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Increased level of urinary 8-oxo-Gsn in patients with heart failure

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ABSTRACT

Background: The level of RNA oxidation in patients with heart failure (HF) and its effect have not been well established. The aim of this study was to investigate the relationship between urinary 8-oxo-7, 8-dihydroguanosine (8-oxo-Gsn), a marker for oxidative stress to RNA and HF.

Methods: A total of 191 HF patients from March 2014 to March 2018 and 155 healthy controls were included in this study, and their morning urine was collected. The level of urinary 8-oxo-Gsn was measured by ultra high-performance liquid chromatography mass spectrometry/mass spectrometry and adjusted by urinary creatinine. Heart failure with reduced ejection fraction (HFrEF) was defined as left ventricular ejection fraction (LVEF) <40%. Heart failure with preserved ejection fraction (HFpEF) was defined as LVEF ≥50% with diastolic dysfunction.

Results: 8-oxo-Gsn in HF patients was significantly increased compared with age- and sex-matched healthy control subjects [4.20 (3.09–5.77) *vs.* 2.75 (2.59–2.85), $p < 0.001$]. The level of 8-oxo-Gsn was positively correlated with age ($r = 0.257$, $p = 0.001$) and N-terminal pro brain natriuretic peptide ($r = 0.257$, $p < 0.001$) and negatively correlated with diastolic blood pressure ($r = -0.147$, $p = 0.049$), hemoglobin ($r = -0.181$, $p = 0.013$), and creatinine clearance rate ($r = -0.215$, $p = 0.004$). Patients with HFrEF and HFpEF exhibited both the higher levels of 8-oxo-Gsn than control subjects (all $p < 0.001$) while there was no difference between HFrEF and HFpEF [4.44 (3.12–7.12) *vs.* 4.02 (3.10–5.67), $p = 0.406$].

Conclusions: The level of urinary 8-oxo-Gsn is increased in patients with HF, indicating that HF may be associated with increased oxidative damage to RNA, and urinary 8-oxo-Gsn be useful in the diagnosis and evaluation of HF.

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Introduction

Oxidative stress is defined as the production of reactive oxygen species (ROS), exceeding the anti-oxidative capacity of the cell and causing cellular oxidative damage. ROS directly impairs cellular structure including DNA and RNA, actives inflammation signal pathway, and induces apoptosis. A series of the studies have found that RNA oxidation is one sign of aging and related to many diseases, such as Alzheimer's disease [1], diabetes mellitus (DM) [2], and coronary heart disease (CAD) [3].

Heart failure (HF) is a complex clinical syndrome with a diverse of etiologies and results in substantial morbidity and mortality. The prevalence of HF may increase because of the aging of population. Oxidative stress plays an important role in the development of HF and related diseases [4,5], but the relationship between RNA oxidation and HF is still unknown. An oxidative stress to RNA can be measured as the urinary excretion of 8-oxo-7, 8-dihydro-guanosine (8-oxo-Gsn). In this study, we analyzed the urinary level of 8-oxo-Gsn in patients

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with HF and healthy controls to investigate the association between RNA oxidation and HF. We searched the relationship between urinary 8-oxo-Gsn and N-terminal pro brain natriuretic peptide (NT-pro-BNP) as well as other parameters to see the influence factors of 8-oxo-Gsn and to search if urinary 8-oxo-Gsn could be used to evaluate HF.

Materials and Methods

Patients

The study group consisted of 191 HF patients aged ≥18 years old recruited from Beijing Hospital from March 2014 to March 2018, including 117 (61.3%) males and 74 (38.7%) females. The average age is 70.8 ± 13.0 years old. Informed consent was obtained for the use of their urine. HF diagnosis criteria included HF symptoms and/or signs and elevated NT-pro-BNP. Left ventricular ejection fraction (LVEF) was assessed with echocardiography. Heart failure with reduced ejection fraction (HFrEF) was defined as LVEF < 40%. Heart failure with preserved ejection fraction (HFpEF) was defined as LVEF ≥50% with relevant structural heart disease (left ventricular hypertrophy and/or left atrial enlargement) or diastolic dysfunction. Patients with infection, malignant cancers, severe psychiatric disorders, or refusing urinary test were excluded from the study.

