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ORIGINAL RESEARCH

Effects of Ozone Therapy on the Bladder of Rats with Streptozocin-Induced Diabetes

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Purpose: Diabetes mellitus (DM), an increasingly prevalent condition worldwide, leads to complications in many organs. Diabetic cystopathy is one of the most frequent urological complications impairing the quality of life. Ozone is used therapeutically in various conditions due to its regulatory effects on oxidant status, as well as its microbicidal and immune-modulatory properties. We aimed to determine the effects of ozone on diabetic bladders, focusing on morphological alterations, inflammatory changes, and serum oxidant status.

Methods: Twenty-four male Wistar Albino rats were randomly assigned to four groups (n=6 each): control (C), ozone (O), DM, and ozone-administered DM (DM+O). Four weeks after diabetes induction by intraperitoneal streptozotocin injection, O and DM+O rats received daily rectal ozone insufflation for 7 days. At the end of the fifth week, serum samples were collected to evaluate oxidant status, while bladders were analyzed by light and electron microscopy and investigated immunohistochemically for inflammatory cytokines.

Results: Compared with the DM group, the DM+O group showed lower serum glucose and TOS levels and higher TAS levels, along with lower OSI values. Histologically, the DM+O group had a higher connective tissue-to-bladder wall ratio, a lower muscle tissue-to-bladder wall ratio, and a lower muscle-to-connective tissue ratio than the DM group. TNF-α and TGF-β immunostaining intensities were lower in the DM+O group compared to the DM group. IL-10 immunostaining intensity was higher in the DM+O group than in the DM group. The thickness of the urothelial basement membrane in the DM+O group was greater than in the DM group.

Conclusion: Study findings suggest ozone may limit oxidative stress, inflammation, and structural alterations in the diabetic bladder, indicating a potential supportive role alongside standard diabetes treatment; further studies are needed to confirm its clinical relevance.

Keywords: diabetic cystopathy, ozone, oxidative stress, inflammation, urothelium, transmission electron microscopy

Introduction

Diabetes, one of the most common metabolic disorders worldwide, leads to microvascular and macrovascular complications due to hyperglycemia, and among the well-known complications of diabetes are neuropathy, nephropathy, and retinopathy.[1–3] On the other hand, lower urinary tract complications involving bladder dysfunction — diabetic cystopathy — in diabetic individuals were reported to be more prevalent than neuropathy and nephropathy complications, which are most commonly screened in diabetic patients.[1,2,4] Although diabetic cystopathy is not a life-threatening condition, the detrimental effects of it on the quality of life bear social and economic issues.[5] However, diabetic bladder dysfunction may lead to serious complications, including hydroureteronephrosis, pyelonephritis, and urosepsis.[4,6]

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Morphological alterations in diabetic bladders involve increased wall thickness and lumen area, due to hypertrophy and dilation of the wall.[1,6] The suggested fundamental mechanism underlying the alterations in the early stages of the diabetic cystopathy is polyuria led by hyperglycemia.[1,7,8] Morphological reflection of this extreme functionality of the bladder in the early stages of diabetes is hypertrophy of the detrusor muscle.[9,10] In the chronic period, on the other hand, apart from excessive urine production, oxidative stress induced by hyperglycemia causes neuropathy, detrusor muscle dilation, and decreased contractility.[1,11] Besides morphological alterations in the smooth muscle of the bladder wall, urothelial changes have also been demonstrated in various experimental studies, revealing the functional importance of urothelium in the regulation of bladder function beyond being a simple barrier.[2,5,12] Mediators secreted by the bladder epithelium in diabetes affect bladder capacity, impacting the function of the smooth muscle layer of the bladder wall.[6,12]

More recent studies focusing on diabetic cystopathy have revealed its highly complex pathophysiology extending beyond polyuria-induced mechanical stress.[13–15] Increased expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the activation of the nucleotide-binding oligomerization domain (NOD)-, Leucine-rich repeat (LRR)- and pyrin domain-containing protein 3 (NLRP3) inflammasome cascade are emphasized among the primary drivers of diabetic cystopathy.[14–16] Emerging molecular evidence demonstrates the crucial role of hyperglycemia-induced mitochondrial structural alterations and dysfunction in diabetic cystopathy, as evidenced by reduced mitochondrial membrane potential, decreased AMP-activated protein kinase (AMPK) phosphorylation, and downregulation of mitophagy- and autophagy-related proteins.[13] Multiple signaling pathways trigger persistent inflammation, epithelial barrier disruption, and progression to fibrosis characterized by aberrant extracellular matrix remodeling in the bladder wall during the chronic period of diabetes.[15,17,18] Despite its high prevalence and the recent mechanistic understanding underlying the pathophysiology, standard treatment algorithms for diabetic cystopathy have yet to be established, making the current treatment management of this patient largely symptomatic and supportive —no different from that of patients without diabetes— offering limited efficacy in halting the underlying structural remodeling and neurogenic deterioration.[2,13] Therefore, targeting the upstream oxidative, inflammatory, and fibrotic pathways represents a critical clinical necessity to prevent the irreversible progression of diabetic bladder complications. Furthermore, animal models that mimic diabetic complications are crucial for exploring the complex pathophysiological processes underlying diabetes and its complications, as well as for developing novel therapeutic strategies.[13,19,20]

Ozone, a gas consisting of three oxygen atoms, has been shown to exhibit modulatory effects in many acute and chronic detrimental conditions, such as wounds, infections, sepsis, diabetes, musculoskeletal disorders, cardiovascular and neurodegenerative diseases, by improving antioxidant defense mechanisms and regulating inflammatory mediators.[21–26] It does not cause oxidative imbalance, instead exerts improvement in antioxidant defense when applied at a proper low-dosage.[27] Because reactive oxygen species contribute to the development of diabetic complications, ozone, owing to its antioxidant properties, has been investigated in both experimental and clinical diabetes research, revealing its beneficial effects on glycemia, oxidative status, endothelial damage, and eventually diabetic complications.[21,28,29] However, to the best of our knowledge, no previous study has addressed the therapeutic or protective effects of ozone on diabetic bladder morphology, particularly regarding its remodeling, inflammation, and ultrastructural integrity. This study addresses this specific gap by providing the first comprehensive evaluation of rectal ozone therapy on bladder remodeling, cytokine expression, and urothelial ultrastructure in streptozotocin-induced diabetic rats, highlighting its translational potential for clinical diabetic cystopathy management.

