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Tabriz University of Medical Sciences
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Entitled:
Study of the effect of metformin on the level and expression of endocan in diabetic conditions

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In The name Of GOD
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>BM</td>
<td>Basement membrane</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CT</td>
<td>Cycle of threshold</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Dil-Ac-LDL</td>
<td>1, 10-dioctadecyl-3, 3, 30, 30-tetramethyl indocarbocyanine-labeled acetylated low-density lipoprotein</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ESM-1</td>
<td>Endothelial cell specific molecule-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HER2</td>
<td>Epithelial growth factor receptor-2</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible factor 1α</td>
</tr>
<tr>
<td>HPF</td>
<td>High-power fields</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemical</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>mTORC</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Noninsulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PGs</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>PI3 kinase</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-segment elevation myocardial infarction</td>
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<tr>
<td>STZ</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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Abstract

Background: Diabetes mellitus is a chronic metabolic disease with complicated vascular injuries. Endocan is a novel endothelium-derived proteoglycan and may play a role in endothelial cells activity under diabetic conditions.

Objective: Here, we evaluated the effect of high glucose concentration on endocan level in presence or absence of metformin.

Methods: Human Umbilical Vein Endothelial Cells (HUVECs) viability was assessed by MTT. Cell migration was studied by scratch test. Endocan expression and protein levels were evaluated by RT-PCR, ELISA and flow cytometry. Griess reaction was used to measure nitric oxide (NO) levels. Functional activity of cells was monitored using Dil-Ac-LDL uptake. BALB/c mice (35-40 g) were divided into 4 groups (n=6): Control, diabetic group was injected Streptozocin, and groups were given 50 and 100 mg/kg metformin orally, once daily for 2 weeks. Endocan was detected in tissues by Immunofluorescence (IF) analysis. Phosphorylation of AMPK was assessed using western blotting. Histological examination was performed to follow the von Willebrand factor (vWF) expression.

Results: High glucose concentration (30mM) reduced endothelial cells’ viability and migration, whereas these features were improved with metformin. Metformin increased endocan transcription and protein levels, NO production, and LDL uptake capacity in diabetic condition (p<0.05). Western blotting confirmed the increase of p-AMPK/AMPK ratio in metformin-treated cells. ELISA assay showed elevated levels of endocan by metformin in tissues. IF and Histological examination of kidneys showed that the increase of endocan protein coincided with the promotion of vWF factor in metformin-treated mice (p<0.05). Western blotting confirmed the phosphorylation of AMPK by metformin in tissues.

Conclusion: Metformin could change the endocan levels possibly by the modulation of p-AMPK/AMPK axis and through its effect on vWF promotes angiogenic potential of endothelial cells.

Key words: Metformin, Endocan, Angiogenesis, High glucose condition
Chapter 1: Introduction

1 Introduction

Endothelial cells are the important components of the vascular system and have critical roles in many physiological and pathological conditions (1). It has been demonstrated that chronic high glucose concentration in bloodstream leads to critical cardiovascular diseases in diabetic patients and high glucose-related endothelial disorders can take part in diseases such as atherosclerosis and hemostatic dysfunctions (2). Recent studies have shown that vascular complications because of high glucose concentration in diabetic patients can be categorized into two main groups; those that are because of excessive angiogenesis such as retinopathy, nephropathy, and atherosclerosis and those results from insufficient angiogenesis such as impaired wound healing in diabetic foot ulcer (3).

The endothelium is covered by a layer called glycocalyx, which is composed of proteoglycans (PGs) and glycoproteins. It has been shown that these macromolecules have an essential impact in regulating some physiological functions such as differentiation, cell migration, and angiogenesis (4).

Endocan or endothelial cell-specific molecule-1 (ESM-1) is a PG secreted from endothelial cells. Some studies have shown that endocan is overexpressed in some cancers like the lung, brain, colon, liver, kidney, and pituitary and therefore it can be used as a marker for cancers (5). Recent studies have reported that endocan can take part in molecular interactions that are essential for proliferation, cell adhesion, migration, and neovascularization (6).

Metformin is one of the most important oral hypoglycaemic agents and studies have reported that it has beneficial effects in the improvement of macrovascular disease (7). The most important cellular target of metformin is AMP-activated protein kinase (AMPK) that plays a crucial role in protecting cellular functions under energy-restricted conditions (8). On the other hand, it has been indicated that metformin could take part in proliferation, growth, and
angiogenesis through inhibition of the mammalian target of the rapamycin complex (mTORC) (8).

Since angiogenic disorders have an important role in diabetes-related complications, the main aim of the current study was to evaluate the expression and release of endocan under high glucose condition from Human Umbilical Vein Endothelial Cells (HUVECs) and tissues of diabetes-induced mice. To study whether metformin could affect the endothelial-derived endocan, we treated the HUVECs with metformin after being exposed to high glucose concentration and the effect of metformin on the cellular content of endocan and angiogenic capacity was investigated. Also, in vivo studies were done in order to evaluate the dynamic of endocan in diabetic mice.

1.1 Main Objective

Study of the effect of metformin on the level and expression of endocan under high glucose condition

1.2 Specific Objectives

1) Evaluation of the effect of high glucose content on the HUVECs viability.
2) Evaluation of the effect of high glucose content on the angiogenesis capacity of HUVECs.
3) Evaluation of the level and expression of endocan under high glucose condition.
4) Evaluation of the effect of metformin on the level and expression of endocan under high glucose content.
5) Evaluation of the effect of metformin on the angiogenesis under high glucose conditions.
Literature review
2 Literature review

2.1 Diabetes

Diabetes mellitus is determined as a chronic metabolic disorder that happens because of impairment of secretion or function of insulin and so increased systemic glucose level, impairing endothelial cells function, and promoting vasculopathy. Both genetic and environmental factors contribute to the pathogenesis of diabetes (9).

Diabetes prevalence is rising rapidly throughout the world. Based on the statistics released by the international diabetes federation, it has been estimated that near to 425 million adults are diabetic in 2017, of which 340 million lived in low- and middle-income countries and 1 in 2 remains undiagnosed (10). Aging, inappropriate lifestyle, and urbanization are the most important factors that could increase the prevalence of diabetes worldwide (11).

Diabetes can be categorized into four classes: (I) type 1 diabetes is due to impaired insulin secretion because of pancreatic beta cell destruction. This type of diabetes, which is also referred to as insulin-dependent diabetes mellitus (IDDM) or Juvenile diabetes, is more common among children and young adults. For the treatment of patients with type 1 diabetes insulin injections are used, (II) type 2 diabetes or noninsulin-dependent diabetes mellitus (NIDDM), occurs when insulin production is normal but there is a resistance to insulin function and this resistance develops in response to high glucose levels. This type of diabetes usually develops in older adults, (III) gestational diabetes that occurs during pregnancy and (IV) diabetes that is caused by genetic defects of pancreatic beta cells or chemical agents (12).

2.2 Diabetes-related complications

The main feature of diabetes is the increased blood glucose level. Hyperglycemia is the leading cause of complications in diabetic patients. It seems that not only chronic hyperglycemia but also transient, acute hyperglycemia can
impair endothelial function in vascular beds affecting the heart, eyes, kidneys and nerves. Diabetes is one of the leading causes of cardiovascular disease, blindness, and kidney failure and lower-limb amputation. Diabetes-related complications can be classified into two categories, microvascular and macrovascular complications (13). Microvascular complications include retinopathy, nephropathy, and neuropathy in which small blood vessels are affected. Macrovascular complications that are because of atherosclerosis and associated with the damage in arteries include peripheral vascular diseases, stroke and heart disease (14). Unfortunately, chronic complications of diabetes because of prolonged hyperglycemia are the principal sources of morbidity in diabetic patients. However, it has been demonstrated that continuous blood glucose monitoring can alleviate these complications (13).

The endothelium is a barrier between circulating blood and underlying tissues that can modulate vascular functions. The endothelium regulates the passage of hormones, nutrients, and macromolecules into the surrounding tissue. Furthermore, it can regulate hemostasis by preventing blood coagulation and the formation of a platelet thrombus (15). It has been proposed that endothelium can take part in modulating many vital physiological functions by producing some mediators such as vasodilators and vasoconstrictors, pro-coagulants and anti-coagulants, adhesion molecules, and cell growth and differentiation factors (15).

In addition to regulating glucose metabolism, insulin has crucial role in modulating some important vascular functions including stimulation of sympathetic activity, and production of nitric oxide (NO) from endothelium (16). Insulin induces NO production after binding to its receptor tyrosine kinase on endothelial cells and following phosphorylation of endothelial nitric oxide synthase (eNOS) via activation of the phosphoinositide-3 kinase (PI3 kinase)/Akt system (17). Increased eNOS activity and NO production lead to enhanced vasodilation, anti-platelet activity, anti-proliferative, and anti-inflammatory effects (18). On the other hand, it has been suggested that defects in insulin signalling and function that occurs during diabetes may affect insulin impact on endothelial cell function that finally can lead to diabetes-related vascular complications (15).
Endothelial dysfunction is the main complication of hyperglycemic changes, leading to vascular insufficiencies (16) and dysfunctions such as impaired vasodilation, increased arterial stiffness, impaired arterial remodeling, increased atherogenesis, and impaired angiogenesis (19). In addition, it has been proven that hyperglycemia can also affect the structure of endothelial cells (16). Structural changes because of hyperglycemia include increased basal membrane thickness, reduced glycocalyx, and formation of advanced glycation end products (AGEs) (16). Mechanisms of endothelial dysfunction in insulin resistance and diabetes include changes in endothelial cells signaling, increased oxidative stress, pro-inflammatory activation of the endothelium, activation of protein kinase C (PKC), and mitochondrial dysfunction (19).

The most important defect in endothelial cells signaling in the setting of diabetes is impaired eNOS activation and NO production. To induce its physiological functions, NO diffuses in the arterial wall and activates guanylyl cyclase in endothelial cells (20). In diabetic patients, insulin-mediated stimulation of eNOS through PI3 kinase/Akt system is impaired that may have wide implications for diabetes-related vascular dysfunction (19). Also, it has been demonstrated that the levels of endogenous inhibitors of eNOS are increased in diabetes mellitus (21). Another mechanism involved in endothelial dysfunction in diabetes mellitus is increased oxidative stress in the vascular system. The elevated level of glucose are exposed to arterial tissue induces superoxide production that impairs eNOS activation and reduces NO bioavailability (22).

It has been indicated that pro-inflammatory factors such as tumor necrosis factor alpha (TNF-α) can activate endothelial cells to promote an atherogenic phenotype (23). As a result of this activation, expression of eNOS and the activity of NO are decreased. On the other hand, since in patients with diabetes the level of inflammatory markers are increased, the endothelial function is impaired and atherosclerosis risk is elevated (19).

PKCβ is an important isoform in endothelial cells which is activated under high glucose concentrations. It has been demonstrated that PKCβ inhibits PI3 kinase/Akt system, therefore reducing eNOS phosphorylation. Also, PKCβ can
stimulate inflammatory factors production (24). In conclusion, activation of PKCβ in patients with diabetes may explain the links between inflammation, endothelial dysfunction, and insulin resistance (19).

Another mechanism that has role in the occurrence of endothelial dysfunction is mitochondrial dysfunction. Under physiologic condition, mitochondrion produces small amounts of superoxide anion that has essential function in normal signaling. Whereas increased levels of superoxide anion have pathological effects in diabetes (25).

One of the most important characteristics of diabetes mellitus is aberrant angiogenesis, which plays key role in the incidence of diabetes-related vascular complications. Angiogenesis is a term that refers to the formation of new blood vessels from pre-existing plexus and it has been proven that endothelial cells migration and proliferation are essential in the angiogenesis process (14). In diabetes, two paradoxical changes occur in the blood vessels. Excessive and uncontrolled angiogenesis results in diabetic retinopathy and nephropathy, while deficient angiogenesis causes impaired wound healing and impaired coronary collateral vessel development (26).

