



Research Article

Metformin Had Potential to Increase Endocan Levels in STZ-Induced Diabetic Mice

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Abstract

Background: Type 2 diabetes mellitus is a chronic metabolic disorder with prominent vascular injuries. In this condition, the levels of multiple pro- and anti-angiogenic factors have been shown to change. This study aimed to investigate the possible effect of metformin on pro-angiogenic factor, endocan levels, via the modulation of p-AMPK/AMPK axis in diabetic mice.

Methods: Mice were randomly assigned to one of 4 groups (n=6): Control (normal saline) and the diabetic group was injected streptozotocin and two groups were given 50 and 100 mg/kg metformin orally, once daily for two weeks after diabetes induction. Endocan protein levels were detected in the liver and kidneys by ELISA and immunofluorescence analysis. Phosphorylation of AMPK was assessed using western blotting. Histological examination was performed to follow the metformin effect on Von Willebrand factor expression and diabetes-related pathologies.

Results: ELISA assay showed an elevated levels of endocan in the renal and hepatic tissues of diabetic mice following treatment with metformin (p<0.05). Immunofluorescence and immunohistochemistry examination of kidneys showed that the increase of endocan protein coincided with the promotion of vWF factors in mice treated with metformin (p<0.05). We did not find endocan factor in hepatic tissue of diabetic mice pre- and post-treatment with metformin. Western blotting confirmed the phosphorylation of AMPK by metformin in kidneys (p<0.05), but these changes did not reach statistically significant levels in hepatic tissues (p>0.05).

Conclusion: Metformin could change the endocan levels during diabetic condition possibly by the modulation of p-AMPK/AMPK axis.

Introduction

Diabetes mellitus, a chronic disorder, occurs in response to the high glucose level and insulin absence or resistance.¹ One of the most characteristics of diabetes mellitus are micro- and macro-vascular complications indicated in renal and hepatic tissues due to abnormalities in endothelial cell function and aberrant angiogenesis rate.¹ In addition to different organs, both hepatic (central regulation of glucose metabolism) and renal (glomerular filtration of glucose) tissues are sensitive to chronic high glucose levels and thereby these conditions could affect the physiological activity of kidneys and liver.²

Defective angiogenesis in patients with diabetes causes an impaired wound healing rate and diabetic foot ulcer, whereas excessive angiogenesis predisposes the onset of diabetic retinopathy.¹ It has been demonstrated that endothelial dysfunction is the main complication of

hyperglycemic changes, leading to vascular insufficiencies.^{3,4} As an extracellular matrix, endothelial glycocalyx takes part in essential physiologic functions such as regulation of inflammation process, enzymes bioactivity and cellular signaling, blood clotting cascade and protecting endothelium against shear stress and oxidative damages.^{5,6} High glucose condition has been shown to have a detrimental effect on the endothelial glycocalyx dynamics thereby promoting vascular complications.⁷

Endocan, also known as endothelial cell-specific molecule-1, is a proteoglycan that is described by Lassalle *et al.* for the first time.⁸ Structurally, endocan is composed of a core protein of 165 amino acids and a glycosaminoglycan chain of dermatan sulfate.⁹ Endocan is secreted from the endothelium and participates in the regulation of some physiologic functions such as cell proliferation, adhesion,

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and migration.¹⁰ This biofactor is integral to endothelial proliferation and angiogenic potential in different tissues.¹⁰ Recent studies have proved the expression of endocan in various cancer types in favor of tumor angiogenesis thereby this factor could be considered as a valuable biomarker to follow the tumor response to anti-angiogenic drugs.¹¹⁻¹⁴ Metformin, as one of the most common anti-diabetic drugs, efficiently reduces the tissue damages in response to hyperglycemia.^{15,16} As an AMPK-activator and mTOR-inhibitor, metformin has been proposed to modulate angiogenesis and vascularization.¹⁷ Metformin has the potential to act via insulin-like growth factor and HER2 axis, contributing to aborted angiogenesis.¹⁷ In contrast, some authorities discovered metformin ability to induce vascular endothelial growth factor (VEGF) expression and subsequently increased angiogenesis in the breast cancer model.¹⁸ To our knowledge, there is no report related to the close relation of metformin with endocan under normal and high glucose contents *in vivo*. Considering inherent sensitivity of both hepatic and renal tissues to hyperglycemic changes,² the current study aims to investigate the modulatory effect of metformin on serum level of endocan and transcription rate in liver and kidneys in diabetic mice. Data from this study could highlight the potent role of metformin in modulating angiogenesis via the control of endocan in the different tissues in diabetes.