Material and Methods

Experimental Animals

Following approval by the Gazi University Local Ethics Committee for Animal Experiments (approval number G.Ü. ET-22-103), the rats used in the study were obtained from the Gazi University Experimental Research Center (GÜDAM). The study was conducted at the same center in accordance with the ARRIVE 2.0 guidelines. All procedures adhered to the accepted standards outlined in the Guide for the Care and Use of Laboratory Animals. 24 Wistar Albino male rats weighing 225–300 g were used for this study. Prior to the commencement of the experimental procedures, all animals were allowed to acclimatize to the laboratory conditions for 7 days to minimize stress-induced physiological variations. Animals were kept in an environment with 45–55%

humidity at 20–21°C and a 12-hour light/12-hour dark cycle, with free access to food and water until 2 hours before the anesthesia procedures.

Study Design

Twenty-four Wistar Albino male rats weighing 225–300 g were used for this study. Animals were randomly allocated to four groups as Control (Group C), Ozone (Group O), Diabetes Mellitus (Group DM), and Diabetes Mellitus + Ozone (Group DM + O), each containing 6 rats. After a 5-week maintenance in normal conditions, animals in group C were sacrificed by intracardiac blood collection, following anesthesia administered via intramuscular injection of ketamine hydrochloride at a dose of 50 mg/kg (Ketalar® Pfizer PFE İlaçları, İstanbul, Turkey) and xylazine hydrochloride at a dose of 5 mg/kg (Alfazyne, 2%, Ege Vet). The rats in Group O were cared for over a 4-week period. After 4 weeks, these rats were administered 4 mL of rectally insufflated ozone (20 µg/mL) (Medozon Compact, Herrmann Apparatebau GmbH, Germany) via an 18-gauge cannula for 1 minute daily for 7 days. At the end of 35 days, they were sacrificed by intracardiac blood collection under ketamine and xylazine anesthesia as the animals in Group C.

To induce diabetes mellitus (DM), rats were injected with a single dose of 55 mg/kg streptozotocin intraperitoneally, a dose that leads to fasting permanent hyperglycemia, relative low-level of blood insulin, glycosuria, polyuria, and a prominent reduction in pancreatic insulin.[30,31] Blood glucose levels were measured via the tail vein 72 hours after injection, and rats with blood glucose levels of 250 mg/dL or higher were considered diabetic.[31,32] The rats were maintained for 4 weeks after streptozotocin injection, the duration required for the effects of diabetes on the organs to emerge, as bladder wall remodeling, including detrusor muscle thickening, has been shown to occur as early as 4 weeks after diabetes induction.[10] After completing 4 weeks, diabetic rats were administered either 4 mL of medical air or 4 mL of ozone (20 µg/mL) (Medozon Compact, Herrmann Apparatebau GmbH, Germany) in Group DM and Group DM + O, respectively. Medical air or ozone (20 µg/mL) was insufflated rectally via an 18-gauge cannula for 1 minute daily for 7 days.

The ozone concentration (20 µg/mL) and administration route were selected based on well-established protocols that represent the therapeutic low-dose ozone concept, which has been proven to stimulate endogenous antioxidant pathways without inducing oxidative stress and exerts anti-inflammatory effects.[27,33,34] Non-fasting blood glucose levels were measured once a week and at the end of the 5th week prior to sacrification to monitor the diabetic status.[35,36] At the end of 35 days, the rats were sacrificed by intracardiac blood collection under ketamine and xylazine anesthesia, like the animals in Groups C and O.

Following sacrification, the bladders of each animal were excised and cut into two parts at the equatorial midline. One half was immersed in 10% neutral buffered formalin for light microscopic examination, whereas the other half was taken into 2.5% glutaraldehyde for transmission electron microscopic (TEM) evaluation. Serum samples harvested from the blood samples of animals were stored at −80 °C until the day of the experiment to measure total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI) values. Histopathological, immunohistochemical, and transmission electron microscopic evaluations were performed by two histologists blinded to group allocation to minimize observer bias, as well as serum samples were coded with randomized identifiers by an independent researcher.

Biochemical Analysis

TAS/TOS Measurement

The TAS/TOS test kit (RelAssay Diagnostic®, Turkey) was used according to the manufacturer's instructions to measure the TAS/TOS levels. TAS/TOS was studied using the method specified in previous publications.[37,38]

Oxidative Stress Index

The OSI, which is shown as an indicator of oxidative stress, is expressed as the ratio of TOS to TAS levels. When calculating the OSI of the samples, TAS levels were multiplied by 100 to equalize the TOS levels and the units. The results were expressed as arbitrary units (AU).

$$\mathrm{OSI} = \frac{\mathrm{TOS}, \mu\ \mathrm{mol}H_2O_2\mathrm{Equiv.}\ /\mathrm{L}}{\mathrm{TAS}, \mathrm{mmol\ Trolox\ Equiv.}\ /\mathrm{L} \times\ 100}$$

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Histopathological Assessments

After being fixed in 10% neutral buffered formalin, the bladder tissue samples were dehydrated through an increasing grade series of alcohol, then cleared in xylene and embedded in paraffin after infiltration with liquid paraffin. From each paraffin tissue block, 4 μm-thick sections parallel to the equatorial plane were cut by using a rotary microtome (Leica RM2245, Germany) and mounted on glass slides. To evaluate the proportions of collagen, smooth muscle, and epithelium, sections were stained with Masson trichrome stain. Trichrome-stained sections of bladder samples were observed under a light microscope (Axioscope A1, Zeiss, Germany) equipped with a computer, and panorama images were captured at 50× magnification using ZEN 2.6 (Blue Edition, Zeiss, Germany) software. Color segmentation was performed using the image analysis module of Zeiss Zen 3.9 software to determine the proportions of blue-, red-, and pink-colored areas, which represent the collagen, smooth muscle, and epithelial content of the section area, respectively.[39,40]

