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The effects of ozone therapy on caspase pathways, TNF-α, and HIF-1α in diabetic nephropathy

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Abstract
Background Accelerated apoptosis plays a vital role in the development of diabetic vascular complications. Ozone may attenuate diabetic nephropathy by means of decreased apoptosis-related genes. The aim of our study was to investigate the effect of ozone therapy on streptozotocin-induced diabetic nephropathy in rats. Also the histopathological changes in diabetic kidney tissue with ozone treatment were evaluated.
Methods The rats were randomly divided into six groups ($n = 7$): control (C), ozone (O), diabetic (D), ozone-treated diabetic (DO), insulin-treated diabetic (DI), and ozone- and insulin-treated diabetic (DOI). D, DI, and DOI groups were induced by a single intraperitoneal injection of streptozotocin. Ozone was given to the O, DO, and DOI groups. Group DI and DOI received subcutaneous (SC) insulin (3 IU). All animals received daily treatment for 6 weeks.
Results Expressions of caspase-1-3-9, HIF-1α, and TNF-α genes were significantly higher in D group compared to C group ($p < 0.05$ for all). Ozone treatment resulted in significant decrease in the expressions of these genes in diabetic kidney tissue compared to both C and D group ($p < 0.05$ for all). Caspase-1-3-9, HIF-1α, and TNF-α gene expressions were found to be lower in DOI group compared to C group ($p < 0.05$ for all). Also adding ozone treatment to insulin therapy resulted in more significantly decrease in the expressions of these genes in diabetic tissue compared to only insulin-treated diabetic group ($p < 0.05$ for all). Regarding histological changes, ozone treatment resulted in decrease in the renal corpuscular inflammation and normal kidney morphology was observed. Both insulin and ozone therapies apparently improved kidney histological findings with less degenerated tubules and less inflammation of renal corpuscle compared to D, DO, and DI groups.
Conclusion Ozone therapy decreases the expressions of apoptotic genes in diabetic kidney tissue and improves the histopathological changes.

Keywords Diabetic nephropathy · Ozone therapy · Caspase · HIF-1α · TNF-α

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Introduction

Diabetic kidney disease has become the most common cause of end-stage renal disease (ESRD) in the world [1]. Recently, instead of the classical view that metabolic and hemodynamic change is the main factor for diabetic nephropathy development, emerging evidences have shown that a number of factors including inflammation, hypoxia, apoptosis act a part in pathogenesis and progression of both

microvascular and macrovascular complications of diabetes [2–4]. Apoptosis is defined as programed cell death. Although apoptosis is a natural phenomenon in multicellular organisms, accelerated apoptosis in endothelial cells plays a vital role in the development of diabetic vascular complications [5]. Hyperglycemia has also been shown to induce in vitro apoptosis of several cells [6, 7]. Apoptosis is mediated by the activation of the caspases and results in the cleavage of protein substrates and DNA fragmentation in diabetic kidney disease [8–10]. TNF-α and HIF-1α are shown to be related with apoptosis [11, 12]. It is shown that HIF-1α is related with serum creatinine value and renal fibrosis in diabetic nephropathy [13, 14]. TNF-α is important in pathogenesis of diabetic nephropathy [15]. The effect of ozone therapy on apoptosis is shown in the orbitofrontal cortex of neuropathic mice and intestinal ischemia–reperfusion injury in rats [16, 17]. Also Morsy et al. [18] showed that ozone therapy improved renal oxidative stress markers in diabetic nephropathy. However, the effect of the ozone therapy on diabetic nephropathy has not been explained yet.

The aim of our study was to investigate the effect of ozone therapy on streptozotocin-induced diabetic nephropathy in rats.