Materials and Methods

Animals

Mature male BALB/c mice were obtained from the Razi Institute (Tehran, Iran). The mice were kept in the room temperature (20°C) with a 12 hours light/dark cycle. Mice were randomly divided into four groups: (I) Control group: mice were injected normal saline intraperitoneally (i.p.); (II) Diabetic group: mice were administrated by 150 mg/kg Streptozocin (STZ; dissolved in citrate buffer, pH=4.5, cat number: S0130; Sigma-Aldrich) i.p.;¹⁹ (III) Met 50 group: diabetic mice received 50 mg/kg metformin, and (IV) Met 100 group: diabetic mice received 100 mg/kg metformin. Metformin was used orally and provided as a gift from Osveh Pharmaceutical Inc., Tehran, Iran. Seventy-two hours after STZ administration, blood glucose levels were measured using glucometer (Accu-Chek Performa Blood Glucose Meter) and mice with the blood glucose level ≥ 200 mg/dl selected as diabetic samples. Blood glucose levels were monitored every three days up to two weeks. After induction of diabetes, mice were treated with metformin daily at respective doses for two weeks. On day 17, all the animals were euthanized by the combination of Ketamine (100 mg/kg) and Xylazine (15 mg/kg). Blood, liver, and kidneys samples were collected for subsequent analyses. The blood serum and tissues were kept at -80°C until use.

ELISA assay

The endocan protein level in the serum and tissues was measured by ELISA. For this purpose, 5 mg of samples were

chopped and transferred into microtubes including 300 μ l of protein Lysis Buffer (50 mM Tris HCl; 50 mM NaCl; 5 mM sodium pyrophosphate; 50 mM NaF; 1 mM dithiothreitol; 1 mM EDTA; 0.1% w/v SDS; 1% w/w Triton-X100) supplemented with protease inhibitor cocktail (Cat no: 11873580001; Roche). To extract total protein, the samples were centrifuged at 14000 rpm for 15 minutes at 4°C and the supernatant was separated. The protein content of each sample was quantified using NanoDrop™ One^C (Thermo Scientific). The level of endocan was assessed using an ELISA kit according to the manufacturer's instructions (Cat no: ab193722; Abcam). In this experiment, 100 μ l of each sample was used to detect endocan levels. The absorbance was measured by the spectrophotometer at 450 nm and the values were expressed in ng/mg tissue. In serum, the data were represented as ng/ml. This experiment was performed in triplicate (each in two wells).

Western blotting

Protein samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Cat no: IPVH20200; Merck). Membrane blocking was done by 2% bovine serum albumin (BSA, Cat no: A7906, Sigma). The Antibodies against phospho-AMPK α (Cat no: 2535; Cell Signaling Technology) and AMPK α (Cat no: 5832; Cell Signaling Technology) were used at 4°C overnight. The membranes were washed with Tris-buffered saline solution containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Cat no: 7074; Cell Signaling Technology) for 1 hour at room temperature.²⁰ Immuno-reactive bands were detected using the ECL BM Chemiluminescence kit (Cat no: 11520709001; Roche) and imaged by Gel Documentation System (Sabz Biomedicals). Band density was calculated (ImageJ software, NIH, USA) and compared with the control. The values of AMPK α and phospho-AMPK α were normalized to β -actin and the mean phospho-AMPK α /AMPK α ratio presented.

Immunofluorescence assay (IF)

The endocan protein levels were also studied in samples by IF assay in 5- μ m frozen tissue sections from renal and hepatic tissues.²¹ To this end, tissue sections were washed three times with phosphate-buffered saline (PBS) and then permeabilized with 1% Triton X-100 (Cat no: T8787; Sigma-Aldrich) for 15 minutes. Slides were then blocked with 1% BSA for 30 minutes. Upon the completion of this stage, tissue slides were incubated with primary endocan antibody (Cat no: sc-515304, Santa Cruz Biotechnology) for 1 hour followed by the addition of FITC-conjugated secondary antibody (Cat: 406001; Biologend). 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) solution was used to stain the nucleus. An inverted microscope (Model: BX51, Olympus) was used to image the slides and data analyzed using CellSense software version 1.4.

