Analysis of the oxidative damage of DNA, RNA, and their metabolites induced by hyperglycemia and related nephropathy in Sprague Dawley rats

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Abstract

We used a sensitive and accurate method based on isotope dilution high-performance liquid chromatography–triple quadrupole mass spectrometry (ID-LC-MS/MS) to determine the levels of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxo-dGsn) and 8-oxo-7,8-dihydroguanosin (8-oxo-Gsn) in various tissue specimens, plasma, and urine of hyperglycemic Sprague Dawley rats induced by streptozotocin (STZ). The oxidative DNA and RNA damages were observed in various organs and the amounts of 8-oxo-dGsn and 8-oxo-Gsn derived from DNA and RNA were increased with hyperglycemic status. In contrast to the results of the nucleic acid samples derived from tissues, the levels of 8-oxo-Gsn in urine and plasma were significantly higher compared with that of 8-oxo-dGsn, which most likely reflected the RNA damage that occurs more frequently compared with DNA damage. For the oxidative stress induced by hyperglycemia, 8-oxo-Gsn in urine may be a sensitive biomarker on the basis of the results in urine, plasma, and tissues. In addition, high levels of urinary 8-oxo-Gsn were observed before diabetic microvascular complications. Based on that the 8-oxo-dGsn was associated with diabetic nephropathy and RNA was more vulnerable to oxidative stress compared with DNA. We also propose that 8-oxo-Gsn is correlated with diabetic nephropathy and that 8-oxo-Gsn in urine could be a useful and sensitive marker of diabetic nephropathy.

Keywords: ID-LC–MS/MS, guanosine, hyperglycemia, biomarker, 8-oxo-7,8-dihydroguanosin

Introduction

Diabetes mellitus (DM) is a chronic disease that affects 382 million people worldwide and is expected to rise to 592 million by 2035 [1]. The increasing prevalence of diabetes together with the associated morbidity and mortality calls for additional preventive and therapeutic strategies.

Hyperglycemia-induced oxidative stress is associated with long-term damage, dysfunction, and failure of various organs, which more likely result in the development of diabetic complications [2]. In fact, increased reactive oxygen species (ROS) and superoxide generated by dysfunctional mitochondria in diabetes have been viewed as the primary initiating event in the development of diabetic complications [3,4]. Normalizing levels of mitochondrial ROS (MROS) prevents the three major pathways known as the causes of hyperglycemic damage: glucose-induced activation of protein kinase C, increased formation of glucose-derived advanced glycation end products, and increased glucose flux through the aldose reductase pathway [3,5].

Increased ROS can cause oxidative base modifications in DNA or RNA and the oxidation of DNA and RNA generates a range of free nucleosides. More than 20 different types of base adducts have been found in DNA exposed to oxidative agents [6]. Since guanine exhibits the lowest oxidation potential, the guanine residues of nucleic acids are more vulnerable to free radicals and ultimately turn into 8-oxo-7,8-dihydro-deoxyguanosine (8-oxo-dGsn) and 8-oxo-7,8-dihydroguanosin (8-oxo-Gsn). Oxidatively damaged guanine can be mismatched to adenine both in DNA and RNA, which compromises the accuracy of cellular processes and may result in the...
production of abnormal proteins. Although the counterparts in DNA can be subjected to corresponding cellular mechanisms of base excision repair, nucleotide excision repair, and mismatch repair [7], RNA repair mechanisms may be not efficient or remain elucidated when compared with DNA. Importantly, the partly single-stranded RNA may in fact have an enhanced potential for oxidatively generated damage because of its widespread cytosolic distribution and the lack of protective histones [8-11]. RNA oxidations are presently recognized as disease-relevant mechanisms [12].

The diabetic state is associated with increased levels of markers of oxidative stress, and evidence derived from mechanistic studies has suggested that oxidative stress exhibits an important role in the pathogenesis of diabetes complications. Recently, Broedbaek et al. have found that urinary excretion of the RNA oxidation marker 8-oxo-Gsn could independently predict diabetes-related mortality, whereas the DNA oxidation marker 8-oxo-dGsn did not [13,14]. This finding also indicates that RNA oxidation damage is more intimately associated with hyperglycemia compared with DNA oxidation damage and that 8-oxo-Gsn is more closely related to the progression of diabetic complications.