The normal angiogenesis process relies on the balance between pro- and anti-angiogenic factors. Some stimulatory factors involved in angiogenesis include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and upregulation of integrin. Mechanisms involved in deficient angiogenesis include inadequate extracellular matrix (ECM)/ basement membrane (BM) degradation, growth factor imbalances, and cytokine imbalances (3).

Some suggest that reduced VEGF, FGF, degradation of ECM, and increased AGEs lead to reduced angiogenesis and collateral formation. Also, reduced VEGF and growth factors play main role in reduced wound healing and transplant failure in patients with diabetes. On the other hand, increased VEGF and integrins enhance vascular permeability and capillary sprouting that are seen in excessive angiogenesis (9).

VEGF is the potent pro-angiogenic factor among the growth factors. It has been reported that hypoxia, hyperglycemia, and some cytokines can induce the
production of VEGF (27). VEGF secreted from podocytes together with a low bioavailability of NO, leads to the enhanced vascular permeability of the glomerulus and glomerular damage in diabetic nephropathy (28). The upregulation of VEGF in the early stage of diabetic nephropathy induces excessive blood vessel formation and development of disease (26).

Diabetic retinopathy, which is the leading cause of blindness throughout the world, is described by retinal vessel edema, hemorrhages, and exudates. In patients with diabetic retinopathy, high levels of VEGF at the site of aberrant angiogenesis and vitreous and aqueous humor has been reported. VEGF can stimulate endothelial cells to produce matrix metalloproteinases (MMPs), which have an essential role in the degradation of BM and endothelial cell migration (29).

The development of foot ulceration because of aberrant angiogenesis in diabetic patients impairs the quality of life in these patients and sometimes results in amputation (30). The normal wound healing process can be divided into a series of stages that include hemostasis, inflammation, proliferation, and remodeling. The important step in the proliferation stage is angiogenesis. Wound healing requires the activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. Epithelial cells and macrophages release VEGF, which induces the phosphorylation of the eNOS resulting in increased NO levels. It has been proven that VEGF is one of the most vital pro-angiogenic factors in the wound healing process (31). Hyperglycemia, oxidative stress and reactive oxygen species (ROS), AGEs production, and neuropathy in diabetes lead to insufficient angiogenesis and the impairment of the diabetic wound healing (32). Gallagher et al. (33) showed that in the animal model of diabetes, wound healing process is reduced because of the impaired eNOS phosphorylation.

2.3 Endocan

The endothelium surface and extracellular matrices are covered by a monolayer of glycocalyx formed by PGs and glycoproteins. PGs consist of proteins and glycosaminoglycan side chains and have an essential role in
regulating physiological and physiopathological functions such as regulation of inflammation process, angiogenesis, wound healing, enzymes bioactivity and cellular signaling, blood clotting cascade and protecting endothelium against shear stress and oxidative damages (34, 35). It has been suggested that high glucose conditions have a negative impact on endothelial glycocalyx dynamics. Also, cell distribution of glycocalyx is decreased in diabetic patients compared to healthy subjects. In particular, glycocalyx destruction results in endothelial dysfunction and associated with many typical phenomena of diabetes-related vascular complications, such as decreased arterial vasodilation, increased endothelial permeability, and in the retinal vessels, fluid leakage and neovascularization. Diabetes-induced endothelial glycocalyx damage is also associated with activation of coagulation, increased leukocyte adhesion, development of albuminuria, and deterioration of the blood-brain barrier (35, 36).

Endocan, also known as ESM-1, is a soluble PG that is described by Lassalle et al. for the first time. Endocan is continuously secreted by endothelial cells and circulates in the blood. This PG has been detected with an average concentration of 1.08 ng/ml in the serum of healthy subjects. Structurally, endocan is composed of a core protein and a glycosaminoglycan chain of dermatan sulphate. The protein part is composed of 165 amino acids and possesses an N-terminal cysteine-rich region of 110 amino acids, which also includes an endothelial growth factor-like region, a phenylalanine-rich domain and a C-terminal region. It is linked to the glycosaminoglycan chain via serine 137 during posttranslational modification (Figure 2.1) (37, 38). It seems that both the protein core and glycosaminoglycan of endocan have been implicated in interactions with ECM components, cell surface proteins, intracellular molecules, and soluble mediators (6).
The exact regulatory mechanism of endocan production is not well documented, but there are studies have shown the relationship between endocan expression and some signaling pathways. It has been suggested that endocan production is regulated by a series of cytokines or cell factors and the impact of inflammatory cytokines. For example, studies have shown that endocan expression is down-regulated by PI3K and interferon-\(\gamma\) (INF-\(\gamma\)), while VEGF, IL-1, TNF-\(\alpha\), transforming growth factor-\(\beta1\), and FGF-2 can result in up-regulation (37, 39). VEGF- induced production of endocan is positively regulated by PKC/NF\(\kappa\)B and negatively regulated by PI3K/Akt signaling pathways (40).

Endocan participates in regulating some biological functions such as cell proliferation, adhesion, migration, neovascularization, and development of vascular tissues in health and diseases (6). It has been shown that after the disruption of endothelial function under pathological conditions, endocan expression is induced; hence, we can consider endocan as a biomarker of endothelial cell function and activity also as a factor that have a potential application in diagnosis of diseases associated with neovascularization (41).

Angiogenesis is an important event in tumor progression because it delivers oxygen and nutrients to proliferating tumor cells. When a tumor rapidly grows during cancer progression, eventually becomes hypoxic resulting in the activation of the hypoxia-inducible factor signaling and VEGF secretion from tumor cells and to provide tumor’s oxygen requirements. In this process, the action of VEGF is mediated by endocan (6). It has been shown that endocan production is
associated with an angiogenic switch of tumor cells in different types of cancer (42). Recent studies have indicated that endocan level is elevated in several types of cancers and there is a close correlation between the systemic content of endocan and angiogenic response during the onset of tumors. Since endocan affects the immunological characteristics of tumor vascular endothelial cells and also has a close association with tumor angiogenesis, it could be considered a valuable biomarker to follow the tumor response to anti-angiogenic drugs (40, 43).

The endocan level not only is increased in cancers but also its level is elevated during sepsis. During inflammation and sepsis, endothelial dysfunction occurs and some inflammatory mediators that induce endocan expression are elevated. Therefore, endocan level in blood of septic patients could be related to the severity of sepsis and endocan could be considered as a marker of sepsis (6, 44).

Some researchers have studied endocan regulation in diabetic conditions. Abu El-Asrar et al. (45) investigated the expression of endocan in the vitreous fluid and epiretinal membranes from patients with proliferative diabetic retinopathy. Results of the Abu El-Asrar et al. study showed that there was a positive correlation between VEGF and endocan and also endocan expression was elevated in patients with proliferative diabetic retinopathy compared to non-diabetic patients. In another study, Qiu et al. (46) investigated the serum level of endocan in stress hyperglycemia patients with acute ST-segment elevation myocardial infarction (STEMI) and found that endocan level correlated with stress hyperglycemia in patients and was associated with short-term prognosis.

In 2016, a preliminary study was carried out to assess the effect of glycemic regulation on endocan levels in patients with diabetes (47). The results of Arman et al. study revealed that the endocan level was significantly higher in diabetic patients with poor glycemic control. On the other hand, drug treatment and lifestyle change decreased the serum level of endocan in the patients. In a clinical study, Cikrikcioglu et al. (48) evaluated the role of endocan as a marker for diabetic nephropathy and showed that endocan levels were lower in patients with
Chapter 2: Literature review

Macroalbuminuria compared to patients with normoalbuminuria. In addition, they concluded that endocan was produced in the early phase of diabetic nephropathy and its secretion was decreased in advanced nephropathy, thereby endocan could be a marker in the monitoring of the development of diabetic nephropathy.

2.4 Metformin

To date, many studies have investigated the effects of different drug treatments, such as allopurinol, glucagon-like peptide-1, rosiglitazone, dipeptidyl peptidase-4 inhibitor, and sodium glucose co-transporter inhibitors on diabetes-related vascular dysfunction to restore endothelial function in patients with diabetes (49-52).

Metformin, a member of the biguanide class of anti-diabetic drugs, is an antihyperglycemic agent and the first-line therapy for patients with type 2 diabetes mellitus. It inhibits hepatic glucose production and reduces insulin resistance in peripheral tissue leading to enhanced glucose uptake and utilization in skeletal muscle. Reduced levels of circulating glucose and the plasma insulin levels following metformin administration improve long-term glycaemic control and reduce tissue damage in response to hyperglycemia (53). Studies have reported that treatment with metformin reduced the risk of diabetes-related mortality in overweight diabetic patients, and was associated with fewer hypoglycaemic attacks compared to insulin and sulfonylureas (8, 54). The beneficial effects of metformin are mediated through indirect activation of AMPK in target cells (55). AMPK is a cellular energy regulator that is activated by phosphorylation in response to the elevated ratio of AMP/ATP, resulting in protective responses under energy-restricted conditions. Based on evidences, metformin phosphorylates AMPK through its effect on the mitochondria, which is the primary target of the drug. In accordance with the stimulation of AMPK, metformin could also inhibit the mTOR signaling pathway directly or indirectly through the suppression of the PI3K/Akt signaling pathway (8). It has been shown that the mTOR signaling pathway plays a pivotal role in cell growth, regulating endothelial cell functions and behaviour like migration, proliferation, and
vascularization. Activation of the PI3K/Akt/mTOR pathway can increase VEGF secretion. The PI3K/AKT pathway also modulates the expression and production of other factors such as NO and angiopoietins, leading to increased angiogenesis (56).

Metformin has the potential to act via an insulin-like growth factor and epithelial growth factor receptor-2 (HER-2) axis, contributing to aborted angiogenesis (53). On the contrary, Phoenix et al. (57) showed metformin ability to induce VEGF expression and subsequently increased promotion of the angiogenic phenotype and increased tumorigenic progression in the breast cancer model.

Considering different therapeutic approaches in the context of diabetes mellitus, metformin is being used widely, and many authorities indicated the beneficial impact of the drug on vascular pathologies by mechanisms independent from the regulation of blood glucose level (55, 58).
Materials and methods
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3 Materials and methods

Materials and equipments that were used in the present study are listed in Table 3.1.

Table 3.1 Chemicals and equipments

<table>
<thead>
<tr>
<th>Chemicals and equipments</th>
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<td>β-Actin antibody</td>
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3.1 *In vitro* studies

3.1.1 Cell culture and treatment protocol

HUVECs were prepared from Iran National Cell Bank and cultured in 5 mM glucose content Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) as growth supplement and 1% Penicillin/Streptomycin. The culture flasks were incubated in 5% CO₂ at 37°C under a humidified atmosphere. HUVECs were treated with high glucose level (30 mM) for 72 hours to induce the hyperglycemic condition. To exclude the possible effect of osmolarity on HUVECs, 25 mM of Mannitol was used as osmolarity control.

3.1.2 Treatment of the HUVECs with Metformin

To prepare a stock solution, metformin was dissolved in cell culture media and filtered by using 0.22 μm microfilter. In the current experiment, HUVECs
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were incubated with various concentrations of metformin including 2.5, 5, 10, 15, 20, 25, 50, 100, 250, and 500 µM for 72 hours and cell survival was determined by MTT ([3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay. Based on MTT results the 25, 50 and 100 µM doses were selected for the subsequent analysis, which did not have an effect on cell growth. We also treated the HUVECs with 10 µM compound C as an inhibitor of AMPK in the presence and absence of metformin.

