The Effects of L-Arginine in Patients with Type 2 Diabetes by Experimental Methods and Molecular Dynamics Simulations

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ABSTRACT

One of the most important global health problems is diabetes mellitus (DM), which is a glucose metabolism disorder and causes various dangerous symptoms. Two types, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the dominant forms of DM. According to estimations, in 2013, 382 million people had DM, of which about 90% were T2DM cases. In the present study, primarily, interventional, and experimental studies on 45 patients with T2DM were occupied and the effects of limited and controlled diet, metformin, and insulin in the presence or absence of L-Arginine were assessed on some blood biochemical parameters. Experimental data showed that the combination of metformin/L-Arginine or insulin/L-Arginine treatments can decrease fasting blood glucose. 2-hour glucose of patients' serums was reduced in all three treated groups. L-Arginine in combination with insulin decreases insulin resistance and increases insulin sensitivity in patients with T2DM. Glut 4, which is not seen in the lymphocytes of T1DM patients or healthy individuals, was found in the patient's peripheral lymphocytes. L-Arginine supplementation in all 3 groups led to a decrease in the level of mRNA of GLUT 4, and also it's trafficking from cytosol to plasma membrane. Furthermore, the structural change of nitric oxide synthase (NOS) in the absence and presence of metformin was investigated by molecular dynamics (MD) simulation. MD data revealed that in the presence of metformin, L-Arginine is run away from the NOS active site. So, the conversion of L-Arginine to NO can be decreased. On the other side, overproduction of NO by NOS leads to pathological conditions in stroke and shock, and the medication by metformin could be assigned worthwhile.

Key Words: *type 2 diabetes mellitus, metformin, L-Arginine, molecular dynamics, nitric oxide synthase.*

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INTRODUCTION

The most wide-spread metabolic disorder in humans is diabetes mellitus (DM), which is characterized by dysregulation of insulin and blood sugar [1-3]. It has been estimated that by 2030, DM will affect 300-400 million people worldwide with a global prevalence of about 9% [4, 5]. DM is also known as silent death, and it is not treatable up to now. This metabolic disorder has been classified into 2 types; T1DM or insulin-dependent diabetes mellitus, which is associated with the destruction of pancreatic β-cells and is identified with inadequate insulin secretion, and T2DM, which is characterized by the resistance to the secreted insulin. T2DM is responsible for 90% of the total prevalence of diabetes [6]. The appearance of T2DM is increased with aging and is associated with long-term

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malfunction and failure of different organs, including blood vessels, heart, nerves, kidneys, and eyes [7]. It has been estimated that the treatment or control of T2DM consumes 5-10% of the budget assigned to the health system [8]. By the change of patient's lifestyle [9] or consuming therapeutic agents [10], the development of T2DM can be prevented or at least delayed. It seems that the most convenient and side-effect-free strategy to control T2DM is changing the lifestyle including the relevant diets and exercises. Routinely, risk factors of T2DM are classified into two groups: the first factors are modifiable risk factors and comprise of smoking, sedentary behavior, physical inactivity, a negative intrauterine environment, inflammation, vitamin D deficiency, hypertension, and overweight/obesity [11, 12]. The second factors, non-modifiable risk factors, include ethnicity, age, gender, family history of T2D, gestational diabetes, and polycystic ovary syndrome [13-15]. If the blood sugar levels are not adequately decreased by the corrected lifestyle, medications and prescription of drugs will be the obligatory items. Due to the undesirable side effects and limited efficacy of current medications, unfortunately, anti-diabetic drugs and pharmaceutical treatments are far from being satisfactory in the control of T2DM. With regard to this fact, T2DM still is an untreatable disease with high mortality rate and high poor life quality [16]. So, the developments of new potent and effective drugs and treatments are highly required. It is also important that the new treatments have the lowest side effects and toxicity. Metformin is a well-known drug and is consumed by millions worldwide to cure various conditions, including T2DM, Pre-diabetes, gestational diabetes mellitus (GDM), polycystic ovarian syndrome, obesity, cancer, etc. at physiological pH, metformin exists in a cationic form and therefore, the absorption, distribution, and excretion of Metformin rely on the transporters such as organic cation transporters, plasma membrane monoamine transporter, and toxin and multidrug extruders [17]. Due to the excretion of non-metabolized and intact metformin to the urine by the renal system, it is not expected to be involved in many drug-drug interactions [18]. It has been thought that metformin inhibits the hepatic gluconeogenesis and hence, causes lactic acidosis [19]. Furthermore, metformin affects the tyrosine kinase activity of insulin plasma membrane receptors and decreases the resistance of cells to the insulin [20]. Glucose, as a major source of energy in the body of mammalian cells, is passed on across the plasma membrane by facilitated diffusion mediated by glucose transporters or GLUT. Up to now, 14isoforms of GLUT have been recognized in human cells including HMIT, GLUT1, GLUT12, and GLUT14 [21]. These receptors are unique in the term of substrate specificities, kinetic properties, and tissue expression profiles. GLUT4 contains unique sequences in its C- and N-terminals that direct its characteristic membrane trafficking capability [22]. This transporter is translocated from cytosol to the plasma membrane in reaction to insulin and other stimuli [23]. Studies have shown that GLUT4 is not expressed in the lymphocytes of healthy people. L-Arginine, a semi-essential amino acid, is a substrate for the NOS, which is responsible for the production of nitric oxide (NO) [24]. This amino acid also used as a substrate for some enzymes, including arginase, Arginine:glycine aminotransferase, and Arginine decarboxylase, yielding citrulline, ornithine and urea, creatine, and agmatine, respectively [25]. Despite the importance of the L-Arginine homeostasis in mammals, few studies have assessed the potential effect of its combination with other anti-diabetic drugs on human factors with T2DM. Moreover, the mechanism of such effects is completely unknown. Therefore, this study was planned and performed into 2 phases. In the first phase, the effects of combinations of L-Arginine:metformin and L-Arginine:insulin were experimentally evaluated on patients with T2DM. In the second phase, for the first time, by employing the computational biology and molecular modeling methods, the interaction of L-Arginine with NOS in the absence or presence of metformin was examined. Furthermore, the conformational changes of NOS were evaluated and analyzed by MD studies.

