Al-Azhar Med. J. *Vol. 45(2), April 2016, 319-329*
DOI: 10.12816/0029132/ORG. No. 91225 ***Issues_title_English:*** 2016 Vol.45 Issue 2

EFFECT OF ATORVASTATIN ON RENAL INFLAMMATORY CYTOKINES AND SERUM CYSTATIN-C IN TYPE-1 DIABETIC RATS

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ABSTRACT

Background: Diabetic nephropathy is one of the major causes leading to end-stage renal failure. There are conflicting reports about the impact of treatment with statins on the diabetic nephropathy through their anti-inflammatory effect. Local cytokine levels and activity are of great value for monitoring of pathological events in a target tissue. So, cytokines measurements in tissue or peripheral circulation have been an important part of the process of defining their role in health and disease. Cystatin-C is an alternative serum biomarker that has been proposed for estimating renal function that can replace or supplement serum creatinine.

Objective: Evaluation of the possible effect of atorvastatin treatment on the renal damage caused by type-1 diabetes mellitus in the terms of renal inflammatory cytokines and serum Cystatin-C.

Materials and Methods: Thirty two male albino rats were divided into four equal groups, Group A: non-diabetic rats (negative controls), Group B: type-1 diabetic rats (positive controls), Group C: non-diabetic rats received oral atorvastatin for 4 weeks, and Group D: type-1 diabetic rats received oral atorvastatin for 4 weeks. At the end of the designated period, animals were sacrificed, kidneys and blood samples were collected; IL-1β, IL-6, IL-10 and prostaglandin E2 were determined in kidney homogenate, while Cystatin-C, creatinine and urea were assayed in serum.

Results: IL-1β, IL-6, IL-10 and PGE2 in diabetic rat kidney significantly elevated above control. In addition, serum Cystatin-C significantly elevated in the diabetic rats. Treatment of diabetic rats with atorvastatin caused decreases in all determined cytokines as well as Cystatin-C to levels near control values.

Conclusion: Atorvastatin has the potential for protecting diabetes-induced renal injury in terms of decreasing renal inflammatory cytokines and serum Cystatin-C. However, the possible protective effect of atorvastatin should be supported by clinical studies.

Keywords: Atorvastatin, Diabetes mellitus, Cystatin-C, Interleukins.

INTRODUCTION

Diabetic nephropathy is one of the most common microvascular complications of type 1 and type 2 diabetes mellitus, and the leading cause of end-stage renal disease worldwide. It is the most frequent cause of mortality in patients with diabetes **(Lopes, 2009).** Many factors contribute to the development of diabetic nephropathy including hyperglycemia, hypertension, obesity, a sedentary lifestyle, hereditary, smoking, and advancing age **(Romero-Aroca *et al.*, 2010)**.

Both oxidative stress and inflammation are intimately linked with the develop-ment of diabetic nephropathy. Increases in

oxidative stress can increase the production of inflammatory cytokines and, likewise, an increase in inflammatory cytokines can stimulate the production of free radicals **(Jeong *et al.*, 2009).** The importance of oxidative stress in diabetic nephropathy is underscored by the finding that inhibition of oxidative stress ameliorates the manifestations associated with streptozotocin-induced diabetic nephropathy **(Thallas-Bonke *et al.*, 2008).**

Diabetic nephropathy has traditionally been considered a nonimmune disease. However, evidence shows an increase in macrophage infiltration and overproduction of leukocyte adhesion molecules in kidneys from diabetic humans and in experimental animal models of diabetes **(Nguyen *et al.*, 2006)**. As a result, there is a growing support for the notion that inflammation plays a key role in the pathogenesis of diabetic nephropathy. Circulating inflammatory markers and proinflammatory cytokines are strongly associated with the risk of developing of diabetic complications **(King, 2008).**

Cytokines are a group of pharmacologically active low molecular weight proteins or glycoproteins that mediate interactions between the immune and the neuroendocrine systems. They are produced throughout the body by cells of varied embryological origin and serve in autocrine, paracrine and at times endocrine fashion as intercellular messengers. Some cytokines clearly promote inflammation and are called proinflammatory cytokines like interleukin-1 (IL-1), both α and β isoforms, IL-6 and tumor necrosis factor- α (TNF-α), whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines such as IL-10 **(Whiteside, 2007).** Inflammatory cytokines exert an important diversity of actions implicated in diabetic nephropathy, from development of the initial stages of diabetes to progression and to late stages of renal failure **(Navarro-Gonzalez and Mora-Fernandez, 2008).**