Immunohistochemical Assessments

Four μm-thick paraffin sections were also immunostained to evaluate tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), transforming growth factor beta1 (TGF-β1), and interleukin 10 (IL-10) expression. To that end, paraffin sections were mounted on positively charged slides, then deparaffinized in xylene and rehydrated through a decreasing-grade series of ethanol. To expose epitopes, sections were subjected to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) (AP-9003-999, Thermo Fisher Scientific, USA) in a water bath at 85 °C for 2 hours, and then cooled at room temperature. Blocking endogenous peroxidase activity with 3% hydrogen peroxide for 30 minutes at room temperature was followed by incubation with protein-blocking solution (PL-125-PBQ, PatoLab, Türkiye), to prevent non-specific binding of the antibodies. Afterward, sections were left in incubation with rabbit polyclonal anti-TNF-α (1:100 dilution, bs-2081R, Bioss, USA), rabbit polyclonal anti-IL-1β (1:100 dilution, E-AB-66749, Elabscience, USA), rabbit polyclonal anti-TGF-β1 (1:300 dilution, bs-0086R, Bioss, USA), and rabbit polyclonal anti-IL-10 (1:300 dilution, bs-0698R, Bioss, USA) primary antibodies at +4 °C overnight. Sections were then incubated with biotinylated goat anti-polyvalent secondary antibodies (PL-125-BN, PatoLab, Türkiye). After the sections were incubated with streptavidin-peroxidase enzyme complex (PL-125-HR, PatoLab, Türkiye) for 30 minutes at room temperature, immunoreactivity was visualized using 3,3'- diaminobenzidine (DAB) chromogen-substrate complex (PL-125-HDX, PatoLab, Türkiye), which produces a brown color. Mayer hematoxylin (05–06002/L, Bio-Optica, Italy) was used for background staining. All immunostained sections were examined under a light microscope (Leica DM 4000B, Germany) equipped with a computer, and images of all sectional areas were captured as non-overlapping fields at 100× magnification using the Leica LAS V4.12 software. TNF-α, IL-1β, TGF-β1, and IL-10 immunoreactivities in the bladder specimens were evaluated based on immunostaining intensities of the sections, measured from captured images using ImageJ software (1.53a, National Institutes of Health, United States), and the results were expressed as "% area".[41,42] To perform measurements, brown-colored DAB staining was separated from hematoxylin staining, and then a consistent threshold adjustment was applied across all groups to isolate the brown-colored positive immune-staining area from the background.

Transmission Electron Microscopic (TEM) Examination

Half of the bladder samples from the control and study groups were fixed in 2.5% glutaraldehyde at +4°C for 24 hours, followed by washing in phosphate buffer. Specimens were further fixed in 1% osmium tetroxide for 2 hours in the dark, then washed in phosphate buffer again. After the samples were washed with distilled water, they were incubated in a solution of 0.05% uranyl acetate and 1% phosphotungstic acid in 70% ethanol for 2 hours in the dark for block staining. For the dehydration steps, a series of 70%, 96%, and 100% ethanol and propylene oxide was used. Afterward, tissue samples were embedded in Araldite 6005. Afterwards, the blocks were left to cool. Thin sections of 70–90 nm thickness from the tissue blocks were cut using an ultramicrotome (Ultracut R, Leica, Solms, Germany) and stained with uranyl acetate and lead citrate. The prepared specimens were examined with a transmission electron microscope (HT 7800 TEM, Hitachi High-tech, Tokyo, Japan) and analyzed using EMIP digital software.

Statistical Analysis

The SPSS 26.0 for Windows was used for statistical analyses. The Shapiro–Wilk test and Q–Q plot test were used to assess data distribution. The results were analyzed using the one-way ANOVA followed by Tukey's test. The results were expressed as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

Results

Results of Biochemical Analysis

Analysis of oxidative stress parameters in bladder tissue revealed significant differences among experimental groups. TAS levels varied significantly across groups (p=0.004). Specifically, TAS levels in the DM group were notably lower than those in the C and O groups (p=0.002, each). Conversely, treatment with ozone (Group DM + O) significantly increased TAS levels compared to the DM group (p=0.015) (Table 1).

TOS also exhibited significant variability among groups (p<0.001). Notably, the DM group showed significantly elevated TOS levels compared with both the C and O groups (p<0.001, each). Importantly, ozone treatment significantly reduced TOS levels in the DM + O group compared with the DM group (p<0.001) (Table 1).

Furthermore, the OSI, reflecting the balance between oxidative and antioxidative processes, showed significant intergroup differences (p<0.001). OSI values were significantly higher in the DM group than in both the C and O groups (p<0.001, each). However, OSI levels in the DM + O group were significantly lower compared to the DM group (p<0.001) (Table 1).

Glucose levels varied significantly across groups (p<0.001). Glucose levels were significantly higher in the DM and DM + O groups compared to the C group (p<0.001 and p=0.003, respectively). Similarly, glucose levels in the DM and DM + O groups were significantly higher than in the O group (p<0.001, both). In the O group, glucose levels were significantly lower than those in the C group (p=0.041). Similarly, glucose levels in the DM + O group were significantly lower than in the D group (p<0.001) (Table 1).

Results of Histopathological Assessments

There was a significant difference between the groups in terms of connective tissue proportions in the bladder sections (p=0.001). The ratio of connective tissue to the wall of the bladder was found to be notably diminished in group DM compared to groups C and O (p<0.001, each). However, it was found to be significantly elevated in group DM + O compared to group DM (p=0.043) (Table 2 and Figure 1).

Statistical analysis revealed a significant difference in the proportion of muscle tissue in the bladder wall among the groups (p=0.014). In group DM, the ratio of muscle tissue was found to be significantly increased compared to groups C and O (p=0.022 and p=0.002, respectively). On the other hand, the ratio of muscle tissue to the bladder wall appeared to be attenuated in the DM + O group compared to the group DM (p=0.045) (Table 2 and Figure 1).

Similarly, the ratio of the epithelial component of the bladder wall differed significantly between the groups (p=0.001). The proportion of the epithelial tissue to the wall was greater in group DM than in groups C and O (p<0.001 and p=0.004, respectively). Although the ratio of the epithelial tissue to the bladder wall diminished in

Table 1 Serum TAS, TOS, OSI, and Glucose Parameters [Mean ± SD]

	Group C (n=6)	Group O (n=6)	Group DM (n=6)	Group DM + O (n=6)	P**
TAS (mmol/L)	1.32±0.18	1.41±0.89	1.09±0.17*,&	1.27±0.70†	0.004
TOS (μmol/L)	8.19±3.62	9.34±2.45	26.37±11.98*,&	12.12±2.85†	<0.001
OSI	0.61±0.19	0.66±0.17	2.46±1.12*,&	0.96±0.26†	<0.001
Glucose (mg/dL)	213.08±26.46	170.20±19.87*	398.20±39.03*,&	279.62±44.55*,&,†	<0.001

Notes: p**: Significance level with ANOVA test p< 0.05. *p<0.05: Compared to Group C; andp<0.05: Compared to Group O; †p<0.05: Compared to Group DM.
Abbreviations: C, control; O, ozone; DM, diabetes mellitus; DM + O, diabetes mellitus + ozone.