A total of 155 healthy members who took part in the health physical examination in Beijing Hospital were selected as the control group, with normal hepatic and renal functions. Members with HF, arrhythmia, acute coronary syndrome, neoplasm, infection, or uncontrolled diseases were excluded.

Preparation of urine samples

About 10 ml of early morning urine samples were obtained from each subject and stored at 4°C for 2 hours. Then, these samples were centrifuged, and the supernatants of 1.0 ml were transferred to Eppendorf tubes and stored at −80°C until they were analyzed. The frozen samples were thawed at 37 stored minutes and then centrifuged at 7,500 g for 5 minutes at 4tesn centrifug µl aliquot of the supernatant, 200 µl of working solution (70% methanol and 30% water with 0.1% formic acid and 5 mmol/l ammonium acetate) and 10 µl of 8-oxo- [$^{15}N_{21}{}^{3}C_{1}$]Gsn as an internal standard (480 pg/µl) were added. The mixture was incubated at 37°C for 10 minutes to redissolve the analytes from precipitate and was then centrifuged at 12,000 g and 4°C for 15 minutes. Finally, 5 µl of the supernatant was injected for ultra high-performance liquid chromatography mass spectrometry/mass spectrometry (UHPLC-MS/MS) analysis. Samples from patients and healthy control subjects were placed on the assay plate in a randomly assigned sequence. Considering the variability among the urinary volumes and the significant differences in the renal glomerular function, the concentrations of 8-oxo-Gsn were normalized by the amount of urinary creatinine. An automatic biochemical analyzer 7,600 series was used for determining the concentrations of urinary creatinine. Laboratory personnel performing the analysis were blinded to the category of participants and the clinical state of patients with HF.

Chromatographic and mass spectrometric analyses

All samples were determined by an Agilent 1,290 Infinity UHPLC instrument. An UHPLC separation was performed on an Agilent C18 (3 µm, 3.00 × 100 mm) column at 35°C using mobile phase A (5 mM NH_4AC with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid). The flow rate was 0.4 ml/minutes. The UHPLC conditions are shown in Supplementary Table 1. We discarded early and late eluting components to reduce interruptions. Moreover, the sample room temperature was kept at 4°C to reduce the loss of sample solution.

The infinitely liquid chromatography was equipped with an Agilent triple-quadrupole mass spectrometer with a Jet Stream electrospray

Table 1. Clinical and demographic data of the study groups.

		HF (n = 191)	Healthy controls (n = 155)	*p*
Male (%)		117 (61.3)	86 (55.5)	0.278
Age (years)		70.8 ± 13.0	70.6 ± 6.6	0.856
8-oxo-Gsn/Cre	Total	4.20 (3.09–5.77)	2.75 (2.59–2.85)	<0.001
	Male	4.02 (3.04–6.48)	2.67 (2.39–2.82)	<0.001
	Female	4.37 (3.15–5.43)	2.77 (2.70–2.86)	<0.001

Cre = creatinine, 8-oxo-Gsn = 8-oxo-7, 8-dihydroguanosine.

ionization (ESI) source and iFunnel (Agilent, 6490, USA). Mass spectrometric data acquisition was in the positive-ion detection mode. Multiple reaction modes were monitored for the quantitative analysis. The optimum nitrogen pressure of the nebulizer was 30 psi, and ESI needle voltage was adjusted to 2,000 V. The temperatures and flow rates of dry gas and sheath gas were set at 200°C (16 l/minutes) and 400°C (12 l/minutes). The high-pressure ratio frequency and low-pressure ratio frequency were 120 and 50 V, respectively. The UHPLC conditions and optimized parameters are shown in Supplementary Tables 1 and 2.

