

## DIABETES-INDUCED RENAL FAILURE IS ASSOCIATED WITH TISSUE INFLAMMATION AND NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN: EFFECTS OF RESVERATROL

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**Abstract:** Diabetes mellitus is a chronic inflammatory disease characterized by high blood glucose levels due to the absence of secretion of insulin or its inefficient use in the body. In this study, we investigated how resveratrol administration affects the renal functions and pro-inflammatory signaling pathway components in streptozotocin-induced diabetes in male Wistar rats. Rats were randomly divided into four groups: (1) control/vehicle; (2) control/20 mg/kg resveratrol; (3) diabetic/vehicle; and (4) diabetic/20 mg/kg resveratrol. In addition to renal glucose, lipid, angiotensin-1 (ANG-1), asymmetric dimethylarginine (ADMA), erythropoietin (EPO), malondialdehyde (MDA) and neutrophil gelatinase-associated lipocalin (NGAL) content, the gene expressions of pro-inflammatory markers, including inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF- $\kappa$ B), nuclear factor (erythroid-derived 2) like-2 (Nrf2), and the protein contents of interleukins-1 $\beta$ ,6,8 (IL-1 $\beta$ ,6,8) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were analyzed using qRT-PCR and ELISA, respectively. The rats in the diabetes group demonstrated significantly lower terminal body weight and renal ANG-1, but significantly higher renal glucose, cholesterol, triglyceride, ADMA and MDA concentrations. Diabetes triggered inflammation in kidney tissues, reflected as an increase in NGAL level. The renal inflammation observed in the diabetes group was associated with significant upregulation of components of the pro-inflammatory pathway, iNOS, NF- $\kappa$ B, Nrf2, IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ . To some extent, resveratrol administration reversed the diabetes-induced changes in renal tissues, suggesting that resveratrol partially protected from diabetes-induced renal failure due to its restorative activities in tissue inflammation.

**Key words:** Diabetes; resveratrol; inflammation; kidney; NGAL

**Abbreviations** ADMA – asymmetric dimethylarginine; ANG-1 – angiotensin-1; Diab - diabetic; EPO – erythropoietin; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; HRP – horseradish peroxidase; iNOS – inducible nitric oxide synthase; IL-1 $\beta$ ,6,8 - interleukin-1 $\beta$ ,6,8; MDA - malondialdehyde; NGAL – neutrophil gelatinase-associated lipocalin; NF- $\kappa$ B – nuclear factor kappa B; NO – nitric oxide; Nrf2 – nuclear factor (erythroid-derived 2) like-2; qRT-PCR – quantitative real-time PCR; Res - resveratrol; STZ - streptozotocin; TNF- $\alpha$  – tumor necrosis factor- $\alpha$

## INTRODUCTION

Diabetes mellitus (DM), a complex clinical condition, is accompanied by an inflammatory response to high glucose level [1]. Adipose tissues also contribute to the development of a systemic inflammatory state in obesity-associated type 2 diabetes [2]. Enlarged adipocytes themselves produce pro-inflammatory cytokines and chemokines [3]. In diabetes, microvascular complica-

tions may arise due to the combination of pro-inflammatory structures and impaired angiogenic response [4]. Neutrophil gelatinase-associated lipocalin (NGAL) has recently attracted much attention as a critical regulator of renal functions associated with diabetes [5]. Normally, NGAL is found in the blood but in inflammation, renal and cardiovascular complications, its serum and urinary levels increase [6]. Hence, it can serve as a potential biomarker [7] for renal inflammation.

In recent years, our understanding of the development and progression of diabetes at the genetic and molecular levels has improved, and recent studies have indicated that immune-mediated inflammatory processes have a significant role in the pathophysiology of diabetes [8]. However, data on the relationship between NGAL and the inflammatory response associated with diabetes do not exist. It has been established that the plasma levels of NGAL and the inflammatory response increase in hyperglycemia [9], however, the renal tissue levels of angiogenesis and inflammatory markers are still unknown.

Studies demonstrating the effects of resveratrol, a phytoalexin with anti-inflammatory, antitumor and anti-oxidant properties, have been conducted in various animal models [10,11]. With its low renal toxicity, it is known to beneficially affect diseases of the kidneys, including sepsis-induced renal injury [12,13]. The NF- $\kappa$ B pathway and many other molecular pathways are influenced by the anti-inflammatory effects of resveratrol [14]. For instance, one of the effects of resveratrol includes suppression of IL-1 $\beta$  activation and reduction of cytokine production in macrophages [15]. We investigated the mechanisms involved in diabetes-induced local immune responses and NGAL in kidney tissues. Our results indicate that resveratrol acts as a suppressant of the inflammatory response in several pathways, possessing properties of a potential therapeutic agent in diabetes-induced kidney failure.