Cystatin-C is an alternative endogenous serum biomarker that has been proposed for estimating renal function that can replace or supplement serum creatinine. In multiple studies it has been shown to be more sensitive for predicting adverse events than serum creatinine or eGFR **(Vigil *et al.*, 2014).** This parameter also showed greater sensitivity to detect mild reductions in renal function and improved the identification of patients with higher cardiovascular risk in epidemiological studies **(Zahran *et al.*, 2007, Eriksen *et al.*, 2010, and Peralta *et al.*, 2011).** The association of cystatin-C with metabolic syndrome and classic cardiovascular risk factors is also well documented. This association may reflect the inflammatory nature of the syndrome. A positive correlation between cystatin-C and other inflammation parameters including IL-6, resistin, TNF-α, and C-reactive protein has been reported **(Balta *et al.*, 2013)**. All the above-mentioned may suggest that cystatin-C could be useful for predicting adverse clinical events, and becomes a clinical tool to optimize the estimation of glomerular filtration rate **(Peralta *et al.*, 2011).**

There are conflicting reports about the impact of treatment with statins on the

diabetic state. New-onset diabetes has been observed in clinical trials and meta-analyses involving statin therapy **(Brault *et al.*, 2014).** On the other hand, there have been claims that rosuvastatin improved the renal function in diabetics through its antioxidant properties **(Abe *et al.*, 2011)** and that cerivastatin prevented glomerular injury in diabetic rats **(Ota *et al.*, 2003).**

Since statins are used as hypolipidemic agents in diabetics, and in view of the conflicting reports on the effect of this group in diabetes, it would be of importance to evaluate their effect on changes in renal biochemical mediators in this disease. The possible effects of atorvastatin treatment on renal inflammatory cytokines and serum cystatin-C and hence the kidney damage were evaluated in the present study.

MATERIALS AND METHODS

Animals: Adult male albino rats of local strain weighing 150–200 g, were supplied from animal house unit of Medical Research Institute, Alexandria University, Egypt. All animals were kept under observation for one week prior to study with free access to commercial rat diet and water *ad libitum.* Rats were housed at 22 ± 2 °C with a 12 h light-dark cycle. All procedures were performed in accordance with international regulations for the care and use of laboratory animals and approved by the Local Ethics Committee of Medical Research Institute.

Experimental Design: Thirty two adult male albino rats were randomly divided into four equal groups: **Group A:** Non-diabetic rats (negative controls) received oral PBS as vehicle, **Group B:** Type-1 diabetic rats with no treatment (positive controls) received oral PBS of as vehicle (1 mL/kg/day), **Group C:** Non-diabetic rats received oral atorvastatin (10 mg/kg/day) for 4 weeks, and **Group D:** Type-1 diabetic rats received oral atorvastatin (10 mg/kg/day) for 4 weeks.

Experimental Induction of Diabetes: Food was withdrawn from 12 to 14 hours before the experiment, and diabetes was chemically induced by a single *i.p.* dose (50 mg/kg) of freshly prepared streptozotocin (STZ - Sigma Aldrich Co., USA) in citrate buffer (pH 4.5) **(El-Awdan *et al.*, 2013).** Successful induction of diabetes was confirmed by measuring the fasting blood sugar (FBS) from tail blood (using Glucometer and glucose testing of Roche Switzerland) in rats 48 h after injection of STZ. The FBS level above 250 mg/dl was considered diabetic and included in the present study.

Atorvastatin administration: Atorvastatin (Biocon limited, Bangalore, India) was suspended in a sterile PBS pH 6.8 and orally administered by gastric gavage at dose of 10 mg/kg/day **(Zhou *et al.*, 2014)**. Freshly atorvastatin suspension was prepared every day.

At the end of the designated period, animals were sacrificed by cervical dislocation. The kidneys were excised, washed with ice-cold saline, and stored at -80°C until homogenized. Blood samples were collected after cervical dislocation by heart puncture, and the sera were separated and stored at -80°C.