It has been indicated that not only are oxygen radicals involved in the cause of diabetes, but also the diabetic status itself is associated with increased production of ROS. For the prediction and prevention of diabetic complications, the assessment of oxidative stress in diabetic patients may be important [15]. Since we cannot directly measure ROS production in various tissues in vivo, 8-oxo-dGsn and 8-oxo-Gsn derived from oxidative DNA and RNA damage can be assessed as new biomarkers because a number of studies have demonstrated that urinary markers of nucleic acid oxidation can be useful biomarkers in diabetes [14].

In this study, we performed isotope dilution high-performance liquid chromatography–triple quadrupole mass spectrometry (ID-LC–MS/MS) to determine the levels of oxidized forms of guanine nucleoside in various tissue specimens, plasma, and urine of hyperglycemic Sprague Dawley rats induced by streptozotocin (STZ). We found that 8-oxo-Gsn in urine is a sensitive and useful biomarker for oxidative stress caused by hyperglycemia and hyperglycemia nephropathy.

Materials and methods

Materials

STZ was purchased from Sigma (St. Louis, MO). The 8-oxo-dGsn (>98% purity), 2′-deoxyguanosine or dGsn (>98% purity), Gsn (98% purity), deferoxamine mesylate (DFOM), and HPLC-grade methanol were purchased from Sigma-Aldrich (USA). The 8-oxo-Gsn (>98% purity) was purchased from Alexis Biochemicals (San Diego, CA, USA). The [15N2]8-oxo-dGsn, [15N3]dGsn, and [15N3]Gsn were obtained from Cambridge Isotope Laboratories (Andover, MA, USA), and [15N2,13C1]8-oxo-Gsn was customized from Toronto Research Chemicals (Canada). TRizol, nuclease P1, and alkaline phosphatase were purchased from Invitrogen, Wako (Japan), and NEB (New England Biolabs Inc., USA), respectively. Hematoxylin and eosin were purchased from ZSGB-BIO. The ammonium acetate was HPLC grade (Fisher Scientific, USA), and the water used in this study was deionized at 18.2 MΩ.

Animals and establishment of diabetic rat model

Sprague Dawley rats were purchased from the Shanghai Animal Experimental Center, Chinese Academy of Sciences, and maintained under specific pathogen-free (SPF) animal facilities in a temperature-controlled room (24 ± 2°C) with automatic illumination on a 12-h cycle at Wenzhou Medical University. The animals had free access to water and standard rat pellets. All experiments were approved by the Wenzhou Medical University Committee, and all of the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In this study, we used rats weighing 249–281 g and aged 2 months. The animals were randomly assigned to 3-month control group, 3-month diabetic group, 6-month control group, and 6-month diabetic group (abbreviated to 3CR, 3DR, 6CR, and 6DR, respectively) and received an intraperitoneal (i.p.) saline injection (n = 8) or a single i.p. injection of STZ (70 mg/kg body weight; n = 20) [16]. At the time of injection, the mean body weights were identical for the STZ and saline groups (Table I). Blood glucose levels were monitored using a glucometer once a week, and the final measurements were recorded at the end of the experiment immediately prior to euthanasia. Rats exhibiting fasting glucose levels in excess of 16.7 mmol/L were designated as diabetic rats. STZ-injected rats not reaching this criterion were excluded from the experiments.

Animal handling and sample collection

Twenty-four hours prior to termination, food was removed from the cages. Accordingly, all plasma values presented are fasting values. The rats were then placed in standard rodent metabolic cages for a 24-h urine collection. The collected urine was immediately aliquoted and stored at –80°C until further analysis.