## MATERIALS AND METHODS

### Chemicals

Trans-resveratrol was purchased from Molekula (Gillingham, Dorset, UK) and streptozotocin (STZ) was obtained from Sigma (St. Louis, MO, USA). Total RNA isolation kits were obtained from Qiagen (Venlo, Netherlands) and the reagents for cDNA synthesis were from Thermo Scientific (Burlington, Canada). SYBR Green I Master Mix was obtained from Roche (Foster City, CA, USA). Buffers were prepared using sterile distilled water. All other chemicals used in this study were of the highest analytical grade available.

### Animals and treatment procedure

Study protocols were approved in advance by the Local Ethics Committee for Animal Research Studies at the Karamanoglu Mehmetbey University (K.M.U. ET-11/01-02). This study was carried out in strict adherence to the rules of the Guide for the Care and Use of Laboratory Animals as published by the US National Institute of Health (NIH Publication No: 85/23, revised in 1986). All efforts were made to minimize animal suffering.

Experiments were performed on 8-week-old adult male Wistar rats weighing between 300-350 g. They were maintained under temperature-controlled conditions (20-22°C) with a 12-h light-dark cycle and fed with standard rodent diet: 62% starch, 23% protein, 4% fat, 7% cellulose, standard vitamins and salt mixture (chow pellet). After 1 week, the rats were randomly separated into 4 groups. The control group included 12 rats that were injected only with vehicle, 10% dimethyl sulfoxide (DMSO), for 4 weeks. The resveratrol group (12 rats) was administered a daily dose of 20 mg/kg resveratrol in vehicle intraperitoneally (i.p.) throughout the 4-week period. The diabetes group (12 rats) received a single i.p. dose of STZ (55 mg/kg) dissolved in 0.05 M citrate buffer (pH 4.5) and daily injections of vehicle for 4 weeks. The diabetes+resveratrol group contained 9 rats that received a daily dose of 20 mg/kg resveratrol i.p. throughout the 4-week period, starting from day 2 after STZ administration. Blood glucose concentrations from the blood of the tail veins were determined weekly using Accu-Chek Go (Roche, Germany) glucometer. A blood glucose concentration higher than 200 mg/dL served as the criteria for diabetes. At the end of the study period, all rats were decapitated and the kidney tissues were blotted dry, frozen in liquid nitrogen, and stored at -85 °C for further use.

### Measurement of renal glucose, lipids, EPO, ANG-1, ADMA, MDA, NGAL and inflammatory markers

Kidney tissues were homogenized in phosphate buffer 1:10 (w/v), 0.1 M, pH 7.4, centrifuged at 10000xg for 10 min and the supernatants were collected. Total protein contents were determined using the Lowry [16] method. The levels of renal glucose, total cholesterol, triglycerides (Spinreact, Santa Coloma, Spain) and

MDA (HPLC; Chromsystems Diagnostics, Munich, Germany) were determined by standard enzymatic techniques. EPO (East Biopharm, Hangzhou, PRC), ADMA (Immundiagnostic AG, Stubenwald-Allee, Bensheim), ANG-1, NGAL and IL-8 (Uscn Life Science Inc., Wuhan, Hubei, PRC), IL-1 $\beta$ ,6 and TNF- $\alpha$  (eBioscience, Bender Med. Systems GmbH, Vienna, Austria) concentrations were measured using commercially available rat-specific ELISA kits according to the manufacturer's protocols.

### Determination of gene expression of *Inos*, *Nf- $\kappa$ b* and *Nrf2* by qRT-PCR

Total RNAs were isolated from the kidney tissues using the RNeasy total RNA isolation kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. After isolation, the amount and quality of the total RNA were determined using spectrophotometry at 260/280 nm. One  $\mu$ g of total RNA was reverse-transcribed to cDNA using the commercial first strand cDNA synthesis kit (Thermo Scientific, USA). Gene expressions were determined by mixing 1  $\mu$ L cDNA, 5  $\mu$ L SYBR Green Master mix (Roche FastStart Universal SYBR Green Master Mix) and primer pairs (Table 1) at 0.5- $\mu$ M final concentrations

**Table 1.** Primer sequences of *Inos*, *Nf- $\kappa$ b* and *Nrf2* and internal standard *Gapdh*, used for the determination of mRNA expression by qRT-PCR.

