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RESEARCH ARTICLE

The Histologic Evaluation of Atorvastatin and Melatonin Treatment on Oxidative Stress and Apoptosis of Diabetic Rat Pancreas [1]

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Summary

In the diabetic state, there is an enhanced oxidative stress due to excessive production of reactive oxygen compounds and decreased bioavailability of nitric oxide. Antioxidant treatment has been used to prevent oxidative damage in diabetes. The objective of the present study was to explore the effects of atorvastatin (AT) and melatonin (MLT) on oxidative stress in diabetic rat pancreas. We also assessed nitric oxide synthase (NOS) activity and apoptosis. Diabetes was induced by an alkylating agent steptozotocin (STZ, 55 mg/kg, IP). Six weeks later rats were divided into five groups: STZ-induced diabetic group received atorvastatin (STZ+AT), STZ-induced diabetic group received melatonin (STZ+MLT) and STZ-induced diabetic group received atorvastatin and melatonin (STZ+AT+MLT). The vehicle-treated non-diabetic (CT) and diabetic group (STZ-CT) served as normoglycemic and diabetic controls. AT was given 8 mg/kg orally and MLT was given 10 mg/kg/IP once a day for 2 weeks beginning from the sixth week. Pancreatic tissue was examined by immunohistochemical methods. Although no significant difference was observed with respect to antioxidant status, NOS activity was tended to be higher in the untreated diabetic rats than in the treated rats. We observed that AT and MLT treatment improved the histopathological changes including apoptosis and oxidative stress in diabetic pancreas.

Keywords: *Diabetes, Atorvastatin, Melatonin, Oxidative stress, Apoptosis*

Atorvastatin ve Melatonin Tedavisinin Diyabetik Sıçan Pankreasında Oksidatif Stres ve Apoptozise Etkilerinin Histolojik İncelemesi

Özet

Diyabette reaktif oksijen bileşiklerinin aşırı üretimi ve nitrik oksidin azalmış biyoyararlanımına bağlı olarak oksidatif stres artmaktadır. Antioksidan tedavi diyabette oksidatif hasarı önlemek için kullanılmaktadır. Bu araştırmanın amacı, atorvastatin (AT) ve melatonin (MLT) tedavisinin oksidatif stres üzerine etkilerini diabetik sıçan pankreasında araştırmak, nitrik oksid sentaz (NOS) aktivitesi ve apoptoz ile ilişkisini değerlendirmektir. Diyabet oluşumu tek doz streptozotozin (STZ, 55 mg/kg, IP) ile indüklendi. 6 hafta sonra sıçanlar 5 gruba ayrıldı. AT uygulanan diyabetik grup (STZ+AT), MLT uygulanan diyabetik grup (STZ+MLT), AT ve MLT uygulanan diyabetik grup (STZ+AT+MLT). Kontrol ve diyabetik kontrol grupları da oluşturulmuştur. AT 8 mg/kg oral yoldan uygulanmış, MLT ise 10 mg/kg/gün olarak 6. haftadan başlayarak 2 hafta boyunca IP yolla uygulanmıştır. Pankreas dokusu immunohistokimyasal yöntemlerle incelenmiştir. Antioksidan durumla ilgili olarak belirgin bir farklılık gözlenmemekle birlikte, NOS aktivitesi tedavi almayan diyabetik sıçanlarda tedavi alan gruplara göre daha yüksek olma eğilimi gösterdi. AT ve MLT tedavisinin pankreasta histopatolojik olarak apoptosis ve oksidatif stresi de içeren iyileşmeye neden olduğu gösterildi.

Anahtar sözcükler: *Diyabet, Atorvastatin, Melatonin, Oksidatif Stres, Apoptoz*

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INTRODUCTION

Diabetes mellitus is a common chronic disorder associated with many complications [1]. It is accepted that diabetes is accompanied by oxidative stress, which is regarded as the main cause of this complications [2]. Streptozotocin (STZ), is an alkylating agent has been used widely to induce diabetes mellitus in animals [3]. STZ causes ß-cell death by inducing poly-ADP-ribose synthetase activation, followed by lethal nicotinamide adenine dinucleotide (NAD) depletion [4,5]. STZ has also imparied of the antioxidative defence system and increased free radical formation contribute to the development of oxidative stress in diabetes [6]. In the diabetogenic action of STZ, involvement of spontaneous nitric oxide (NO) production has been reported [7]. NO has a significant role in modulation of oxidative damage and significantly involved in pancreatic destruction, and seems to be closely related to the events leading to oxidative stress. It also seems that enhancement of NO production may be involved in the induction of apoptotic cell death [8,9]. Therefore, antioxidant treatment is an important therapeutic option for preventing complications and reduce tissue damage in diabetes mellitus [10,11].