MATERIALS AND METHODS

Study design and human groups

Interventional and experimental studies were performed on 50-60 year adult diabetic patients. 45 patients, 22 males and 23 females were included in this study. The study was conducted under the supervision of an endocrine and metabolism physician in Shahid Rajaie Hospital of Qazvin during the period from February 2017 to March 2017 for two months. All experimental stages of this study were done based on ethical considerations and patient' consent letter. According to the type of treatment, patients were randomly divided into 3 groups (15 patients/group):

Group I: patients who were treated with diet containing fibers and proteins without carbohydrates or lipids.

Group II: patients who were treated with metformin 500 mg daily.

Group III: patients who were treated with 30U NPH insulin daily. After the first measurements, L-Arginine (in the morning and at night, a dose of three grams) was added to the three groups for two months. Then, the measurements were repeated again. The results after and before the treatments with L-Arginine were analyzed and compared with each other. All patients were interviewed and detailed history was obtained.

Biochemical methods

- **Quantitative biochemical parameters of serum**

Phosphorous, calcium, and glucose were measured by PARS AZMOON kits and auto analyzer SELECTRA E. The levels of vitamin D3 in the serum were calculated by Pishtazteb kit and stat fax 4200 microplate reader. The level of insulin, interleukin-8 (IL-8) and interleukin-10 (IL-10) in the serum were calculated with Quantikine ELISA Kit (R&D Systems, USA). The levels of NO in the patient serum and urine were measured by Cib Biotech Co kit. HbA1c was calculated by a colorimetric Pishtazteb kit. All parameters were measured according to the manufacturer's instruction of the relevant kits.

- **Lymphocyte isolation**

5 ml of heparin anti-coagulated blood samples were obtained from 45 adult patient donors. 5 mL normal saline was added to the blood sample and smoothly shaken. Then, 8 mL diluted blood was poured in 3 mL ficoll solution followed by centrifugation at 3000 rpm for 20min. The layer containing lymphocytes was isolated and the equal volume of cold normal saline was added to it. After centrifugation at 2500 rpm for 5min, and discarding the supernatant, the cells were resuspended in 0.5 mL of cold normal saline. Finally, the isolated cells were kept in a cold room for further analysis.

- **Lymphocyte viability test**

The test was done to check the cell survival during the experiment. 1% trypan blue solution was added to the isolated suspension cells at a 1:1 volume ratio. The number of dead lymphocytes in a sample of 500cells was counted using the hemocytometer and a light microscope.

- **Investigation of the presence of GLUT4 on the lymphocyte surface with flow cytometry**

The isolated lymphocytes T (about 2.5×10^5) were washed in 2 mL of the washing buffer for FACS (PBS without $Ca^{2+}$and Mg^{2+}, with the addition of 0.002% sodium azide and 2% fetal bovine serum) by centrifugation (4 °C, 250 g) and suspended in 100mL of the buffer. The cells were incubated for 5min with 0.1 mL Perm 2 (Becton–Dickinson) to achieve permeabilization enabling antibodies to the intracellular domain of glucose transporters to enter the cytosol. Then, 2 µL of the monoclonal antibody, labeled with FITC (GLUT4 IF8: SC-53566 SANTA CRUZ BIOTECHNOLOGY, INC), was added to the cells and incubated. Then, the cells were washed in the buffer for FACS. After incubation, 2 mL of washing buffer for FACS were added into each sample, the resulting suspension was centrifuged (at 4 °C, for 250 g), the supernatant was discarded and 1% formaldehyde and 0.5 ml washing buffer for FACS were added. The analysis of the samples was done using the FACS Calibur flow cytometer (Becton–Dickinson) fitted with an argon laser (488 nm), using the CellQuest software [26].

- **Total RNA extraction**

Total RNA was extracted from isolated lymphocytes using RNA Extraction kit (IraiZol) (Zist Fanavaran, Iran) according to the manufacturer's instruction. A NanoDrop spectrophotometer (Thermo Scientific Co) was used to measure the purity and concentrations of RNA.

- **Complementary DNA (cDNA) synthesis**

4 µg of total RNA was mixed with 1 µL (1µg/µL) random primers, and 10 mM dNTP mix in the final volume of 15 µL. The mixture was incubated at 56 °C for 5 min and then placed on ice for 3 min. The reverse transcriptase master mix containing 3 µL of 5× RT buffer, and 0.5 µL of M-MLV RT (100 U/µL) was added to the mixture and was incubated at 50 °C for 50 min followed by 15 min at 72 °C.

- **Real-time quantitative PCR**

Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction contained SYBR Green qPCR Mix (Applied Biosystems), gene-specific primer pairs for GLUT 4, which are shown in Table 1 and were designed with Allele ID v. 6.0 from RNA sequences from the gene bank. The relevant primers for β-actin were taken from the literature [6]. Quantitative RT-PCR was conducted in a 25-µl reaction volume consisting of 2X RB SYBR Green qPCR Master Mix (Applied Biosystems), 10 pM of each primer, and 3µl of cDNA. Amplification conditions were: 10 min at 95°C, 15s at 95°C, 20s at 55°C, 20s at 65°C and 40 cycles of denaturation. Data from real-time assays were calculated using the v1·7 sequence detection software from PE Biosystems (Foster City, CA). Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalized to the beta-actin, which was used as the control housekeeping gene.

Table 1. Oligonucleotide primers' sequences of the studied genes. The primer of the β-actin gene was taken from literature [6].

Sequence (5´ to 3´)	Oligonucleotide Name
GLUT 4 Forward	GAC TAC TCA GGG CTG ACA TCA G
GLUT4 Reverse	TCA CCT GGG CGA TCA GAA TG

Beta Actin Reverse	TGCTGGTGCTGAGTATGTCG
Beta Actin Reverse	TTGAGAGCAATGCCAGCC

- **Calculation of insulin resistance and insulin sensitivity**

To estimate the degree of insulin resistance, the formula of homeostatic model assessment (HOMA-IR) was employed [27]:

HOMA-IR = fasting plasma insulin × fasting plasma glucose/22.5

To compute the degree of insulin sensitivity, the formula of quantitative insulin-sensitivity check index (QUICKI) was occupied [28]:

QUICKI = 1/ [log (fasting insulin, lU/mL) log (fasting glucose, mg/dL)]

Statistical analyses

Data analysis was performed using SPSS version 16 (SPSS Inc., Chicago, IL). P-values <.05 were considered statistically significant. The analysis was conducted using the chi-square test for the categorical variables.