Methods

Study design

All experimental protocols conducted on the animals were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and approved by the Local Ethical Committee. Forty-two adult male Sprague–Dawley rats weighing approximately 300 g were used. All of the rats were maintained in a 12-h light/dark cycle environment (lights on 7:00–19:00 h) at 22 ± 1 °C and 50 % humidity, and they were kept in transparent plastic cages (42 × 26 × 15 cm), each containing three rats. The rats had access to food and water ad libitum. The rats were randomly divided into six groups ($n = 7$): control (C), ozone (O), diabetic (D), ozone-treated diabetic (DO), insulin-treated diabetic (DI), and ozone- and insulin-treated diabetic (DOI). Diabetes was induced by a single intraperitoneal injection of freshly prepared STZ (Sigma-Aldrich Co., Taufkirchen, Germany) solution (60 mg/kg body weight in 0.09 M citrate buffer, pH 4.8) in the D, DO, DI, and DOI groups. The animals in the C and O groups received the same volume of vehicle. Hyperglycemia was confirmed 48 h after STZ injection by measuring tail vein blood glucose levels using a glucometer (Accu-Chek, Roche Diagnostics Co., Mannheim, Germany). Only animals with mean plasma glucose levels above 300 mg/dl were classified as diabetic [18, 19]. The weights and blood glucose levels of all of the rats were measured before the experimental procedure and at the end of the experiments. The ozone was generated by an ozone generator (Dr. J. Hänsler OZONOSAN GmbH, Iffezheim, Germany). A 50 µg/ml concentration of ozone was given to the O, DO, and DOI groups (1.1 mg/kg intra peritoneal, 1-min injection period once a day for 6 weeks). This dosage and schedule of ozone was in a previous study. This dose of ozone has been shown to achieve oxidative preconditioning without appreciable toxicity [18, 20]. Insulin (3 IU) (Novo Nordisk Co., Bagsvaerd, Denmark) was administered to the DI and DOI groups (intraperitoneal in 1 ml saline, once a day for 6 weeks). Oxygen was injected as the vehicle for the ozone in the C, D, and DI groups every day for 6 weeks. In addition, 1 ml saline was injected in the C, O, D, and DO groups every day for 6 weeks. At the end of the experimental period, the rats were anaesthetized with ketamine/xylazine (90 and 10 mg/kg, respectively, intraperitoneally. Then the abdomens of the rats were opened using a midline incision under anesthesia. Kidney tissues were removed for genetic and histological analyses. The animals were then euthanized by exsanguination while under ketamine and xylazine anesthesia.

RNA isolation and analyses of apoptosis-related gene expression

The kidney divided into small pieces in trizol solution by a lancet on ice. Total RNA was extracted from the tissues using an RNA isolation reagent (Tri reagent) procedure (Sigma, St. Louis, MO, USA). An EasyScript Plus cDNA Synthesis kit was used for the synthesis of complementary DNA (cDNA) from the tissues.

Relative quantification of apoptosis-related gene expression

Real-time quantitative polymerization chain reaction (RT-PCR) analyses of all genes were performed using a Step One Plus RT-PCR instrument and software. Rat primers were used for gene expression analyses. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene was chosen as a standard to control the variability in amplification. RT-PCR was performed by using an Eva Green qPCR master mix kit according to the instructions of the manufacturer. The experiments were repeated twice using duplicates in each group.

Analysis setup for delta–delta CT method

In the "Basic Setup" section, assign samples to different groups. At least two groups are needed, where one of those

Table 1 Primers used for one-step RT-PCR

Primer name	Sequence
rCaspase1	F: 5′-CCACTCCTTGTTTCTCTC-3′
rCaspase1	R: 5′-CCTTCCTTGTATTCATGTC-3′
rCaspase3	F: 5′-TGAGCATTGACACAATACAC-3′
rCaspase3	R: 5′-AAGCCGAAACTCTTCATC-3′
rTNFalpha	F: 5′-TACTGAACTTCGGGGTGATTGGTCC-3′
rTNFalpha	R: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
rCaspase9	F: 5′-ATGGACGAAGCGGATCGGCGGCTCC-3′
rCaspase9	R: 5′-AAGCCGAAACTCTTCATC-3′

groups must be the control group. Click “Update” when finished. You may exclude samples from the analysis by selecting “Exclude” on the drop-down menu.

Review the “Data QC” section to assess each groups’ PCR reproducibility, reverse transcription efficiency, and the presence of genomic DNA contamination.

The “Select Housekeeping Genes” section allows you to remove or add preferred housekeeping genes for data normalization by clicking the appropriate checkboxes. Click “Update” when finished.

Review the “Data Overview” section to see each group’s distribution of threshold cycle values and the average of the raw data in each group.

Analysis

See the “Average Ct,” “2^(−Ct),” “Fold Change,” “*p* value,” and “Fold Regulation” sections for the results processed by the software from your data. The “Fold Change” and “*p* value” results are used by the software in subsequent graphical analyses.

Histological evaluation

The study included formalin-fixed and paraffin-wax-embedded kidney specimens of rats of control and experimental groups. For histopathological examination, routine paraffin-wax-embedded method was used. Briefly, at the end of experiment, the kidneys were removed from the rats and fixed in 10 % formaldehyde, embedded in paraffin. Thick paraffin sections (5 µm) were cut from each specimen. After deparaffinization and rehydration, all sections stained with periodic acid-Schiff (PAS) to provide a morphological overview and to show the brush border of the epithelial cells of the proximal tubule and the thickness of basal lamina. Tissues were examined, and images were captured using a Olympus BX51 microscope (Olympus BX-51, Japan). Histological evaluation was evaluated according to a previous study [21].