The rats were anesthetized with chloral hydrate at 0.3 ml/0.1 kg. Whole blood was collected (via postcaval exsanguination) into sodium salt of ethylenediaminetetraacetic acid (Na4EDTA) anticoagulant-pretreated vacuum tubes and centrifuged at 3000 rpm for 10 min at 4°C. Plasma samples were immediately separated from whole blood and were frozen at –80°C until further analysis. All organs, including the brain, heart, lungs, liver, kidneys, testes, and epididymis, were immediately removed from the animals on ice (0°C) and quickly frozen in liquid nitrogen and then stored at –80°C.
Preparation and hydrolysis of nucleic acids

To prevent artificial oxidation of samples during genomic DNA preparation, we followed the protocol recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD) with some modifications [10,11,17]. In addition, we added a metal chelator, DFOM, to reduce the background levels of oxidized DNA and RNA.

For DNA preparation, the samples were prepared on ice (0°C) throughout the procedure. The tissues were lysed in 1 ml buffer A (10 mM Tris, 0.32 M sucrose, 5 mM MgCl₂, and 1 mM DFOM, at pH: 7.5; 1% Triton X-100 added) and homogenized. Next, the reactions with sodium dodecyl sulfate or SDS, RNase A, and proteinase K were performed in 300 μl of buffer B (10 mM Tris, 5 mM Na₂EDTA, and 1 mM DFOM, at pH: 8.0). To prevent the artificial oxidation of samples, 600 μl of NaI solution (40 mM Tris, 20 mM Na₂EDTA, 7.6 M NaI, and 1 mM DFOM, at pH: 8.0) was prepared, and the reaction was performed in the presence of 1 mM DFOM [11]. For DNA hydrolysis, 20 μg of DNA was dissolved in 79 μl of 1 mM DFOM solution and denatured by heating at 100°C for 3 min, followed by rapid chilling. Next, the samples were incubated with 10 units of nuclease P1 (dissolved in 0.3 M sodium acetate and 1 mM ZnSO₄, at pH: 5.3, at 1 U/μL) at 37°C for 2 h, followed by an additional incubation with 10 units of alkaline phosphatase at 37°C for 1 h. Next, the samples were centrifuged at 12,000 × g for 10 min at 4°C, and 5 μl of 50 pg/μl [¹⁵N₂,¹³C₁] 8-oxo-Gsn and 5 μl of 2.5 ng/μl [¹⁵N₃] Gsn were added to 90 μl of the RNA hydrolysate supernatant. The values of RNA oxidation were expressed as the number of 8-oxo-Gsn per 10⁶ residues of Gsn (8-oxoGsn/10⁶ Gsn).

Preparation of plasma samples

The frozen plasma samples were thawed by incubation at 37°C for 5 min. To each 300 μl of plasma, 6 μl of 50 pg/μl [¹⁵N₂] 8-oxo-dGsn and 6 μl of 50 pg/μl [¹⁵N₂,¹³C₁] 8-oxo-Gsn were added. After brief mixing, 900 μl of acetonitrile was added to the mixture. The mixture was further mixed for 1–2 min and centrifuged (12,000 × g for 10 min at 4°C) to obtain the supernatant. Next, the supernatant was transferred to a new tube and evaporated until dry under a gentle stream of nitrogen. After that, the dried residue was dissolved in 100 μl of 10 mM ammonium acetate solution and centrifuged at 12,000 × g for 20 min at 4°C. Finally, 50 μl of the supernatant was used for LC–MS/MS analysis.

Preparation of urine samples

The frozen urine was thawed at 37°C for 5 min and then centrifuged at 12,000 × g for 5 min at 4°C. Each sample was divided into two aliquots, one for LC–MS/MS analysis to quantify 8-oxo-dGsn and 8-oxo-Gsn and the other to quantify the creatinine for normalization. The oxidized guanine nucleoside level was normalized against the corresponding creatinine level.

For the oxidized guanine nucleoside analysis, 100 μl of urine was mixed with 400 μl of 30% methanol solution to obtain a 1:5 diluted urine sample. 10 μl of 80 pg/μl [¹⁵N₂] 8-oxo-dGsn and 10 μl of 80 pg/μl [¹⁵N₂,¹³C₁] 8-oxo-Gsn were added to the urine sample and then centrifuged at 12,000 × g for 15 min at 4°C. Next, 100 μl of the supernatant was collected for LC–MS/MS analysis of 8-oxo-dGsn and 8-oxo-Gsn.