Molecular modeling phase

- **MD simulation on NOS**

The 3D structure of zinc, heme, and L-Arginine-bound domain of human inducible NOS, with PDB code of 1NSI, was taken from the protein data bank [29] and employed for MD studies. Two independent MD runs were conducted for NOS: L-Arginine and NOS: L-Arginine: metformin complexes. In both systems, zinc, tetrahydrobiopterin, and heme were included. In the last complex, a total of six metformin molecules were assigned in the MD box. All MD simulations were accomplished implementing AMBER 99SB force field and GROMACS 5.1 package [30]. In order to allocate appropriate ionization states of NOS ionizing groups, the pKa of protein residues was calculated using PROPKA 2.0 server [31]. The partial charges and topology parameter files of L-Arginine, metformin, tetrahydrobiopterin, and heme were generated via ACPYPE, a tool established on ANTECHAMBER [32-34]. TIP3P water model in a cubic periodic box was applied to solve every single molecular system. To neutralize each system, one and seven chlorine ions were added to metformin-free and -bound NOS, respectively. Minimizing energy was done with the steepest descent integrator and the conjugate gradient algorithm consecutively to achieve a maximum force of <1000 kJ $mol^{-1}nm^{-1}$ on any atom. A twin-range cutoff scheme was assigned to evaluate electrostatic interactions truncated at 0.9nm, as well as non-bonded, short-range interactions with van der Waals interactions truncated at 1.4nm. Then, particle mesh Ewald (PME) method was used to treat the Long-range electrostatic interactions [35, 36]. The temperature was fixed at 300K (ref_t) using velocity rescaling with a stochastic term and coupling time constant (tau_t) of 0.1 ps [37]. This thermostat is comparable with Berendsen coupling, with the same scaling using tau_t, but the stochastic term ensures that an appropriate canonical ensemble is produced [30]. The pressure was set at 1.0atm using a Parrinello-Rahman barostat with a coupling constant of 2 ps [38]. Simulations were done with a time step of 2fs, and all bonds involving H atoms were constrained by a Linear Constraint Solver (LINCS) [39]. The highest order in the expansion of the constraint coupling matrix (lincs_order) and the number of iterations to correct the rotational lengthening in LINCS (lincs_iter) and were assigned 4 and 1, respectively. For "normal" MD simulations, an order of 4 is typically enough. Each of the systems were equilibrated under a constant pressure (NPT) ensemble (100 ps) and a constant volume (NVT) ensemble (100 ps). All MD simulations were performed for 30ns. The trajectories were analyzed using VMD software [40] and standard tools were implemented in the GROMACS package.

RESULTS AND DISCUSSION

Biochemical analysis

This study was conducted in two independent phases. In the 1st phase, 45 T2DM patients were selected for the interventional experiment. The effects of L-Arginine were evaluated on the biochemical parameters of three groups: patients with a controlled diet, patients with metformin treatments, and finally, patients with insulin treatments. In the second phase, MD simulations were employed to study the NOS, L-Arginine, and metformin, computationally. Our biochemical analysis showed that L-Arginine supplementation used did not change the plasma or urine levels of NO in groups I and II (Table 2). In group III, NO levels of urine and serum were increased. As seen in Table 2, L-Arginine supplementation used did not alter the fasting blood sugar (FBS) in group I. Conversely, FBS was reduced in groups II and III. L-Arginine supplementation employed reduced the glucose 2 hours in all 3 groups. Hyperglycemia speeds up the formation of advanced glycation end products (AGE), including glycated hemoglobin (HbA1c), albumin, and other plasma proteins and consequently changing their binding affinity for NO [41]. Thus, protein glycation can change NO binding affinity of hemoglobin and plasma proteins, hence,