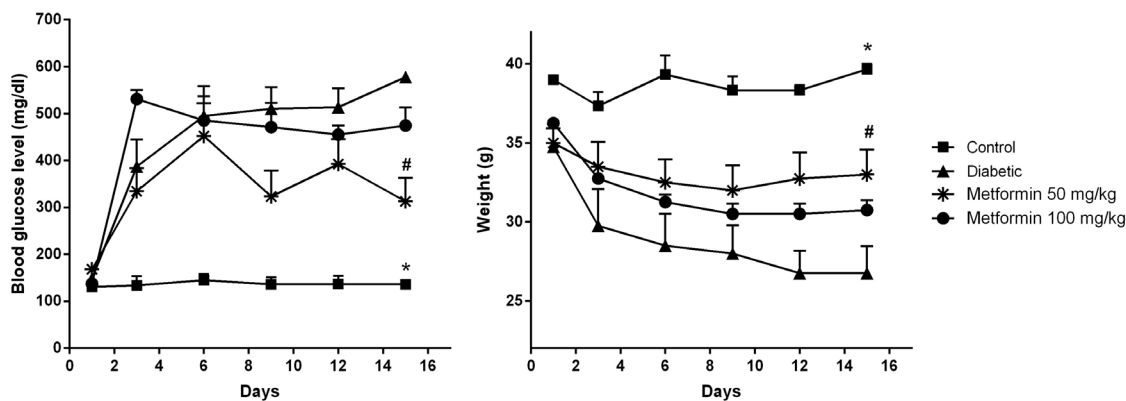


Figure 1. Body weight and blood glucose level variation in different experimental groups during two weeks. Results are expressed as the mean ± SEM. One-Way ANOVA with Tukey post hoc test. Symbol * indicates statistical significant difference compared to control group and # compared to diabetic group.

Pathological analysis

Hematoxylin and Eosin (H&E) staining

To study the protective effect of metformin on diabetic-related pathological changes in the renal and hepatic tissues, samples were fixed in 10% formalin solution and paraffin-embedded blocks were prepared. Samples were cut into 5 μm thick-sections using a Microtome (Model: Leica® RM2135) and stained with H&E solution according to previously published data.²² Slides were examined by light microscope (Model: BX51, Olympus) and compared to the control.

Immunohistochemical (IHC) analysis

To evaluate the vascular density, IHC analysis was performed on formalin-fixed and paraffin-embedded samples from renal and hepatic tissues.²¹ To validate the microvascular density, we monitored the protein content of the Von Willebrand factor (vWF). For this propose, 5 μm-thick slides were prepared, exposed to a 3% H₂O₂ solution for 30 minutes. For antigen retrieval, we put the slides in 15 psi in citrate buffer (pH= 6.0) and then incubated with an anti-vWF antibody (Cat no: A0082; Dako) for 1 hour. Samples were washed twice with PBS and incubated with

EnVision + Dual Link System HRP kit (Cat no: K5007; Dako) solution. We used 3, 3'-Diaminobenzidine (DAB) as the chromogen. Mayer's Hematoxylin solution was used as a counterstain. The vascular intensity was measured in slides from different groups in 3 random serial high-power fields (HPF).

Statistical analysis

One-way Analysis of variance (ANOVA) with Tukey post hoc test was used to show significant levels between groups. $p < 0.05$ was considered to be statistically significant.

Results

Effect of metformin on the blood glucose level and body weight of diabetic mice

Figure 1 shows the variation of the weight and blood glucose level of different experimental groups during the respective time (two weeks). The blood glucose level in STZ-induced diabetic mice was significantly higher than that of the control group ($p < 0.001$). According to our data, administration of 150 mg/kg (single dose) STZ contributed to 9.7% mortality rate in diabetic mice. Treatment of diabetic mice with 50 mg/kg metformin caused a

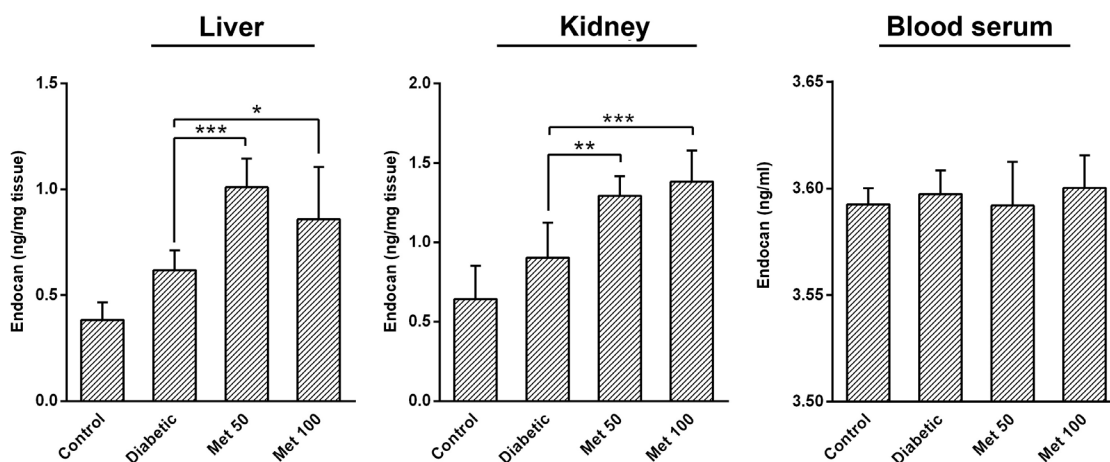


Figure 2. The level of endocan protein in the blood serum and lysate of renal and hepatic tissues of different experimental groups (n=3). Results are expressed as the mean ± SD. One-Way ANOVA with Tukey post hoc test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

significant decrease in blood glucose levels compared to the diabetic mice ($p < 0.01$) (Figure 1). Calling attention, the body weight of diabetic mice was significantly decreased compared to the control ($p < 0.001$) and administration of 50 mg/kg metformin significantly decreased the detrimental effect of diabetes ($p < 0.05$). These effects were not reached significant levels in Met 100 group (Figure 1).