3.1.3 MTT assay

Cell viability and cytotoxic effects of high glucose conditions were studied using the MTT assay. Briefly, 1.5×10^4 HUVECs were plated in 96-well plates one day before the treatment. Three days after the incubation with metformin, glucose and compound C, supernatant media were removed and cells exposed to 100 µl of MTT solution (dilution: 2 mg/ml) for 3 hours. Finally, 100 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. The absorbance was measured by a spectrophotometer at a wavelength of 570 nm. Cell viability was expressed as % of the non-treated HUVECs.

3.1.4 In vitro scratch assay

To investigate the effect of metformin on HUVECs migration under high glucose concentration, cell migration was assessed using in vitro scratch assay. Three ml of culture media with a total of 5×10^5 cells were incubated in medium with 0.5% FBS and transferred into each well of 6-well plates. After the formation of a confluent cell monolayer, a sterile yellow tip was used to create a straight line and phosphate-buffered saline (PBS) was used to remove the debris. Cells were imaged at the time of scratch (0 h) and 72 hours by an inverted light microscope and the distance of scratch edges analyzed by using AxioVision software Version Rel 4.8 software package (Carl Zeiss MicroImaging, Inc.). The data were represented as a percentage rate migration to the values from matched control following initial scratching time.
3.1.5 NO measurement

In order to evaluate the NO production, Griess reagent was used. This method is a colorimetric assay to measure the content of nitrite which is the stable form of NO. Griess A solution was prepared by adding 50 mg sulfanilamide in 5 ml of 5% phosphoric acid. Griess B was prepared by dissolving 6.95 mg N-(1-Naphtyl) ethylenediamine.2HCl (=NED) in 5 ml of Milli Q water. 200 μl of each sample was plated in 96 well plates in duplicate and 20 μl Griess A solution was added in each well. After 10 minutes incubation in room temperature, 20 μl Griess B solution was added and the plate incubated for 2 minutes in room temperature and protected from light. The absorbance was read by a microplate reader at 540 nm. The amount of NO was calculated based on the sodium nitrite standard curve and reported as micromole/liter.

3.1.6 ELISA assay

Endocan protein level was analyzed by ELISA. Briefly, a total of 1×10^5 cells/well were cultured in 24-well plates. After 72-hours incubation time, cells were collected and centrifuged at 1500 rpm for 5 minutes. To extract total cell protein, cells re-suspended in 200 μl of RIPA Lysis Buffer and centrifuged at 14000 rpm for 15 minutes at 4°C. Finally, the level of endocan in supernatants was measured using ELISA endocan commercial kit, according to the manufacturer's instructions. We also measured the level of endocan in cell supernatant collected from different groups. The absorbance was measured by a spectrophotometer at 450 nm and the values were expressed in pg/ml.

3.1.7 Real-Time Quantitative PCR of endocan mRNA

To evaluate the mRNA level of endocan, a total of 5 × 10^5 cells were plated in 6-well plates and treated over a period of 72 hours. Total RNA extracted by using an RNA extraction kit according to the manufacturer’s instructions and extracted RNA was quantified by measuring OD (optical density) A260/A280 and A260/230 using Nano Drop. The quality of extracted RNA evaluated using gel electrophoresis by visualizing intact 28srRNA and 18srRNA on a 2.0% agarose
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cDNA synthesis was done via a cDNA synthesis kit. Primers were designed by Oligo 7 software (Table 3.2). Prior to running real-time PCR, conventional PCR was done to find the optimum annealing temperature of primers. Then, to run real-time PCR reaction, a cocktail including target cDNA (1 µl), SYBER Green qPCR Master Mix (7 µl), nuclease free water (4 µl) and specific primer (1 µl with a final concentration of 10 pM) was prepared and loaded on real-time PCR cycler. CT (cycle of threshold) values were extracted using the corresponding software. Expression folding of each sample in relation to control values were calculated using ΔΔCT method, in which ΔCT represents the difference between the CT value of target genes and the CT value of GAPDH, an endogenous housekeeping gene.

Table 3.2 Primer list designed for detection of endocan mRNA expression

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<tr>
<th>Gene</th>
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<th>Product size</th>
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<td>ESM-1</td>
<td>Endothelial Cell Specific Molecule 1; Endocan</td>
<td>F: 5’-AACTTGCTACCGGCACAGTCTCA-3’ R: 5’-CTGGCAGTTGCAGGTCTCTCT-3’</td>
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<td>166 bp</td>
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<tr>
<td>GAPDH</td>
<td>Aging-Associated Gene 9 Protein; Oct1 Co-activator In S Phase, 38 Kd Component</td>
<td>F: 5’-TGACCTCAACTACATGTTTACA-3’ R: 5’-GCTCTGGAAGATGGTGATG-3’</td>
<td>59</td>
<td>129 bp</td>
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</table>

F= Forward; R= Reverse; bp= base pair

3.1.8 Functional analysis of the HUVECs by Dil-Ac-LDL uptake

To investigate the function of endothelial cells, uptake of Ac-LDL, the low-density lipoprotein (LDL), was performed using Dil-Ac-LDL (1, 10-dioctadecyl-3, 3, 30, 30-tetramethyl indocarbocyanine-labeled acetylated low-density lipoprotein) uptake assay. A total of $1 \times 10^3$ cells were plated in 8-well chamber slides. After 72-hours, the supernatant was removed and cells incubated with medium containing Dil-Ac-LDL (10 µg/m) at 37°C for 4 hours. Subsequently, the Dil-Ac-LDL solution was removed and cells washed by PBS.
and fixation in pre-chilled 4% paraformaldehyde solution for 20 minutes were done. 4′, 6-diamidino-2-phenylindole (DAPI) was applied to stain the nuclei. Finally, the slides were examined using an inverted microscope and the images were processed using CellSense software version 1.4.

### 3.1.9 Flow cytometry

In order to detect the distribution of endocan in HUVECs, flow cytometry was used. A total of 5×10⁵ cells were plated in 6-well plates. Briefly, cells were collected and centrifuged at 1500 rpm for 5 minutes 72 hours after treatment, and then washed twice with PBS. Then, the cells were permeabilized with 1% Triton X100 for 30 minutes and blocked by 1% FBS for 1 hour. Primary antibody against endocan was used by incubating cells at 4°C for 1 hour. After washing with PBS, endothelial cells were incubated with a secondary antibody (FITC-conjugated anti-mouse antibody) for 30 minutes at room temperature. Flow cytometry was performed using the FACSCalibur system, and the raw data analyzed with FlowJo software version X.0.7.

### 3.1.10 Western blot analysis

A total of 3×10⁶ cells were plated in culture plates (SPL). In order to extract total protein, endothelial cells were collected after treatment, re-suspended in 200 μl of RIPA Lysis Buffer [50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide] supplemented with protease inhibitor, and kept overnight at 4°C. The next day samples were roughly mixed and supernatants were collected after centrifugation at 14000 rpm. The protein content of each sample was quantified using NanoDrop™ OneC. Protein homogenates were resolved by 10% SDS-Polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane. Nonspecific antibody binding areas were inhibited by 2% bovine serum albumin (BSA; Sigma) in TBST [20 mM Tris-buffered saline (pH=7.5) with 0.1% Tween 20] for 1 hour at room temperature. The membranes were then incubated overnight with specific phospho-AMPKα (Thr172) and
AMPKα antibodies at 4°C. Membranes were washed with TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibodies for 1 hour at room temperature. BM Chemiluminescence kit was used to visualize immunoreactive bands and photographed by Gel Documentation System. The density of each band was calculated using ImageJ software version 1.41o (Wayne Rasband, NIH, USA). For normalization, we measured the β-actin level. The mean phospho-AMPKα/AMPKα ratio was calculated in each group and compared to each other.

3.2 In vivo studies

3.2.1 Animals

BALB/c male mice (35-40 g) were obtained from the Razi Institute. Animals were kept under a 12:12 h light/dark schedule at an ambient temperature of 23±2 °C. All the experiments were carried out according to the guidelines published by the local ethics committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1395.145). The animals were randomly divided into four groups; 1) control mice were injected normal saline intraperitoneally (i.p.), 2) diabetic mice were injected 150 mg/kg Streptozocin i.p. (STZ; dissolved in citrate buffer, pH=4.5), 3) diabetic mice received 50 mg/kg metformin, and 4) diabetic mice received 100 mg/kg metformin. Metformin was used orally for 2 weeks. Seventy-two hours after STZ injection, blood glucose levels were measured using glucometer and the mice with blood glucose level ≥200 mg/dl considered as diabetics. Blood glucose levels were monitored every 3 days up to 2 weeks. After induction of diabetes, the 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 studies by keeping them at -80°C until use.

3.2.2 ELISA assay

The endocan protein level in the serum and tissues was measured by ELISA. In order to extract total protein 5 mg of samples were chopped and
transferred into microtubes including 300 μl of protein lysis buffer (50 mM Tris HCl; 5 mM sodium pyrophosphate; 50 mM NaCl; 1 mM dithiothreitol; 50 mM NaF; 1 mM EDTA; 0.1% w/v SDS; 1% w/w Triton-X100) including protease inhibitor cocktail. The samples were centrifuged at 14000 rpm for 15 minutes at 4°C and the supernatant was separated. The protein content of samples was measured using NanoDrop™ OneC. The level of endocan in 100 μl of each sample was assessed using an ELISA kit according to the manufacturer's instructions. The absorbance was measured by the spectrophotometer at 450 nm and the values were expressed in ng/mg tissue and ng/ml serum. This experiment was performed in triplicate (each in two wells).

3.2.3 Western blotting

To measure the level of endocan in the liver and kidneys 5 mg of samples were chopped and transferred into microtubes including 300 μl of protein Lysis Buffer. Then, the western blot analysis was completed as mentioned in part 3.1.10.

3.2.4 Immunofluorescence (IF) assay

Endocan protein levels were also studied in renal and hepatic tissues by IF assay in 5-μm frozen tissue sections. For this purpose, tissue sections were washed three times with PBS and then permeabilized with 1% Triton X-100 for 15 minutes. Slides were then blocked with 1% BSA for 30 minutes. Finally, primary endocan antibody was used for 1 hour followed by the addition of FITC-conjugated secondary antibody. 1 μg/ml DAPI solution was used to stain the nuclei. In order to image the slides, an inverted microscope was used and data analyzed using CellSense software version 1.4.

3.2.5 Hematoxylin and Eosin (H & E) staining

In order to evaluate the effect of metformin on diabetic-related pathological changes in tissues, samples were fixed in 10% formalin solution and paraffin-embedded blocks were prepared. Tissue samples were cut into 5 μm thick-sections using a microtome and stained with H&E. The existence of hepatic
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and renal tissues injuries was monitored using qualitative analysis. Slides were examined by light microscope and compared to the control.

3.2.6 Immunohistochemical (IHC) analysis

In order to study the vascular density in samples from renal and hepatic tissues, IHC analysis was performed on formalin-fixed and paraffin-embedded sections to monitor the protein content of the Von Willebrand factor (vWF). For this purpose, 5 μm-thick slides were prepared and exposed to a 3% \( \text{H}_2\text{O}_2 \) solution for 30 min. For antigen retrieval, the slides were put in 15 psi (pound-force per square inch) in citrate buffer (pH=6.0) and then incubated with an anti-vWF antibody for 1 hour. Samples were washed twice with PBS and incubated with EnVision+Dual Link System HRP kit solution. 3, 3'-Diaminobenzidine (DAB) was used as the chromogen. Mayer's Hematoxylin solution was used as a counterstain. The vascular intensity was measured in slides in 3 random serial high-power fields (HPF).

3.2.7 Statistical analysis

The results are expressed as mean ± standard deviation (SD). To investigate the significant difference between groups, one-way ANOVA analysis was performed followed by Tukey post hoc test. \( p<0.05 \) was considered to be statistically significant.
Results
4 Results

4.1 Results of in vitro studies

4.1.1 The effects of metformin on the viability of the HUVECs

In order to determine optimum concentrations with no effect on cell viability, the effect of different concentrations of metformin was assessed on HUVECs viability, under the normal concentration of glucose using MTT assay. As indicated in Figure 4.1, metformin reduced the cell viability slightly in a dose-dependent manner which reached a significant level at high concentrations above 100 µM ($p<0.05$). Doses of 25, 50 and 100 µM were selected for subsequent analysis.