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Table 2 Result of Histological Evaluation of Masson's Trichrome-Stained Bladder Specimens [Mean ± SD]

	Group C (n=6)	Group O (n=6)	Group DM (n=6)	Group DM + O (n=6)	P**
Ratio of Connective Tissue to the Wall of the Bladder	0.50±0.04	0.50±0.08	0.37±0.02*,&	0.43±0.06†	0.001
Ratio of Muscle Tissue to the Wall of the Bladder	0.44±0.07	0.40±0.06	0.51±0.03*,&	0.45±0.04†	0.014
Ratio of Epithelial Tissue to the Wall of the Bladder	0.07±0.04	0.09±0.02	0.14±0.02*,&	0.11±0.03	0.002
Muscle Tissue/Connective Tissue Ratio	0.89±0.20	0.86±0.31	1.39±0.14*,&	1.07±0.22†	0.002

Notes: p**: Significance level with ANOVA test p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group O; †p<0.05: Compared to Group DM.
Abbreviations: C, control; O, ozone; DM, diabetes mellitus; DM + O, diabetes mellitus + ozone.

the group DM + O compared to the group DM, the difference between the groups did not reach a significant level (p=0.069) (Table 2 and Figure 1).

Furthermore, a significant difference was observed among the groups in the comparison of the muscle tissue-to-connective tissue ratio of the bladder wall (p=0.002). The ratio of muscle tissue content to the connective tissue content of the bladder wall was notably higher in group DM compared to groups C and O (p<0.001, each). In contrast, it was found to be significantly lower in group DM + O than in group DM (p=0.023) (Table 2 and Figure 1).

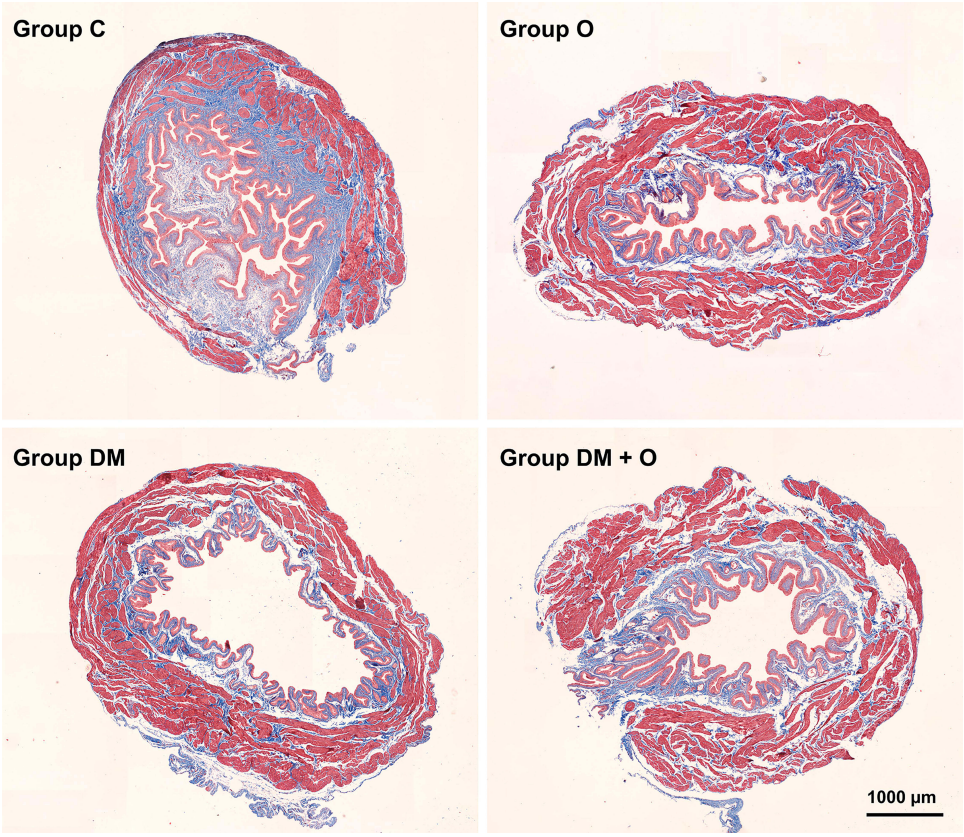


Figure 1 Representative Masson's trichrome-stained sections of the bladder from the Control (C), Ozone (O), Diabetes Mellitus (DM), and Diabetes Mellitus + Ozone (DM + O) groups, demonstrating the proportional distribution of urothelial (pink), connective tissue (blue), and detrusor muscle (red) components of the bladder wall. Images were acquired using the live panorama tool of the imaging software. Scale bar is 1000 µm for all images. Images from 6 animals per group were analyzed.

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Table 3 TNF-α, IL-1 β, TGF-β, and IL-10 Immunopositivity of Bladder Specimens [Mean ± SD]

	Group C (n=6)	Group O (n=6)	Group DM (n=6)	Group DM + O (n=6)	P**
TNF-α immunostaining intensity (% area)	7.75±3.15	6.65±3.28	11.88±1.33*,&	8.27±2.29†	0.010
IL-1β immunostaining intensity (% area)	9.08±1.80	9.72±0.90	11.90±1.59*,&	10.80±1.63	0.022
TGF-β immunostaining intensity (% area)	8.13±3.28	7.49±2.84	12.27±1.73*,&	9.19±2.56†	0.025
IL-10 immunostaining intensity (% area)	12.58±2.39	12.38±3.47	7.16±1.99*,&	10.38±4.21†	0.013

Notes: p**: Significance level with ANOVA test p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group O; †p<0.05: Compared to Group DM.
Abbreviations: C, control; O, ozone; DM, diabetes mellitus; DM + O, diabetes mellitus + ozone,

Results of Immunohistochemical Assessments

A statistically significant difference was found among the groups in terms of TNF-α immunostaining intensity in the bladder specimens (p=0.010). TNF-α immunostaining intensity in the DM group was notably higher than that of the C and O groups (p=0.010 and p=0.002, respectively). On the contrary, TNF-α immunostaining intensity of the specimens from the DM + O group was found to be notably decreased compared to the DM group (p=0.011) (Table 3 and Figure 2).