TUNEL immunofluorescence staining

To detect apoptosis within the cells of the kidneys, in situ TdT-mediated X-Dutp nicked labeling (TUNEL) reaction to the paraffin sections was applied by using ApopTag® Fluorescein In Situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany) in accordance with the manufacturer’s recommendations. Briefly, serial 5-µm-thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and incubated 5 min in phosphate-buffered saline (PBS) at room temperature. Slides were incubated 15 min with proteinase K and washed with distilled water. After wash with several times in PBS, they were pre-treated with 3 % hydrogen peroxide for 10 min. The specimens were incubated with fluorescein-labeled deoxy-UTP at 37 °C for 1 h at humidity ambient. The nucleus was visualized with 4, 6-diamino-2-phenylindole (DAPI). The images were taken randomly for evaluating the TUNEL-positive cells by using immunofluorescence microscope (Olympus BX51, Tokyo, Japan). For quantitative analysis, ten visual fields were randomly photographed for each TUNEL-stained section from each experimental groups under microscope at 400× magnification. The number of TUNEL-positive cells nuclei (apoptotic nuclei) was counted with Image J software.

Statistical analysis

Data from related mRNA expressions have been analyzed with the delta–delta CT method and quantitated with a computer program named LightCycler Quantification Software. The comparisons were performed using the Student’s *t* test, one-way ANOVA, and post hoc Tukey test.

Results

Rat primers were used for gene expression analyses (Table 1). Glucose levels of groups are shown Table 2. Expression of caspase-1 gene was determined to be 9.98-fold higher, caspase-3 gene 1.89-fold higher, caspase-9 gene 19.91-fold higher, HIF-1α gene 2.12-fold higher, and TNF-α gene 2.12-fold higher in D group when compared with C group ($p < 0.05$ for all).

Expression of caspase-1 gene was determined to be 9.52-fold lower, caspase-3 gene 1.56-fold lower, caspase-9 gene 25.52-fold lower, HIF-1α gene 1.00-fold lower, TNF-α gene 1.65-fold lower in O group when compared with C group($p < 0.05$ for all). Ozone treatment resulted in significant decrease in the expression

Table 2 Glucose levels of groups

	DOI	DI	DO	D	O	C
Glucose at fist	103.41 ± 17.79	87.72 ± 15.12	101.14 ± 17.02	97.15 ± 9.06	105.00 ± 7.63	100.01 ± 9.57
Glucose at death	248.42 ± 40.75*	300.71 ± 30.51*	406.57 ± 46.80*	556.57 ± 24.27	112.85 ± 12.7*	105.85 ± 2.26*

C control, *O* ozone, *D* diabetic, *DO* ozone-treated diabetic, *DI* insulin-treated diabetic, *DOI* ozone- and insulin-treated diabetic

$p < 0.001$ versus diabetic group

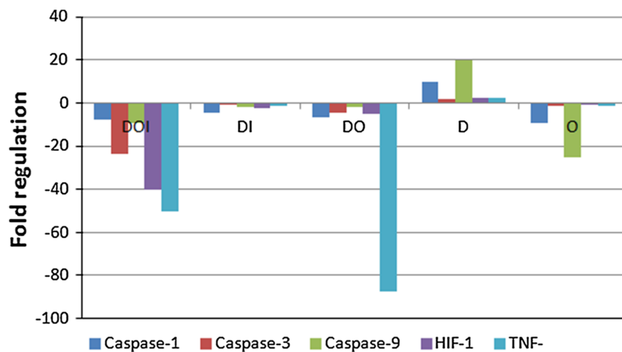

Fig. 1 Apoptosis associated with gene expression in all groups: comparing to control group $p < 0.05$. *C* control, *O* ozone, *D* diabetic, *DO* ozone-treated diabetic, *DI* insulin-treated diabetic, *DOI* ozone- and insulin-treated diabetic, *HIF-1α* hypoxia-inducible factor alpha, *TNF-α* tumor necrosis factor alpha