Metformin effect on the endocan serum level and content in the renal and hepatic tissues

The level of endocan in the blood serum and tissues of experimental groups was measured by ELISA (Figure 2). Our results revealed that the endocan level in the kidneys and liver of diabetic mice was higher compared to the control but did not reach statistically significant levels. However, treatment of the diabetic mice with metformin caused a significant increase in the level of endocan in the renal and hepatic tissues compared to the control and diabetic groups ($p < 0.05$) (Figure 2). In this study, we found a non-significant difference regarding serum endocan levels between groups ($p > 0.05$).

AMPK phosphorylation in the renal and hepatic tissues

Based on our results, we showed that AMPK activation was statistically diminished both in the kidneys and liver of diabetic mice which statistically attained a significant level

of $p < 0.05$ in the kidneys (Figure 3). Two-week treatment of diabetic mice with metformin at doses 50 and 100 mg/kg significantly induced the phosphorylation of AMPK only in kidneys, showing endocan biological activity related to tissue type during diabetic changes ($p < 0.01$).

Endocan localization in the renal and hepatic tissues

Immunofluorescence imaging showed the existence of endocan protein in the renal and hepatic tissues of the control mice (Figure 4). As it is illustrated in Figure 4, there were no endocan-positive cells in samples from the diabetic mice tissues, showing the decrease of endocan by endothelial cells following diabetes. Based on the results, metformin treatment potentiated endothelial cells to retrieve endocan synthesis under the diabetic condition in the kidneys. These features stand for the fact that metformin could improve the angiogenic potential and endothelium repair possibly by the synthesis of endocan.

Effect of metformin on the histology of renal and hepatic tissues

Histological analysis revealed the injury of kidneys and liver in diabetic mice (Figure 5 and 6). In diabetic condition, massive tubular cell necrosis was detected. Besides, we found the existence of fibrin infiltration in interstitial space, the formation of hyaline casts and tubular

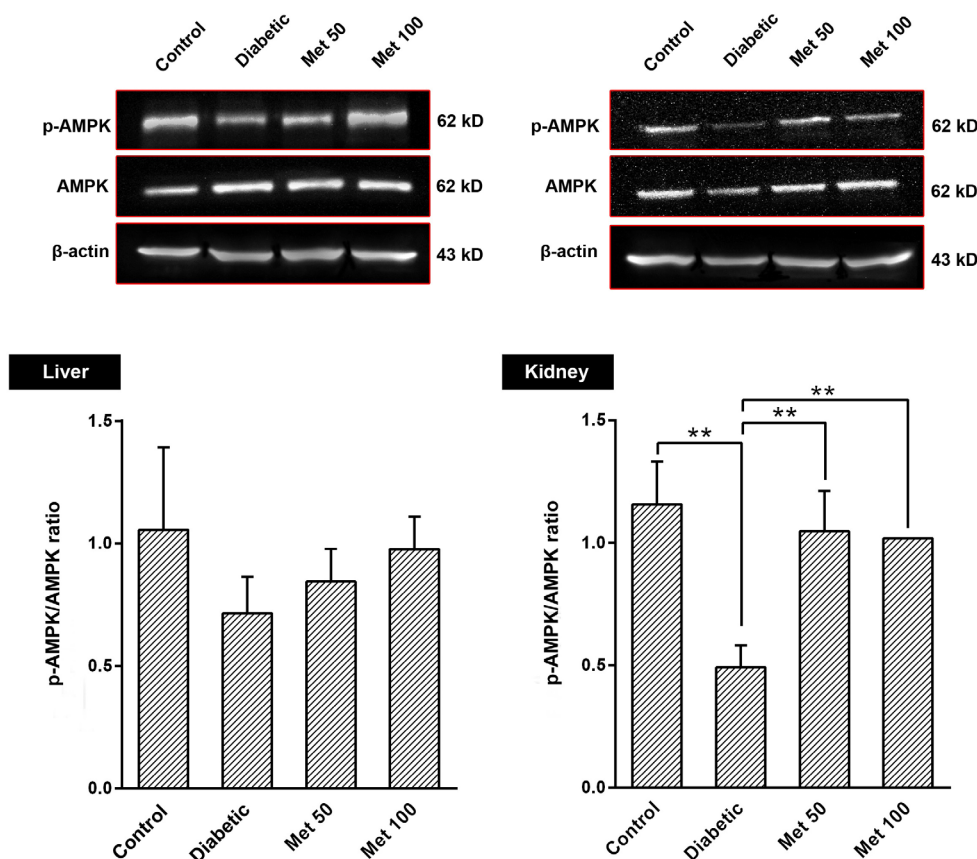


Figure 3. Western blot analysis of AMPK, p-AMPK and Beta actin in the renal and hepatic tissues of different experimental groups ($n=3$). Results are expressed as the mean \pm SD. One-Way ANOVA with Tukey post hoc test. $**p < 0.01$. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