![Figure 4.1 Effects of metformin on HUVECs survival after 72 hours (n=3). HUVECs were treated with serial concentrations of metformin. Results are expressed as the mean ± SD of three independent experiments. *$p<0.05$, and ***$p<0.001$.](image)
4.1.2 The effects of metformin on the viability of the HUVECs exposed to high glucose concentration

As demonstrated in Figure 4.2, viability of endothelial cells significantly decreased at 30 mM concentration of glucose compared to normal glucose concentration (5 mM) \( (p<0.05) \). Metformin did not affect the endothelial cells viability both in normal and high glucose conditions. Compound C alone had no action on the high glucose concentration-induced cell death and when used along with metformin caused more anti-proliferative effects \( (p<0.001) \). To rule out the possibility of high concentration of glucose osmotic pressure on cell death, mannitol was used and it was indicated that at 25 mM, as an inert sugar, did not affect endothelial cells survival.

Figure 4.2 Effects of high glucose condition, metformin and compound C on HUVECs survival after 72 hours \( (n=3) \). Results are expressed as the mean ± SD of three independent experiments. \( *p<0.05 \), and \( ***p<0.001 \). (Glu = glucose; Met = metformin; Man= Mannitol; and Com. C = compound C).
4.1.3 The effects of metformin on the migration of the HUVECs

In order to reveal the effect of metformin on endothelial cells migration capacity, an in vitro scratch assay was performed. An inhibitory effect of high concentration of glucose (30 mM) on endothelial lineage migration was found compared to the condition supplemented with normal glucose content of 5 mM after 72 hours (p<0.001; Figure 4.3 A-B). Metformin was found to accelerate endothelial cells migration under the condition containing 30 mM glucose (p<0.001). As indicated in the figure, compound C prohibited cell motility in condition containing 30 mM glucose and metformin (p<0.001). The combination of metformin with compound C increased cell migration rate compared to compound C treated cells in 30 mM glucose concentration (p<0.05).
Figure 4.3 Representative images of HUVECs migration rate evaluated by scratch analysis after 72 hours (A-B). The rate of migration was measured by quantifying the distance that the HUVECs migrate from the edge of the scratch. Results are expressed as the mean ± SD. *p < 0.05, and ***p < 0.001. (n=3) (Glu = glucose; Met = metformin; and Com. C = compound C).
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4.1.4 The effect of metformin on endocan release from the HUVECs

The concentration of endocan in the endothelial cell lysate and the supernatant was measured after 72 hours by ELISA (Figure 4.4 A-B). No statistical differences were observed between groups regarding the endocan level inside the cells. However, there was a trend toward a decrease in the cell lysate concentration of endocan in high glucose-exposed endothelial cells. On the other hand, treatment with metformin increased the level of endocan in the cells exposed to a high concentration of glucose. It seemed that the glucose-induced increase of endocan in the cell lysate was blocked by compound C. The concentration of endocan in the supernatant was measured and results showed that the ratio of the supernatant level of endocan to cell lysate counterpart was higher in the 30 mM glucose concentration and metformin-treated groups compared to 5 mM glucose concentration. These results confirmed that as compared to the control condition, the endocan release was increased in the endothelial cells exposed to high glucose conditions alone and high glucose conditions plus metformin. Compound C can prohibit the synthesis of endocan in the endothelial lineage.
Figure 4.4 The level of endocan protein in the lysate (A) and supernatant (B) of HUVECs exposed to experimental condition. Results are expressed as the mean ± SD. (Glu = glucose; Met = metformin; and Com. C = compound C).
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4.1.5 Metformin effect on LDL uptake in the HUVECs

To study the endothelial cells functional behaviour, we analyzed the uptake of Dil-Ac-LDL, showing the lipoprotein lipase (LPL) activity (Figure 4.5 A-B). The results showed the ability of endothelial cells in the uptaking of Dil-Ac-LDL at a normal concentration of glucose and this capacity was diminished following 72-hours incubation of HUVECs with 30 mM glucose ($p<0.001$). Interestingly, treatment of HUVECs exposed to 30 mM glucose with metformin improved LDL uptake and LPL activity ($p<0.01$). Compound C had the potential to suppress the uptake of Dil-Ac-LDL in HUVECs both in normal and high glucose concentrations, confirming the irreversible inhibition of AMPK. Nevertheless, co-treatment of endothelial cells with metformin and compound C caused to reduce in the extent of uptake compared to metformin alone in high glucose concentration ($p<0.05$). These data show that the addition of compound C to metformin-exposed cells under high glucose conditions could decrease LPL activity.
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Figure 4.5 LDL uptake analyses by Dil-Ac-LDL assay (A-B). Nuclei were stained with DAPI. Results are expressed as the mean ± SD. *p<0.05, **p<0.01, and ***p<0.001 (n=3). (Glu = glucose; Met = metformin; Com. C = compound C; and HPF= high power field).
4.1.6  **Metformin effect on the synthesis of endocan in the HUVECs**

The results of flow cytometry analysis showed that endothelial cells exposed to 30 mM glucose had a very low but not significant propensity to increase in the expression of endocan, in contrast to the cells exposed to a normal concentration of glucose (**Figure 4.6 A-B**). Metformin increased the expression of endocan significantly in the high glucose content compared to the control metformin group ($p<0.05$). Treatment with compound C did not yield any significant results compared to cells from control and high glucose concentration groups. Metformin administration decreased the inhibitory effect of compound C on the endocan level after 72 hours. It could be considered that metformin administration causes the induction of endocan under diabetic conditions.
Figure 4.6 Flow cytometric-based analysis of endocan content in HUVECs exposed to 5 and 30 mM glucose (A-B). Results are expressed as the mean ± SD. *p<0.05. (Glu = glucose; Met = metformin; and Com. C = compound C).
4.1.7 Effect of metformin on endocan expression in the HUVECs

The quality of extracted RNA was confirmed by 28s and 18s rRNA gel electrophoresis (Figure 4.7A). Conventional PCR analyses showed that the best annealing temperature was 62°C (Figure 4.7B). Real-time PCR analysis showed that high glucose conditions caused the 3-fold expression of endocan compared to the control cells ($p<0.05$) (Figure 4.7C). Treatment with metformin in high glucose condition potentiated the ability of HUVECs to up-regulate endocan expression compared to metformin treated cells in normal glucose condition ($p<0.01$). Treatment with compound C reduced the expression of endocan in high glucose concentration alone or high glucose concentration supplemented with metformin ($p<0.01$), compared to the control. It seems that the addition of metformin to compound C-treated endothelial cells reduced the inhibitory effect of compound C on the endocan transcription level.
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Figure 4.7 Results of gene expression analysis. Quality control of extracted RNA assessed by visualizing 28srRNA and 18srRNA (A). Optimization of annealing temperature of primers based on conventional PCR analyses (B) (a=62°C, b=63°C). Relative mRNA expression of endocan gene in endothelial cells after 72 hours (C). Expression levels in non-treated cells (control) were set to 1, and all other values were compared to the control. Results are expressed as the mean ± SD. *p<0.05, and **p<0.01. (Glu = glucose; Met = metformin; and Com. C = compound C).
4.1.8 Effect of metformin on the level of NO in HUVECs

Griess test results revealed that the level of NO was increased in the endothelial cells exposed to metformin compared to the control cells ($p<0.001$; Figure 4.8). Treatment with metformin increased the level of NO in the HUVECs exposed to high glucose content compared to the control-matched group ($p<0.001$). Compound C was shown to reduce cell NO production significantly both in normal and high glucose conditions as compared with endothelial cells from the groups treated with metformin ($p<0.01$). Based on the results, we found a neutral effect of metformin on NO levels in endothelial cells pre-treated with compound C ($p>0.05$).

![Figure 4.8 Detecting NO level in HUVECs by Griess test. Results are expressed as the mean ± SD. **$p<0.01$, and ***$p<0.001$. (Glu = glucose; Met = metformin; and Com. C = compound C).](image-url)
4.1.9 AMPK phosphorylation in the HUVECs treated metformin

AMPK phosphorylation was increased under high glucose conditions, but it did not reach significant levels ($p>0.05$; Figure 4.9). Treatment with metformin *per se* induced the phosphorylation of AMPK especially in endothelial cells exposed to 30 mM glucose but the values were not statistically significant ($p>0.05$). It seems that compound C was able to blunt the stimulatory effect of metformin on AMPK phosphorylation. Addition of metformin to compound C-exposed cells increased AMPK phosphorylation under high glucose conditions but these changes were not significant.

Figure 4.9 Western blot analyses of AMPK, and p-AMPK in HUVECs exposed to 5 and 30 mM glucose. Results are expressed as the mean ± SD. Glu = glucose; Met = metformin; and Com. C = compound C.)
4.2 Results of in vivo studies

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

The blood glucose level in STZ-induced diabetic mice was significantly higher than mice in the control group ($p<0.001$; Figure 4.10). Treatment of diabetic mice with 50 mg/kg metformin caused a significant decrease in blood glucose levels compared to diabetic mice ($p<0.01$). STZ injection caused a significant reduction of the body weight in animals ($p<0.001$) and administration of 50 mg/kg metformin significantly decreased the detrimental effect of STZ injection ($p<0.05$). These differences were not significant in mice treated with 100 mg/kg metformin.
Figure 4.10 Body weight and blood glucose level variation in different experimental groups during two weeks. Results are expressed as the mean ± SEM. Symbol * indicates statistical significant difference compared to control group and # compared to diabetic group (p<0.05).
4.2.2 Effect of metformin on the endocan serum level and content in the renal and hepatic tissues

The level of endocan was measured by ELISA (Figure 4.11). Results showed that the endocan level in the kidneys and liver of diabetic mice was higher compared to the control but the differences between groups were not statistically significant ($p>0.05$). However, the level of endocan in the renal and hepatic tissues of metformin-treated diabetic mice was significantly higher compared to control and diabetic groups ($p<0.05$). In this study, we found a non-significant difference regarding serum endocan levels between groups ($p>0.05$).

![Figure 4.11](image)

Figure 4.11 The level of endocan protein in the blood serum and lysate of renal and hepatic tissues. Results are expressed as the mean ± SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).
4.2.3 AMPK phosphorylation in the renal and hepatic tissues

Based on the results, it was indicated that AMPK activation was decreased both in the kidneys and liver of diabetic mice which statistically attained a significant level of \( p < 0.05 \) in the kidney (Figure 4.12). Administration of metformin at doses 50 and 100 mg/kg for two weeks in diabetic mice significantly induced the phosphorylation of AMPK only in kidneys \( (p < 0.01) \). It seems that endocan biological activity is related to tissue type during diabetic changes.