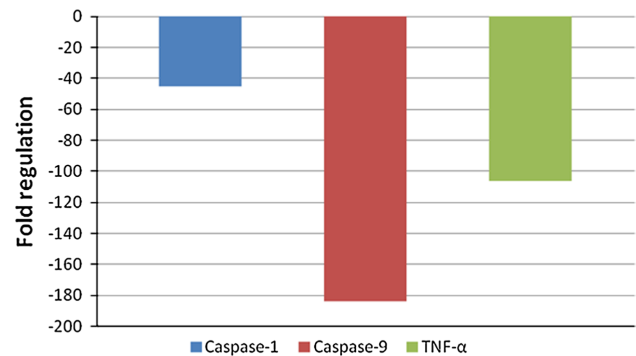

Fig. 2 Apoptosis associated with gene expression in diabet + ozone therapy group: comparing to diabetic group $p < 0.05$. *TNF-α* tumor necrosis factor alpha

of these genes in diabetic group compared to C group (caspase-1 6.72-fold lower, caspase-3 4.45-fold lower, caspase-9 1.78-fold lower, HIF-1α 5.38-fold lower, and TNF-α 87.50-fold lower; $p < 0.05$ for all). Expression of caspase-1 gene was determined to 4.54-fold lower, caspase-3 gene 1.11-fold lower, caspase-9 gene 1.98-fold lower, HIF-1 α gene 2.430-fold lower, and TNF-α gene 1.20-fold lower in DI group when compared with C group ($p < 0.05$ for all). Expression of caspase-1 gene was determined to be 7.93-fold lower, caspase-3 gene 23.82-fold lower, caspase-9 gene 9.22-fold lower, HIF-1α gene 40.17-fold lower, and TNF-α gene 50.07-fold lower in DOI group when compared with C group ($p < 0.05$) (Fig. 1).

Expression of caspase-1 gene was determined to be 44.95-fold lower, caspase-9 gene 183.95-fold lower, and TNF-α gene 105.99-fold lower in DO group when compared with D group ($p < 0.05$ for all) (Fig. 2).

Expression of caspase-1 gene was determined to be 50.65-fold lower, caspase-9 gene 8.98-fold lower, HIF-1α gene 413.07-fold lower, and TNF-α gene 73.01-fold lower in DOI group when compared with DI group ($p < 0.05$ for all) (Fig. 3).

Significant differences were not detected gene expression of BAD, BAX, BID, BCL2, and BCL-XL in all groups (Figs. 4, 5, 6).

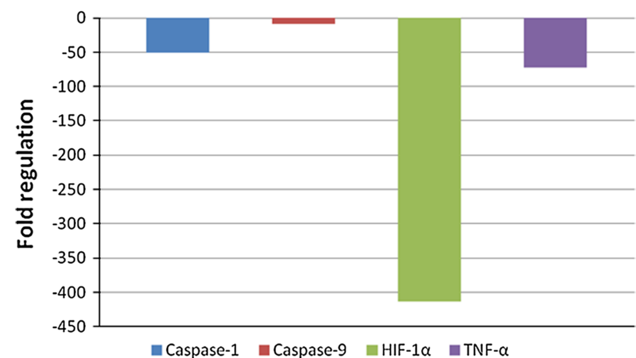

Fig. 3 Apoptosis associated with gene expression in diabet + insulin + ozone therapy group: comparing to diabet + ozone therapy group $p < 0.05$. *HIF-1α* hypoxia-inducible factor alpha, *TNF-α* tumor necrosis factor alpha

Histological findings

As shown in Fig. 7, histological study of the normal kidney of the nondiabetic rats revealed normal glomerulus surrounded by the Bowman's capsule, proximal, and distal convoluted tubules without any inflammatory changes. No histopathological alterations were observed in control kidney. Many histopathological changes were observed in the STZ-induced diabetic kidney compared with control groups. The histopathological examination of STZ-induced diabetic kidney showed degenerated glomeruli,

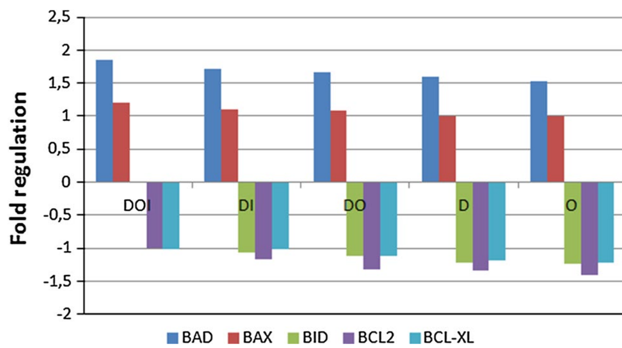

Fig. 4 Apoptosis associated with gene expression in all groups: comparing to control group $p > 0.05$. *C* control, *O* ozone, *D* diabetic, *DO* ozone-treated diabetic, *DI* insulin-treated diabetic, *DOI* ozone- and insulin-treated diabetic