Gene	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')
<i>Inos</i>	CTTCAGGTATGCGGTATTGG	CATGGTGAACACGTTCTTGG
<i>Nf-<math>\kappa</math>b</i>	GGGTCAGAGGCCAATAGAGA	CCTAGCTTCTCTGAACTGCAAA
<i>Nrf2</i>	GATTCGTGCACAGCAGCA	GCCAGCTGAACTCCTTAGAC
<i>Gapdh</i>	TCCTTGGAGGCCATGTGGGCCAT	TGATGACATCAAGAAGGTGGTGAAG

in a total volume of 10  $\mu$ L [17]. The relative expression of genes with respect to the internal control; *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) was calculated with the efficiency corrected advance relative quantification tool provided by the LightCycler<sup>®</sup> 480 SW 1.5.1 software.

### Statistical analysis

Collected data were analyzed using Statistical Package for Social Sciences version 21.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as means $\pm$ standard error and Student's t-test or one-way analysis of variance followed by Tukey's honestly significant difference (HD) post-hoc analysis was used where appropriate. P values less than 0.05 were considered statistically significant.

## RESULTS

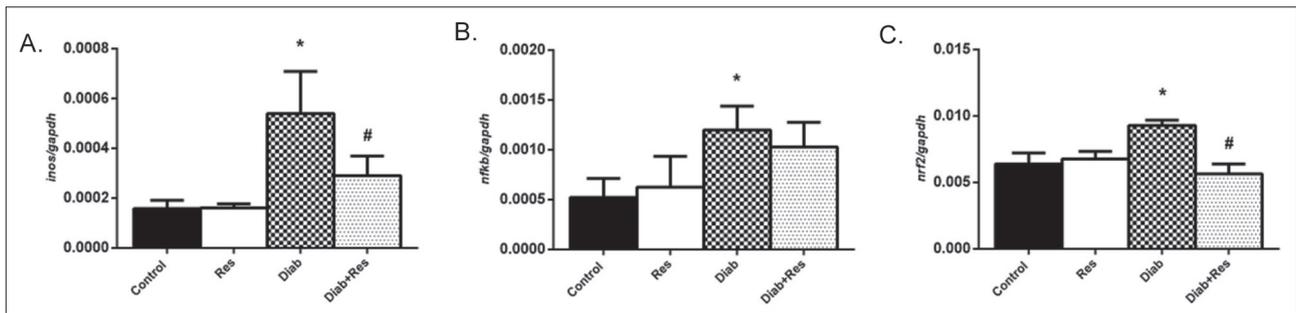
### Effects of resveratrol on renal metabolic characteristics of the rats

Recently, we showed that STZ-treated rats displayed a significant induction in fasting blood glucose concentration compared to age-matched controls, and that resveratrol did not affect the diabetic blood glucose levels significantly [18]. When compared to those in the control group, rats in the diabetes group had significantly lower terminal body weights and significantly higher renal glucose concentrations. Table 2 shows the comparison of the metabolic characteristics of the control, resveratrol, diabetes and

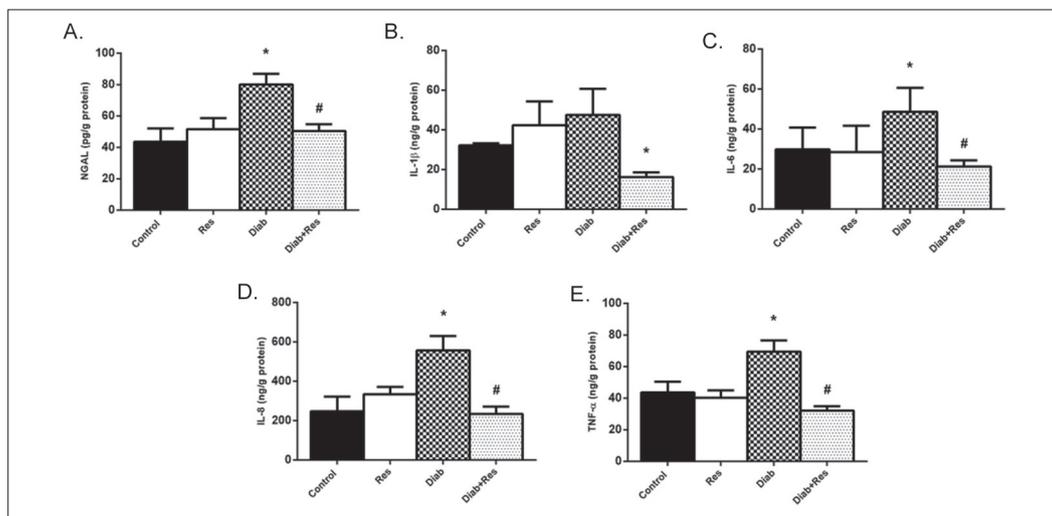
**Table 2.** Effects of diabetes and resveratrol on body weight and other metabolic parameters in the kidney tissues of STZ-induced diabetic rats.