For creatinine quantification, 100 μl of urine was mixed with 400 μl of 30% methanol to obtain a 1:5 diluted urine sample. Next, the samples were centrifuged (12,000 × g for 10 min at 4°C). Twenty microliters of the supernatant was injected into the HPLC-ultraviolet (UV) system (Waters, USA) to measure creatinine using the method developed.
by our laboratory. Briefly, a Waters SunFire C18 column (5 μm, 4.6 × 250 mm) was used with a mobile phase consisting of 20 mM ammonium acetate (A) and 100% methanol (B). Gradient elution was performed from 0 to 6.0 min with 8% B at 0.8 ml/min, from 6.0 to 6.01 min changing from 8% B to 50% B at 1 ml/min (sixth curve), from 6.01 to 8 min in 50% B at 1 ml/min, from 8.0 to 8.01 min changing from 50% B to 8% B (sixth curve), and then from 8.01 to 10.0 min in 8% B at 0.8 ml/min (sixth curve). The injection volume was 20 μl, and the UV-detecting wavelength was set at 233 nm. The results were calculated based on the values for standard solutions.

H&E staining
Kidney sections were cut using a Thermo Scientific Cryotome (serial no. CC0561C1204) at a thickness of 3 μm and mounted onto glass slides coated with poly-L-lysine. Slides were immersion-fixed in 4% formaldehyde for 5 min and rinsed in distilled water for 5 min. Hematoxylin was used to stain the sections for 1 min, followed by a 5-min wash in water. Next, the sections were decolorized in acid alcohol and rinsed in 37°C water for 10 min and exposed to eosin for 2 min. After thorough washes in water, the sections were dehydrated with graded ethanol steps and xylene and coverslipped in neutral balsam. Observations were made using a Leica microscope (Leica, Wetzlar, Germany).

Chromatographic and mass spectrometric analyses
A Shimadzu Prominence LC-20A (Shimadzu, Japan) was connected to an Applied Biosystem API 5000 triple quadrupole mass spectrometer (Applied Biosystems–SCIEX, USA) equipped with a TurboIonSpray source controlled by an Analyst software program, version 1.5 (Sciex, Thornhill, Canada). Chromatography was performed on a Waters Atlantis dC18 (2.1 × 150 mm, 5 μm) column with the mobile phase consisting of 10 mM ammonium acetate at pH of 3.75 (A) and 100% methanol (B). The sample was maintained at 4°C. To reduce contamination of the ion source, the early and late eluting components were discarded. The mass spectrometer was operated in the positive-ion detection mode. Nitrogen was used for nebulizing, turbospray, and curtain gas, with optimum values. According to various samples, we applied different elution conditions, flow rates, and column temperatures. All the optimized conditions were summarized in Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1033416.

Results
Characteristics of the animals
Rats exhibiting fasting glucose levels in excess of 16.7 mmol/L were designated as hyperglycemic rats; otherwise, they were excluded from further experiments. The plasma glucose levels were higher in DR than those in CR (3CR: 5.89 ± 0.42 mmol/L vs. 3DR: 25.06 ± 1.82 mmol/L, P < 0.01; 6CR: 5.73 ± 0.50 mmol/L vs. 6DR: 25.58 ± 2.33 mmol/L, P < 0.01) (Table I). Polydipsia, polyphagia, polyuria, emaciation, and weakness, which are major features of DM, were easily observed in DR. There were also significant differences in body weight (3CR: 599.25 ± 26.00 g vs. 3DR: 222.13 ± 16.70 g, P < 0.01; 6CR: 752.5 ± 53.15 g vs. 6DR: 247.80 ± 11.73, P < 0.01) or urinary microalbumin normalized against the corresponding creatinine level (6CR: 12.24 ± 5.43 g/mol vs. 6DR: 25.33 ± 15.68 g/mol, P < 0.01).

Hyperglycemic status-related increases in oxidized guanine in DNA
We extracted DNA from various DR or CR tissue specimens according to the recommended procedure of ESCODD with some modifications. A metal chelator, deferoxamine methylate, was applied to reduce the background level of oxidation [18]. DNA was heat-denatured and hydrolyzed to nucleosides by successive treatments with nuclease P1 and alkaline phosphatase. [15 N]8-oxo-dGsn—an internal standard, was added to the mixtures. The two types of deoxyguanosine were determined using LC–MS/MS to quantify 8-oxo-dGsn in the hydrolyzed DNA samples. The values were expressed as the number of 8-oxo-dGsn per 10^6 residues of dGsn (8-oxo-dGsn/10^6 dGsn).