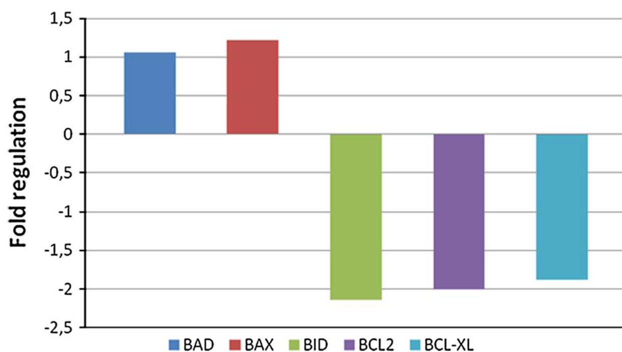

Fig. 5 Apoptosis associated with gene expression in ozone treated diabetic group. $p > 0.05$: comparing to diabetic group

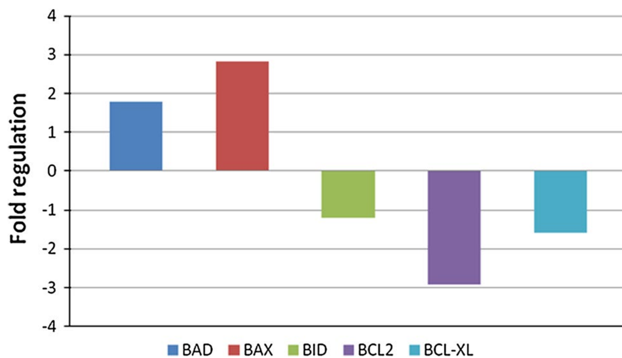

Fig. 6 Apoptosis associated with gene expression in diabet + insulin + ozone therapy group: comparing to diabet + ozone therapy group $p > 0.05$

the inflammatory cells in the glomeruli, and thickening of the basement membrane. PAS staining indicated that epithelium of proximal and distal convoluted tubules exhibited edematous changes. There were damages at brush border edges of apical membranes of proximal tubules by PAS staining, and bowman cavity was enlarged compared to control group. The only ozone administered animals did not induce any pathological changes, and the kidney tissues appeared similar to the control group. There were normal glomeruli and normal tubules in only ozone therapy group. Moreover, ozone treatment resulted in little pathological alterations when compared with STZ-induced diabetic group. Only some edematous changes were observed in some proximal and distal convoluted tubules. This group showed features of healing as normal glomerulus, absence of inflammatory cells, and normal basement membrane. The group that was treated with insulin showed normal basement membrane, absence of inflammatory cells in the glomeruli, and morphologically normal tubules except some proximal convoluted tubule exhibited edematous changes. Both the insulin and ozone therapies improved kidney histological pictures with less degenerated tubules compared to STZ group. The degree of renal changes to the number of kidney glomerulus in all groups is shown in Table 3.

TUNEL findings

We investigated the influence of ozone and insulin on the diabetic kidney in each experimental group. To evaluate apoptotic cell number, we used the TUNEL method. The number of the TUNEL-positive cells more increased in the diabetic group compared to control group, while the TUNEL-positive cell number was nearly same with control group in ozone group. TUNEL-positive cell number decreased in co-treatment of STZ administered with ozone and insulin groups. According to our recent findings, we detected an increased number of apoptotic cell within the kidney tissue of diabetic group, but these apoptotic cell number downregulated in the group of ozone + insulin-administered diabetic group (Fig. 8). The number of apoptotic cells in all groups is shown in Table 4.

Discussion

In our study, we showed for the first time that ozone therapy significantly decreased the gene expressions of caspases-1-3-9, HIF-1α, and TNF-α and improved histopathological outcomes of the diabetic nephropathy. Also apoptotic cell number downregulated in the group of ozone + insulin-administered diabetic group.

Apoptotic cell death was demonstrated to be important in diabetic nephropathy by Kumar et al. [22]. Wong et al. [23] have shown that high glucose levels stimulated expression of apoptosis genes. In line with the study, we demonstrated that expression of caspases-1-3-9 genes was increased in diabetic group compared to control group. Liadis et al. [24] have shown that caspase-3-mediated