Kidney tissue homogenization: Kidney (washed in ice-cold saline) was excised into small pieces and homogenized (1:9 wt/vol) in cell lysing buffer (R&D Systems Inc. USA, Cat. #: 895347)

containing protease inhibitor cocktail (Promega corporation. USA, REF. #: G6521) using Teflon homogenizer (Potter-Elvehjem) on ice. The lysate was then cold-centrifuged at 12,000 *g* for 10 minutes at 4°C. Supernatants were collected and stored at -80°C until analyzed. Protein concentrations of supernatants of homogenized kidneys were determined by Lowry's method **(Lowry *et al.*, 1951)**.

Interleukin-1β (Cat. #: RLB00), IL-6 (Cat. #: R6000B), IL-10 (Cat. #: R1000) **(O'Bryan *et al.*, 2005),** and PGE2 (Cat. #: KGE004B) **(Souza Mdo *et al.*, 2008)** were determined in the supernatant of kidney homogenate by ELISA according to the manufacturer instructions (R&D Systems, Inc. USA), and the concentrations were expressed as ng/g protein. Creatinine and urea were measured in serum spectrophotometrically using automatic biochemistry analyzer by Biolabo reagents (Biolabo SAS, France). Cystatin-C (Cat. #: MSCTCO, R&D Systems, Inc. USA) was determined in the serum by ELISA and the concentrations were expressed as µl/mL **(Kong *et al.*, 2013)**.

Statistical analyses: All data were expressed as mean ± standard deviation (SD). All analyses and graphics were performed using GraphPad Prism version 5, 2007 (Graph Pad Software, San Diego, USA). Data passed the normality test (D'Agostino test) and treated with the parametric tests. One way Analysis of Variance (ANOVA) followed by Tukey's procedure was used to compare between variables. Differences between means were considered statistically significant at $P < 0.05$.

RESULTS

Error! Reference source not found.) showed levels of blood glucose, serum creatinine, urea and cystatin-C in control and diabetic rats with and without atorvastatin treatment, it was observed that serum cystatin-C signifi-cantly increased in diabetic group, while it returned around the normal level in the atorvastatin-treated group. Serum creatinine and urea significantly increased in diabetic rats, while atorvastatin did not change the level of both.

Changes in the renal cytokines as a result of induction of type-1 diabetes and treatment with atorvastatin were presented in **(Table 2)**. Induction of diabetes resulted in statistically significant elevations of all determined ILs in kidney tissues. The pro-inflammatory IL-1 increased by 50.7% from negative control **(Fig. 1),** and IL-6 by 43.2% **(Fig. 2).** In the meantime, the increase in the anti-inflammatory IL-10 reached 41.2% **(Fig. 3)**. Moreover, an increase of 30.8% was detected for PGE2 **(Fig. 4)**.

No statistically significant differences were detected between the levels of all determined parameters in negative control group and those treated with atorvastatin. However, following treatment of diabetic rats, all determined parameters tended to go back to levels comparable to those found in control animals, with no statistically significant differences detected **(Table 2)**. IL-1β decreased by 30.4% below the mean level of the diabetic animals **(Fig. 1)** and a decrease of about 28.4% was found with IL-6 **(Fig. 2).** The anti-inflammatory IL-10 decreased by 23.1% **(Fig. 3)**. The level of PGE2 went

down by 20.3% below that of the diabetic animals **(Fig. 4).**

Table (1): Fasting blood sugar (FBS), serum creatinine, urea and cystatin-C (Mean ± SD) in control and diabetic rats with and without atorvastatin treatment.

Parameters / Groups	FBS (mg/dl)	Serum creatinine (mg/dl)	Serum urea (mg/dl)	Serum Cystatin-C (μg/mL)
Group A (control)	95.0 ± 7.15	0.67 ± 0.095	35.0 ± 9.48	1.56 ± 0.215
Group B (DM)	398 ± 131*	0.70 ± 0.178	54.9 ± 17.6*	2.24 ± 0.229*
Group C (atorvastatin)	93.6 ± 11.4#	0.64 ± 0.187	54.6 ± 14.1*	1.65 ± 0.372
Group D (DM + atorvastatin)	389 ± 133*	0.74 ± 0.241	59.1 ± 9.51*	1.71 ± 0.177

** Significant different from group A (negative control)*
#: Significant different from group B (positive control)

Table (2): Changes in the concentrations of ILs and PGE2 (Mean ± SD) in the kidney of control and diabetic rats with and without atorvastatin treatment.