reducing NO availability in groups I, II, and III. Therefore, the fact that we did not find increments in NO levels even with L-Arginine supplementation could be elucidated by the rapidly binding of NO to blood proteins, preventing the appearance of NO in the plasma of group I and II (Table 2). On the other hand, in groups I and II, hyperglycemia induced the oxidative inactivation of NO by free radicals [42, 43]. One possibility for the increment of NO in the serum and urine of patients in group III is that in the presence of exogenous insulin, the pigment epithelium-derived factor (PEDF) plasma level is increased, and subsequently, the NO production is increased. It has been shown that PEDF may stimulate the conversion of L-Arginine to NO via NOS [44]. This stimulation is related to the reduction of asymmetric dimethylarginine, an endogenous NOS inhibitor, by PEDF. The plasma level of PEDF is increased in individuals with the metabolic syndrome [45, 46] and patients with T2DM [47, 48]. Based on Table 2, the serum level of calcium was increased in groups II and III. It seems that in the combination of L-Arginine with metformin or insulin, the elevated level of calcium is seen. It has been pointed out that all amino acids, especially L-Arginine, accelerate the biosynthesis of growth hormone. The produced growth hormone mimics the vitamin D functions including a) increased absorption of calcium from the gut, b) increased urinary excretion of calcium, a lowering of plasma Ca^{+2}, and d) a rise in plasma calcium [49]. With regard to Table 2, in group II and III, the fasting blood sugar (FBS) was decreased significantly. However, L-Arginine supplementation was not able to diminish the FBS in patients treated with just a controlled diet. The same result was achieved by Jablecka et al. in 2012 [50]. They found that in diabetic individuals with atherosclerotic peripheral arterial disease, HbA1c and FBS did not change significantly after treatment with L-arginine. Also, our data proved that 2h-Glucose was reduced in all three groups. Our data suggest that L-Arginine has temporal and short effects on the reduction of serum glucose. L-Arginine supplementation led to a significant decrease in HOMA-IR in group III from 2.97±1.1 to 2.06±1.11 ($p < 0.05$; Table 2). The same result also was achieved for QUICKI in group III (increment in QUICKI). Therefore, the combination of insulin and L-arginine reduces insulin resistance and increases insulin sensitivity. It has been proposed that there is a relationship between the impairment in NO synthesis and insulin resistance [51, 52]. So, the reduction in HOMA-IR and increment in QUICKI in group III can be related to the elevated level of serum NO (Table 2). Several studies have demonstrated that T2DM is associated with higher circulating levels of TNF-α, C-reactive protein (CRP), and interleukin 8 (IL-8) [53-56]. This pro-inflammatory polypeptide, IL-8, is a member of CXC chemokine superfamily and is characterized by the presence of 2 cysteine residues separated by an intervening amino acid in the first 3 positions [55]. IL-8 is secreted by several cell types including adipocytes, monocytes/macrophages, T-lymphocytes, endothelial and epidermal cells in a tissue-specific manner [57, 58]. In addition, in vivo studies figured out that IL-8 causes insulin resistance due to the inhibition of insulin-induced Akt phosphorylation in human [59]. So, the reduction of IL-8 levels in the serum can be assigned as a good strategy to diminish insulin resistance. Figure 1 demonstrates the serum IL-8 level before and after L-Arginine supplementation in the three studied groups. In the group I, the serum IL-8 level is reduced from 56.2 (before L-Arginine supplementation) to 45.6 pg/ml (after L-Arginine supplementation). The same results were found for groups II and III (Figure 1). The most reduction in IL-8 level was found for L-Arginine/insulin combination treatment (from 69.1 to 43.3 pg/ml). As increased levels of IL-8 are associated with unfavorable systemic inflammatory pattern and worse glycemic profile [55], the induced reduction of IL-8 by L-Arginine supplementation can be assigned worthwhile in T2DM control and treatment. In contrast with IL-8, IL-10 is known as an anti-inflammatory cytokine, which shows potent suppressive effects in preventing autoimmune disease [60]. IL-10 is an important immunoregulatory cytokine that is produced by activated T cells, monocytes, B cells and thymocytes [61]. It has been shown that IL-10 genetic variants are associated with the risk of T2DM [62]. It is believed that diabetes is an immune-dependent disease in which the pattern of cytokine expression changes [63]. For instance, in T2DM, the peripheral blood monocytes produce inflammatory cytokines more than those in normal individuals [63]. Figure 2 exhibits the serum level of IL-10 in groups I, II, and III. In the group I, serum IL-10 level is increased from 2.4 to 3.15 pg/ml after L-Arginine supplementation. In groups II and III, the IL-10 levels increased from 1.9 and 1.8 to 2.9 and 2.5 pg/ml, respectively (Figure 2). As the other researchers showed that the serum levels of IL-10 decrease in T2DM patients compared to controls [64, 65], the L-Arginine supplementation can be used as a useful medication to increase the protectant cytokine like IL-10. The possible mechanism of the protective role of IL-10 is mediated by reducing caspase-3 activity [66] and finally, the β cells are protected from death. After the increase in the glucose level of plasma by exogenous glucose, its level is rapidly decreased to the normal level. The main mechanism of such reduction is related to insulin-stimulated glucose transporter (GLUT). One of the most important GLUT is named GLUT 4 and is a key regulator of whole-body glucose homeostasis and a critical mediator of glucose removal from the circulation. This transporter belongs to

the family of sugar transporter proteins containing 12 transmembrane domains [22]. Conversely, the insufficient expression of GLUT 4 in the skeletal muscle or adipose tissue of animal models leads to insulin resistance and an approximately equivalent prevalence of animals with diabetes [67, 68]. However, GLUT 4 has not been found and expressed in circulating lymphocytes of healthy people or patients with T1DM [21]. The glucose indirectly increases the level of GLUT 4 in lymphocytes; the elevated level of glucose in blood causes the release of insulin from the pancreatic β-cell. Insulin stimulates the trafficking of GLUT4 from the cytosol to the surface of plasma membrane. With regard to our results, combination treatment of patients with L-Arginine decreases the trafficking (Figure 3) and expression level of GLUT 4 (Figure 3) on the circulating lymphocytes. The reduction in the GLUT 4 level in blood cells is directly associates with healthy condition. The highest expression level of GLUT 4 was observed in group III (Figure 4). This phenomenon can be attributed to the higher concentration of NO in group III, as it has been shown that NO increases GLUT4 expression via a c-GMP and AMPK-dependent mechanism [69].

MD simulations

In this phase, for the first time, we studied the conformational changes and structural behavior of NOS in the absence and presence of metformin. Figure 5 shows the interaction mode of L-Arginine in the active site of NOS in the presence of zinc, heme, and pterin after 30 ns MD simulation. As seen in this figure, the side-chain carboxyl group of Glu 377 interacts with the guanidino group of L-Arginine via three H-bonds. The backbone of Trp 372 and the side chain of Gln 263 make hydrogen bonds with the guanidino group and carboxyl moiety of L-Arginine, respectively. Also, two hydrogen bonds were formed between the carboxyl moiety of heme and L-Arginine (Figure 5). Our MD data showed that the L-Arginine remained confined in the heme pocket (Figure 6). Also, our MD results exhibited the approaching of a loop containing Ser 118, Ile 119, Met 120, and Thr 121 into the L-Arginine (Figure 6). This loop has a critical role in the binding of NOS to its ligand [29] and Ser 118 form a gate for interring the L-Arginine. The MD simulation of L-Arginine bound NOS in the presence of zinc, heme, pterin ,and five molecules of metformin was done for 30 ns (Figure 7). Based on Figure 7A, one of the metformin molecules interacts with Lys 497, His 499, and Glu 285 via three hydrogen bonds. The second metformin makes a hydrogen bond with Asp 292 (Figure 7B). However, our MD data showed that upon binding the NOS to metformin, the L-Arginine is run away from the enzyme active site (Figure 8). This ligand escape is due to the wide conformational changes of NOS that is induced by metformin attachments (Figure 8).