As shown in Figure 1A, we found that 8-oxo-dGsn and dGsn were eluted at distinct positions and could be assayed without cross-contamination, though there were a few miscellaneous chromatographic peaks. Figure 1B displays oxidative DNA damage observed in all the organs examined. The levels of 8-oxo-dGsn/10^6 dGsn were similarly increased in various tissues of diabetic rats as compared with those from the control rats, though there were some organ-specific differences in the levels of 8-oxo-dGsn/10^6 dGsn from the control rats. Moreover, the epididymis exhibited the highest levels of 8-oxo-dGsn/10^6 dGsn from all the observed tissues in both DR and CR. There were increases of 8-oxo-dGsn/10^6 dGsn value in all the examined organs from DR compared with those of control. However, only 8-oxo-dGsn/10^6 dGsn in kidney and lung from 6-month DR showed significant increases compared with their age-matched control. In addition, the levels of 8-oxo-dGsn/10^6 dGsn in different tissues of 6-month diabetic rats were not significantly increased compared with those of 3-month diabetic rats.

Hyperglycemic status-related increases in oxidized guanine in RNA
Total RNA was extracted using TRIZol (Invitrogen, USA) reagent (with 1 mM DFOM) according to the manufacturer’s recommended protocol. We further applied the RNase inhibitor DEPC to minimize the effects of degradation. As shown in Figure 2A, 8-oxo-Gsn and Gsn were eluted in distinct fractions under the conditions used for
liquid chromatography. In addition, the two types of ribonucleosides were different from those for deoxyribonucleosides in the retention time and molecular weight, which facilitated the examination of both the RNA- and DNA-derived materials without the fear of cross-contamination. Since the isotopically labeled internal standards further increased the level of sensitivity, we included $^{15}\text{N}$-substituted compounds as internal standards. The values were

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**Figure 1.** LC–MS/MS measurement of the concentrations of 8-oxo-dGsn and dGsn in the tissues of diabetic Sprague Dawley rats. (A) LC–MS/MS chromatograms of 8-oxo-dGsn, $[^{15}\text{N}_5]\text{8-oxo-dGsn}$, dGsn, and $[^{15}\text{N}_5]\text{dGsn}$, and # denotes the target chromatographic peak. (B) DNA oxidation (8-oxo-dGsn/10^6 dGsn) in the tissues of diabetic Sprague Dawley rats induced by STZ for 3 months, 6 months, and their corresponding control groups. The data are expressed as the mean ± standard deviation (SD); *$P<0.05$, **$P<0.01$. (□) CR, (■) DR.
similarly expressed as the number of 8-oxo-Gsn per 10^6 residues of Gsn (8-oxo-Gsn/10^6 Gsn).

Figure 2B showed the 8-oxo-Gsn contents of RNAs derived from various tissues of the two types of rats. Similar hyperglycemic status-related effects in DNA were observed in RNAs derived from various tissues of diabetic rats. However, the differences in the levels of 8-oxo-Gsn/10^6 Gsn between DR and CR were slightly more significant and extensive in comparison to those observed with 8-oxo-dGsn/10^6 dGsn in DNA. The
diabetic rats exhibited higher levels of 8-oxo-Gsn/10^6 Gsn compared with the values for the same-age groups of control rats, and the organ-specific differences in the levels of 8-oxo-Gsn/10^6 Gsn were also found in the different tissues of control rats. The levels of 8-oxo-Gsn in the testis were the highest among all the observed tissues in both DR and CR. Early significant increases of 8-oxo-Gsn/10^6 Gsn value were observed in kidney, heart, and testis from 3-month DR compared with their age-matched CR. Most of the organs from 6-month DR exhibited significant increases of 8-oxo-Gsn/10^6 Gsn in comparison with those from 6-month CR, except for brain and lung. In contrast, there was no significant increase in the 8-oxo-Gsn/10^6 Gsn from tissues between 6-month DR and 3-month DR.