Parameters / Groups	IL-1β (ng/gm protein)	IL-6 (ng/gm protein)	IL-10 (ng/gm protein)	PGE2 (ng/gm protein)
Group A (control)	34.9 ± 2.66	76.8 ± 4.51	41.0 ± 2.74	1.17 ± 0.08
Group B (DM)	52.6 ± 7.59*	110 ± 19.5*	57.9 ± 12.7*	1.53 ± 0.12*
Group C (atorvastatin)	34.0 ± 4.16#	71.9 ± 8.76#	37.6 ± 4.87#	1.08 ± 0.13#
Group D (DM + atorvastatin)	36.6 ± 3.86#	78.8 ± 5.67#	44.5 ± 5.18#	1.22 ± 0.12#

** Significant different from group A (negative control)*
#: Significant different from group B (positive control)

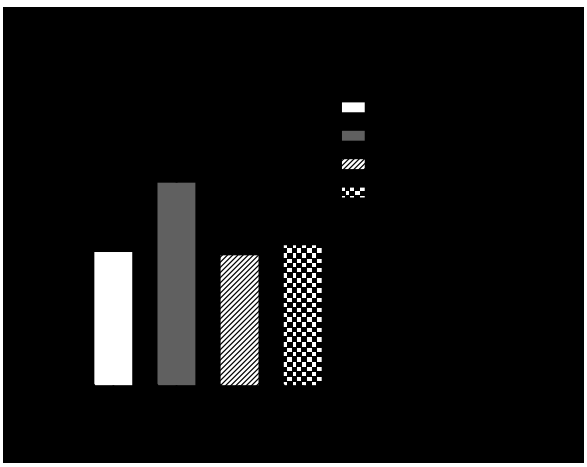

Figure (1): Effect of treatment with atorvastatin on the level of IL-1β (Mean ± SD) in the kidney of control and diabetic rats

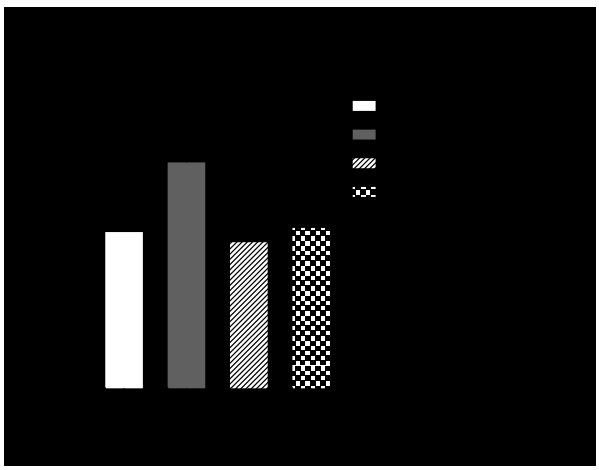

Figure (2): Effect of treatment with atorvastatin on the level of IL-6 (Mean ± SD) in the kidney of control and diabetic rats

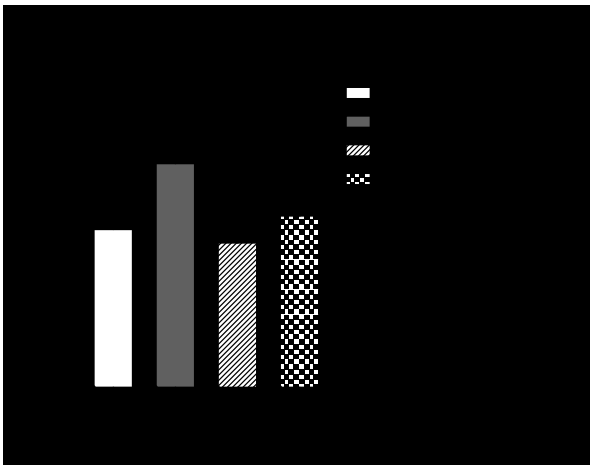

Figure (3): Effect of treatment with atorvastatin on the level of IL-10 (Mean ± SD) in the kidney of control and diabetic rats