Figure 4.12 Western blot analyses 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 ± SD. **\( p < 0.01 \). (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg).
4.2.4 Endocan localization in the renal and hepatic tissues

Immunofluorescence imaging showed the existence of endocan protein in the renal and hepatic tissues of control mice (Figure 4.13 and 4.14). There were no endocan-positive cells in the liver and kidney tissues of diabetic animals, indicating the decrease of endocan by endothelial cells following diabetes. The results showed that treatment with metformin potentiated endothelial cells to retrieve endocan synthesis in the kidneys of diabetic mice.
Figure 4.13 Immunofluorescence staining of the hepatic tissue 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 4.14 Immunofluorescence staining of the renal tissue 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).
4.2.5 Effect of metformin on the histology of renal and hepatic tissues

Histological analysis revealed the injury of kidneys and liver in diabetic mice (Figure 4.15). 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 epithelial cells atrophy compared to the control (Figure 4.15 A). The results showed that the diabetic-related damages were reduced in mice received metformin 50 and 100 mg/kg. It seems that the anti-diabetic effects of 50 mg/kg metformin in mice were more than that of mice given 100 mg/kg metformin. Monitoring the hepatic microstructure revealed the interruption of liver sinusoidal integrity and necrosis of hepatic cells with focal accumulation of fibrin-like depositions (Figure 4.15 B). Similar to kidneys, the intensity of diabetic changes and related injuries were reduced in groups received metformin. The detrimental effects of diabetes were less in mice treated with 50 mg/kg metformin. These data demonstrated that the administration of metformin could alleviate diabetic-derived kidneys and liver injuries.
Figure 4.15 Representative imaging of renal and hepatic tissues stained with H&E from different experimental groups (A-B). 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).
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4.2.6 vWF localization in the renal and hepatic tissues

To investigate the impact of the diabetic condition on the vascular structure in the liver and kidneys, vWF staining was performed (Figure 4.16). Results of the analysis showed that the intensity of cells encoding the vWF factor was decreased in the tissues of diabetic animals compared to the control group ($p<0.001$). In the diabetic liver, the endothelial cells were detached and shed to the lumen of central veins. In samples obtained from the control mice, vWF positive cells were found inside Bowman's capsules microvascular system while these features reduced intensively in diabetic mice. Treatment diabetic mice with metformin (50 and 100 mg/kg) reversed the inhibitory effects of hyperglycemic condition on the expression of vWF in kidneys and the protein levels of vWF in the liver showed a similar trend as seen in kidneys ($p<0.001$). The administration of 50 mg/kg metformin showed a superior effect to blunt hyperglycemic detrimental effects on the number of hepatic vWF positive cells ($p<0.001$).
Figure 4.16 Immunohistochemical staining of the renal and hepatic tissues. In diabetic kidneys, the intensity and number of vWF positive cells reduced. By using metformin, the Bowman’s 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 ± SD. ***p<0.001. Red arrows: vWF positive cells. (Met 50 = Metformin 50 mg/kg; Met 100 = Metformin 100 mg/kg, and HPF= high power field).
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5 Discussion

As a chronic disease, diabetes prevalence is growing globally and associated with the increase in mortality and morbidity and also with a financial burden on the health care system (11). It has been demonstrated that endothelial cells function is affected in patients with diabetes mellitus and diabetes-related endothelial dysfunction is the most important cause of vascular complications in patients with diabetes (9). In the last decade, considerable attention has been paid to evaluate endocan changes as a biomarker of endothelial cell functions and angiogenesis during the onset of various diseases. To alleviate diabetes-related pathologies, various drugs from different types of pharmacological classes have been studied to improve endothelial cell functions in diabetic subjects.

In the present study, the level of endocan, synthesized or released by HUVECs, was monitored as a marker of endothelial cells activation, under high glucose content and the modulatory effect of metformin on endocan was investigated in vitro. Also, the effect of metformin was evaluated on serum, hepatic and renal contents of endocan in STZ-induced diabetic mice model.

The results of the current study showed that the exposure of HUVECs to high glucose concentration caused a significant reduction in cell viability. Previous studies have also shown that high glucose content promotes cell cytotoxicity through the induction of oxidative stress mechanisms (59, 60). As reported previously by Arunachalam et al. (61), and Detaille et al. (62), the results of the present study confirmed that metformin has potential to improve high glucose-induced endothelial cell death. The supplement of compound C, an AMPK inhibitor, to high glucose concentration-exposed HUVECs exacerbated cytotoxic effects showing the essential role of AMPK and related signaling pathway in the diabetic condition.

As a common cellular process, angiogenesis takes part in pathological and physiological phenomena and seems to be modulated after the onset of diabetes mellitus. In diabetic patients insufficient angiogenesis contributes to a reduced
wound healing and diabetic foot ulcer whereas, excessive aberrant angiogenesis could lead to diabetic induced retinal capillary occlusion and nephropathy (9). In the present study results of the scratch assay showed that exposing of HUVECs to high glucose concentration for 72 hours reduced the migration capacity of the cells which is consistent with Hamuro et al. (63) and Yu et al. (64) reports. Previously, it has been demonstrated that treatment of diabetic endothelial lineage with metformin improved the wound healing process and angiogenic function of endothelial progenitor cells via activating AMPK/eNOS pathway in the diabetic mouse model (64). The results indicated that metformin had a superior effect on improving the migration of HUVECs under high glucose concentration. In addition, supplement of compound C for inhibition of AMPK activation remarkably decreased the stimulatory effect of metformin on endothelial cell migration. These data show that mTOR axis takes part in regulating endothelial cells migration by engaging AMPK and mTOR cross-link (64).

The results of the ELISA assay showed that high glucose concentration diminished the potency of endothelial cells to store endocan inside cells while the release of endocan to the supernatant medium was increased. After treatment HUVECs with metformin under high glucose condition, a measurable but not significant change in cell ability to secrete endocan was found. Consistent with our results, it seems that metformin could increase the cellular distribution and secretion of endocan by the modulation of the AMPK signaling pathway. The administration of metformin after the inhibition of AMPK phosphorylation by compound C could partly, but not completely, increase the synthesis of endocan. Lassalle et al. study (37) regarding the endocan expression showed that the addition of TNF-α or IL-1β caused a time-dependent upregulation of endocan mRNA. Also, their study showed that the combination of IFN-γ with TNF-α inhibited the stimulatory effects of TNF-α on endocan mRNA level. These data possibly demonstrate the dynamic of endocan under the pro-inflammatory conditions in addition to metabolic disorders. It seems that the induction of VEGF signaling pathways simultaneously with pro-inflammatory response exacerbates the synthesis and secretion of endocan in the vascular tissue (45). Rennel et al.
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(39) identified that VEGF induction of endocan expression in endothelial cells was regulated via the PI3K pathway. Besides, Abid et al. study (38) showed that VEGF-mediated induction of endocan was positively and negatively regulated by PKC/NFκB and PI3K/Akt signaling pathways, respectively. Based on the results, it seems that metformin could not regulate the secretion of endocan under a diabetic condition, indicating the participation of other signaling pathways in the secretion of endocan during the physiological and pathological conditions.

One of the most critical endothelial cell deficiencies seen during diabetes is the reduction of LPL activity. It has been indicated that LPL deficiency is related to insulin resistance in diabetic patients (65). The results of LDL uptake analyses revealed a significant protective role of metformin in the activation of LPL under high glucose conditions by the increase of fluorescent Ac-LDL uptake inside endothelial cells. Consistent with these data, the activation of LPL activity and expression could be related to the increase of p-AMPK/AMPK ratio post-metformin administration (65, 66). Moreover, the administration of compound C was found to diminish the effects of metformin.

NO is one of the most important endothelium-derived molecules that could trigger endothelial bioactivity (18). Based on results endogenous p-AMPK/AMPK complex was found to induce NO production in metformin-treated HUVECs. In support of this claim, the presence of compound C faint the positive effects of metformin on AMPK phosphorylation and NO production. It has been suggested that the increase of NO not only could induce the angiogenic potential of endothelial cells but also prevents the generation of harmful oxygen radicals (67). It seems that the induction of NO production by metformin under normal and high glucose conditions is at the range of physiological concentration.

STZ-induced diabetes model is a common method to induce diabetes in animals and leads to body weight loss in animals (68). In the current study, the body weight of diabetic animals was significantly lower than the healthy mice. As expected, the blood glucose level of metformin-treated groups was lower than the non-treated diabetic group. The results revealed that 50 mg/kg metformin exerted a superior effect compared to 100 mg/kg metformin in the case of lowering blood
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glucose level and body weight retrieval. On the contrary, Zhou et al. (69) found statistically non-significant changes in body weight of diabetic mice treated with 200 mg/kg metformin after 8 weeks follow up. Han and colleagues (70) also reported a slight but non-significant increase in weight of diabetic mice treated with 250 mg/kg metformin for 2 weeks. Some authors’ study showed a dose-dependent action of metformin in reducing blood glucose level in diabetic mice (71, 72). In this study, low doses of metformin for 2 weeks were administered in diabetic mice that yielded prominent weight changes compared to the previous experiments. Martin-Montalvo et al. study (73) indicated that the high doses of metformin had no more beneficial effects and not only shortened the lifespan of mice but also induced oxidative stress, lactic acidosis and renal failure. It seems that, the less effectiveness of high doses of metformin may be as a result of lactic acidosis and inability to activate related effectors.

Diabetes is associated with different types of vascular complications and intensive blood glucose control is a key point in managing these types of vascular events (74). It has been previously shown that the normal endothelial cells function and vascularization are impaired due to high blood glucose levels (9). Defective angiogenesis in diabetic patients causes important complications affecting the life quality and in some critical conditions contributes to mortality (26). Since endocan participates in the progression of cancer and induces vascularization it can be considered as a marker of angiogenesis and it seems that there might be a close relationship between endocan and diabetes-related vascular complications (5, 75). In the present study, we found that metformin had a marked effect on the endocan level in the kidneys of diabetic mice where metformin had the greatest effect on phosphorylation of AMPK. However, there are some paradoxes about metformin function in the field of angiogenesis. Previously, it has been demonstrated that metformin increased the proliferation of vascular endothelial cells and further promoted the angiogenic potential of HUVECs (76). Additionally, metformin can restore vascular compliance and repair and prevent of endothelium damage caused in diabetic foot ulcer and nephropathy. Inconsistent with our data, Dallaglio, and co-worker (77) found that metformin
had a modulatory effect on HUVECs pro-angiogenic activity in a time-dependent manner. Their study revealed that short-term administration of 2 mg/mice/day metformin, induced angiogenesis process by promoting VEGF and related signaling pathways in diabetic mice.

As an AMPK activator, metformin could suppress the mTORC1 signaling pathway and thereby affect some important physiological pathways including protein synthesis (78). Since mTORC1 induces angiogenesis through regulating hypoxia-inducible factor 1α (HIF1α), it seems that metformin should inhibit angiogenesis (54, 78). Also, our results indicated that metformin which can decrease the vascular insufficiencies in diabetic kidneys, significantly elevated the level of endocan in tissues. It seems that metformin probably produces at least part of its protective effect by endocan. According to histological findings, there was a reduction in the number of vWF positive cells inside the Bowman's capsules in diabetic kidneys because of the accelerated and uncontrolled proliferation of the mesangial matrix that leads to glomerular capillaries occlusion and regression (79). Peairs et al. (80) study showed that metformin, via activation of the AMPK signaling pathway, decreased the regression of glomerular capillaries in response to the decreased proliferation of the mesangial matrix. In the current study, metformin reduced diabetes-related pathological injuries due to the regulation of angiogenesis and endocan levels.

Roudnicki et al. (81) found a close association between the dynamics of endocan and vWF levels in human endothelial cells. Their study showed that the suppression of endocan by siRNA decreased the endothelial vWF and phosphorylation of VEGF receptor 2 which regulates the angiogenic potential of endothelial lineage. Notably, 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 (81). Our study confirmed the ability of metformin to increase endocan and vWF levels in kidneys of diabetic mice. Consistent with our data, Dallaglio et al. (77) found the positive effect of metformin on VEGF synthesis in HUVECs after 24 hour.
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Conclusion

We noted that the administration of metformin possibly promotes endothelial cells’ bioactivity through engaging the AMPK signaling pathway. In particular, some beneficial effects of metformin on angiogenesis could be related to the regulation of endocan in diabetic vascular cells. 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 vascular system. One possible mechanism for the therapeutic effect of metformin in diabetic subjects correlates with the modulation of the p-AMPK/ AMPK axis. It seems that the results of this study could shed light on the protective effects of metformin on angiogenesis under diabetic conditions through the modulation of endocan. More investigation is needed to find the relationship between endocan and metformin to predict angiogenic potential during physiological and pathological conditions.
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Suggestions

1. It seems that the use of endocan peptide as a positive control could reflect the modulatory effects of metformin on endothelial lineage after exposure to 30 mM glucose.