The levels of oxidized guanine nucleoside in plasma

As shown in Figure 3A, plasma 8-oxo-dGsn and 8-oxo-Gsn concentrations in DR were significantly higher compared with those in control subjects (8-oxo-dGsn (nM) — 3DR: 0.086 ± 0.013 vs. 3CR: 0.033 ± 0.009, P < 0.01; 6DR: 0.105 ± 0.007 vs. 6CR: 0.071 ± 0.004, P < 0.01. 8-oxo-Gsn(nM) — 3DR: 0.469 ± 0.037 vs. 3CR: 0.179 ± 0.018, P < 0.01; 6DR: 0.701 ± 0.034 vs. 6CR: 0.292 ± 0.012, P < 0.01). In contrast, the levels of oxidized guanine nucleoside in plasma from tissues between 6-month DR and 3-month DR, the organ-specific differences in the levels of 8-oxo-Gsn/10^6 Gsn were also found in the different tissues of control rats. The levels of 8-oxo-Gsn in the testis were the highest among all the observed tissues in both DR and CR. Early significant increases of 8-oxo-Gsn/10^6 Gsn value were observed in kidney, heart, and testis from 3-month DR compared with their age-matched CR. Most of the organs from 6-month DR exhibited significant increases of 8-oxo-Gsn/10^6 Gsn in comparison with those from 6-month CR, except for brain and lung. In contrast, there was no significant increase in the 8-oxo-Gsn/10^6 Gsn from tissues between 6-month DR and 3-month DR.

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The levels of oxidized guanine nucleoside in urine

We maintained the rats in standard rodent metabolic cages for spontaneous metabolism, which was superior to the manual bladder palpation method. Fresh urine was collected from rats and stored at −80°C until analysis. LC–MS/MS method was performed to analyze oxidized guanine nucleoside in urine and the results were shown as oxidized guanine nucleoside levels normalized with the corresponding creatinine concentration.

The tendencies of 8-oxo-dGsn and 8-oxo-Gsn in urine were in consistent with those in plasma. As shown in Figure 3B, much higher levels of 8-oxo-dGsn, as well as 8-oxo-Gsn, were present in the urine of DR compared with age-matched CR (8-oxo-dGsn (μmol/mol) — 3DR: 6.44 ± 1.34 vs. 3CR: 1.39 ± 0.27, P < 0.01; 6DR: 8.16 ± 0.64 vs. 6CR: 2.42 ± 0.99, P < 0.01. 8-oxo-Gsn(μmol/mol) — 3DR: 38.52 ± 3.35 vs. 3CR: 11.40 ± 2.04, P < 0.01; 6DR: 44.66 ± 4.47 vs. 6CR: 12.40 ± 1.56, P < 0.01; Figure 3B). The levels of 8-oxo-dGsn and 8-oxo-Gsn were also significantly increased in 6-month DR in comparison with 3-month DR (8-oxo-dGsn (μmol/mol) — 3DR: 6.44 ± 1.34 vs. 6DR: 8.16 ± 0.64, P < 0.05; 8-oxo-Gsn (μmol/mol) — 3DR: 38.52 ± 3.35 vs. 6DR: 44.66 ± 4.47, P < 0.05). Furthermore, the value of 8-oxo-Gsn in urine was much greater than those of 8-oxo-dGsn.
Hyperglycemia-related nephropathy

The index of renal hypertrophy, microalbumin, and H&E staining of diabetic Sprague Dawley rats induced by STZ for 6 months, and their corresponding control groups were evaluated to confirm hyperglycemia-related nephropathy.

The index of renal hypertrophy was the ratio of kidney weight to rat weight. We found that the ratios were greater in DR than in CR (Figure 4A), which indicated that hyperglycemia is one of the major factors that facilitates nephropathy, as also shown from the results obtained with H&E staining. Since microalbumin was used as a well-accepted predictor of overt diabetic nephropathy, we determined the microalbumin of 6-month DR and CR (Figure 4B). Briefly, as shown in Figure 4, the rats induced by STZ indeed exhibited renal damage. Furthermore, as early as 3 months after receiving the STZ injection, a few microalbumin and desquamated epithelial cells were observed in the renal tubules of rats as assessed using H&E staining (Figure 5). 6-month DR demonstrated abundant microalbumin and desquamated endothelial cells (except for the kidney, the obvious physical damage did not occur in other organs; data not shown). Thus, we proposed that aggravated damage in the kidney was caused by oxidative stress induced by hyperglycemia and that the kidney was potentially more vulnerable to this damage compared with other issues.