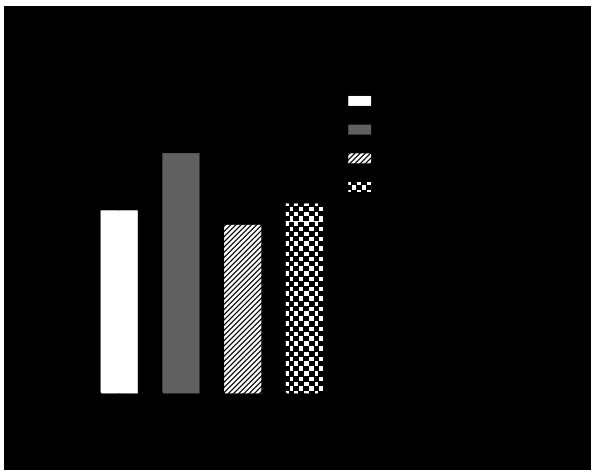

Figure (4): Effect of treatment with atorvastatin on the level of PGE2 (Mean ± SD) in the kidney of control and diabetic rats

DISCUSSION

In our study, all determined cytokines (IL-1β, IL-6, IL-10) and PGE2 in the diabetic kidney, significantly elevated above control. However, following treatment of diabetic rats with atorvastatin, all determined parameters tended to go back to levels comparable to those found in control animals, with no statistically significant differences detected That suggested the protective role of atorvastatin in this respect.

Several studies have confirmed the usefulness of cystatin-C determinations as a marker of early deterioration of GFR, being more sensitive than serum creatinine **(Tomino *et al.*, 2001, Shimizu *et al.*, 2003, and Balta *et al.*, 2013).** Currently, serum creatinine concentration is widely used to estimate GFR because this estimation is simple and inexpensive. However, serum creatinine can be affected by age, gender, ethnicity, dietary protein intake, and muscle mass, and its measurement requires a variety of analytical interferences and is associated with significant standardization **(Woo *et al.*, 2014).**

Our study results also confirmed that cystatin-C could be one of the additional biomarkers which represented kidney state of diabetic patients. In the present study, serum cystatin-C in diabetic rats was significantly higner than non-diabetic, while serum cretinine and urea were not sensitive to the renal damage caused by diabetes. In accordance to our results, previous studies have shown that serum cystatin-C is more sensitive than serum creatinine for the detection of GFR diabetic patients. **El-Shafey *et al.* (2009)** and **Jeon *et al.* (2011)** showed that serum cystatin-C is more sensitive than serum creatinine for the estimation of GFR in type-2 diabetic patients. **Tan *et al*. (2002)** showed the same in type-1 diabetic patients.

After adjustment for age, **Christensson *et al.* (2004)** also demonstrated that serum cystatin-C is better in detection of mild

diabetic nephropathy. **Shimizu *et al.* (2003)** showed a significant relationship between serum cystatin-C levels and the prognostic stage in patients with type-2 diabetic nephropathy.

Cytokines measurements in tissue or in the peripheral circulation have been an important part of the process of defining their role in health and disease. It has been suggested that local cytokine levels and activity are of considerably greater value for monitoring of pathological events in a target tissue than are systemic serum cytokine levels **(Mathey *et al.*, 2003).**

Proinflammatory cytokine-mediated inflammation is a cascade of gene products. The expression of these genes is stimulated by IL-1, while anti-inflammatory cytokines, including IL-10, block or at least suppress the intensity of the cascade. It has been proposed that the biological activities of IL-10 in modulating inflammation may be caused, in part, by down-regulation of pro-inflammatory cytokines and the expression of their receptors and up-regulation of cytokine inhibitors **(Glocker *et al.*, 2011).**

Prostaglandin E2 is one of the most abundant PGs produced in the body and exhibit versatile biological activities. Under physiological conditions, PGE2 is an important mediator of many biological functions including regulation of immune responses **(Ricciotti and FitzGerald, 2011).** Dysregulated PGE2 synthesis or degradation has been associated with a wide range of pathological conditions **(Legler *et al.*, 2010).** It may, therefore, be involved in the mechanism of diabetes-induced renal injury.