CONCLUSION

In this study, we discussed the effect of L-Arginine and its synergism influence on some biochemical parameters in patients with T2DM by an interventional and experimental observation. Furthermore, we investigated the possibility of effects of metformin on NOS function by employing MD. In the experimental phase, our data showed that the combination of L-Arginine / metformin or L-Arginine/insulin treatments caused a decrease in fasting blood glucose. In all three groups, the 2hour-glucose was decreased significantly. In groups I and III, the percentage of HbA1C was reduced after L-Arginine supplementation. Furthermore, the serum and urine NO concentrations were diminished in just group III. The plasma proteins glycation can alter the affinities of these proteins to bind to NO in blood. However, it has been illustrated that in the presence of exogenous insulin, the PEDF plasma level is increased, and subsequently, the NO production is increased via stimulating the NOS activity. The insulin resistance was just decreased in patients who treated with the combination of insulin and L-Arginine. It has been suggested that there is a relationship between insulin resistance and the impairment in NO synthesis. Therefore, the decrease in HOMA-IR and increment in QUICKI in group III can be related to the elevated level of serum NO. In all three groups, the IL-8 and IL-10 concentrations were decreased and increased, respectively. The elevated IL-8 level is related to insulin resistance. Also, IL-10, as an anti-inflammatory cytokine, can be used to control the T2DM via the reducing of caspase-3 activity and protecting the β cells from death. The level of GLUT 4 expression and its trafficking have not been found in the lymphocytes of healthy people or patients with T1DM. However, our flow cytometry and real-time PCR data demonstrated that the patients with T2DM exhibited an increase in the expression of GLUT 4 and its emerging on the surface of the lymphocytes. The level of GLUT 4 mRNA and its translocation from the cytosol to the lymphocyte plasma membrane were decreased significantly in all three groups after L-Arginine treatment. In this study, for the first time, the conformational changes and structural behaviors of NOS containing zinc, pterin, and heme in the presence or absence of metformin were investigated by MD simulations. Our MD data showed that in the presence of metformin, L-Arginine is an escape from the enzyme active site. This effect can lead to a decrease in NO production by NOS. On the other hand, since the overproduction of NO by NOS brings pathological

conditions in stroke and shock, the medication by metformin could be useful in such conditions.

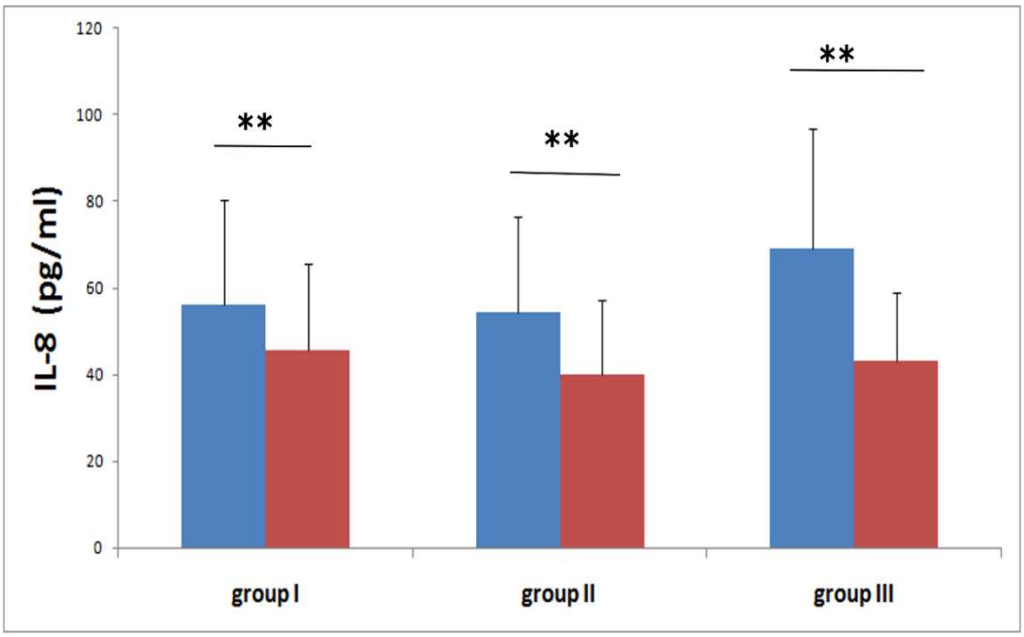

Figure 1. The serum level of IL-8 in patients with T2DM.

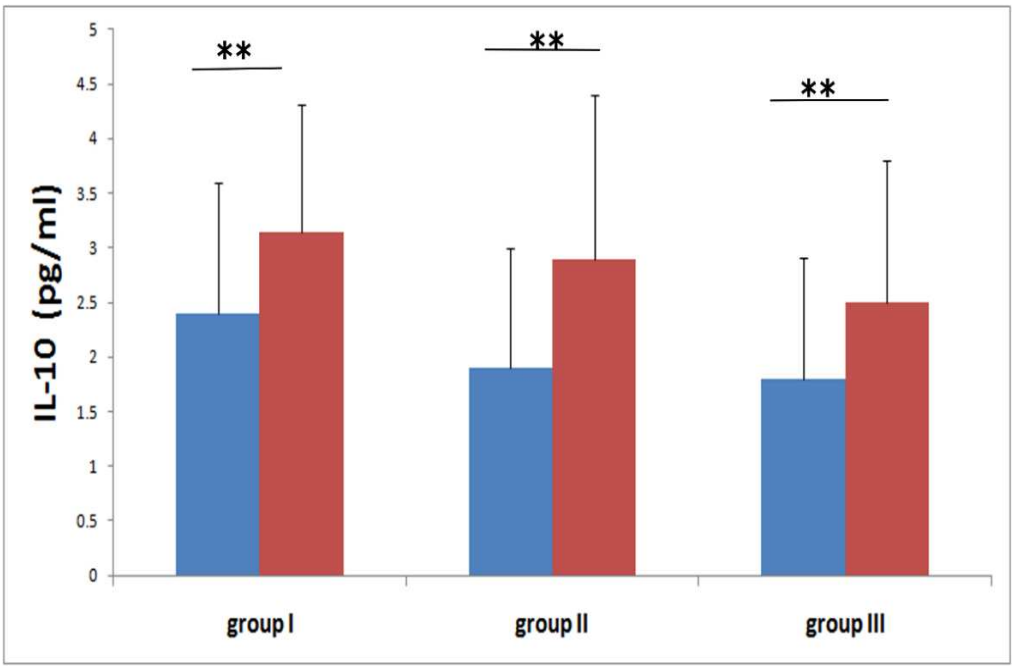

Figure 2. The serum level of IL-10 in patients with T2DM.