2. The examination of metformin on the endothelial lineage from different tissues in particular, retinal vascular cells is helpful to show tissue-dependent differences in endocan biogenesis during the diabetic changes and modulation by metformin.

3. In the in vivo 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 two weeks.

4. Monitoring the diabetic changes over a long period would be beneficial to address the metformin effect on endocan dynamic during the diabetic condition.

5. Precise and sophisticated experiments are needed to address the exact modulatory effect of metformin and relevant signaling pathway/s participated in the endocan biogenesis under the high glucose condition.
References

خلاصه فارسی

1. مقدمه و مروری بر متن

سلول های اندولیال از اجزای مهم سیستم عروقی بوده و در پروز یکسره و قبیع
فیزیولوژیک و بیانویژیولوژیک نقش اساسی دارند (1). گلزنشانی گلکوز در خون به طور
مزمن با افزایش خطر وقوع بیماری های قلبی عروقی همرا پیدا و اختلالات اندولیالی ناشی از
هایپرگلیکمی منجر به پروز بیماری عروقی به از جمله آرواسکلوزوز و عدم توانایی صحیح
اندولیوم در حفظ هموستان عروقی می‌شود به همین علت به نظر می‌رسد که اندولیوم نقش
کلیدی در حفظ سلامتی عروقی دارد (2). مطالعات نشان داده است که اختلالات عروقی ناشی از
بیماری دیابت به دو دسته کلی قابل تقسیم است دسته اول اختلالاتی هستند که به علت رگ
زاپای بیش از اندازه اتفاق می‌افتد از جمله این اختلالات میتوان به رتینوپاتی و نفروپاتی اشاره
کرد دسته دوم اختلالات به علت رگ زاپای ناپاکی رخ می‌دهند و مثال بارز آن‌ها اختلال در
پهپاد زخم پای دیابتیک است (3).

اندولیوم عروقی توسط لایه ای تحت عنوان گلیکوکالیکس پوشیده شده است و این لایه از
پروتئین‌ها و گلیکوپروتئین‌ها تشکیل یافته است. پروتئین‌ها ماکرومولولکول‌های
پیچیده ای هستند که در سطح سلول‌ها، ماتریکس خارج سلولی احاطه کننده سلول‌ها و
همچنین در مایعات بدن حضور دارند. مطالعات نشان داده است که این ماکرومولولکول‌ها در
تنظیم فعالیت‌هایی مثل تماز، مهاجرت سلول‌ها و رگ زایی نقش اساسی دارند (4).

اندوزنه‌کننده‌ی پروتئین‌ها درمانان سولفات‌های است که از 165 اسید آمید تشکیل یافته و از
سلول‌های اندولیال ترشح می‌شود برخی مطالعات نشان داده‌اند که تولید اندوزنه‌کننده در برخی
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از سرطان‌ها مثل سرطان ریه، مغز، روده، کبد و کلیه افزایش می‌یابند، در نتیجه میتوان انوکوان را به عنوان شناختی از وقوع سرطان در نظر گرفت مفادی بالای این پروتوتیپ‌ها با وقوع معنی‌دار و افزایش مرگ و میر در برخی از سرطان‌ها از جمله سرطان سینه و ری به رابطه مستقیم دارد (5). مطالعات اخیر ثابت کرده‌اند که انوکوان در وقوع یکسپری از تعاملات مهم مولکولی مثل تکثیرسلولی، اتصالات سلولی، مهاجرت و رگ زایی نقش اساسی دارد (6).

متفورمین‌یکی از مهم‌ترین داروهای ضد دیابت بوده و مطالعات نشان داده که در بهبود بیماری‌های عروقی نقش مهمی دارد (7). مهم‌ترین هدف داخل سلولی متفورمین است که نقش مهمی در محافظت از سلولی در هنگام محدود شدن ذخایر انرژی دارد پروتئین‌ها، آنزیم سرین ترنتونین باعث افزایش می‌شود. در داخل سلولی کم می‌شود و نسبت AMP/ATP فعال شدن مسیرهای مصرف انرژی افزایش می‌یابد. در این افزایش HIF-1α نقش دارد که از طریق مهر HIF-1α در واحد پروتئین mTORC1 و سال خاصی این بیماری همچنین متفورمین که در تکثیر، رشد و رگ زایی نقش مهمی ایفا کند (8).

با توجه به اهمیت اختلالات آنزیم‌پذیری در بیماری دیابت؛ نقش انوکوان در این اختلالات و نیز تأثیر مسیری در جلوگیری از این اختلالات هنوز شناخته نشده است. هدف این مطالعه بررسی نقش انوکوان در رگ زایی و القای مرگ و آسیب سلولی ناشی از غلظت بالای گلوکز در سلول‌های اندوئدین و اثرات انگیژس و همچنین مطالعه اثر تحریک مسیر AMPK توسط متفورمین در نقش انوکوان بود.
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2. مواد و روش‌ها

2.1. روش کشت سلول و درمان با متغورمین

سلول‌های اندوتلیال در محیط کشت حاوا (FBS) و در غلظت 5 mM گلوکز کشت داده شده و در غلظت 5% CO2 در دمای 37 درجه سانتی‌گراد نگهداری شدند. در ادامه به منظور ایجاد شرایط دیابتی، سلول‌ها به مدت 72 ساعت تحت غلظت 30 mM گلوکز قرار گرفتند. به منظور بررسی اثرات متغورمین غلظت‌های سریالی دارو (500-2/5 میکرومولار) به روش‌های مذکور اضافه شد.

2.2. روش (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

جوهت بررسی زنده‌ماندن سلول‌ها به سuliar مطالعه محلول MTT به سلول MTT جهت بررسی زنده‌ماندن سلول‌ها. 72 ساعت بعد از شروع مطالعه محلول ha اضافه شد. بعد از 3 ساعت انکوباسیون در دمای 37 درجه سانتی‌گراد محلول اضافه شده و جذب نوری توسط استکتروفتوپتروس در 570nm اندازه‌گیری شد.

2.3. بررسی مهاجرت سلولی از طریق مدل النیم زخم

سلول‌ها در پلیت‌های کشت داده شده و بعد از حصول تراکم 100% توسط سر سمال phosphate-buffered saline (PBS) زرد استریل بر روی سلول‌ها خراشی ایجاد شد. بعد از شستشو با سلول‌ها با گلوکز، متغورمین و compound C درمان شدند. بعد از 72 ساعت، میزان مهاجرت سلولی در محل ایجاد خرائش بعد از عکس‌برداری از طریق نرم افزار AxioVision Version Rel 4.8 برآورد شد.

2.4. اندازه‌گیری سطح Nitric Oxide (NO)
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به منظور اندوزه گیری نیتريک اکساید از روش Griess که بر مبنای رنگ شناختی باشد، استفاده شد. به طور خلاصه محیط کشت رویی سلول ها بعد از گذشته 72 ساعت جمع آوری شده و به ۲۰۰ میکرولیتر محلول Griess B و Griess A اضافه شد. جذب نوري توسط اسکیتروفومتر در نرخ ۵۴۰ nm خوادن شده و به صورت micromole/liter تعریف گردید.

گزارش شد.

25. اندوزه گیری پروتئین انوکنان

اندوزه گیری مقدار پروتئین انوکنان در محلول رویی سلول ها و همچنین محلول حاصل از لیز کردن سلول ها به روش ELISA و توسط کیت مربوطه طبق پروتوکل استاندارد انجام شد. جذب نوري توسط اسکیتروفومتر اندوزه گیری شده و مقدار پروتئین به صورت pg/ml گزارش شد.

26. بررسی بیان زن انوکدان

به منظور بررسی بیان mRNA مربوط به انوکان از روش real-time PCR استفاده شد. در این روش ۷۲ ساعت بعد از شروع آزمایش سلول ها جمع آوری شده و ها توسط کیت مربوطه استخراج گردید. کیفیت و مقادیری استخراج شده از طریق بررسی RNA و اندازه گیری توسط نانودراب مورد ارزیابی قرار گرفتند. در ادامه به RNA cDNA تبدیل گردید و به منظور انجام واکنش PCR برای‌هار مورد استفاده قرار گرفت. در ادامه به منظور انجام واکنش cDNA و در ادامه به منظور انجام واکنش real-time PCR برای‌هار مورد استفاده قرار گرفت. در ادامه به منظور انجام واکنش واکنش برای‌هار مربوط به هر نمونه، سایبرگرین، پرایمرهای مربوط به زن هدف و آپ در یک میکروتیوب ریخته شدن نا مورد واکنش قرار گیرند. به منظور آنالیز داده های حاصل از انجام واکنش از روش ΔΔCT استفاده شد.
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27. اندازه‌گیری برداشت

اندازه‌گیری برداشت یک indocarbocyanine-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) استفاده می‌شود. منظور بررسی عملکرد سلول‌های اندوتنیال از روش برداشت استفاده می‌شود. در این روش بعد از 72 ساعت از شروع آزمایش، سلول‌ها به مدت 4 ساعت در معرض قرار گرفته، سپس محلول روبی جمع آوری شده و سلول‌ها توسط Dil-Ac-LDL پارافرمالدهید تنبیت شدند. در نهایت به منظور بررسی سلول‌ها از میکروسکوب inversion استفاده شد و عکس های مربوطه توسط نرم افزار CellSense version 1.4 انجام شد.

28. روش فلوسیتومتری

به منظور بررسی نحوه توزیع پروتئین اندوکان در سلول‌ها از روش فلوسیتومتری استفاده می‌شود. شدت سلول‌ها بعد از جمع آوری از پلیت‌های کشت به مدت 30 دقیقه در معرض PBS و به مدت یک ساعت در مواجعه با X100% FBS قرار گرفته و بعد از شستشو با آنتی بادی اولیه اندوکان و سپس آنتی‌بادی ثانویه مربوطه به سلول‌ها اضافه شدند. روش توسط روش توسط FlowJo version X.0.7 انجام شد و داده‌های حاصله توسط نرم افزار بررسی شدند.

29. روش وسترن بلات

چهار اندازه‌گیری میزان فسفوتراکسیون AMPK از روش وسترن بلات استفاده شده در این روش پروتئین‌های استخراج شده از سلول‌ها توسط زلیا آگریلامید جداسازی شده. سپس محل bovine serum albumin پروتئین‌های موجود در زل به غشاء متنقل شده و توسط اتصالات غیر اختصاصی آنتی‌بادی پوششی داده شد. در ادامه غشاء حاوی پروتئین در مواجعه با آنتی‌بادی اولیه و سپس ثانویه قرار گرفت. به منظور آشکار سازی باندهای مربوط به پروتئین
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استفاده شد و باندهای حاصله توسط Gel Documentation System از p-AMPK و AMPK مورد ارزیابی قرار گرفتند. نرم افزار 1.41o ImageJ version 1.41o مورد افزایش و باندهای حاصله توسط نرم افزار ImageJ version 1.41o مورد ارزیابی قرار گرفتند.

### 2.10 مدل اتقان دیابت

موش های سوري نر (40-35 گرم) به 4 گروه به شرح زیر تقسیم بندی شدند: (1) گروه کنترل، دریافت کننده نرمال سالین، (2) گروه دیابتیک، دریافت کننده استرسیوزین به صورت تزریق صفا، (3) گروه های دیابتیک تحت درمان با متفورمین هفتاد و 100 mg/kg متفورمین، هفتاد و دو ساعت بعد از تزریق استرسیوزین گلوكوز خون حیوانات اندازه گیری شده و مقدار بالاتر از 200 mg/dl به عنوان دیابتی در نظر گرفته شد. بعد از اینجا دیابت گروه های تحت درمان با متفورمین به مدت دو هفته دارو را به صورت خوراکی دریافت کردند. بعد از بایان آزمایش موش ها کشته شدند و نمونه خون و بافت های کبد و کلیه جهت انجم آزمایشات بعدی جداسازی و در دمای 80 درجه سانتیگراد تغذیه شدند.