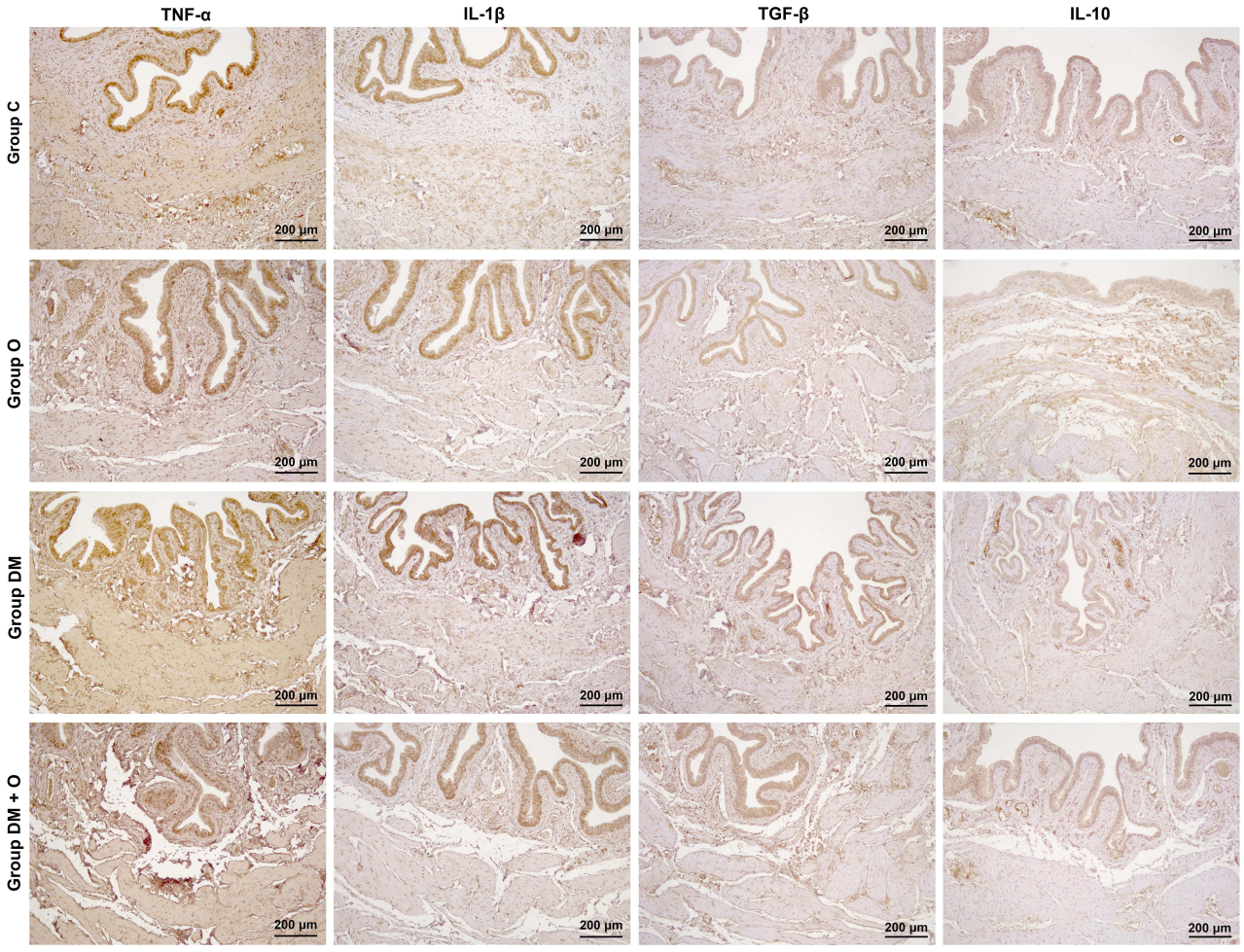


Figure 2 Representative micrographs showing the TNF-α, IL-1 β, TGF-β, and IL-10 immunostaining intensities of the bladder specimens from the Control (C), Ozone (O), Diabetes Mellitus (DM), and Diabetes Mellitus + Ozone (DM + O) groups. Immunoreactivity for all markers was visualized with DAB chromogen (brown) for all markers. Scale bars are 200 µm for all images. Images from 6 animals per group were analyzed.

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Similarly, there was a notable difference in the immunostaining intensities of IL-1β between the groups (p=0.022). IL-1β immunostaining intensity was found to be significantly higher in the DM group compared to the C and O groups (p=0.004 and p=0.022, respectively). Although the IL-1β immunostaining intensity in the DM + O group was lower, the difference between the DM group was not significant (p=0.224) (Table 3 and Figure 2).

Additionally, a comparison of the TGF-β immunostaining intensity in the bladder specimens of the groups revealed a significant difference (p=0.025). There was a significant increase in TGF-β immunostaining intensity in the DM group compared to the C and O groups (p=0.014 and p=0.005, respectively). TGF-β immunostaining intensity was found to be attenuated in the DM + O group compared to the DM group (p=0.048) (Table 3 and Figure 2).

A significant difference was also noted among the groups regarding the immunostaining intensity of IL-10 in the bladder specimens (p=0.013). Specimens from the DM group exhibited notably lower levels of IL-10 immunostaining compared to the C and O groups (p=0.004 and p=0.005, respectively). Conversely, IL-10 immunostaining intensity was greater in the DM + O group than that in the DM group (p=0.027) (Table 3 and Figure 2).

Results of TEM Examination

Compared with the control group, the hinge regions of umbrella cells in the DM group appeared more curved rather than angular, and hence their surface area seemed reduced. Umbrella cells at the surface exhibit vacuolization and attenuated fusiform vesicles in the DM group, in addition to vacuolization in the intermediate cells. However, vacuolization in the DM + O group appeared attenuated, along with improved fusiform vesicles and hinge regions on the surface of the umbrella cells (Figure 3).

Epithelial basement membrane thickness varied significantly among the groups (p<0.001). Thickness of the basement membrane was notably attenuated in the DM group compared to the C and O groups (p=0.025 and p<0.001, respectively). Whether the thickness of the basement membrane in the DM + O group was thinner than that in the C and O groups (p<0.001, both), it was greater compared to the DM group (p<0.001) (Table 4).