Melatonin (MLT) is considered to be the one of strongest antioxidative agent [11,12]. MLT might regulate the activities of antioxidant enzymes of STZ-diabetic rats and it is capable of protecting cells from free-radical damage via its free-radical-scavenging and antioxidant properties [12,13]. Recent studies evidenced that MLT receptors are present on the pancreatic islet cells [14] and they seem to protect against the damaging consequences of hyperglycemia [15]. MLT administration has also prevents the increase in NO levels in blood plasma during STZ-induced diabetes and reduced apoptosis and damage in pancreas [16,17].

3-Hydroxy-3methylglutaryl coenzyme A (HMG-CoA) catalyses inhibitors (statins) are widely used in hyper-lipidemia [18]. Hovewer, statins have not only lipid-lowering properties but also have significant effects on inflammation and oxidative stress [19]. These are called pleiotropic effects and they are independent the effects on the lipid profile [20,21]. Moreover, statins have been shown to modulate endothelial nitric oxide synthase (eNOS) [22] and induce apoptosis in different studies. It has been shown that atorvastatin (AT) was the most effective statin commonly used in lowering cholesterol compared to other statins. AT has also improving on the markers of oxidative stress and inflammation in diabetic rats [23,24]. Statins as well as MLT were shown to possess antioxidant and free radical scavenging properties. But to our knowledge their combined effects are not evaluated in the diabetic rat pancreas yet.

The aim of the present study was to determine the effects of AT, MLT and combination of MLT with AT treatment on histopathology of pancreatic β-cells and apoptosis in STZ-induced diabetic rats

MATERIAL and METHODS

Present study was carried out in the Labroatories of Departments of Physiology and Histology & Embryology of Celal Bayar University Faculty of Medicine between 2006 and 2008. Male Wistar rats 3 months old (250-350 g) received laboratory food and water ad libitum. The ethics committee of Celal Bayar University approved the design and protocol of the experiments (27.11.2006 - 2006/47).

Experimental Procedures

Diabetes was induced by a single intraperitoneal (IP) injection of STZ (Sigma, St. Louis, MO, USA, 55 mg/kg, freshly dissolved in distillated water). Twenty-four h after STZ treatment, development of diabetes in three experimental groups was confirmed by measuring blood glucose levels in a tail vein blood samples and rats with blood glucose levels of 250 mg/dl or higher were considered to be diabetic. 6 weeks after STZ induction, rats were treated with MLT (Sigma, St. Louis, MO, USA USA, IP at a dose of 10 mg/kg) with or without AT (by gavage orally at a dose of 8 mg/kg/d) for two weeks.

Animal Study Groups

The animals were randomly assigned to one of the experimental groups as follows:

1. The vehicle-treated non-diabetic control group (CT)
2. Diabetic control group (STZ-CT)
3. STZ-induced diabetic group received atorvastatin (STZ+AT),
4. STZ-induced diabetic group received melatonin (STZ+MLT)
5. STZ-induced diabetic group received atorvastatin and melatonin (STZ+AT+MLT).

Histologic Evaluations

The pancreas was used for histological analysis. The samples were removed after the rats were euthanized by pentobarbital overdose (200 mg/kg). The specimens were fixed with 10% neutral formalin for 48-78 h. Following fixation the samples were embedded in paraffin. Four to five micron thick serial sections were obtained. Sections were stained with hematoxylene-eosin (H&E). Immunocytochemistry for TGF-beta1, e-NOS and TUNEL was also applied to the sections. Tissues were examined with a light microscope (Olympus BX40,

Japan) images were captured with a digital video camera (Olympus OlPM-CD35X) and transferred to a computer. Microscopic evaluations were performed by a single investigator who was unaware of the group assignment of the animals.