### 2.11 اندازه گیری پروتئین اندرکاوان

اندازه گیری پروتئین اندرکاوان در سرم و بافت های کبد و کلیه انجام شد. به منظور اندازه گیری پروتئین در بافت های مذکور ابتدا آن ها در معرض محلول لیزری کننده قرار گرفتند و بعد از انجم سانتریفیوز محلول رژیم جمع آوری شده و مقدار پروتئین موجود در آن به روش و طبق پروتوكول استاندارد کیت مربوطه اندازه گیری شد. جذب نوری نمونه ها و توسط ELISA اسپکتروفیتوتر در طول 450 nm تعیین شد و مقدار پروتئین به صورت ng/ml گزارش گردید.

مقدار پروتئین اندرکاوان در بافت ها به روش Immunofluorescence (IF) کیت مربوطه اندازه گیری نیز انجام شد. شد و بعد از تهیه مقاطع بافتی به روش مقاطع بافتی منجمد (frozen tissue section)، اسلایدها در معرض آنتی بادی اولیه اندرکاوان و سپس آنتی بادی ثانویه قرار گرفتند به منظور رنگ
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آمیپی هسته‌ها از 6-diamidino-2-phenylindole (DAPI) استفاده شد. عکس‌های همبند نیز توسط نرم‌افزار invert از میکروسکوپ اریزیبای قرار گرفتند.

1.4 مورد از این ثابت شد.

Hematoxylin- Eosin رنگ آمیپی

به منظور مطالعه اثرات متغیرهای روزی تغییرات پاتولوژیک، مورد به ترتیب استفاده از پسینه و توسط خاص Eosin و Hematoxylin رنگ آمیپی شدند و در نهایت اسلاید‌های حاصله توسط میکروسکوپ نوری مورد بررسی قرار گرفتند.

2.2 آنالیز ایمونوهیستوشیمی‌ای (Immunohistochemical)

جهت دانستن عروقی روش Immunohistochemical (IHC) به منظور تغییرات شده با فرمولین سه به ترتیب به شکلی که پسینه، Trichromatic 3, 5 EnVision+Dual Link System HRP kit توسط باور فسفات با نشان داده شده و 3 دیامینوبنزنیدین به عنوان ماده رنگ زا استفاده شد. در نهایت دانستن عروق در سه منطقه مختلف از هر اسلاید مورد بررسی قرار گرفت.

2.3 تجزیه و تحلیل آماری داده‌ها

One way ANOVA (آنالیز تجزیه و تحلیل آماری) به منظور دستیابی به شیوه‌بندی Mean ± SD داده‌ها به‌صورت (انالیز واریانس یکطرفه) به همراه تست تعقیبی باینترین تمايز مورد استفاده قرار گرفت و اختلاف معنی‌داری در تلخی گردید.
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3. نتایج

مطالعه غلظت‌های مختلف متغیرومین بر روی میزان زنده مانی سلول‌های اندوتوتیلیال نشان داد که این دارو در غلظت‌های بالایی آ100 میکونیده به صورت واپسینه به دوز و به طور معنی‌داری دار زنده مانی سلول‌ها را کاهش دهد (p<0.05) بر طبق نتایج حاصل، غلظت 10 mM که تأثیری روی زنده مانی سلول‌ها نداشت، برای ادامه کار برگردیده شد.

نتایج این مطالعه نشان داد که گلکوز با غلظت 30 mM می‌تواند به طور معنی‌داری در مقایسه با غلظت‌های نرمال‌‌گلکوز (5 mM) و میر سلول‌های اندوتوتیلیال را افزایش دهد (p<0.05) به منظور بررسی اثرات ایمنی‌نوعی حاصل از غلظت بالایی گلکوز از مانیتول به عنوان کنترل استفاده شد و نتایج نشان دهنده عدم تأثیر مانیتول بر روی زنده مانی سلول‌ها بود.

جهت بررسی میزان و نحوه اثر متغیرومین بر روی مهار جر و سلول‌های اندوتوتیلیال از روش خراش استفاده شد. نتایج حاصل نشان داد که گلکوز با غلظت 30 mM به طور معنی‌داری دار باعث کاهش قدرت مهار جر و سلول‌های اندوتوتیلیال در مقایسه با غلظت‌های نرمال‌گلکوز بعد از 72 ساعت شد (p<0.001، متغیرومین به طور معنی‌دار دار باعث تسهیل مهار جر و سلول‌های در معرض با غلظت بالایی گلکوز گردید (p<0.001)، ترکیب باعث مهار جر و سلول‌های گلکوز باعث مهار جر و سلول‌های گلکوز گردید (p<0.001)، مصرف هم‌زمان درمان شده با متغیرومین در حضور غلظت بالایی گلکوز شد (p<0.001، متغیرومین و compound C در سلول‌های اندوتوتیلیالی که در معرض غلظت بالایی گلکوز بودند باعث شد تا مهار جر و سلول‌های در مقایسه با گروه‌های کنترل در دوباره کرده و در compound C حضور غلظت 30 mM گلکوز بودن افزایش بیابد (p<0.05) غلظت اندوزکان در محلول حاصل از لیزر سلول‌ها و همچنین محیط کشت روبی سلول‌ها بعد از 72 ساعت از شروع مطالعه اندوزه‌گیری شد. هیچ تغییر معنی‌داری از نظر آماری در
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مقدار انوکوان بین گروه های مورد مطالعه دیده نشد. با این حال یک روند رو به کاهش در غلظت انوکوان موجود در محلول حاصل از لیز سلولی در گروهی که در معرض غلظت بالایی گلکوز بود مشاهده گردید. از طرف دیگر متفومنین باعث افزایش غلظت انوکوان در سلول های compound C مقایسه گردد. به نظر می رسد مصرف 30 mM گلکوز گردید. به نسبت میزان غلظت انوکوان در شرایط غلظت بالایی گلکوز و همچنین میزان غلظت در آنها از سلول های انوکولار را افزایش دهد و ترکیب باعث مهار سنتز انوکوان در سلول های انوکولار شده است.

برای مطالعه نحوه فعالیت و عملکرد سلول های انوکولار میزان برداشت Dil-Ac-LDL توسط سلول ها بررسی شد. نتایج نشان داد که غلظت گلکوز تولاناپی سلول های انوکولار در زمینه برداشت Dil-Ac-LDL را کاهش داده و در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوزبودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول های انوکولار شده (p<0.01) در غلظت compound C باعث کاهش برداشت Dil-Ac-LDL بوده که در معرض غلظت بالایی گلکوز بودند توسط متفومنین باعث بهبود عملکرد سلول Hای انوکولار و بالایی گلکوز گردید. این نتایج نشان دهنده این است که اضافه کردن به سلول های درمان شده با متفومنین تحت شرایط غلظت بالایی گلکوز باعث کاهش فعالیت و عملکرد لیپوپروتئین لپاز (LPL) می شود.

نتایج تست فلوسیتوپاتری نشان داد که در سلول هایی که در محیط حاوی غلظت بالایی گلکوز بودند مقادیر این انوکوان بیشتر از میزان کمی در مقایسه با سلول های موجود در محیط کشته شده، افزایش یافت. متفومنین میزان نشان داد که با طور معنی داری در گروه با
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درمان با compound C غلظت 30 mM گلوکزر افزایش داد (p<0.05). درمان با منتفورمین در افزایش بیان اندرکان را کاهش داد ولی این تغییرات از نظر آماری معنی دار نبود. مصرف توأم اثرات مهاری ناشی از compound C را تا حدی کاهش داد.

نتایج حاصل از روش RT-PCR نشان داد که غلظت بالای گلوکزر میزان بیان اندرکان را حدوداً 3 برابر در مقایسه با گروه کنترل افزایش داد (p<0.05) در درمان سلول ها با منتفورمین در محیط کشت حاصل غلظت 30 mM گلوکزر باعث تقویت توانایی سلول های انرژوالیا در زمینه افزایش بیان اندرکان در مقایسه با محیط کشت حاصل 5 mM گلوکزر شد (p<0.01). استفاده از منتفورمین و compound C مصرف کاهش داد (p<0.01). افزودن منتفورمین به گروه حاوی compound C منتفورمین کاهش داد (p<0.01). اثرات منفی شدید را در درمان حاوی compound C روي میزان بیان اندرکان گردید.

نتایج حاصل از روش Griess نشان داد که مقدار NO در سلول های درمان شده با منتفورمین در مقایسه با گروه کنترل افزایش یافت (p<0.001) از طرفی در درمان با منتفورمین NO را در سلول های مواده شده با غلظت بالای گلوکزر نیز دچار افزایش کرد (p<0.001) تولید NO را هم در گروه نرمال و هم در گروه غلظت بالای گلوکزر 30 mM گلوکزر در مقایسه با گروه های درمان شده با منتفورمین کاهش داد (p<0.01).

مقدار فسفیلیاسیون AMPK در شرایط غلظت بالای گلوکزر افزایش یافت درمان با منتفورمین باعث افزایش فسفیلیاسیون AMPK مخصوصا در سلول های گروه 30 mM گلوکزر گردید. با این حال هیچ کدام از این تغییرات از نظر آماری معنی دار نبودند. به نظر می رسید توانسته است اثرات تحریکی ناشی از منتفورمین را مهار کند.

نتایج حاصل از مطالعات حیوانی نشان داد که مصرف منتفورمین توانست به طور معنی دارد غلظت قند خون موش های دیابتی را کاهش دهد (p<0.01). تزیک استرپتوزوسین به طور
قابل توجهی وزن موش‌ها را کم کرده و مصرف متفورمین با دوز 50 mg/kg توانست این کاهش وزن را جبران کند ($p<0.05$).

نتایج حاصل از روش ELISA در راستای اندازه‌گیری مقدار اندوکاکان، نشان دهنده این بود که مقدار اندوکاکان موجود در بافت‌های کبد و کلیه در گروه‌های مبتلا به دیابت بیشتر از گروه کنترل بود. اما این تفاوت از نظر آماری معنی‌دار نبود. در مقابل مقدار اندوکاکان در گروه‌های درمان شده با متفورمین در مقایسه با گروه دیابتی به طور معنی‌داری در هر دو بافت کبد و کلیه بیشتر بود ($p<0.05$). در این مطالعه تفاوت معنی‌داری بین گروه‌ها از نظر غلظت سرمی اندوکاکان یافت نشد ($p>0.05$).

بر اساس نتایج حاصل از روش western blot مشخص شد که میزان فسفریلاسیون AMPK در هر دو بافت کبد و کلیه موش‌های سالم کاهش یافته و لی این تفاوت فقط در بافت کلیه از نظر آماری معنی‌دار بود ($p<0.05$)، مصرف متفورمین مقدار فسفریلاسیون AMPK را در بافت کلیه به طور معنی‌داری در مقایسه با گروه دیابتی افزایش داد ($p<0.01$).

نشان دهنده حضور اندوکاکان در هر دو بافت کبد و کلیه موش‌های سالم بود، در بافت‌های موش‌های دیابتی هیچ سلولی که از نظر حضور اندوکاکان مشبی باشد دیده نشد. درمان موش‌های سالم مبتلا به دیابت توسط متفورمین باعث تقویت سلول‌های اندوتنیال بافت کلیه از نظر تولید اندوکاکان گردید.