![Figure 4](image1.png)

**Figure 4.** The index of renal hypertrophy and the levels of microalbumin in diabetic Sprague Dawley rats. (A) Index of renal hypertrophy and (B) microalbumin normalized to their corresponding creatinine levels in urine of diabetic Sprague Dawley rats induced by STZ at 6 months and their corresponding control groups. The data are expressed as the mean ± SD; *P < 0.05, **P < 0.01. (□) CR, (■) DR.

![Figure 5](image2.png)

**Figure 5.** H&E staining of the kidneys in diabetic Sprague Dawley rats induced by STZ at 3 months (3DR), 6 months (6DR), and their corresponding control groups (3CR and 6CR). The black arrows indicate pathological change.
Discussion

Many kinds of method have been developed to determine the levels of oxidized guanine nucleoside metabolites from DNA and RNA, such as enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, high-performance liquid chromatography-electron capture detector (HPLC-ECD), gas chromatography-mass spectrometry (GC–MS), and HPLC–tandem mass spectrometry (LC–MS/MS) [19–22]. However, the immunological methods often provide high and biased results and cannot be recommended at present. HPLC-ECD method lacks specificity of the detector for complex matrices like urine, while GC–MS method may have severe artificial oxidation during vaporization. For the highest specificity, the preferred method is LC–MS/MS [23,24]. Therefore, we used a method developed by our laboratory [9], namely ID-LC–MS/MS, with a few modifications to determine the levels of 8-oxo-dGsn and 8-oxo-Gsn in various organs, plasma, and urine of hyperglycemic Sprague Dawley rats. It was found that levels of 8-oxo-dGsn and 8-oxo-Gsn in various samples were increased with hyperglycemic status. This is consistent with the previous finding that increased levels of 8-oxo-dGsn and 8-oxo-7,8-dihydroguanine or 8-oxoGua are present in samples from DNA and RNA, such as enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, high-performance liquid chromatography-electron capture detector (HPLC-ECD), gas chromatography-mass spectrometry (GC–MS), and HPLC–tandem mass spectrometry (LC–MS/MS) [19–22]. However, the immunological methods often provide high and biased results and cannot be recommended at present. HPLC-ECD method lacks specificity of the detector for complex matrices like urine, while GC–MS method may have severe artificial oxidation during vaporization. For the highest specificity, the preferred method is LC–MS/MS [23,24]. Therefore, we used a method developed by our laboratory [9], namely ID-LC–MS/MS, with a few modifications to determine the levels of 8-oxo-dGsn and 8-oxo-Gsn in various organs, plasma, and urine of hyperglycemic Sprague Dawley rats. It was found that levels of 8-oxo-dGsn and 8-oxo-Gsn in various samples were increased with hyperglycemic status. This is consistent with the previous finding that increased levels of 8-oxo-dGsn and 8-oxo-7,8-dihydroguanine or 8-oxoGua are present in samples of diabetic rats, which have been determined by HPLC-ECD method [15,25,26].

It is interesting that the levels of 8-oxo-dGsn and 8-oxo-Gsn in tissue were slightly increased with hyperglycemic status and there were also tissue-specific patterns in the basal levels of DNA and RNA oxidation. The epididymis exhibited the highest levels of 8-oxo-dGsn in all tissues and the levels of 8-oxo-Gsn in testis were the highest. These differences may be attributed to tissue-specific variations in the extent of exposure to ROS, as well as to the efficiency of defense mechanisms against oxidative radicals [9]. However, increased levels of 8-oxo-dGsn and 8-oxo-Gsn in tissue DNA or RNA cannot alone be used to draw conclusions about long-term effects. Snapshots of the level in a specific organ reflect the balance between formation rate and elimination rate at the time of the biopsy of tissue [23].