Groups	Control	Res	Diab	Diab+Res
Initial body weight (g)	441 $\pm$ 21	399 $\pm$ 7	393 $\pm$ 11*	392 $\pm$ 7
Glucose (mg/g protein)	93.7 $\pm$ 6.7	93.2 $\pm$ 3.4	133.4 $\pm$ 14.7*	96.1 $\pm$ 12.6
Triglyceride (mg/g protein)	262.5 $\pm$ 31.5	252.9 $\pm$ 34.0	381.7 $\pm$ 76.6	258.3 $\pm$ 45.1
Total Cholesterol (mg/g protein)	11.9 $\pm$ 2.4	11.6 $\pm$ 2.3	21.6 $\pm$ 3.8	11.1 $\pm$ 1.5#
ADMA ( $\mu$ mol/g protein)	0.19 $\pm$ 0.07	0.19 $\pm$ 0.06	1.59 $\pm$ 0.8	1.35 $\pm$ 0.2
MDA ( $\mu$ mol/g protein)	2.17 $\pm$ 0.7	2.44 $\pm$ 0.2	4.61 $\pm$ 1.3	1.98 $\pm$ 0.2
ANG-1 ( $\mu$ mol/g protein)	23.6 $\pm$ 8.2	20.4 $\pm$ 5.8	15.9 $\pm$ 2.4	12.5 $\pm$ 2.8
EPO (IU/g protein)	0.63 $\pm$ 0.06	0.66 $\pm$ 0.25	0.63 $\pm$ 0.21	0.64 $\pm$ 0.15

Values are expressed as means $\pm$ SEM, n = 6-12. All parameters, except the body weight, were measured immediately after the decapitation of animals. Diab – diabetes, Res – resveratrol. \* Indicates that the means were significantly different (p<0.05) compared to the control group. # Indicates that the means were significantly different (p<0.05) compared to the diabetes group.



**Fig. 1.** Levels of *Inos* (A), *Nf-kb* (B) and *Nrf2* (C) mRNA expression in kidney tissues of rats from Control, Res, Diab and Diab+Res groups. Data were normalized by *Gapdh*. Values are expressed as means $\pm$ SEM, n=6-8. \*p<0.05, significantly different from control; #p<0.05, significantly different from diabetic rats. Diab – diabetes, Res – resveratrol.



**Fig. 2.** Effect of diabetes and resveratrol on NGAL (A), IL-1 $\beta$  (B), IL-6 (C), IL-8 (D) and TNF- $\alpha$  (E) protein expressions, as measured by ELISA in kidney tissues of rats from Control, Res, Diab and Diab+Res groups. Values are expressed as means $\pm$ SEM, n=6-8. \*p<0.05, significantly different from control; #p<0.05, significantly different from diabetic rats. Diab – diabetes, Res – resveratrol.

diabetes+resveratrol groups. Renal triglyceride, total cholesterol, ADMA and MDA levels were generally higher compared to the control but the differences were not significant. Renal total cholesterol was significantly decreased by resveratrol in STZ-induced diabetic rats. ANG-1 levels were lower in the diabetes group. When compared to the control group, NGAL levels were significantly higher in the diabetes group and normalized by resveratrol in the diabetes+resveratrol group (Fig. 2A).