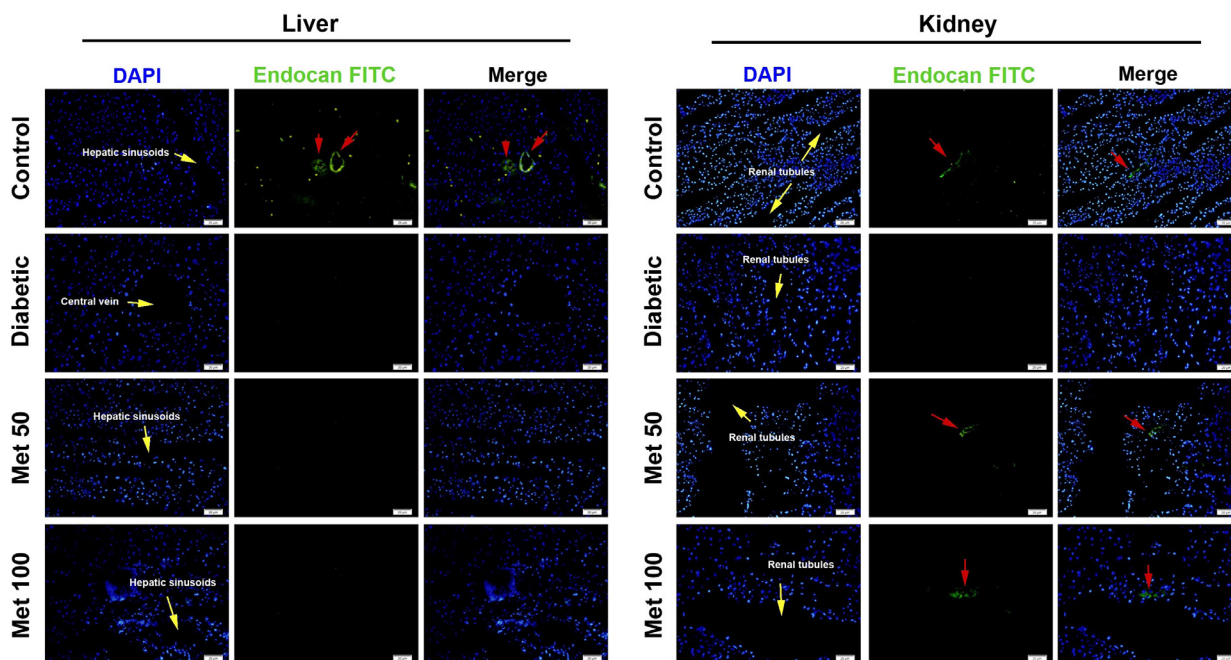


Figure 4. Immunofluorescence staining of the renal and hepatic tissues of different experimental groups. Red arrows indicate the fluorescence intensity of endocan. Nuclei were stained with DAPI. Green=Endocan, Blue=DAPI. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

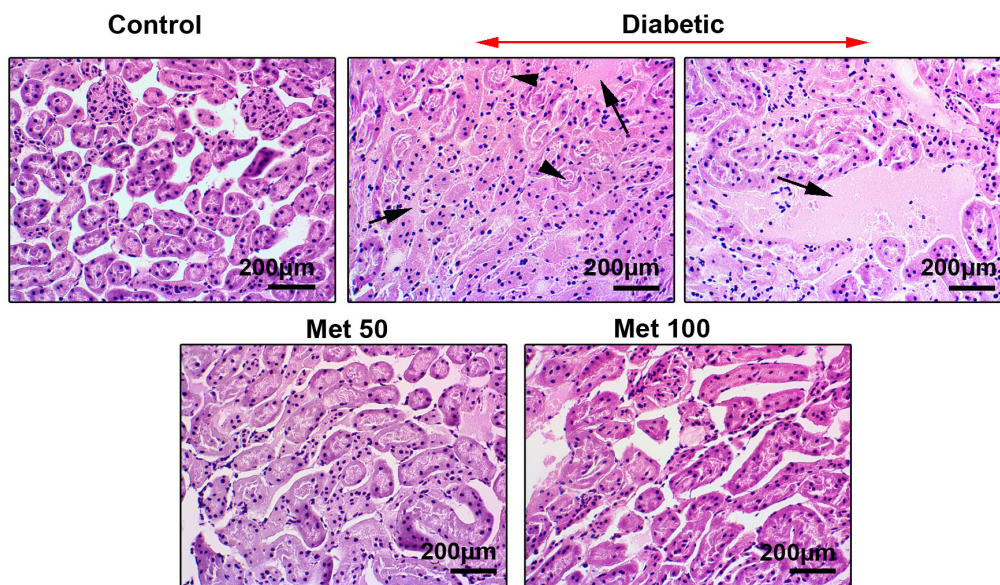


Figure 5. Representative imaging of renal tissue stained with H&E solution from different experimental groups. In diabetic kidneys, prominent tubular necrosis, hyaline cast formation and fibrinous exudates were detected. The detrimental effects of diabetic condition were reduced after metformin administration. Arrow head: hyaline casts; arrows: fibrinous exudate. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