Statistical analysis

The characteristics of the participants were expressed as mean ± SD for normally distributed variables and were compared between groups by independent *t*-test. Abnormally distributed continuous variables were expressed as median [interquartile range (IQR)] and were compared by rank-sum test. The Chi-square test was used to determine the significance of the differences in the distribution of categorical data. Pearson's (for continuous variables) or Spearman's (for categorical variables) correlation coefficient was performed to examine the relationships between 8-oxo-Gsn and indicators. A two-sided p value < 0.05 was considered to be statistically significant. For all parametric tests, the statistical analyses were performed using SPSS 19.0 software (IBM Corporation, New York, NY).

Results

In this study, 191 patients with HF and 155 age- and sex-matched healthy controls were included. All of the study participants were Chinese. The ratios of male/female were 117/74 and 86/69 for patients and controls, respectively. The age of the patients ranged from 22 to 90, and the average age was 70.8 ± 13.0 years. There were no significant differences in age and gender between healthy controls and HF patients. The urinary levels of 8-oxo-Gsn were significantly higher in patients with HF than those in the healthy controls [4.20 (3.09–5.77) vs. 2.75 (2.59–2.85), $p < 0.001$]. The levels were also higher in male and female patients compared to those in controls as shown in Table 1. The distribution of urinary 8-oxo-Gsn and serum NT-pro-BNP among different age periods is shown in Figure 1.

The correlation analysis (Table 2) showed that the level of urinary 8-oxo-Gsn was positively correlated with age ($r = 0.257$, $p = 0.001$) and NT-pro-BNP ($r = 0.257$, $p < 0.001$) and negatively correlated with diastolic blood pressure ($r = -0.147$, $p = 0.049$), hemoglobin ($r = -0.181$, $p = 0.013$), and creatinine clearance rate ($r = -0.215$, $p = 0.004$).

We enrolled 83 (43.5%) HFrEF and 81 (42.4%) HFpEF patients as shown in Table 3). The patients with HFpEF were older than those with HFrEF (73.4 ± 11.2 *vs.* 67.0 ± 14.7, $p = 0.002$) and suffered more atrial fibrillation and chronic kidney disease but less CAD (all $p < 0.05$). There was no significant difference in NT-pro-BNP between HFrEF and HFpEF [1,604.00 (885.30–340.65) *vs.* 1,662.00 (667.76–3,382.47), $p = 0.767$]. Apparently, there were significant differences in 8-oxo-Gsn compared to HFrEF or HFpEF with healthy controls (all $p < 0.001$). The patients with HFrEF had a little higher levels of 8-oxo-Gsn than those with HFpEF [4.44 (3.12–7.12) *vs.* 4.02 (3.10–5.67), $p = 0.406$].