Quantitation of global oxidative stress to DNA or RNA may be estimated by measuring the urinary excretion of 8-oxo-dGsn or 8-oxo-Gsn, respectively. This method is particularly useful in diabetes where global oxidative stress is increased. In this study, 8-oxo-dGsn and 8-oxo-Gsn concentrations in urine and plasma of DR were consistently higher compared with the concentrations in control subjects. Comparison of the data also shows that the levels of 8-oxo-dGsn or 8-oxo-Gsn from 6-month DR were higher than those of 3-month DR, implying that there is a hyperglycemic status-related increase of RNA/DNA oxidation. Hence, the origin of oxidized urinary nucleic acid metabolites is not finally established. Urinary 8-oxo-dGsn might have originated from the DNA repair processes or oxidation in the nucleotide pool, while the origin of urinary oxidized RNA residues 8-oxoGsn is unresolved whether it is from RNA or the nucleotide pool. It is recently suggested that the origin of the oxidized nucleic acid metabolites in urine is from DNA and RNA, and that the contributions from the nucleotide pools are negligible [24]. Our results also confirm that urinary excretion of 8-oxo-dGsn and 8-oxo-Gsn may be good markers for hyperglycemic status-related DNA/RNA oxidation. Measuring the urinary excretion of 8-oxo-dGsn or 8-oxo-Gsn can give valuable information since it can give prognostic information [14,27].

Actually, oxidative stress caused by increased free radical production plays an essential role in the pathogenesis of diabetes and diabetic complications [3,4,28]. DNA oxidation have been linked to diabetes and its complications for a long time [14,15,25,27,29–31], while RNA oxidation are only recently associated with type 2 diabetes [13,14]. Nonetheless, RNA oxidations are recently recognized as disease-relevant mechanisms [12]. Oxidative damage of RNA primarily leads to oxidative base modifications, strand breaks, and ribosomal dysfunction [32,33], resulting in decreased levels of functional protein as well as the production of abnormal proteins. Ribosomal dysfunction may also signal apoptosis by p53-independent pathways [34,35]. Broedbaek K et al. have recently found that it was the RNA oxidation marker 8-oxo-Gsn in urine that could predict death and death from complications in type 2 diabetes [13,14]. However, there is little direct information about RNA oxidation in comparison to DNA oxidation in diabetes and diabetic complications. In this study, we also found that the amounts of the free forms of 8-oxo-dGsn and 8-oxo-Gsn in plasma and urine were in good agreement with the levels of tissue 8-oxo-dGsn and 8-oxo-Gsn, and the levels of 8-oxo-Gsn in plasma and urine were considerably higher compared with the levels of 8-oxo-dGsn. These results not only confirm previous investigation that RNA is more vulnerable to oxidative stress than DNA in hyperglycemic status [8,14,36,37], but also support the hypothesis that RNA oxidation may be a risk factor in diabetes [14]. Similarly, not only are free radicals involved in the cause of diabetes, but also diabetic status itself is associated with an increased production of free radicals, and this condition in turn has been suggested as one of the pathogenic mechanisms of complications [38,39]. Further investigation is still needed to find out mechanisms responsible for the association between RNA oxidation and mortality in diabetes patients, given that complications are the major causes of morbidity and mortality in patients with diabetes.

There are evidences in favor of increased RNA oxidation levels in many different diseases and very few negative studies so far. For example, mRNA oxidation is viewed as an early event associated with motor neuron deterioration in amyotrophic lateral sclerosis or ALS, and may also be a common early event preceding neuron degeneration in other neurological diseases [40]. In the present study, although microalbumin and desquamated epithelial cells...
were present in the renal system, we still could not observe the macro- and microvascular damage in 6-month DR. However, increased urinary 8-oxo-Gsn levels in advanced diabetic macro- and microvascular complications were observed, which supports the aforementioned idea that increased oxidative stress can potentially contribute to the development of diabetic macro- and microvascular complications [41,42]. Thus, 8-oxo-Gsn in urine can also be a sensitive and useful biomarker for the oxidative stress induced by hyperglycemia and diabetic complications.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Tables I–V to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1033416.