Immunohistocytochemistrical Evaluation

For immunohistochemical staining, sections were incubated at 60˚C overnight and then in xylene for 30 min. After washing with a decreasing series of ethanol, sections were washed with distilled water and phosphate -buffered saline (PBS) for 10 min. Sections were then treated with 2% trypsin at 37°C for 15 min. After washing with PBS, they were incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. Then sections were washed with PBS and incubated for 18 h at +4°C with primary antibodies: a monoclonal anti-e NOS (rabbit Pab, RB-1711-P1, Neomarkers, Fremont, CA), anti-iNOS (rabbit Pab, RB-1605-P, Neomarkers, Fremont, CA), Antibodies against TGF-ß1 (Santa Cruz Biotechnology, SC146). Afterwards, sections were washed 3 times for 5 min each with PBS, followed by incubation with biotinylated goat IgG anti-rabbit IgG and then with streptavidin conjugated to horse-radish peroxidase for 30 min each (Dako LSAB 2 kit, Peroxidase). After washing, 3 times for 5 min with PBS, sections were incubated DAB (Dako) for 5 min to stain immunolabelling and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed light microscopically with a BX 40 microscope (Olympus, Tokyo, Japan). Control samples were processed in an identical manner, but primary antibody was omitted. Two observers blinded to clinical information evaluated the staining scores independently. Detection of the apoptotic cell death in situ using as TUNEL method was used for programme cell death mechanism. Fragmentation of the DNA in the nucleus is one of the first morphological changes of the apoptotic process and can be detected in histological sections using a terminal deoxynucleotidyl-transferase-biotin nick end-labelling method (TUNEL) performed with a commercial kit (DeadEnd Colorimetric TUNEL system, Promega G7130) according to the manufacturer's instructions. Briefly, after proteinase K treatment for 10 min, the sections were incubated at 370˚C with TdT for 60 min. As negative control for TdT was omitted during the tailing reactions. Staining intensity was assigned according to a semiquantitative immuno-histochemical scoring system as follows: absent or few (+1), very mild (+2), mild (+3), moderate (+4), and strong (+5).

Biochemical Evaluations

Blood samples were obtained after an overnight fasting. The blood sample was obtained between 8:00-9:00 via the tail vein. Serums were obtained by centrifugation at 2000 g at a temperature of 4°C for 15 min. The samples were subsquently stored at -80°C until assayed. The levels of serum total cholesterol, triglyceride and fasting blood glucose were measured by a colori-metric method using Integra 800 automatic immuno-analyzer (Roche Diagnostic Systems, Mannheim, Germany).

Statistical Analysis

The data were expressed as mean ± standard deviation (S.D.). The data were analysed using repeated measures of variance. Tukey Kramer multiple comparisons test was used to test for differences among means when ANOVA indicated a significant p value ($P<0.05$) .

RESULTS

Blood glucose levels and serum lipid levels of animals are shown in *Table 1*. The diabetic animals exhibited consistently hyperglycemia *(Table 1)*. MLT and AT treatment did not induce a significant change in the blood glucose levels of the diabetic rats. Two weeks of treatment with AT and MLT siginificantly ($P<0.05$) improved the lipid content in the diabetic rats. The effect of their combination on the lipid content was more obvious and significant ($P<0.01$).

Table 1. *The effects of AT, MLT and their combination on the body weight, blood glucose and lipid content of diabetic animals. Control (CT), diabetic control (STZ-CT), melatonin treated diabetic (STZ+MLT), atorvastatin treated diabetic (STZ+AT), melatonin and atorvastatin treated diabetic (STZ+AT+MLT)*

Tablo 1. *Diyabetik hayvanlarda AT, MLT ve kombinasyonunun vücut ağırlığı, kan glukozu ve kan lipid içeriği üzerine etkileri. Kontrol (CT), diyabetik kontrol (STZ-CT), melatonin uygulanmış diyabetik (STZ+MLT), atorvastatin uygulanmış diyabetik (STZ+AT) ve melatonin ve atorvastatin uygulanmış diyabetik (STZ+AT+MLT)*

Groups	Blood Glucose (mg/dl)	Total Cholesterol (mg/dl)	Trigliserid (mg/dl)
CT	142.00±17.37	152.31±20.60	239.37±44.62
STZ-CT	325.50±16.46	213.24±22.45	311.18 ±50.43
STZ+AT	327.00±21.52	118.17±21.63	203.94±15.14
STZ+MLT	330.25±22.09	125.61±22.64	188.14±36.09
STZ+AT+MLT	312.75±8.05	125.61±24.24	142.75±11.51

There was inflammation, picnotic alterations and apoptotic morphology in beta cell of the diabetic pancreas *(Fig. 1)*. Morphological examinations of the diabetic pancreas treated with AT and MLT alone or their combined therapy showed less damage and reduce inflammation. Moreover, the expression of the iNOS, eNOS, TGF and TUNEL staining was also increased in diabetic pancreas *(Fig. 2)*. AT, MLT and their combination were reduced these parameters for pathologic alterations *(Fig. 3)* but they were not significant ($P>0.05$).