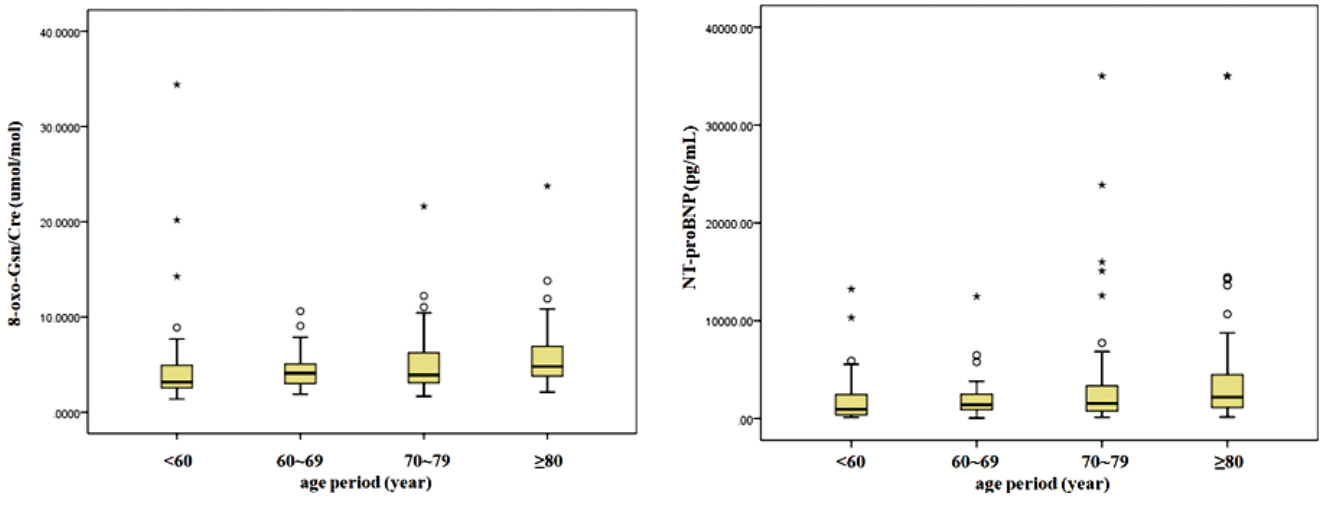

Figure 1. Levels of urinary 8-oxo-Gsn and plasma NT-pro-BNP with age.

Table 2. Correlations between 8-oxo-Gsn and indicators.

Variables		No. or value	Coefficient	*p*
Male		117 (61.3)	0.011	0.885
Age (years)		70.8 ± 13.0	0.257	0.001
CAD		87 (45.5)	−0.010	0.888
Hypertension		120 (62.8)	−0.086	0.238
Atrial fibrillation		93 (48.7)	0.036	0.616
Dyslipidemia		94 (49.2)	−0.007	0.927
DM		63 (33.0)	0.013	0.857
Hyperuricemia		105 (55.0)	0.012	0.871
NYHA functional classification	I	2 (1.1%)	0.042	0.565
	II	44 (23.3)		
	III	104 (55.0)		
	IV	39 (20.6)		
Height (cm)		165.7 ± 8.2	−0.016	0.827
Weight (kg)		70.57 ± 14.00	−0.092	0.225
BMI (kg/m^2)		25.5 ± 4.5	−0.118	0.119
Systolic BP (mmHg)		134.6 ± 23.8	−0.135	0.069
Diastolic BP (mmHg)		76.3 ± 14.2	−0.147	0.049
Heart rate (beat per min)		84 ± 22	0.104	0.164
LVEF (%)		43 ± 15	−0.113	0.118
LAD (mm)		45.0 ± 7.4	0.000	0.999
LVEDD (mm)		53.0 ± 9.9	0.028	0.708
NT-pro-BNP (pg/ml)		1,623.61 (776.00–3,326.00)	0.257	<0.001
Hemoglobin (g/l)		128.4 ± 23.3	−0.181	0.013
Ccr [ml/(minute·m^2)]		69.52 ± 34.79	−0.215	0.004
Serum uric acid (umol/l)		430.1 ± 144.6	0.068	0.367
Serum albumin (g/l)		40 (38–42)	−0.130	0.075
D-dimer (umol/l)		218.50 (100.75–452.25)	0.079	0.284

Values are *n* (%), median (IQR), or mean ± SD.
BMI = body mass index, BP = blood pressure, CAD = coronary artery disease, Ccr = creatinine clearance rate, LAD = left atrial diameter, LV = left ventricle, LVEDD = left ventricular end-diastolic diameter, LVEF = left ventricular ejection fraction, NT-pro-BNP = N-terminal pro-brain natriuretic peptide, NYHA = New York Heart Association.

Discussion

The etiology and pathophysiology of HF are complex and varied. Cardiovascular risk factors induce pro-inflammation cytokines and ROS, which not only play a significant role in cardiac myocyte apoptosis but also active neuroendocrine system, such as angiotensin II and norepinephrine contributing to cardiac hypertension [6]. All these are associated with the progression of HF. RNA distributes widely, and due to its single-stranded nature, unidentified active repair mechanism, and the lesser association with protecting proteins [7], it is more prone to oxidative damage, which leads to mistaken transcription and translation, myocardial dysfunction, and cardiac remodeling. Guanosine is the most vulnerable and oxidized to 8-oxo-Gsn, which circulates in the blood and excretes from the urine. Hence, 8-oxo-Gsn is the most widely used indicator to evaluate the degree of RNA oxidation. The urinary level of 8-oxo-Gsn could reflect RNA oxidative damage. Many studies have found that the level of 8-oxo-Gsn was elevated in many oxidative stress-related diseases, such as CAD [3], obesity [8], and cancer [9].