epithelial cells atrophy compared to the control (Figure 5). Based on the data, the diabetic pathologies were reduced in Met 50 and 100 groups. It seems that the anti-diabetic effects seen in Met 50 group were prominent compared to Met 100 group. Monitoring the hepatic microstructure revealed the interruption of liver sinusoidal integrity and necrosis of hepatic cells with focal accumulation of fibrin-like depositions (Figure 6). Similar to kidneys, the intensity of diabetic changes and related pathologies were

reduced in both groups administrated with 50 and 100 mg/kg metformin. The detrimental effects of diabetes were less in Met 50 group. These data demonstrated that the application of metformin could alleviate diabetic-derived kidneys and liver injuries.

vWF localization in the renal and hepatic tissues

We performed vWF staining to investigate the impact of the diabetic condition on the vascular structure in the liver

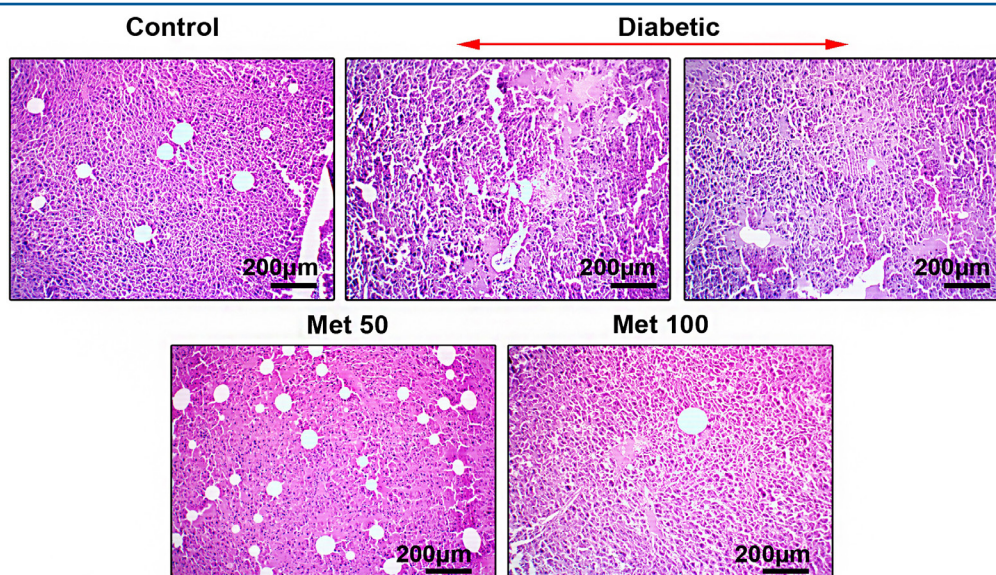


Figure 6. Representative imaging of hepatic tissue stained with H&E solution. The detrimental effects of diabetic conditions on hepatic tissue were reduced after metformin administration. Foci with necrotic changes and fibrinous exudate are commonly seen in diabetic conditions that were reduced post metformin administration. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

and kidneys (Figure 7). Based on IHC analysis, the intensity of cells encoding the vWF factor was decreased following the diabetic changes compared to the control ($p < 0.001$). In samples prepared from the control mice, we found vWF positive cells inside Bowman's capsules microvascular system while these features reduced intensively in the diabetic mice. Administration of two doses of metformin (50 and 100 mg/kg) in diabetic mice reversed the inhibitory effects of hyperglycemic condition on the expression of vWF in kidneys (Figure 7). As expected, the protein levels of vWF in the liver showed a similar trend as seen in kidneys ($p_{\text{Control vs. Diabetic}} < 0.001$). The application of 50 mg/kg metformin, but not 100 mg/kg, showed a superior effect to blunt hyperglycemic detrimental effects on the number of hepatic vWF positive cells ($p < 0.001$; Figure 7). In the diabetic liver, the endothelial cells were detached and shed to the lumen of central veins.

Discussion

Diabetes-related endothelial dysfunction is the most important cause of vascular complications in patients with diabetes.¹ In the last decade, considerable attention has been paid to monitor endocan, as a biomarker of endothelial cells functions, during the onset of various diseases. In the present study, we evaluated the effect of metformin, the first-line medication for the treatment of diabetes, on serum, hepatic and renal contents of endocan in STZ-induced diabetic mice.