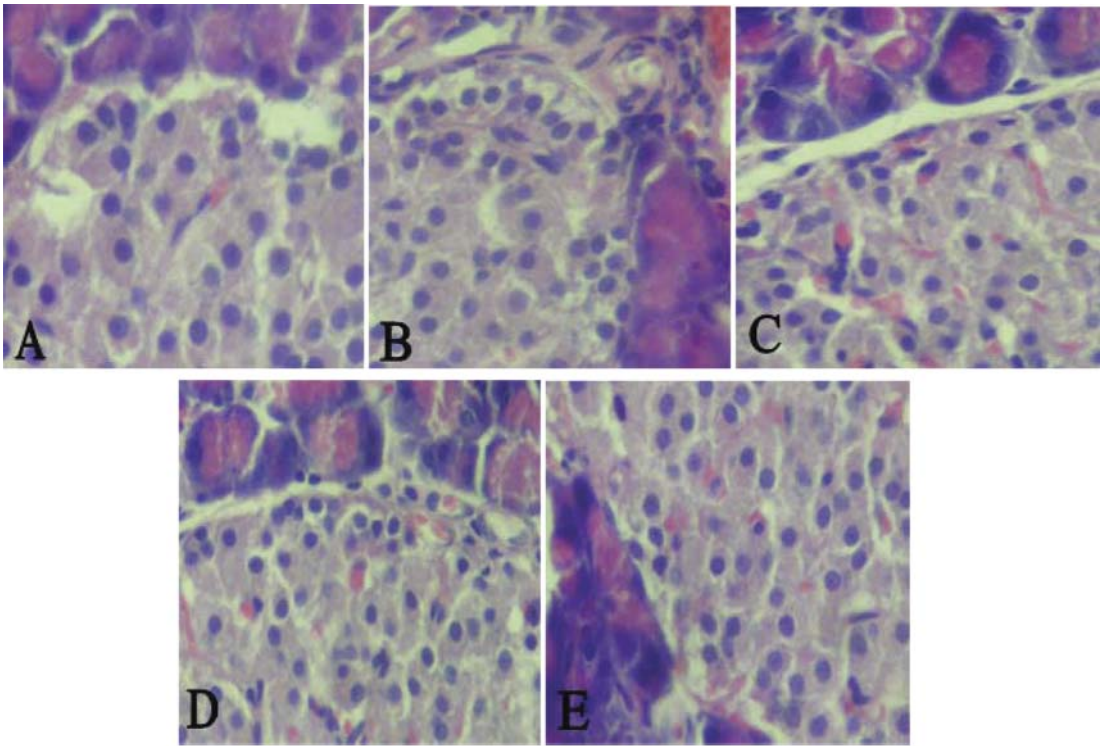

Fig 1. In vivo photomicrographs showing histologic cross sections of pancreas stained with H-E (400x magnification). **A-** Control (CT), **B-** Diabetic control (STZ-CT), **C-** Melatonin treated diabetic (STZ+MLT), **D-** Atorvastatin treated diabetic (STZ+AT), **E-** Melatonin and atorvastatin treated diabetic (STZ+AT+MLT)

Şekil 1. H-E ile boyanmış pancreas histolojik kesitleri. **A-** Kontrol (CT), **B-** Diabetik kontrol (STZ-CT), **C-** Melatonin uygulanmış diabetik (STZ+MLT), **D-** Atorvastatin uygulanmış diabetik (STZ+AT), **E-** Melatonin ve atorvastatin uygulanmış diabetik (STZ+AT+MLT)