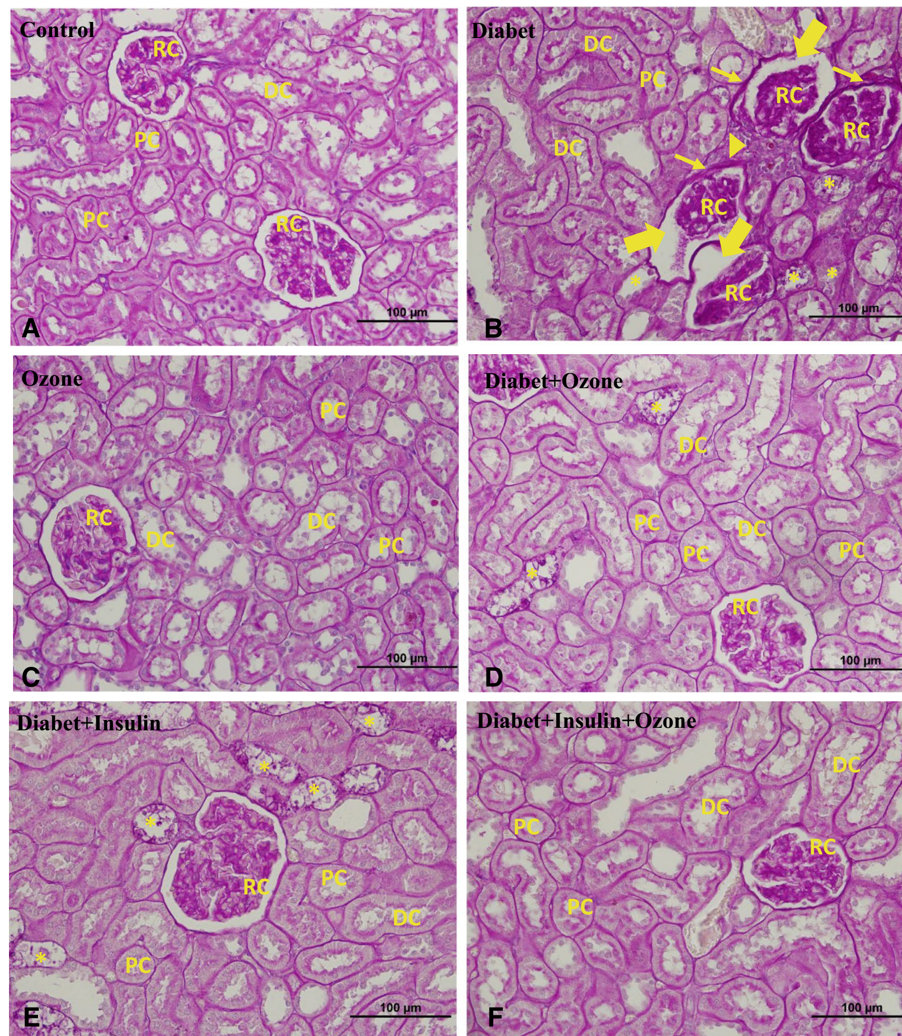

Fig. 7 Representative photomicrographs of PAS-stained kidney tissue sections from experimental groups. **a** Histological kidney sections of normal control group showing normal tubular structure and renal corpuscle. **b** In the rats treated with STZ, the kidney damaged. There were inflammatory cells, degenerated renal corpuscle with thickening of the basement membrane, and the proximal convoluted tubule showed hypercellularity with edema. **c** The general architecture of kidney was also structurally normal in the only ozone therapy group. **d** Ozone treatment apparently alleviated STZ-induced histopathological damage in the STZ + ozone group. **e** Histological picture showed a decrease in the kidney damages in STZ + insulin group. **f** Histological picture of STZ + insulin + ozone group showed a significant amelioration of kidney histoarchitecture. *Arrow* thickening of the basement membrane. *Thick arrow* enlarged bowman cavity. *Star* degenerated tubules with hypercellularity with edema. *Arrow head* inflammation. *RC* renal corpuscle, *PC* proximal convoluted tubule, *DC* distal convoluted tubule. Original magnification, PAS, ×400

Table 3 The degree of these renal changes to the number of kidney (glomerulus) in all groups

Group	Renal changes to the number of kidney (glomerulus) %
C	3
O	4
D	90
DO	65
DI	35
DOI	12

C control, *O* ozone, *D* diabetic, *DO* ozone-treated diabetic, *DI* insulin-treated diabetic, *DOI* ozone- and insulin-treated diabetic

apoptosis plays a role in beta cell destruction. High glucose levels were shown to induce apoptosis in endothelial cells by Ho et al. [25]. Previous studies showed that gene expression of caspase-1 is higher in diabetic nephropathy [26, 27].