STZ-induced diabetes model is a common method to induce diabetes in animals and this type of diabetes contributes to body weight loss.²³ Our results showed that the body weight of diabetic animals was significantly lower than the control mice. As expected, the blood glucose level of metformin-treated groups was lower than the non-treated diabetic group. Based on the results, it seems that 50 mg/kg metformin exerted a superior effect compared

to 100 mg/kg metformin in lowering blood glucose level and body weight retrieval ($p < 0.01$). In contrast to our data, Zhou *et al.* found statistically non-significant changes in weight gain in diabetic mice given 200 mg/kg metformin after 8 weeks.²⁴ Han and colleagues also reported a slight but non-significant increase in weight of diabetic mice treated with 250 mg/kg metformin for two weeks.²⁵ Some authors revealed a dose-dependent action of metformin in reducing blood glucose level in the diabetic mice.^{26,27} In this study, we performed shorter treatment duration and used low doses of metformin in the diabetic mice that yielded prominent weight changes compared to above-mentioned experiments. Consistent with our results, it has been demonstrated that the high doses of metformin have no more beneficial effects and in some studies not only shorten the lifespan of mice but also induce oxidative stress, lactic acidosis and renal failure.²⁸ Even, excessive pharmacological concentrations of metformin do not activate intracellular signaling pathways in the target cells. As a matter of fact, the less effectiveness of metformin at higher doses may be as a result of lactic acidosis and inability to activate related effectors. Diabetes is associated with different types of vascular complications. It has been demonstrated that intensive blood glucose control is a key point in managing diabetes-related vascular complications.²⁹ There are strong pieces of evidence that the normal endothelial cells bioactivity and vascularization are impaired due to high blood glucose levels.¹ Defective angiogenesis causes detrimental effects affecting the life quality of diabetic patients and in some cases contributes to mortality.³⁰ As a biomarker of angiogenesis, the endocan participates in the promotion of cancer expansion and vascularization rate.³¹ Based on the evidence, it seems that there might be a close relationship between endocan and diabetes-related disorders.³²