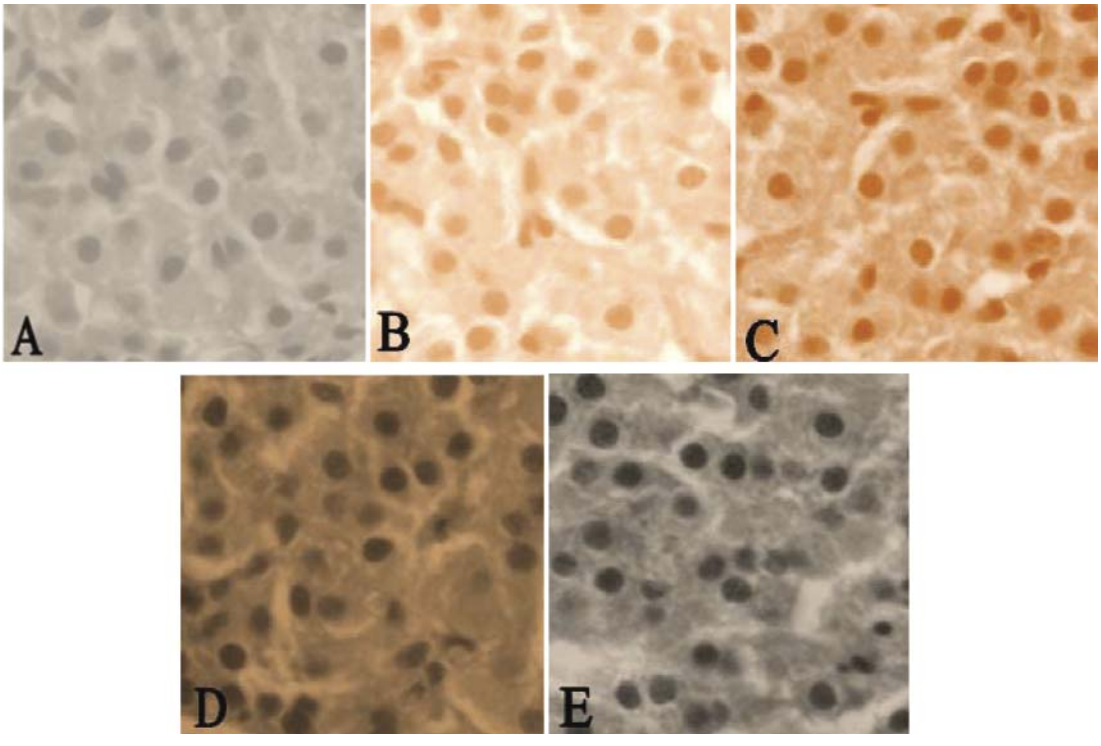

Fig 2. In vivo photomicrographs showing immunocytochemistry of diabetic pancreas (1000x magnification). **A-** Control (PBS), **B-** iNOS, **C-** eNOS, **D-** TGF-β1, **E-** TUNEL

Şekil 2. Diyabetik pankreas immunositohistokimyası (1000 büyütme). **A-** Kontrol (PBS), **B-** iNOS, **C-** eNOS, **D-** TGF-β1, **E-** TUNEL

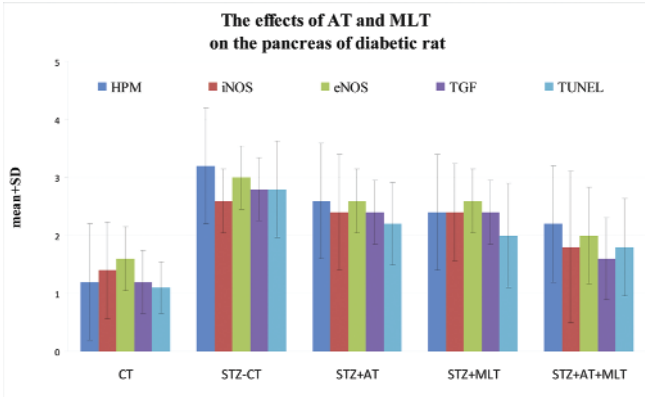

Fig 3. The effects of AT and MLT on the pancreas of diabetic rat. The groups were designed as control (CT, PBS was given IP), diabetic control (STZ-CT, 55 mg/kg, IP), atorvastatin treated diabetic (STZ+AT, AT 8 mg/kg/d orally), melatonin treated diabetic (STZ+MLT, MLT 10 mg/kg/d IP) and melatonin and atorvastatin treated diabetic (STZ+AT+MLT). There was significant (P<0.05) damage to the pancreas by STZ induced diabetic groups compared to the CT and all parameters were increased in diabetic groups by morphometric analyses. AT, MLT and their combination were reduced these parameters for pathologic alterations but they were not significant (P>0.05)