عکس‌سیرداری Immunofluorescence نشان دهنده حضور اندوکاکان در هر دو بافت کبد و کلیه موش‌های دیابتی نکروز سلول‌های توبولی مشاهده شد. همچنین بررسی ها نشان دهنده ایnfیلتراسیون فیبرین در فضای بینلیوم و تشکیل hyaline casts و آتروفی سلول‌های اپی تلیال توبولی در مقایسه با گروه سالم بود. در بافت کبد موش‌های دیابتی یکپارچگی مطالعات هیستولوژیکی نشان دهنده آسیب وارده به بافت‌ها در پی ابتلا به دیابت بود در بافت کلیه موش‌های دیابتی نکروز سلول‌های توبولی مشاهده شد. همچنین بررسی ها نشان دهنده ایnfیلتراسیون فیبرین در فضای بینلیوم و تشکیل hyaline casts و آتروفی سلول‌های اپی تلیال توبولی در مقایسه با گروه سالم بود. در بافت کبد موش‌های دیابتی یکپارچگی
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سینوزونیده‌های کبد از بین رفته، سلول‌ها دچار تغییرات شده و تجمع رسوایی شده فیبرینی مشاهده شد. مصرف متفورمین میزان آسیب‌های وارده به هر دو بافت را کاهش داد و در مورد متفورمین با دوز ۵۰ mg/kg این اثرات بارزتر بود.

نتایج مطالعه حاضر نشان داد که مقدار سلول‌های بیان کننده vWF در بافت های موس های دیابتی در مقایسه با موش های سالم کاهش یافته (p<0.001) در نمونه های سالم سلول های بیان کننده vWF در عروق کپسول بروز قابل مشاهده بودند در حالی که همین سلول ها در موش های دیابتی نشان دهنده درمان با متفورمین اثرات مهیار قند خون بالا بر روی میزان vWF را در هر دو بافت کبد و کلیه جیران کرد و این زمینه مصرف متفورمین با دوز اثرات بهتری در مقایسه با دوز ۵۰ mg/kg از خود نشان داد.

بحث و بررسی

در این مطالعه مقدار اندوکان، همچنین سنتز و ترشح آن در سلول‌های اندورتیلیال نقد

متفورمین بر روی آن بررسی شد. همچنین نتایج مطالعات قبلی نشان داد که غلظت بالای گلوکز مرگ و میر سلول‌ها را افزایش می‌دهد (59, 60). مطابق با نتایج مطالعات قبلی، نتایج به دست آمده از مطالعه ما نیز نشان داد که متفورمین قادر است مرگ و میر ناشی از غلظت بالای گلوکز را بهبود بی‌خانش (61, 62).

نتایج حاصل از تست خراش در مطالعه حاضر نشان داد که مواجیه سلول‌های اندورتیلیال با

غلظت بالای گلوکز به مدت ۷۲ ساعت باعث کاهش میزان مهاجرت سلول‌های ما شود که این نتیجه با نتایج حاصل از مطالعه Yu و همکاران (63) و مطالعه Hamuro و همکاران (64) مطابقت دارد. قبل از این نکته است که درمان با متفورمین باعث بهبود بدیده ترمیم زخم و عملکرد آنزیم‌های سلول‌های اندورتیلیال از طریق فعال کردن مسیر AMPK/eNOS می‌باشد.
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شود (64). مطالعه ما نشان داد که متفورمین در شرایط هایپرگلایسیمی اثرات بیشتری در بهبود مهاجرت سلول ها دارد و مصرف C باعث کاهش این اثرات می شود این نتایج در تنظیم مهاجرت سلول ها می باشد (64).

نتایج ما نشان داد که غلظت بالای گلوکز توانایی سلول های اندوتنیال در ذخیره اندولکان را کاهش داده و در همان حالت باعث می شود تا ریلیز اندولکان از سلول ها افزایش یابد. بنابراین درمان با متفورمین توانایی سلول ها در زمینه ترشح اندولکان افزایش یافته به نظر می رسد. متفورمین می توانند از طریق تنظیم mTOR در نحوه توزیع و تجمع اندولکان در سلول نقش داشته باشند.

یکی از مشکلاتی که در دیابت برای سلول های اندوتنیال به می آید اختلال در نحوه عملکرد LPL است که به دلیل مقاومت به انسلین اتفاق می افتد (65). مطالعه ما نشان دهده نقش مثبت متفورمین در بهبود عملکرد LPL در شرایط دیابتی است در تایید این نتایج قبلا نشان داده شده است که فعال شدن AMPK میتواند به علت افزایش فسفیریلاسیون LPL دربنال مرصف متفورمین باشد (65,66).

بر اساس نتایج حاصل از مطالعه حاضر، مسیر باعث انتقال تولید NO در سلول های AMPK باعث انتقال تولید NO در سلول های دیابتی باعث مهار اثرات compound C درمان شده با متفورمین می شود در تایید این گفتگو، مصرف C ناشی از مصرف متفورمین در تولید NO می شود مطالعات نشان داده است که افزایش میزان بهبود وزن می تواند به علت افزایش قدرت انتزاعی سلول های اندوتنیال می شود، بلکه از تولید رادیکال NO های آزاد اکسیژن نیز جلوگیری می کند (67).

نتایج حاصل از مطالعه حاضر in vivo نشان داد که دیابت باعث کاهش وزن می شود. مصرف متفورمین با دوز 100 mg/kg در زمینه بهبود وزن و 50 mg/kg اثرات بهتری در مقایسه با دوز در زمینه بهبود وزن و همکاران (69) تفاوت معنی‌داری نشان داد در مقایسه مطالعه Zhou که در این نوشتار از خود نشان داد.
خلاصه فارسی

داری در تغییر وزن موش‌های درمان شده با متغیرین دیده نشد. همچنین Han (70) گزارش کردند که وزن موش‌های درمان شده با متغیرین به طور جزئی افزایش پیدا کرد. نتایج بعضی مطالعات نشان داده است که متغیرین در کاهش قند خون به صورت وابسته به دوز عمل می‌کنند (71, 72). در تایید نتایج ما، مطالعه Martin-Montalvo و همکاران (73) نشان داد که غلظت‌های بالای متغیرین تاثیرات مثبت چندانی نداشت و نه تنها باعث کوتاه شدن طول عمر موش‌ها شده بلکه باعث گلخانه‌بودن بود.

فعالیت و عملکرد طبیعی سلول‌های اندوتیال و همچنین رگ‌زایی در شرایط قند خون با دچار اختلال می‌شود (74). از انجاییه‌های اندوکار در پیشرفت سرطان و افزایش رگ‌زایی نشان داده شد که عناوین مارکری برای آنزیم‌زی و اختلالات عروقی ناشی از سرطان، در نظر گرفته شود (5, 75). نتایج مطالعه حاضر نشان داد که متغیرین تاثیر قابل توجهی روی میزان اندوکار در بافت کلیه داشت. قبل از اینکه متغیرین باعث افزایش تکثیر سلول‌های اندوکار در محل بیماری و آنزیم‌زی را افزایش می‌یافت (76). همچنین متغیرین می‌توانند باعث ترمیم آسیب های وارد شده به اندوکار در نفروپاتی و زخم‌پای دیابتی شود مطالعه Dallaglio و همکاران (77) نشان داد که متغیرین به صورت وابسته به زمان باعث تغییر فعالیت‌های angiogenic سلول‌های اندوکار در می‌شود.

متغیرین از طریق مهار مسیر mTORC1 می‌توانند در فرآیندهای مثل سنتز پروتئین، mTORC1 باعث افزایش قند خون بشود. به نظر می‌رسد که مسیر مهار آنزیم‌زی می‌گردد (54, 78). نتایج مطالعه حاضر نشان داد که متغیرین باعث افزایش مقدار اندوکار در بافت‌های مورد مطالعه شد. احتمالاً متغیرین بخشی از اثرات مفید خود را از طریق افزایش اندوکار اعمال می‌کنند.
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هیستولوژیکی، تعداد سلول های مثبت از نظر vWF در کپسول بومن موس های دیابتی کاهش یافته است که به علت تکثیر کنترل نشده ماتریکس مزانشیال می باشد (79). مصرف متفورمین باعث بهبود افراشی عروق گلومورولی در پاسخ به کاهش ماتریکس مزانشیال شد (80).

نتیجه گیری

نتایج مطالعه حاضر نشان داد که مصرف متفورمین احتمالاً از مسیر AMPK باعث بهبود عملکرد سلول های اندوتلیال می شود به علت می رسد که اثرات مفید آن بر روی آنزیم‌های شرایط غلظت بالای گلوکز از طریق اندوکان اعمال می شود مصرف متفورمین باعث کاهش اثرات سوی ناشی از غلظت بالای گلوکز در سلول های اندوتلیال از طریق افراشی بیان vWF شد.

پیشنهادات

1) بهتر است از پیشینه اندوکان به عنوان کنترل مثبت در آزمایش ها استفاده شود.
(2) بررسی اثرات متفورمین در سلول های اندوتلیال بافت های مختلف از جمله سلول های اروری شبکه میتواند در تصمیم گیری بهتر در مورد نقش اندوکان راهگشای باشد.
(3) در این مطالعه اثرات درمانی متفورمین در موس های دیابتی به مدت دو هفته بررسی شد، پیشنهاد می شود برای درک دقیق تر تأثیر متفورمین روی دینامیک اندوکان، مطالعه طولانی تر انجام شود.
چکیده فارسی

چکیده فارسی

مقدمه: دیابت یک بیماری متابولیک مزمن است که همراه با آسیب های عروقی پیچیده می باشد. اندوکان یک پروتئین گلیکان مترشجع از اندوتنیوم است و احتمال می رود که در فعالیت سلول های اندوکن‌دار در دیابت نقش اساسی دارد.

هدف: در این مطالعه اثر غلظت بالای گلوزک روی مقدار اندوکان در حضور و عدم حضور داروی متفورمین به صورت in vivo و in vitro بررسی شد.

روش کار: میزان زنده مانی سلول ها توسط روش MTT و مهاجرت سلولی با روش خراش گیری می‌شود. بررسی شد مقدار بیان زن و پروتئین اندوکان با روش های RT-PCR، Griess، Fluorospectrometric بررسی شد. روش Gries به کار رفت تأیید شده. فعالیت سلول های اندوکنی با روش Dil-Ac-LDL uptake در مطالعه وسایل موش های سوری (40-35 گرم) به 4 گروه تاپی تقسیم بندی شدند. گروه سالم، گروه دیابتی و گروه متفورمین. اندوکان بافت های کبد و کلیه توسط روش Immunofluorescence (IF) در مطالعات western blot بالا میزان vWF از ایالیز گردید. جهت بررسی مقدار بیان vWF از مطالعات ELISA استفاده شد.

نتایج: مصرف متفورمین باعث افزایش میزان زنده مانی و مهاجرت سلول ها، افزایش میزان vWF و پروتئین اندوکان در شرایط دیابتی شد. نتایج حاصل از وسترن بلات نشان دهنده افزایش فسفیریلاسیون AMPK با رویکرد NO و پیشگیری از سلول های درمان شده با متفورمین بود. نتایج ELISA نشان داد که متفورمین توانسته است مقدار
چکیده فارسی

اندوکان بافتی را افزایش دهد (p<0.05) مطالعات IF و هیستولوژیکی در بافت کلیه تأیید کرد. افزایش مقدار اندوکان و vWF در موش های درمان شده با متفورمین بودند. مطالعه وسترن بلات نشان داد که میزان فسفولیازون AMPK در بافت ها توسط متفورمین افزایش یافت است.

نتیجه گیری: احتمالاً متفورمین از طریق مسیر AMPK می تواند باعث تغییر مقدار اندوکان شده و همچنین از طریق تأثیر بر روی میزان vWF می تواند باعث تقویت قدرت انژیوژنیک سلول های انژوئتانال گردد.

کلمات کلیدی: متفورمین، اندوکان، آنزیوژنی، شرایط دیابتیک
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عنوان:
بررسی اثر متفورمین بر روی سطح و بیان اندازه در شرایط دیابتیک

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