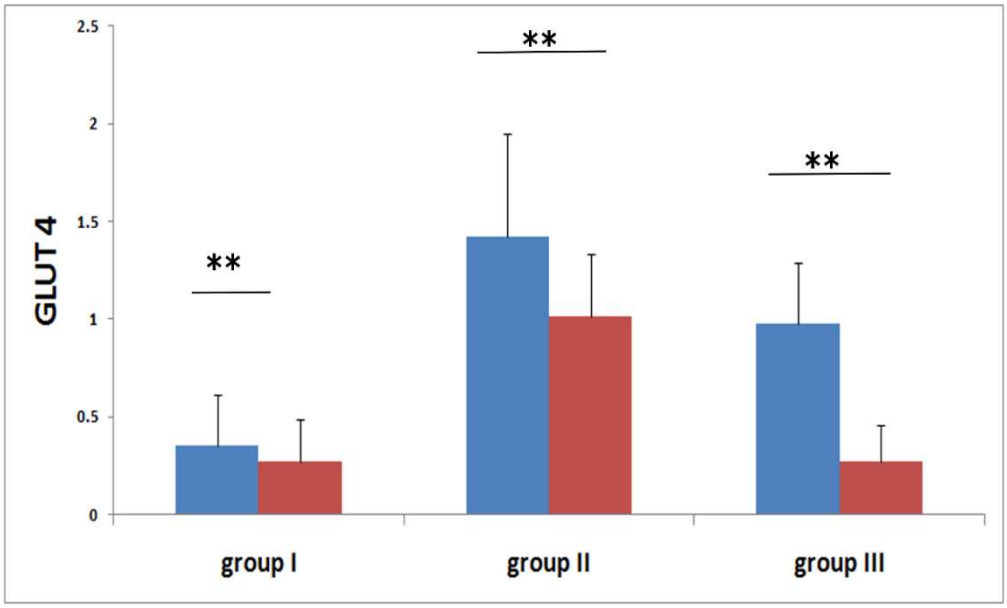

Figure 3. The content of GLUT 4 on the surface of peripheral lymphocytes in patients with T2DM.

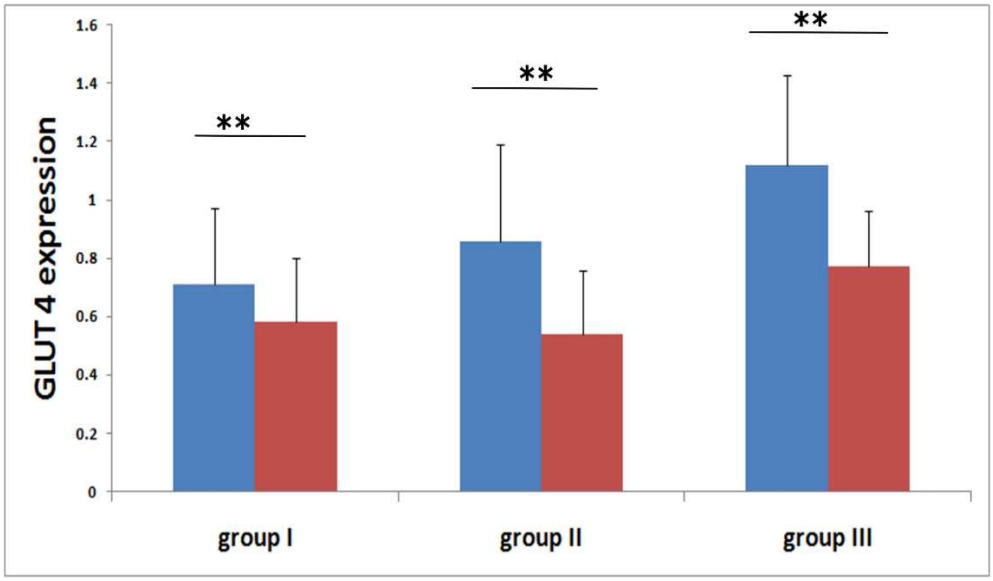

Figure 4. The expression level of GLUT 4 in the peripheral lymphocytes in patients with T2DM.

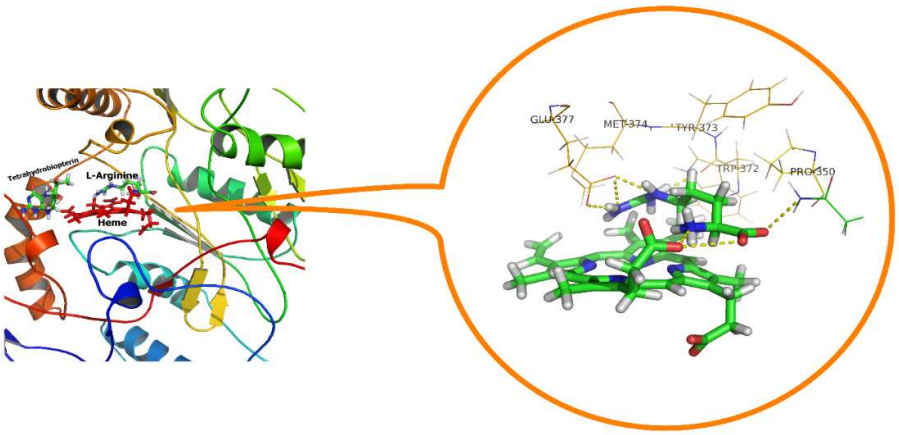

Figure 5. The interaction mode between L-Arginine and the active site of NOS in the absence of metformin after 30 ns MD simulation.