Şekil 3. AT ve MLT nin diyabetik rat pankreasına etkileri. Gruplar; CT (IP PBS verilmiş), diyabetik kontrol (STZ-CT, 55mg/kg, IP), atorvastatin uygulanmış diyabetik (STZ+AT, AT 8 mg/kg/g oral), MLT uygulanmış diyabetik (STZ+MLT, MLT 10 mg/kg/g IP) ve AT+MLT kombinasyonu uygulanmış diyabetik grup olarak oluşturuldu. STZ uygulanmış gruplarda pankreasta CT grubuna göre belirgin hasar izlendi ve morfometrik analizde diyabetik gruplarda tüm parametrelerde artış izlendi (P<0.05). AT, MLT ve kombinasyonu bu parametrelerdeki patolojik değişiklikleri azalttı ancak anlamlı bulunmadı (P>0.05)

DISCUSSION

In this study, we demonstrated the improving effects of MLT and AT on morphological and histopathological changes of the pancreas caused by diabetes and early apoptotic damage produced by STZ in diabetic rats.

STZ is a pancreatic beta cell toxin and it causes irreversible necrosis of beta cells and is widely used in making experimental animal models of diabetes. Studies of Tanaka et al.[25] point out that STZ may spontaneously produce NO, which may, partly responsible for its diabetogenic action. Diabetics and experimental animal models present a high oxidative stress resulting from persistent and chronic hyperglycemia by decreasing the activity of the antioxidative defense system and thus promote free radical generation. We have the evidence gathered in recent years that reactive oxygen species (ROS) and NO contribute to the destruction of pancreatic islets in the pathogenesis of insulin-dependent diabetes mellitus [6,7]. Our results also confirm that the excessive production of NO in STZ pancreatic damage involves an increased activity of NOS whose level is higher in diabetic with respect to the control tissues. This is wellmatched with McDaniel et al.[26] who stated that the ß-cells which are selectively destroyed in diabetes seem to express the inferable isoform of NOS and to excessive production of NO, which performs harmful effects on their function. Pancreatic beta cells have a low antioxidative capacity and they are very susceptible to oxidative changes and NO perform a deleterious effect on the ß-cells. Therefore the induction of nitric oxide (NO) synthesis causes to nuclear damage in both rat and human pancreatic beta cells, induced by peroxynitrite resulting in cell death by apoptosis or necrosis [27]. It was shown earlier that MLT could effectively normalize the impaired antioxidative status in rats with streptozotocin-induced diabetes [28] and also protects the kidney and pancreas tissue of STZ-induced rats [11,17].

In view of our findings, MLT administration prevents tissue damage and ameloriates oxidative stress in diabetes mellitus. Although, in agreement with the other reports MLT treatment does not attenuate diabetic hyperglycaemia [11,12] MLT has been shown to stimulate several anti-oxidative enzymes, such as SOD and GSH-Px, which increases the efficiency of MLT as an antioxidant. Previously, Anwar and Meki [28] also indicated that MLT possessed blood glucose lowering effects in STZ diabetic rats. But in our study two weeks MLT administration was not enough to has significant effect on glucose levels. Statins (3-hydroxy-3-methylglutaryl CoA reductase inhibitors) mainly characterised by their cholesterol lowering effects has also exert beneficial vascular effects. They frequently used in the treatment of patients with diabetes mellitus. Lately, statins have been shown to possess anti-inflammatory properties that might be attributed to inhibition of leukocyte adhesion and migration to sites of inflammation. Pleiotropic effects of statins include up-regulation of nitric oxide synthase and antioxidant or anti-inflammatory effects. Beyond simply lowering cholesterol, it also has beneficial antithrombotic effect by inhibiting platelet aggregation and promoting local NO synthesis. It has been found that treatment of diabetic rats with MLT decreased blood glucose, triglyceride, total lipid and cholesterol levels. Therefore MLT was chosen as an antioxidant in the present study. In our study dose and exposure time of MLT were designed according to previous studies on this field [21,22,29]. However, in our study, statin therapy alone has not shown significant effect on eNOS and iNOS and apoptosis in diabetic rat pancreas but two weeks administration of AT at 8 mg/kg dose which used in previous studies [30-32] . It may be a short period of time for these changes to occur. We believe that the observed effects of these agents may be due to a reduction of STZ-induced NO and free radicals in the pancreatic tissue and especially together with the combination of MLT the beneficial effects of statins may even be extended beyond improving lipid profile.

In conclusion, the presented results suggest that combine treatment with AT and MLT may prevents oxidative damage and NOS damage in the pancreas in diabetic rat. However further investigations are needed to to elucidate the exact mechanism of action and to examine the potential therapeutic effects of MLT and AT on diabetic tissue damage and apoptosis.

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