces blood glucose concentration, but not able to improve insulin resistance. The previous study has shown that magnesium is more effective than insulin in *GLUT4* translocation to the cell membrane [17].

*GLUT4* is the major glucose transporter isoform in tissues that exhibits insulin-stimulated glucose uptake, such as adipose and skeletal muscle tissues. Eliciting the translocation of an intracellular pool of transporters to the plasma

membrane, insulin stimulates *GLUT4* expression primarily in adipose and muscle tissues [21]. It is described that fasting, high-fat feeding, obesity and diabetes initiate lowering the *GLUT4* mRNA concentration in adipose and skeletal muscle tissue [22, 23]. On the other hand, some researchers presented that PPAR- $\gamma$  ligands play a role in metabolism of lipid, lipoprotein and glucose homeostasis [24] and decrease of insulin resistance [25]; this effect of PPAR- $\gamma$  deletion was mediated by both *GLUT4* and *GLUT1* [6]. But undesired side-effects of PPAR- $\gamma$  agonist have limited its utility.

Another study demonstrated that Mg<sup>2+</sup> deficiency impairs PPAR- $\gamma$  activity [26] and our findings in the present study showed post diabetic decrease of Mg<sup>2+</sup> plasma level and PPAR- $\gamma$  gene expression.

Interestingly, treatment with MgSO<sub>4</sub> for 16 weeks in diabetic rats increased the level of PPAR- $\gamma$  mRNA and its protein. According to our previous results [17], MgSO<sub>4</sub> administration in STZ-induced diabetic rats increased the expression of *GLUT4* mRNA about 23 percent. This's why the results of this study showed that magnesium sulphate improved IPGTT and lowered blood glucose levels close to the normal range. The findings of the present research demonstrated that Mg<sup>2+</sup> may induce *GLUT4* gene expression via increasing PPAR- $\gamma$  gene expression. To the best of our knowledge, no study has been done yet on the effect of magnesium on PPAR- $\gamma$  gene expression. But our results showed that insulin administration in diabetic rats could not change both PPAR- $\gamma$  gene expression and its protein level in skeletal muscle. However, studies showed that PPAR- $\gamma$  expressed in muscle and adipose tissues and its level increases by insulin in adipose tissue [27] and it has been shown that PPAR- $\gamma$  activation affects the insulin signaling cascade, through direct effects on the expression or

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phosphorylation of specific signaling molecules such as GLUT4 [9]. Although in another report no effect could be seen on *GLUT4* expression in adipocytes of normal and diabetic animals by PPAR- $\gamma$  ligands [28, 29]. Our previous findings [17] showed that insulin increases the expression of *GLUT4* gene by only 10% in insulin treated diabetic group. Although insulin could increase *GLUT4* gene expression in skeletal muscle, according to our results in the present study it seems that PPAR- $\gamma$  does not play a role in this achievement and it may involves other mechanisms.

It has been suggested that activation of nuclear factor- $\kappa$ B (NFKB) transcription may participate in diabetes and its complications [30]. A recent study demonstrated that hyperglycemia increases *NFKB* gene expression [31] and this pro-inflammatory agent can cause insulin resistance in adipose tissue [32]. Our findings in this study showed for the first time that insulin therapy could decrease *NFKB* gene expression and its protein in skeletal muscle. Although this effect was observed by  $Mg^{2+}$ , administration of  $MgSO_4$  for 16 weeks resulted in reduced *NFKB* (*p65*) protein production. The previous documents revealed that NFKB promote insulin resistance and insulin administration in adipose and endothelial cells suppresses its expression and activity [33].  $Mg^{2+}$  deficiency has been reported to be involved in *NFKB* up regulation [34]. NFKB (*p65*) protein was suppressed successfully by  $Mg^{2+}$  therapy, although its fortification of drinking water was not successful in inhibiting *NFKB* (*p65*) gene expression in diabetic animals. So it seems that 16 weeks administration of  $Mg^{2+}$  increased GLUT4 expression and decrease blood glucose level in diabetic rats by increasing PPAR- $\gamma$  gene expression and decreasing NFKB (*p65*) protein.

## Conclusions

PPAR- $\gamma$  has emerged as a key regulation of glucose hemostasis and increased insulin sensitivity. Although the expression of *NFKB* (*p65*) gene was not changed by  $MgSO_4$  therapy, reduction of *NFKB* (*p65*) protein and PPAR- $\gamma$  enhancement in muscle cells by  $Mg^{2+}$  could regulate plasma glucose level. Regarding the results of this study we may be able to consider  $Mg^{2+}$  as a PPAR- $\gamma$  ligand in future as an agent to decrease insulin resistance.

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## Disclosure of conflict of interest

None.

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