Discussion

Diabetic complications affecting the bladder are insidiously manifested in time and often neglected by patients or health professionals, since bladder assessment is not a part of the examination algorithm of diabetic patients during routine consultation to the healthcare system.[2,12,43] However, urological complications are also very common among diabetic patients, even accounting for a greater percentage of diabetic patients than those diagnosed with neuropathy and nephropathy.[2] Therefore, animal studies are of great importance for exploring the underlying pathophysiological mechanisms and for investigating early attempts to prevent diabetic bladder, given the challenges in conducting studies with human subjects.[2,7,10,11,44,45]

Hyperglycemia triggers a sequence of pathological processes, including the generation of advanced glycation end products (AGE), excessive production of reactive oxygen species, activation of inflammation-related transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and release of certain proinflammatory cytokines and growth factors, such as IL-1, TNF-α, insulin-like growth factor-1 (IGF-1), and TGF-β, all of which contribute to organ damage considered as diabetic complications.[3,46,47] In a recent review, Hughes Jr et al drew attention to the critical role of NLRP3 inflammasome in the development of bladder dysfunction in diabetes[15] Therefore, oxidative stress and inflammation, driven by hyperglycemia, are fundamental mechanisms investigated as pathophysiological processes underlying diabetic cystopathy and primary targets to prevent the development of diabetic bladder dysfunction.[3,11,15,45] In this regard, Poladia and Bauer designed an experiment to examine the time-dependent contribution of reactive nitrogen species to diabetes-related bladder remodeling at early stages of diabetes, as early as 3 days and 2 weeks.[48] The authors reported marked increases in 3-nitro-L-tyrosine expression in the epithelial and smooth muscle layers, demonstrating the existence of oxidative stress as early as the third day after diabetes induction and proceeding progressively.[48] Similarly, Beshay and Carrier examined the effects of oxidative stress on diabetes-induced bladder dysfunction in an eight-week diabetic rat model, revealing marked oxidative stress, evidenced by reduced catalase-like activity and increased thiobarbituric acid-reactive substances (TBARS) levels in diabetic bladder samples, along with an increased number of inducible nitric oxide synthase (iNOS)-positive and apoptotic cells in the smooth muscle layer.[45]

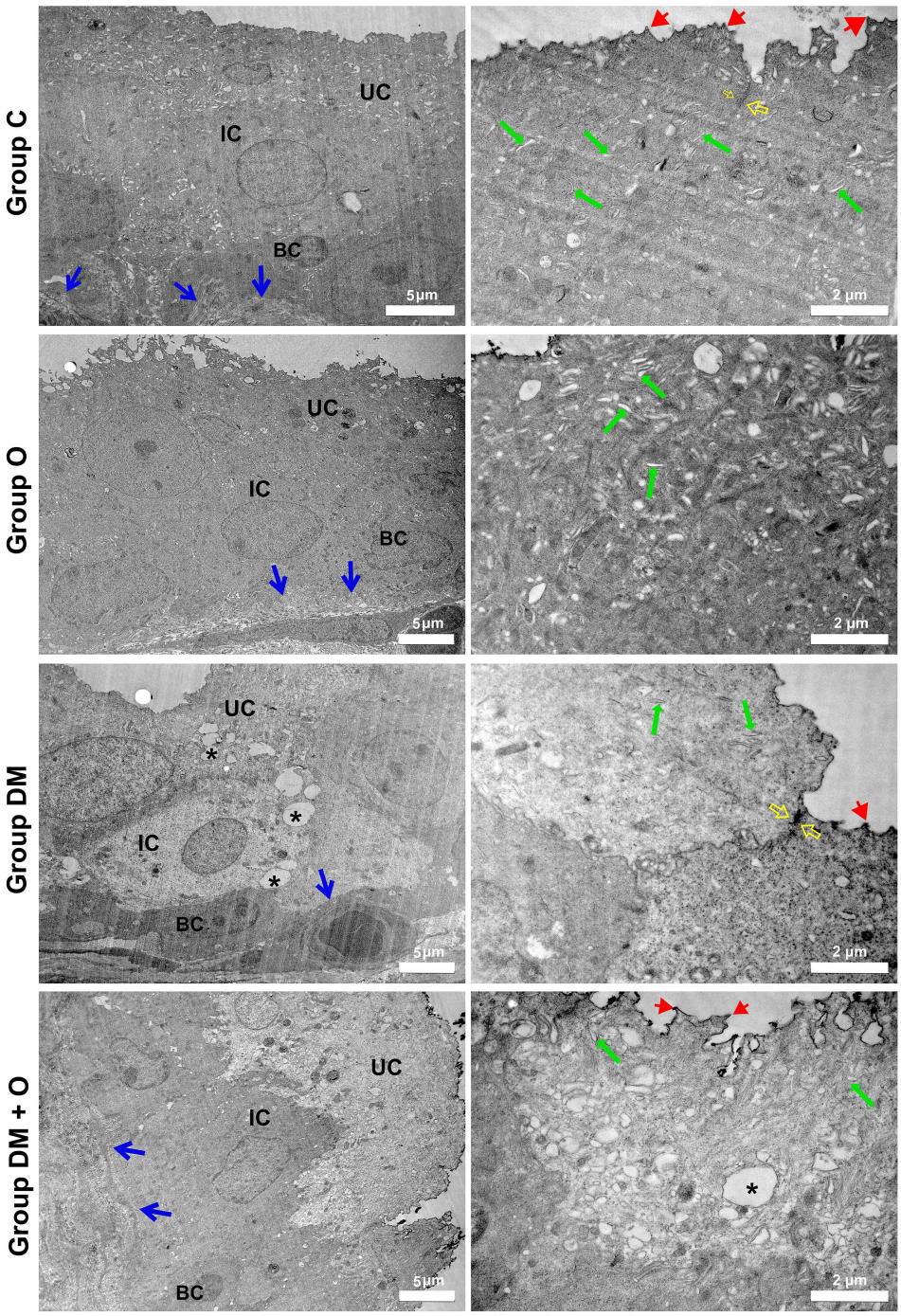


Figure 3 Representative transmission electron micrographic images of the bladder urothelium from the Control (C), Ozone (O), Diabetes Mellitus (DM), and Diabetes Mellitus + Ozone (DM + O) groups. blue arrows, basement membrane; hollow yellow arrows, junctional complexes; green arrows, fusiform vesicles; red arrows, hinge regions; asterisks, vacuolization. Scale bars are 5 µm for all images in the left column, and 2 µm for all images in the right column. Images from 6 animals per group were analyzed.
Abbreviations: UC; umbrella cell; IC, intermediate cell; BC, basal cell.