#### Anti-inflammatory effects of resveratrol on kidney tissues of the rats

Renal *Inos* (Fig. 1A), *Nf-kb* (Fig. 1B) and *Nrf2* (Fig. 1C) mRNA expression was increased in the diabe-

tes group as compared to the control rats. Resveratrol treatment normalized these increases in the diabetes+resveratrol group. When compared to the rats in control group, diabetic rats had significantly higher renal levels of IL-6, IL-8 and TNF- $\alpha$ . Renal IL-1 $\beta$  protein levels were generally higher in the diabetes group but these changes were not statistically significant. The reduction in IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  protein levels with resveratrol treatment is summarized in Fig. 2D, E and F.

#### DISCUSSION

In this study, we evaluated the effects of resveratrol supplementation on diabetes-induced changes in renal tissues. In most studies with a similar aim, resveratrol

was administered orally, however, in this study we also wanted to examine the bioefficiency of resveratrol by i.p. administration to healthy and STZ-induced diabetic rats [19]. The significant increase in glucose concentrations in kidney homogenates in the diabetic group lead to increased oxidative stress, observed as an increase in ADMA. ADMA is excreted by the kidneys and inhibits nitric oxide synthase [20], so that NO levels can be linked to ADMA levels. Thus, it can be assumed that the renal iNOS levels in the diabetic group were induced by free oxygen radicals and associated macrophage infiltration. The ADMA levels increased in response to the increase in iNOS activity. In this respect, our results were in accordance with previous studies [13,21]. Our finding that the levels of MDA were increased, accompanying the increase in renal lipid levels in the diabetes group, was in agreement with the results of a study showing that renal MDA levels are higher in STZ-induced diabetic male rats [22].

Another complication caused by diabetes is the deterioration of the balance between angiogenesis inhibitors and activators. Angiogenesis is a physiological process also known as capillary restructuring. Previous studies argue that the level of ANG-1 is reduced in diabetes [4]. Our study also supports the reduction of renal ANG-1 in diabetes. However, there was no significant difference in the level of erythropoietin, which is known to be produced in large amounts in the kidneys, in kidney homogenates from different test groups. This suggests that there is no relationship between diabetes-induced complications, such as inflammation, and disturbances in homeostasis of blood cells.

The most common indicators of tissue inflammation are the presence of free oxygen radicals, oxidized lipids and cytokines such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [23]. According to our results, despite the absence of difference in renal erythropoietin levels, the levels of IL-6 and TNF- $\alpha$  were increased significantly in diabetic renal tissues. It is possible that hyperglycemia-induced free oxygen radicals, together with the increase of pro-inflammatory cytokines and lipid levels, enhanced the expression of renal NF- $\kappa$ B. The increase in the level of IL-8, which is responsible for the induction of macrophage infiltration into damaged tissues in the diabetic group, could serve as the source of the pro-inflammatory factors. This possibility is also

supported by previous studies [4,24-26]. Furthermore, a rise in renal oxygen free radicals and *Nf-kb* gene expression could lead to a compensatory increase in *Nrf2* levels. It can be inferred from our data that the resveratrol we used in our study normalized renal expression of all pro-inflammatory cytokines.

NGAL is not just a binding transport protein but also a protein synthesized in the renal tubular epithelium with many physiological functions, mainly in the synthesis of prostaglandins [27]. NGAL is usually synthesized from cells under stress conditions. Therefore, it is believed that in cases of ischemia, inflammation and infection, the expression of NGAL increases [6]. In inflammation, acute kidney failure and/or renal epithelium damage, blood and urine have increased concentrations of NGAL. After experimental renal tubular damage, the expression of renal NGAL mRNA and proteins in the urine and plasma were shown to increase [28]. In our study, the level of NGAL was increased parallel with the diabetes-associated inflammation. This suggests that the increase in pro-inflammatory cytokines causes an increase in NGAL expression. The normalization of the level of NGAL with the resveratrol treatment can be associated with the decreased expression of pro-inflammatory cytokines.

In conclusion, in diabetes, kidney tissues are exposed to damage because of the high levels of oxidized lipids, pro-inflammatory cytokines and free oxygen radicals produced by uncontrolled hyperglycemia. These complications lead to higher renal protein levels of NGAL. The rapid change in NGAL levels can be a parameter of acute renal failure and/or acute kidney damage. Since it is known that renal failure is a consequence of oxidative and inflammatory factors, resveratrol could be a supplement of choice for faster and more effective treatment of kidney failure/damage.

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**Author's contributions:** HBK, MBP and SK did the practical research work. GP and GS helped during experimental work and in writing the manuscript. MBP was responsible for the drafting of the manuscript, GS made study conception and design as well as critical revisions to the manuscript.

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