The increase in oxidant activity due to hyperglycemia is great importance in pathogenesis of diabetic nephropathy. A significant increase in oxidant activity was detected in renal proximal epithelial cells after being exposed to high glucose approximately 24 h. Significantly increased activity of caspases-3-8-9 was detected. DNA fragmentation reached a maximum after 48 h of exposure to high glucose. The high glucose-induced increase in caspase activity

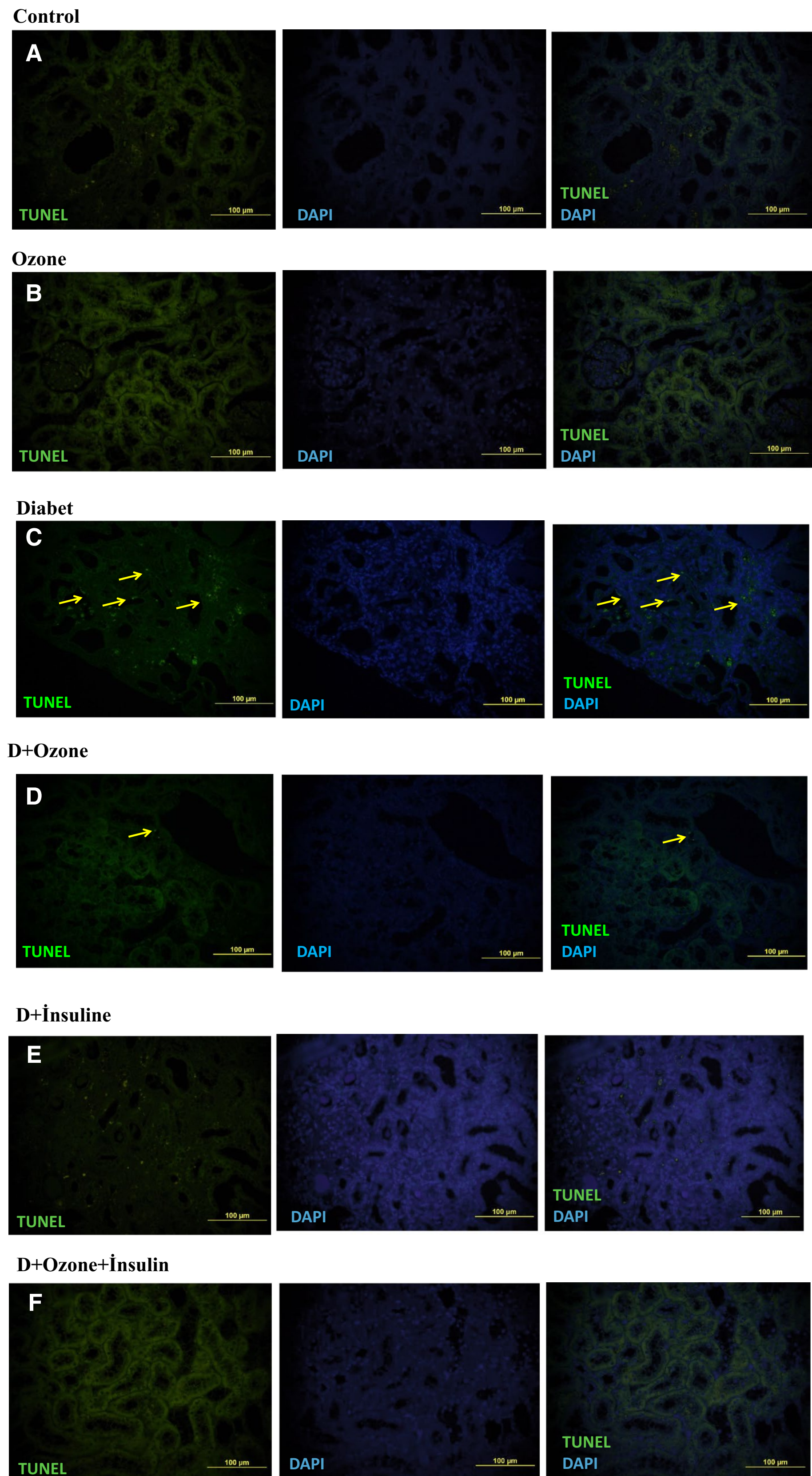

Fig. 8 TUNEL + cells reflective green immunofluorescence. **a** There was only a few TUNEL + cells in kidney of control group. **b** The ozone-treated group had also less TUNEL + cells like control group. **c** Many apoptotic cells in the tubules both cortex and medulla of diabetic group were determined. **d** Effects of ozone on TUNEL + cells in renal diabetic injury in rats. **e** There was TUNEL + apoptotic cells decreased on section profiles of insulin group compared with diabetic group. **f** TUNEL + cell number in kidney tissue of ozone + insulin-administered diabetic group was more less compared to only ozone and insulin therapy groups. TUNEL staining, ×400