We found that the level of 8-oxo-Gsn was higher in HF patients. To the best of the authors' knowledge, the relationship between RNA oxidation and HF has not been investigated adequately. An animal experiment using male Dahl salt-sensitive rats found that the content of 8-oxo-Gsn in urine

Table 3. Clinical characteristics and 8-oxo-Gsn between HFrEF and HFpEF.

	HFrEF	HFpEF	*p*
No.	83 (43.5)	81 (42.4)	
Male	55 (66.3)	43 (53.1)	0.085
Age (years)	67.0 ± 14.7	73.4 ± 11.2	0.002
CAD	47 (56.6)	29 (35.8)	0.008
Hypertension	53 (63.9)	49 (60.5)	0.657
Atrial fibrillation	31 (37.3)	45 (55.6)	0.019
Dyslipidemia	41 (49.4)	39 (48.1)	0.873
DM	28 (33.7)	26 (32.1)	0.824
Hyperuricemia	45 (54.2)	47 (58.0)	0.623
Height (cm)	167.4 ± 8.3	164.3 ± 8.6	0.021
Weight (kg)	72.96 ± 14.86	68.25 ± 13.70	0.046
BMI (kg/cm^2)	25.78 ± 4.31	25.20 ± 4.92	0.440
Systolic BP (mmHg)	130.7 ± 21.0	135.6 ± 25.7	0.184
Diastolic BP (mmHg)	78.1 ± 13.9	73.2 ± 14.4	0.032
Rate (beat per min)	87 ± 22	80 ± 20	0.056
LVEF (%)	27 ± 6	59 ± 4	<0.001
LAD (mm)	46.1 ± 6.4	44.1 ± 8.6	0.094
LVEDD (mm)	57.4 ± 10.0	48.8 ± 8.5	<0.001
NT-pro-BNP (pg/ml)	1,604.00 (885.30–340.65)	1,662.00 (667.76–3,382.47)	0.767
Hemoglobin (g/l)	132.9 ± 23.6	124.9 ± 24.2	0.033
Ccr [ml/(min·m^2)]	79.90 ± 35.60	62.51 ± 33.88	0.004
Serum uric acid (umol/l)	419.9 ± 121.3	443.7 ± 172.9	0.324
Serum albumin (g/l)	41.0 (38.0–42.0)	39.5 (38.0–41.0)	0.154
D-dimer (umol/l)	209.5 (98.0–461.5)	213.5 (122.3–469.3)	0.855
8-oxo-Gsn/Cre	4.44 (3.12–7.12)	4.02 (3.10–5.67)	0.406

Values are *n* (%), median (IQR), or mean ± SD.
BMI = body mass index, BP = blood pressure, CAD = coronary artery disease, Cre = creatinine, Ccr = creatinine clearance rate, LAD = left atrial diameter, LV = left ventricle, LVEDD = left ventricular end-diastolic diameter, LVEF = left ventricular ejection fraction, NT-pro-BNP = N-terminal pro-brain natriuretic peptide, 8-oxo-Gsn = 8-oxo-7, 8-dihydroguanosine.

and heart tissue also increased, which was positively correlated with the related indicators of HF and might be involved through extracellular signal regulated kinase-mitogen-activated protein kinase pathway [10]. The results may suggest that RNA oxidation exists and increases significantly in patients with HF. There are emerging markers related to oxidative stress, such as galectin-3, α1-antitrypsin, and lectin-like oxidized low-density lipoprotein receptor-1 [11]. Compared with these markers, the measurement of urinary 8-oxo-Gsn is simple, rapid, noninvasive, and not expensive, so urinary 8-oxo-Gsn may be a useful biomarker for measuring the oxidative stress in HF patients.