Likewise, Changolkar et al investigated the contribution of oxidative stress to decreased detrusor muscle function in the chronic course of diabetes, and they found that lipid peroxidation byproducts, malondialdehyde (MDA) levels, were elevated in the detrusor muscle of diabetic animals, and the contractility of bladder was decreased, representing a correlation between the factors leading to oxidative stress and a decrease in detrusor contractility[11] Xiao et al studied

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Table 4 Epithelial Basement Membrane Thickness of Bladder Specimens [Mean ± SD]

	Group C (n=6)	Group O (n=6)	Group DM (n=6)	Group DM + O (n=6)	P**
Thickness of basement membrane (nm)	88.03±5.95	97.18±10.83*	55.23±2.58*,&	73.22±3.23*,&,†	<0.001

Notes: p**: Significance level with ANOVA test p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group O; †p<0.05: Compared to Group DM.
Abbreviations: C, control; O, ozone; DM, diabetes mellitus; DM + O, diabetes mellitus + ozone.

a urinary diversion diabetes model to differentiate the effects of polyuria and hyperglycemia on bladder alterations twenty weeks after diabetes induction, and they found that hyperglycemia, independent of polyuria, leads to oxidative stress in the bladder wall and suggested that hyperglycemia-induced oxidative stress in turn may play a crucial role in the development of diabetic bladder dysfunction in the chronic course of diabetes[39] Consistent with the literature, we found notably elevated reactive oxygen species (ROS) levels in contrast to significantly decreased TAS levels in the serum of animals five weeks after diabetes induction, leading to an increased OSI value. These serum TAS and TOS values reveal an oxidative stress driven by hyperglycemia, as evidenced by elevated glucose in the serum samples of diabetic animals. On the other hand, a one-week ozone administration treatment appeared to be significantly effective in improving oxidative status in diabetic animals in the current study. This regulatory effect of ozone on the prooxidative-antioxidative balance in chronic inflammatory conditions was extensively stated in the literature.[27,33,49,50] In a study, Martínez-Sánchez et al compared the effects of anti-thrombotic therapy with and without rectal ozone insufflation on pro-oxidative and anti-oxidative balance in serum samples from patients diagnosed with coronary artery disease[49] They reported a significant decrease in both MDA and advanced oxidation protein products (AOPP) levels, and a higher glutathione (GSH) concentration in the group treated with antithrombotic therapy and ozone combination compared to the group receiving antithrombotic therapy only.[49] Also, ozone therapy via rectal insufflation was shown to be advantageous in regulating oxidative stress by increasing superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST) enzyme activities and GSH levels while decreasing the MDA and AOPP levels in the serum samples of multiple sclerosis patients.[33] Ozon exhibits hormetic effects, such that low-dose ozone acts as a preconditioning agent against overwhelmed oxidative stress by inducing mild oxidative stress; thereby, low-dose ozone restores the redox balance by activating antioxidant mechanisms through nuclear factor erythroid 2-related factor 2 (Nrf2) signaling and regulates the inflammation via 4-hydroxynonenal (4-HNE) action.[51,52]

Although our primary focus was on local bladder changes in the present study, it was noteworthy that a one-week rectal ozone administration resulted in a significant reduction in blood glucose levels in diabetic rats compared with untreated diabetic animals, as well as in ozone-administered healthy animals compared to healthy controls. The effect of low-dose ozone therapy on improving the blood glucose levels in the present study was consistent with existing literature, indicating that controlled ozone administration acts as a moderate therapeutic stressor that triggers positive adaptive responses to oxidative stress.[29,53,54] Specifically, ozone therapy mitigates systemic oxidative stress, thereby restoring insulin sensitivity in peripheral tissues.[29] Furthermore, through its antioxidant effects, medical ozone has been shown to exert robust glycemic regulation by preventing pancreatic beta cell damage and protecting their functions.[53] This dual action — improving pancreatic endocrine function and enhancing insulin sensitivity — explains the significant systemic glucose-lowering effect observed in our ozone-administered diabetic group.

In a study focusing on the anti-inflammatory effects of resveratrol on the detrusor muscle in diabetic rats, Xu et al reported significantly elevated mRNA expressions and protein levels of NF-κB, TNF-α, IL-1β, and IL-6 in the detrusor muscle of 12-week diabetic animals, and they revealed the anti-inflammatory effects of resveratrol by attenuated levels of inflammatory markers in the detrusor samples of 12-week diabetic animals treated with resveratrol[14] Furthermore, a more recent study by Li et al investigating the effects of oridonin on diabetic cystopathy revealed the importance of hyperglycemia-induced mitochondrial structural and functional deterioration in the pathogenesis of diabetic cystopathy in addition to hyperglycemia-induced overexpression of inflammatory cytokines, including TNF-α, IL-1β, and IL-6.[13] They demonstrated reduced mitochondrial membrane potential, altered phosphorylation of AMPK, and downregulation

of autophagy- and mitophagy-related proteins, Beclin1, p62, LC3-II, PTEN-induced kinase 1 (PINK1), and Parkin, in the smooth muscle of diabetic bladder.[13] Although we did not focus on the effects of ozone on mitochondrial dysregulation in the diabetic detrusor muscle, we examined TNF-α, IL-1β, TGF-β, and IL-10 expression in the bladder wall to determine whether ozone administration acts on inflammatory processes, another component of the pathogenesis. We found significantly elevated IL-1β, TGF-β, and TNF-α expression and diminished IL-10 expression in the diabetic bladder. These cytokine levels were regulated in ozone-applied diabetic animals, as the IL-1β, TGF-β, and TNF-α expressions were reduced, although the decrease in IL-1β levels did not reach a significant level. Also, one-week ozone administration improved IL-10 expression in bladder samples of diabetic animals. These results were compatible with research investigating or reviewing the antioxidant and inflammation regulatory effects of low-dose ozone.[24,27,33]