In the present study, we found a marked effect of metformin

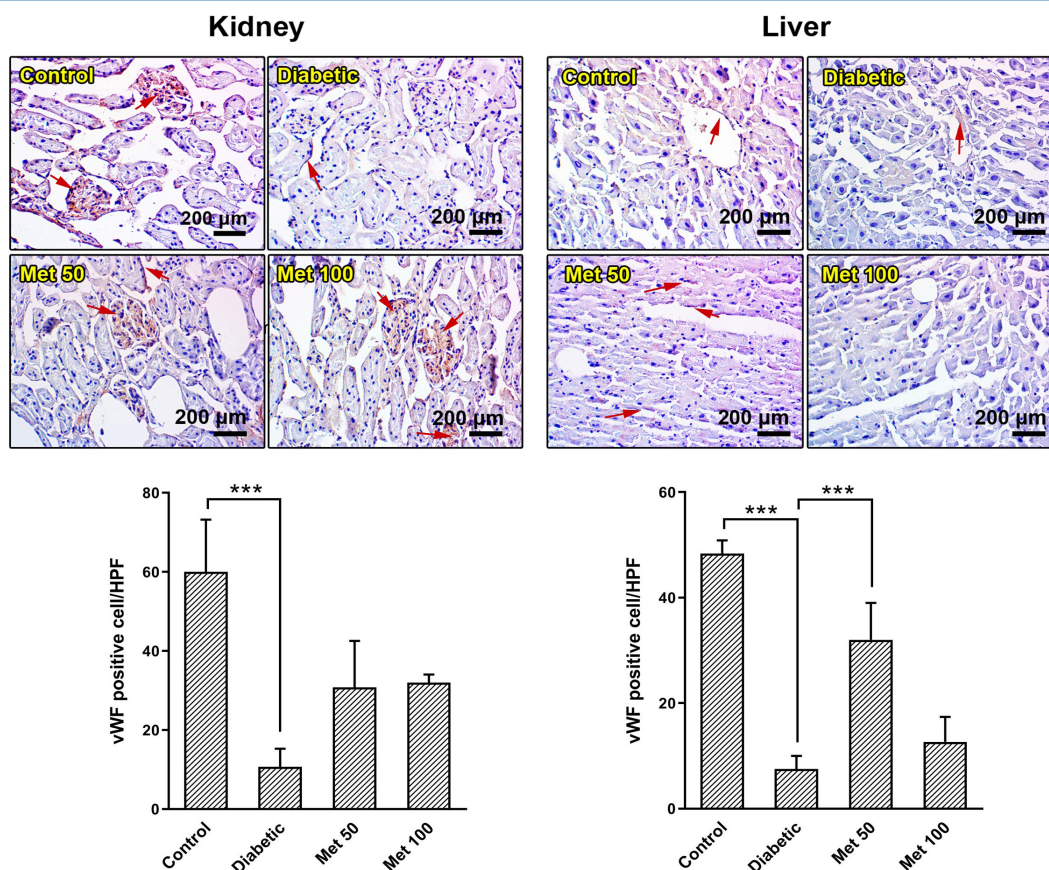


Figure 7. Immunohistochemical staining of the renal and hepatic tissues of different experimental groups. In diabetic kidneys, the intensity and number of vWF positive cells reduced showing endothelial insufficiency. By using metformin, the Bowman microvascular endothelial cells retain the activity to produce vWF. The hepatic tissue also is unable to produce vWF factor. In mice treated with metformin, sinusoidal endothelial cells synthesize vWF. Results are expressed as the mean \pm SD. One-Way ANOVA with Tukey post hoc test. *** $p < 0.001$. Red arrows: vWF positive cells. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).

on the endocan level in the kidneys of diabetic mice where metformin had the greatest effect on phosphorylation of AMPK. However, there is a dichotomy with metformin effects on angiogenesis. Previously, it was found that metformin increased the proliferation of vascular endothelial cells and further promoted the angiogenic potential of human umbilical vein endothelial cells (HUVECs).³³ In support of this claim, metformin can be used in restoring vascular compliance and the repair and prevention of endothelium damage caused by high glucose levels such as in diabetic foot ulcers and nephropathy. Inconsistent with our data, Dallaglio and co-worker found the modulatory effect of metformin on HUVECs pro-angiogenic activity in a time-dependent manner.³⁴ They showed that short-term administration of mice with metformin (2 mg/mice/day) induced angiogenesis by promoting VEGF and related signaling pathways. As an AMPK activator, metformin could affect some important physiological pathways including protein synthesis through suppressing the mammalian target of rapamycin complex 1 (mTORC1) signaling.³⁵ In support of this statement, since mTORC1 induces angiogenesis through regulating hypoxia-inducible factor 1 α (HIF1 α), it seems that metformin should inhibit angiogenesis.^{16,35} Also, we found that metformin which has the potential to decrease

the vascular insufficiencies in diabetic kidneys significantly elevated the level of endocan in tissues. Metformin seems to produce at least part of its protective effect probably by endocan. According to histological examination, we found a decrease in the number of vWF positive cells inside the Bowman's capsules in diabetic kidneys. The reason for this issue would be that diabetic condition could accelerate the uncontrolled proliferation of the mesangial matrix that leads to glomerular capillaries occlusion and regression.³⁶ The increase of glomerular capillaries was improved in response to the decrease of mesangial matrix by using metformin via the activation of the AMPK signaling pathway.³⁷ We, here, found that diabetes-related pathological injuries were reduced after the administration of metformin by the regulation of angiogenesis and endocan levels. Previous works demonstrated a close association between the dynamics of endocan and vWF levels in human endothelial cells.³⁸ In this regard, Roudnicky and colleagues found that the suppression of endocan by siRNA decreased the endothelial vWF and phosphorylation of VEGF receptor 2 (VEGFR-2) which governs the angiogenic potential of endothelial lineage. In particular, the synthesis of endocan is prominent in endothelial tip cells associated with the formation of filopodia and cellular anastomosis by regulating the endothelial cell physical contact with

extracellular matrix proteins mainly fibronectin.³⁸ Our study confirmed the ability of metformin to increase endocan and vWF levels in kidneys. Along with our data, Dallaglio *et al.* found the positive effect of metformin on VEGF synthesis in HUVECs after 24 hour.³⁴ These data stand for a fact that metformin could decrease the anti-angiogenic effect of the diabetic condition by modulating the synthesis of endocan and vWF.

There are some limitations regarding this study. It is suggested to include groups that receive AMPK blockers to precisely address the metformin effects under diabetic conditions. Here, we studied the therapeutic effect of metformin on the hyperglycemic condition for 2 weeks. Monitoring the diabetic changes over a long period would be beneficial to address the metformin effect on endocan dynamic during the diabetic condition.

Conclusion

Consistent with the results from our study, the administration of metformin could decrease the detrimental effect of hyperglycemic condition on the ability of endothelial cells to express angiogenic factor vWF and endocan levels in hepatic and renal vasculature system. One possible mechanism for therapeutic effect of metformin in diabetic subjects correlates with the modulation of p-AMPK/ AMPK axis. More investigation is needed to find the relationship between endocan and metformin to predict angiogenic potential during physiological and pathological conditions.

Ethical Issues

All the experiments were carried out and approved by the guidelines published by the local ethic committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1395.145).

Acknowledgments

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Conflict of Interests

Prof. Alireza Garjani is the Editor-in-Chief of Pharmaceutical Sciences. The peer-review process of the submission was supervised by another member of the editorial board. The authors declare no conflict of interest.

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