Statins are widely used to treat dyslipidemias associated with diabetes mellitus. Pleiotropic effects of statins have important clinical implications, independent of their lipid-lowering effects. Alterations in inflammatory responses, plaque stabilization, and improved endothelial function are thought to be partially responsible for the reduction in cardiovascular morbidity and mortality **(Epstein and Campese, 2005).** The anti-inflammatory effects of statins have been attributed to their capacity to inhibit the production of the proinflammatory cytokines and PGs **(Santodomingo-Garzo *et al.*, 2006).** Some studies have reported that atorvastatin has a protective effect on renal function **(Bianchi *et al.*, 2003, Douglas *et al.*, 2006, Sandhu *et al.*, 2006, and Colhoun *et al.*, 2009).**

Our results showed that levels of cytokines in atorvastatin-treated animals tended to go back to the normal levels found in the control animals, with no statistically significant differences detected. These results were in accordance with **Zhao *et al.* (2014),** who showed that a single dose of atorvastatin was able to decrease IL-6 levels, possibly resulting in an anti-inflammatory effect. Also, atorvastatin significantly decreased the secretory level of TNF-α and IL-6 in adipose tissue. These findings suggest that atorvastatin may inhibit the generation of inflammatory factors **(Zhang *et al.*, 2010).** Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase by atorvastatin not only reduces cholesterol synthesis, but also decreases the levels of geranylgeranyl phosphate and farnesyl pyrophosphate, which have important roles in the post translation modification of proteins **(Lawrence, 2005).** In addition,

Goicoechea *et al.* (2006) concluded that treatment with atorvastatin induced, in addition to its lipid-lowering effect, a significant decrease in inflammatory parameters. Such evidence could suggest a new therapeutic use of statins as anti-inflammatory agents.

CONCLUSION

Despite the lack of clinical data, results of the present study suggested that atorvastatin has the potential for protection or attenuation of diabetes-induced renal injury through preventing elevation of levels of the proinflammatory cytokines, and keeping them at near control values. Also, our results suggested that measurement of serum cystatin-C provided a simple and accurate method for detecting early renal impairment in subjects with diabetes.

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تأثير الأتورفاستاتين على سيتوكينات الكلى وسيستاتين سي فى مصل الدم لدى الجرذان المصابة بداء السكري من النوع الأول

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خلفية البحث: إعتلال الكلى السكرى هو أحد الأسباب الرئيسية التي تؤدى إلى الفشل الكلوي. وبما أن هناك ارتباط بين مستوى السيتوكينات والحالة الباثولوجية للأنسجة، فإن قياس مستوى السيتوكينات فى الكلى يعد جزءا هاما فى تحديد دورها في حدوث الخلل المرضى. بعض الدراسات رجحت دور أدوية خفض الكوليسترول فى الحماية من إعتلال الكلى السكرى.

هدف البحث: دراسة تاثير أحد الأدوية من مجموعة الستاتينات وهو الأتورفاستاتين على سيتوكينات الكلى لدى الجرذان المصابة بداء السكري من النوع الأول.

مواد وطرق البحث: أجريت هذه الدراسة على 32 جرذا قسمت الى أربع مجموعات (8 جرذان فى كل مجموعة): المجموعة الأولى: هى مجموعة الضوابط السلبية، والمجموعة الثانية: هي المجموعة الضابطة الإيجابية وتحتوى على الجرذان المصابة بداء السكرى من النوع الأول، والمجموعة الثالثة: جرذان تم حقنها بالأتورفاستاتين (10 مجم لكل كجم لمدة 4 اسابيع)، والمجموعة الرابعة: جرذان مصابة بالنوع الأول من مرض بالسكري تم حقنها بالأتورفاستاتين (10 مجم لكل كجم لمدة 4 اسابيع). بعد انتهاء مدة البحث تم قتل الجرذان، وفصل الكلى، وتجميع عينات الدم لقياس الكرياتينين، والبولينا، والسيستاتين سي، وقياس الانترليوكينات 1β، 6، 10، وبروستاجلاندين هـ2 فى جناسة الكلى.

النتائج: أظهرت نتائج البحث زيادة كل السيتوكينات والبروستاجلاندين هـ2 فى كلى الجرذان المصابة بداء السكري مقارنة بالمجموعة الضابطة، بينما عاد مستوى السيتوكينات الى الطبيعي فى المجموعة المعالجة بالأتورفاستاتين. وكذلك إرتفع مستوى السيستاتين سي فى مصل دم الجرذان المصابة بالسكرى وعاد إلى الطبيعى فى المجموعة المعالجة بالأتورفاستاتين.

الخلاصة: الأتورفاستاتين له دور فى حماية الكلى من الإعتلال بسبب مرض السكري من النوع الأول عن طريق خفض مستوى السيتوكينات المسؤولة عن التهاب الكلى وكذلك مستوى السيستاتين سي.