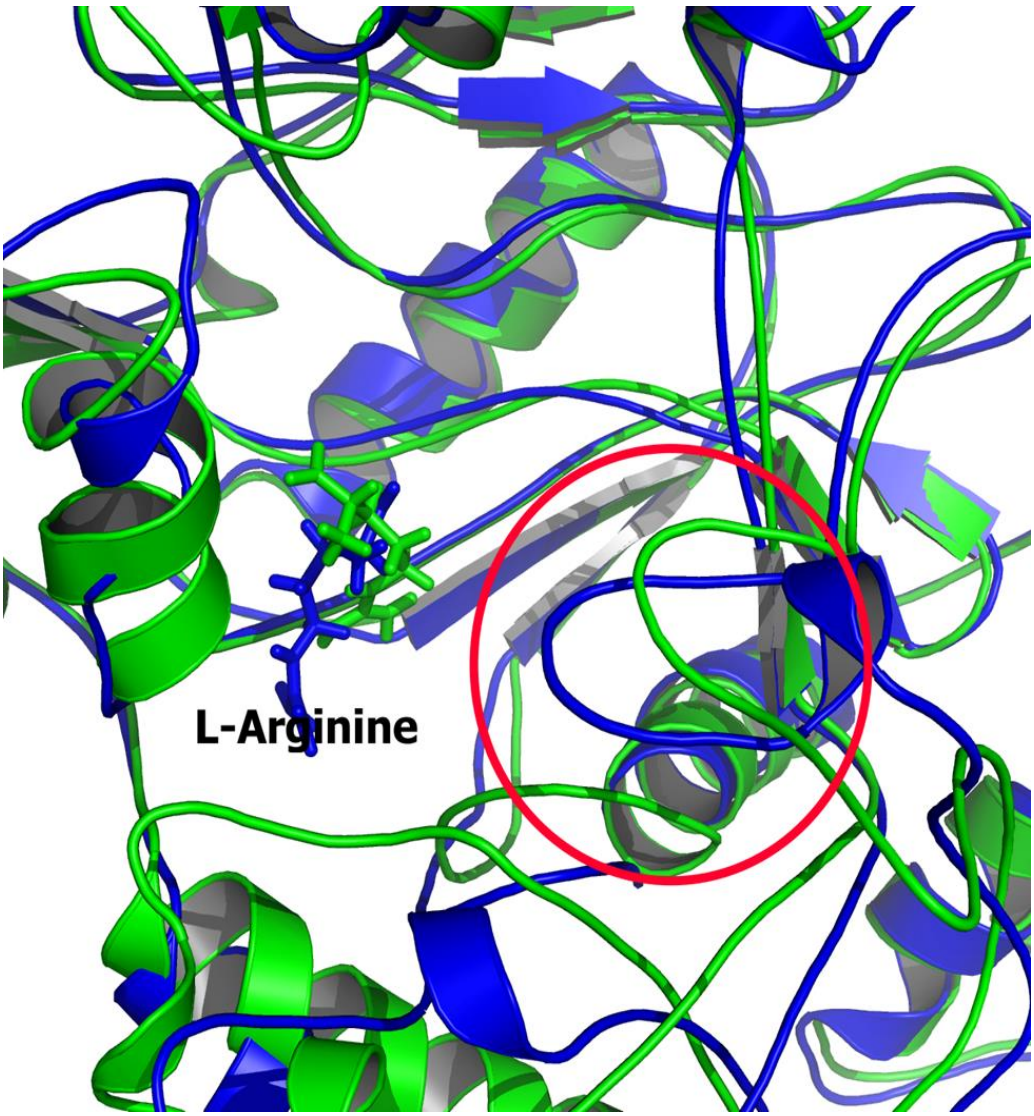

Figure 6. Superposition of L-Arginine and NOS in the crystal structure (green) and computational structure after 30 ns MD simulation (blue).

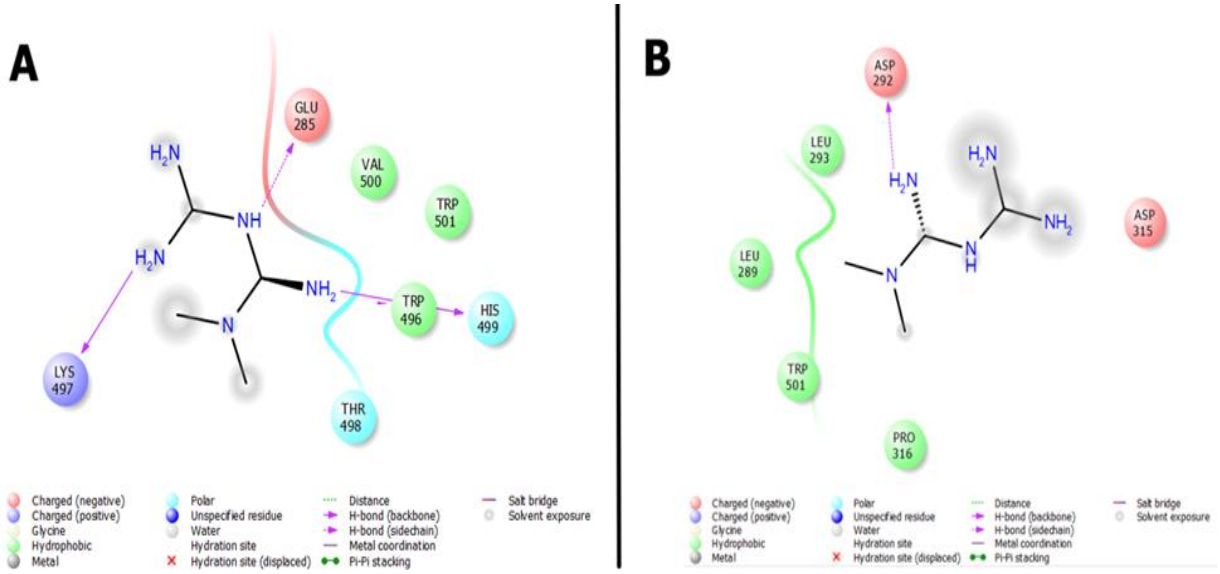

Figure 7. The interaction mode between two metformin molecules and NOS.