Table 4 The number of apoptotic cells in all groups

	DOI	DI	DO	D	O	C
The number of apoptotic cells	1.65 ± 2.19*	1.71 ± 1.88*	2.30 ± 2.40$^{\alpha}$	4.47 ± 3.25	1.63 ± 1.89*	1.38 ± 1.72*

C control, *O* ozone, *D* diabetic, *DO* ozone-treated diabetic, *DI* insulin-treated diabetic, *DOI* ozone- and insulin-treated diabetic

* $p < 0.001$ versus diabetic group

$^{\alpha}$ p 0.005 versus diabetic group

and DNA fragmentation were reduced in the presence of antioxidant therapy [10]. It has been shown that ozone therapy increases the activity of antioxidant enzymes and reduced activity of oxidant enzymes [20, 28]. Erken et al. [19] showed that ozone therapy decreases the total oxidant status and oxidative stress index, and increases total antioxidant status in diabetic rats. Morsy et al. [18] have demonstrated ozone therapy reduces oxidative stress markers and improves renal antioxidant enzyme activity in diabetic nephropathy. Hyperglycemia decreases antioxidant activity and induces oxidative stress. Ozone therapy decreased the blood glucose levels [19]. Several mechanisms have been suggested for this effect of ozone. Glucose uptake is rapidly decreased in the presence of hydrogen peroxide, and the effect was reversed by ozone therapy [29, 30]. It was reported that ozone treatment affected the metabolic actions related to inhibition of glycogen depletion and decrease in the blood glucose levels [31]. These findings indicate that the protective effect of ozone on diabetic nephropathy might be mediated by oxidant/antioxidant mechanisms and glucose lowering effect may result in decrease in caspases levels and apoptosis.

Effects of ozone therapy on apoptosis and caspase pathway have been shown in some studies. Fuccio et al. [16] have shown that ozone therapy inhibits expression of caspase-1 gene in orbitofrontal cortex. Also ozone therapy has been shown to inhibit apoptosis in intestinal ischemia reperfusion injury in rats in a study by Haj et al. [17].

It was demonstrated that expression of HIF-1α is correlated with apoptosis [11, 35]. We have demonstrated that HIF-1α value increased in diabetic rats in comparison with control rats. In accordance with present results, Sagar et al. [13] have shown that over expression of HIF-1α is correlated with renal interstitial fibrosis and serum creatinine value in diabetic nephropathy. Tang et al. [14] have shown that in the 4th, 8th and 12th week, the areas of renal interstitial fibrosis were significantly increased in diabetic nephropathy group, which was accompanied by higher levels of 24-h urinary protein HIF-1α, compared with control group. HIF-1α mRNA expression has been shown to increase significantly in diabetic rats [36]. In our study, HIF-1 α gene expression level decreased in ozone therapy group.

It has been shown that TNF-α induces apoptosis [12, 32]. In this study, ozone therapy decreased TNF-α in diabetic nephropathy. In accordance with our study, Xing et al. [33] have shown that ozone therapy could decrease the mRNA levels of TNF-α in reperfusion injury in rat kidney. Azuma et al. [34] have shown that the intraperitoneal injection of ozonized water decreased the levels of TNF-α and increased the activity of superoxide dismutase. Selective antioxidants attenuated TNF-α-mediated activation of HIF-1α [37, 38]. HIF-1α increases caspase-9 and caspase-3 via BAX which is a proapoptotic protein [39, 40]. Ozone therapy may reduce TNF-α and HIF-1α via its antioxidant properties and thus might decrease caspase-3 and caspase-9.

There are some limitations of this study. Protein expressions should be analyzed in addition to gene expression to clarify the importance of inflammation and HIF-1α in diabetic nephropathy.

In conclusion, this study showed ozone therapy significantly improves the histopathological changes induced by high glucose level in diabetic nephropathy. Possible mechanisms for the protective role of ozone therapy reduced the gene expression levels of caspases-1, 3, 9 and cell apoptosis-related genes such as TNF-α and HIF-1α. We demonstrated for the first time the effects of ozone therapy on expression of apoptosis genes in diabetic nephropathy, and we think that this article will contribute to further studies for new researches.

Study limitations

There are some limitations of this study. Protein expressions should be analyzed in addition to gene expression to clarify the importance of inflammation and HIF-1-alpha in diabetic nephropathy.

Compliance with ethical standards

Conflict of interest None.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experimental protocols conducted on the animals were consistent with the National Institutes of Health Guidelines for the Care and Use of

Laboratory Animals (NIH Publication No: 85-23) and approved by the Local Ethical Committee.

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