NT-pro-BNP is a specific test used to diagnose HF and is associated with the severity and prognosis of HF [12], which is thought to be the most important biomarker. We found that 8-oxo-Gsn was positively correlated with NT-pro-BNP, which may suggest the possibility of 8-oxo-Gsn to evaluate the severity or prognosis of HF to some degree. The New York Heart Association (NYHA) functional classification is also simple and widely used to evaluate the severity of HF. However, this study showed no correlation between 8-oxo-Gsn and NYHA functional classification.

The explanation is that 8-oxo-Gsn is not a specific biomarker and is influenced by many factors. First, 8-oxo-Gsn was originally considered to be an index of aging and might be a potential biomarker to identify the individuals at high risk of developing age-associated disease [13]. In this research, the levels of urinary 8-oxo-Gsn were significantly higher in elderly patients, and 8-oxo-Gsn was correlated with

age. It is a clinical characteristic that HF in elderly patients is more severe, which has been shown in this research that serum NT-pro-BNP was significantly increased with age. One pathophysiologic mechanism of aging is oxidative stress, and elderly patients may also be more vulnerable to this. Second, 8-oxo-Gsn excretes from the kidney and is apparently influenced by renal function represented by creatinine clearance rate, which has also been proven in this research and other studies [14]. Hence, urinary 8-oxo-Gsn may represent the whole-body level of oxidative stress in patients with HF and only partly reflect the severity of HF. Moreover, NYHA functional classification is too simple and subjective, so it is difficult to show the real relationship between them in such a small-scale study. More large-scale prospective studies are needed.

We found that the levels of 8-oxo-Gsn in HFrEF and HFpEF were both elevated significantly, when compared to healthy controls, which indicates that RNA oxidation may occur regardless of the levels of LVEF. The levels of other circulating inflammation markers are also elevated in both HFrEF and HFpEF [15,16]. HFpEF comprises half or even more than half of all HF patients, and the prevalence of HFpEF is increasing. However, its pathogenesis is poorly understood, and therapies which could improve the outcomes of HFrEF have been proven useless in HFpEF [17]. Many studies including our results have proven that HFpEF is more common in old women with a lot of comorbidities such as atrial fibrillation and decreased kidney function [17]. A systemic pro-inflammatory state is induced by these comorbidities and causes coronary microvascular endothelial inflammation [18]. This reduces protein kinase G activity, which favors myocardial hypertrophy and interstitial fibrosis and develops HF [18]. This pro-inflammatory state may also lead to RNA oxidation and protein translation abnormality. Antioxidation could be an effective treatment in HF.

Limitations

There are some limitations in the study. First, the sample size was small, and it was a single-center study in a Chinese population. Second, the excretion of 8-oxo-Gsn via the kidneys is still speculative, and more experimental evidence is needed. Third, there was no direct evaluation about the outcomes of these patients. Therefore, the additional studies are required to confirm the results in the study.

Conclusion

RNA oxidation exists in patients with HF, and the level of urinary 8-oxo-Gsn may be a novel and alternative index to evaluate it.

Acknowledgments

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Conflict of interest

None declared.

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Supplementary Material

Table S1. UHPLC conditions for urine.

Sample	Temperature	Flow	The percentage of A in relation to time				
			90%–70%	70%–2%	2%–2%	2%–90%	90%–90%
Urine	35°C	0.4 ml/minute	0–3 minutes	3–4 minutes	4–5 minutes	5–5.01 minutes	5.01–7 minutes

Table S2. Conditions for urine sample.

Type of sample	Gas temperature	Gas flow	Sheath gas temperature	Sheath gas flow	Nebulizer	Capillary	Nozzle voltage	High pressure RF	Low pressure RF
Urine	200°C	16 l/minute	400°C	12 l/minute	30 psi	2,000 V	0	120 V	50 V