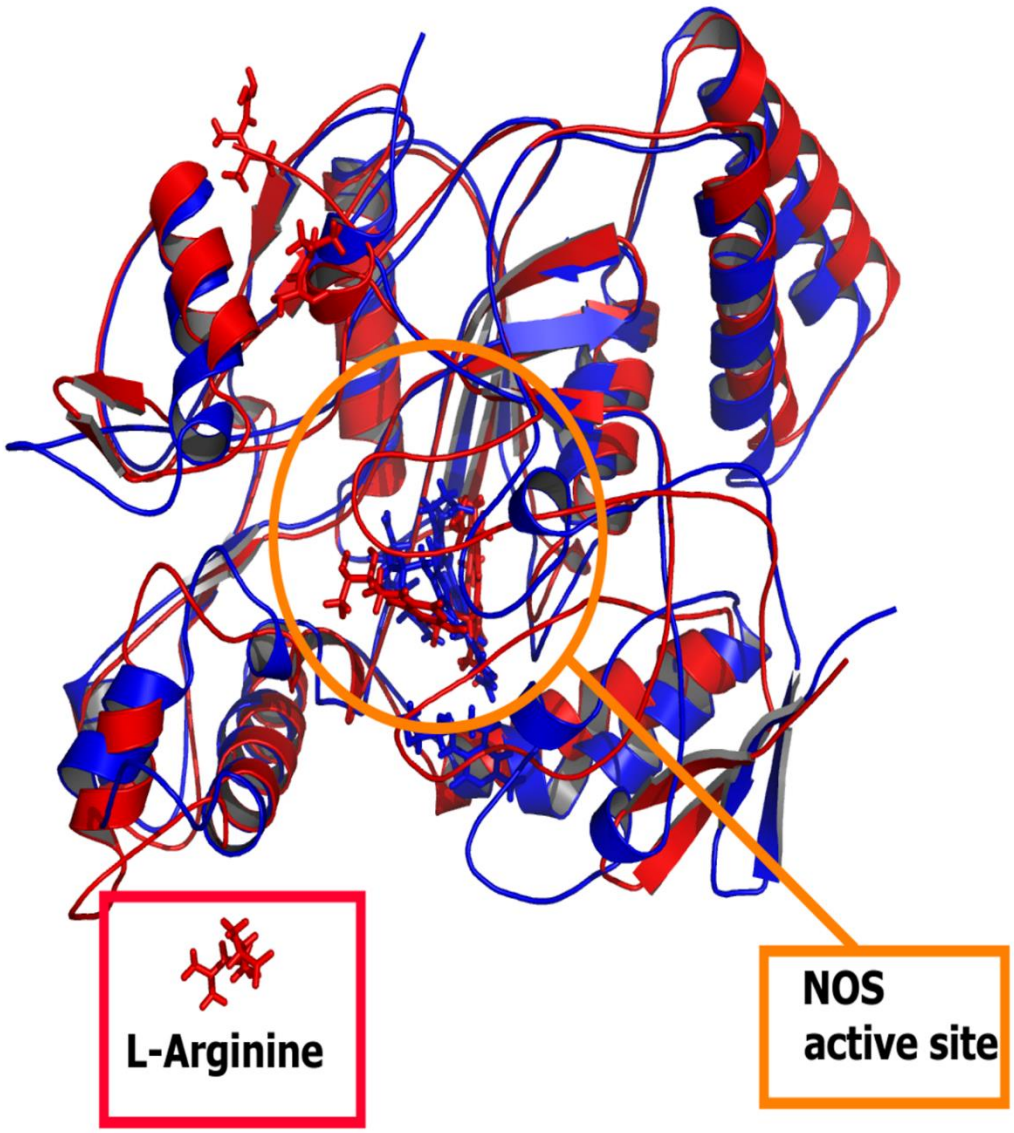

Figure 8. Superposition of L-Arginine and NOS in the presence (red) and absence (blue) of metformin. Orange and red rectangle exhibit the NOS active site and thrown out L-Arginine, respectively.

Table 2: Biochemical parameters' measurements among three studied groups before and after treatment with L-Arginine

	Group I		p-value	Group II		p-value	Group III		p-value
	before	after		before	after		before	after	
Weight (kg)	78.6±7.3	78.5±7.2	0.663	78.7±7.8	78.7±8.8	0.998	82.3±6.1	82.2±6.2	0.744
HbA1C %	7.04±0.28	6.7±0.29	**0.002**	7.2±0.29	7.04±0.29	**0.002**	7.7±0.43	7.5±0.42	**0.009**
Serum nitric oxide (µmol/L)	27.7±7.3	26.1±4.7	0.25	25.4±5.8	24.7±5.3	0.932	19.5±6.2	24.4±6.6	**0.012**
Urine nitric oxide (µmol/L)	1.21±0.28	1.26±0.29	0.272	1.31±0.31	1.18±3370	0.384	0.84±0.27	1.16±0.31	**0.001**
Calcium (mg/dL)	9.5±0.31	9.8±0.73	0.311	9.1±0.51	9.8±0.29	**0.02**	9.1±0.39	9.9±0.62	**0.002**
Phosphorus (mg/dL)	3.7±0.37	4.09±0.12	**0.015**	4.01±0.31	4.17±0.32	0.112	3.5±0.31	4.09±0.22	**0.011**
Insulin (µU/mL)	0.37±0.039	0.3±0.027	0.08	0.26±0.029	0.28±0.021	0.122	0.41±0.024	0.42±0.041	**0.001**
FBS	105.4±14.3	107.2±13.15	**0.01**	129.3±22.1	112.5±17.9	**0.01**	163.06±38.1	110.6±27.5	**0.001**

(mg/dL)									
2hours Glucose (mg/dL)	156±10	138.4±14.7	**0.002**	188.6±16.1	163.3±23.7	**0.003**	243.4±51.9	203.2±33.5	**0.005**
HOMA-IR	1.73±0.67	1.42±0.41	0.068	1.49±0.62	1.4±0.46	0.725	2.97±1.1	2.06±1.11	**0.039**
QUICKI	0.63±0.012	0.66±0.01	0.378	0.66±0.017	0.67±0.01	0.211	0.55±0.005	0.6±0.015	**0.031**
Vitamin D3 (ng/mL)	25.9±7.2	27.2±6.3	0.641	23.1±4.2	24.1±4.6	0.157	22.9±5.3	24.8±6.4	0.066

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