Remodeling of the bladder wall in the early stages of diabetes involves reorganization of the relative composition of the epithelial, smooth muscle, and connective tissue components of the wall as the bladder adapts to polyuria.[9,10,12] To demonstrate the early time-dependent changes in bladder wall morphology, Pitre et al performed morphometric analysis of bladder samples harvested at 3 days, 2 weeks, and 5 weeks after diabetes induction in a rat model.[40] Measurement of the proportional areas of epithelial, smooth muscle, and connective tissue showed a gradual increase in the smooth muscle and epithelial layers, reaching significant levels at the fifth week of diabetes, a finding interpreted as reflecting the known reciprocal regulatory relationship between these two layers.[40] However, the proportion of connective tissue was decreased significantly at the second and fifth weeks after diabetes was induced.[40] Similarly, another study by Liu and Daneshgari documented time-dependent alterations in the diabetic bladder wall, based on evaluations performed 4 days, 1, 2, 3, and 9 weeks after diabetes induction.[9] They reported a gradual increase in urothelial area, reaching significant levels in animals with 3 and 9 weeks of diabetes, but not when evaluated proportionally.[9] They also stated a notably decreased percentage of connective tissue area at 3 and 9 weeks of diabetes, while a marked increase in both absolute and percentage areas of smooth muscle at 2, 3, and 9 weeks of diabetes, concluding the enlargement of the smooth muscle and urothelium areas as primarily responsible for the bladder wall remodeling in the early stages of diabetes.[9] Also, in a study by Liu et al, investigating the activities of cholinergic, purinergic, and nonadrenergic-noncholinergic signaling on the contractility of the diabetic bladder, hypertrophic changes, including increases in bladder wall thickness and luminal areas, were observed after 9 weeks of diabetes, and these morphological changes were attributed to diuresis related to diabetes[7] In a recent study by Cao et al, streptozotocin-induced diabetes caused significant thickening of the bladder muscle layer, even as early as four weeks after diabetes emerged in animals, while the increase in the smooth muscle-to-collagen ratio did not differ significantly from that in bladders of non-diabetic rats[10] To determine whether ozone treatment prevents alterations in the three components of the bladder wall, we evaluated the proportions of urothelium, detrusor muscle, and collagen. Consistent with previous studies investigating early morphological changes, diabetes-induced alterations led to higher percentages of muscle tissue and epithelium during our 5-week study course. In contrast, connective tissue percentage was relatively lower in the animals with diabetes. One-week ozone treatment appeared to regulate the proportional distribution of tissue components that constitute the bladder wall. These findings may be attributed to the regulatory effects of ozone on cytokines and growth factors, such as TGF-β, contributing to inflammation and tissue remodeling. However, an increase in connective tissue could alternatively reflect fibrotic change, which has been reported to arise in the chronic course of diabetic cystopathy due to chronic hyperglycemia-driven inflammation and oxidative stress, culminating in myofibroblast formation and excessive collagen deposition via TGF-β and related signaling pathways.[18] This possibility remains to be further evaluated comprehensively by assessing complex mechanisms relevant to collagen synthesis, connective tissue remodeling, and fibrosis, taking into consideration that ozone has been reported to exert remodeling effects by promoting collagen synthesis and arrangement.[52]

Besides serving as a protective barrier against urine, urothelium interacts with the underlying tissue components through its various receptors and by secreting mediators.[5,6,12] Rizk et al studied ultrastructural alterations in the urinary bladders of 8, 10, and 16-week diabetic rats, and one of the points that they emphasized was the presence of mitochondria with varying degrees of degeneration, including swelling, calcification, and condensation, in the epithelial cells of the bladder in 8-week diabetic animals.[55] They also noted vacuolization or condensation of the epithelial cells in the 16-week diabetic bladder, interpreting the cells with condensed cytoplasm as undergoing apoptosis.[55] Also, another study by Hanna-Mitchell et al demonstrated the time-dependent detrimental impact of diabetes on bladder urothelium at the gene

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expression and ultrastructural levels[5] By means of electron microscopic examination of bladder urothelium at different time points, Hanna-Mitchell et al revealed that surface folds of umbrella cells had disappeared, and they exhibited more curved, rather than angular, hinge regions in the 3-week diabetic bladder[5] They also reported that epithelial alterations became more pronounced in the 9-week diabetic bladder, represented by drastically distorted epithelial surface, decreased numbers of fusiform vesicles in the apical cytoplasm of umbrella cells with inconspicuous hinge regions, and the presence of blebs in the apical membrane surfaces.[5] To further examine the effects of ozone on the epithelial lining of the bladder wall, we investigated the urothelium at the electron microscopic level. We observed that the basement membrane of the urothelium became thinner with vacuolization of the epithelial cells in our 5-week diabetic model animals; however, these findings appeared to be improved with ozone administration. Improvement of these epithelial changes in the diabetic animals with ozone application was consistent with a previous study demonstrating positive effects of ozone therapy on bladder mucosal alterations in patients with chronic cystitis.[56] In that study, Neimark et al examined bladder biopsy specimens from women with chronic cystitis to compare the effectiveness of intravesical and parenteral ozone application with the basic treatment approach[56] Their findings indicate favorable effects of ozone in improving the outcomes of basic treatment approach, by reducing inflammation, and degenerative changes in the urothelium, enhancing the epithelial proliferation and organization, increasing the vascularity in lamina propria; and these improvements were greater when ozone was applied intravesically, while parenteral ozone delivery resulted in much more significant effects on blood circulation of the mucosa.[56]

Despite its novel and comprehensive findings regarding the structural and ultrastructural protection of the bladder wall by rectal ozone therapy, the current study has several limitations, including the relatively short (one-week) ozone treatment period, the absence of direct assessment of molecular pathways such as NF-κB/Nrf2 signaling, and the lack of fibrosis-related comprehensive evaluations to clarify the nature of the observed connective tissue changes. A more comprehensive study would examine whether ozone's effects target mitophagy-related pathways in the treatment of diabetic bladder alterations, thereby revealing its further effects. Additionally, we did not conduct functional urological assessments to directly evaluate the effects of ozone on changes in bladder capacity, compliance, and detrusor muscle contractility. Nevertheless, our findings provide evidence for the use of ozone as a low-cost, low-toxicity adjunctive strategy for oxidative stress-driven conditions, including diabetic complications. Future studies investigating time-dependent changes with longer treatment durations and effects on relevant molecular pathways will be needed before ozone can be considered as a supportive option alongside standard diabetes management for the prevention of diabetic cystopathy.

Conclusion

Diabetic cystopathy is a common yet neglected complication of diabetes, driven largely by oxidative stress and inflammation triggered by hyperglycemia. In the current study, ozone administration attenuated oxidative stress, reduced pro-inflammatory cytokine expression, and partially preserved bladder wall architecture in diabetic rats. These findings suggest that ozone may have potential as a supportive adjunct to primary diabetes management for limiting the development of diabetic cystopathy. However, more comprehensive and multifaceted studies addressing treatment duration, dosage, and underlying molecular mechanisms are needed before its clinical translation can be considered.

Abbreviations

4-HNE, 4-hydroxynonenal; AGE, advanced glycation end products; AMPK, AMP-activated protein kinase; AOPP, advanced oxidation protein products; CAT, catalase; DM, diabetes mellitus; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione-S-transferase; iNOS, inducible nitric oxide synthase; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1 beta; IL-10, interleukin 10; MDA, malondialdehyde; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, nucleotide-binding oligomerization domain (NOD)-, Leucine-rich repeat (LRR)- and pyrin domain-containing protein 3; Nrf2, nuclear factor erythroid 2-related factor 2; OSI, oxidative stress index; PINK1, PTEN-induced kinase 1; ROS, reactive oxygen species; SOD, superoxide dismutase; TAS, total antioxidant status; TBARS, thiobarbituric acid-reactive substances; TEM, transmission electron microscopic; TNF-α, tumor necrosis factor alpha; TOS, total oxidant status; TGF-β1, transforming growth factor beta1.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

Ethical approval for the study was obtained from Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-22-103).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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