博士論文

Development of a rapid method for measuring rat oxidized albumin: verification using a model of proteinuria and hypertension

（ラット血中酸化型アルブミンの迅速測定法の開発：高血圧とタンパク尿を呈するモデルでの検証）

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Abbreviations

BP	Blood Pressure
CKD	Chronic Kidney Disease
CV	Coefficient of variation
DDW	Distilled Deionized Water
Fig.	Figure
HPLC	High Performance Liquid Chromatography
HMA	Human Mercaptalbumin
HNA	Human Non-Mercaptalbumin
HS	High Salt Diet
HST	High Salt Diet + Tempol
I.D.	Inner Diameter
LV	Left Ventricular
NS	Normal Sale Diet
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SD	Sprague-Dawley
Tempol	4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl
UnX	Uninephrectomy

Abstract

Oxidative stress is a risk for and cause of various diseases, and markers such as oxidized albumin were developed to evaluate oxidative stress levels in many disease states.[1–8] However, measurements of oxidative stress with oxidized albumin are either time-consuming or non-specific. In this thesis, a rapid method using high performance liquid chromatography (HPLC) has been established, for measuring oxidized albumin in a rat model of proteinuria and hypertension demonstrating high oxidative stress levels.

HPLC conditions for rat oxidized albumin were optimized. To validate the method, three-week-old male Sprague-Dawley rats were uninephrectomized and fed normal diet, high salt diet or high salt diet with Tempol, a superoxide dismutase (SOD) mimetic. After 4 weeks of treatment, serum oxidized albumin was analyzed.

The main findings are listed as below. (i) The new method of oxidized albumin measurement only takes 16 minutes, with an intra-day and inter-day deviation within 1% and a detection limit concentration of 6.4 mg/ml. (ii) Oxidized albumin levels were significantly higher in the high salt diet group than in the normal salt diet group, and this effect was reversed by Tempol. (iii) Oxidized albumin levels also correlated with urinary protein and 8-isoprostane levels.

In conclusion, a simple method for evaluating rat serum oxidized albumin using HPLC has been established. This method is rapid and has an advantage over conventional methods and may be useful for future studies of animal models of oxidative stress-related diseases.

Keywords: oxidative stress, oxidized albumin, rat serum albumin, high performance liquid chromatography, Tempol

1. Introduction

1.1 Free radicals

A free radical can be defined as an atom, molecule or ion that contains an unpaired electron in its atomic orbit. Free radicals including reducing and oxidizing ones, the former means one who gives an electron to the acceptor, while the latter represents for the one who accepts an electron[9]. Many free radicals are highly reactive and can provide an electron or receive an electron from other molecules, and they are called oxidants or reductants[10]. Most radicals have half-lives as short as 10^{-6} seconds or less in biological systems[11].

1.2 Free radicals and oxidative stress

Due to the high reactivity of free radicals, they can capture electrons from surrounding molecules to keep stability. The molecule which is attacked loses its electron and soon turns into a free radical itself. Then a chain reaction is started and eventually damages the cell [9,12].

Free radicals and oxidants can be produced from body metabolisms or the environment such as air pollution and smoking (Fig 1). For normally physiological functioning, a subtle balance between free radicals and antioxidants exists.

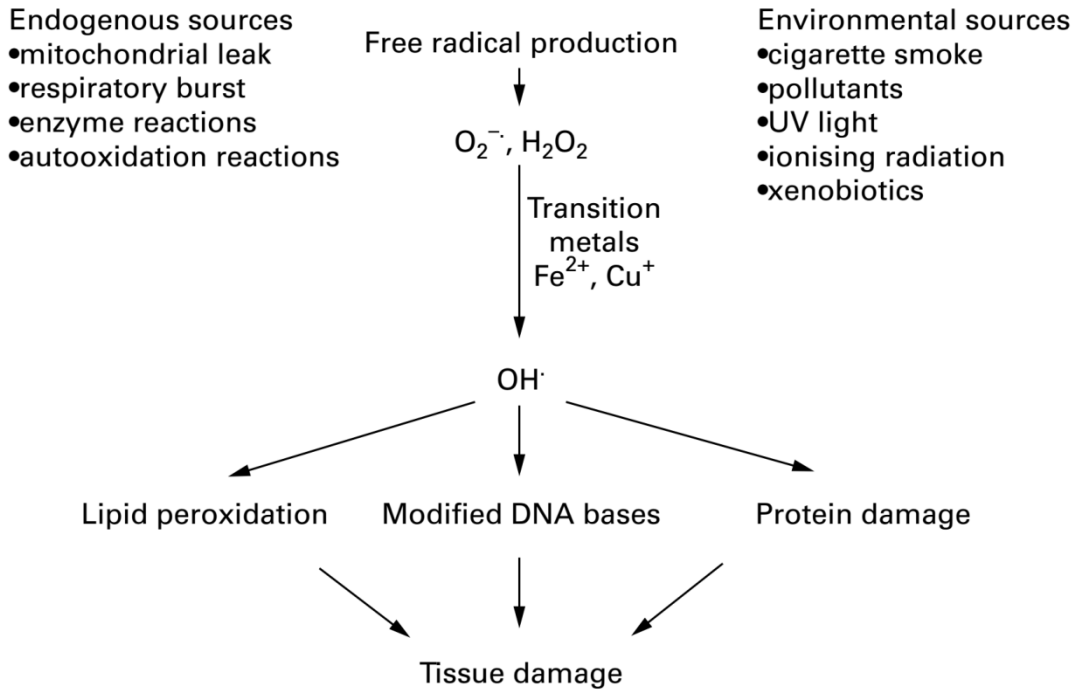

Fig. 1. Major sources of free radicals in the body and the consequences of free radicals' damages. *Reproduced with permission from J Clin Pathol 2001; **54:176**–186*

If the radicals are at low or moderate levels, they have positive effects and participant in many physiological processes (e.g. in immune function and mitogenic responses). If the concentration of free radicals is too high and exceeds the body's capability to adjust them, oxidative stress occurs, which will cause a lot of human diseases[13].

1.3 Oxidative stress and markers

Oxidative stress has elicited high levels of interest in the field of biology for a long time, and is termed as the imbalance between oxidants and antioxidants. Some of the oxidants works as cellular messengers in redox signalling[14], the pathways of ROS formation are shown as Fig 2. It has been reported that oxidative stress plays pivotal roles in a variety of disease conditions such as heart failure[15], cancer[16], DNA damage[17], as well as aging [18–22].

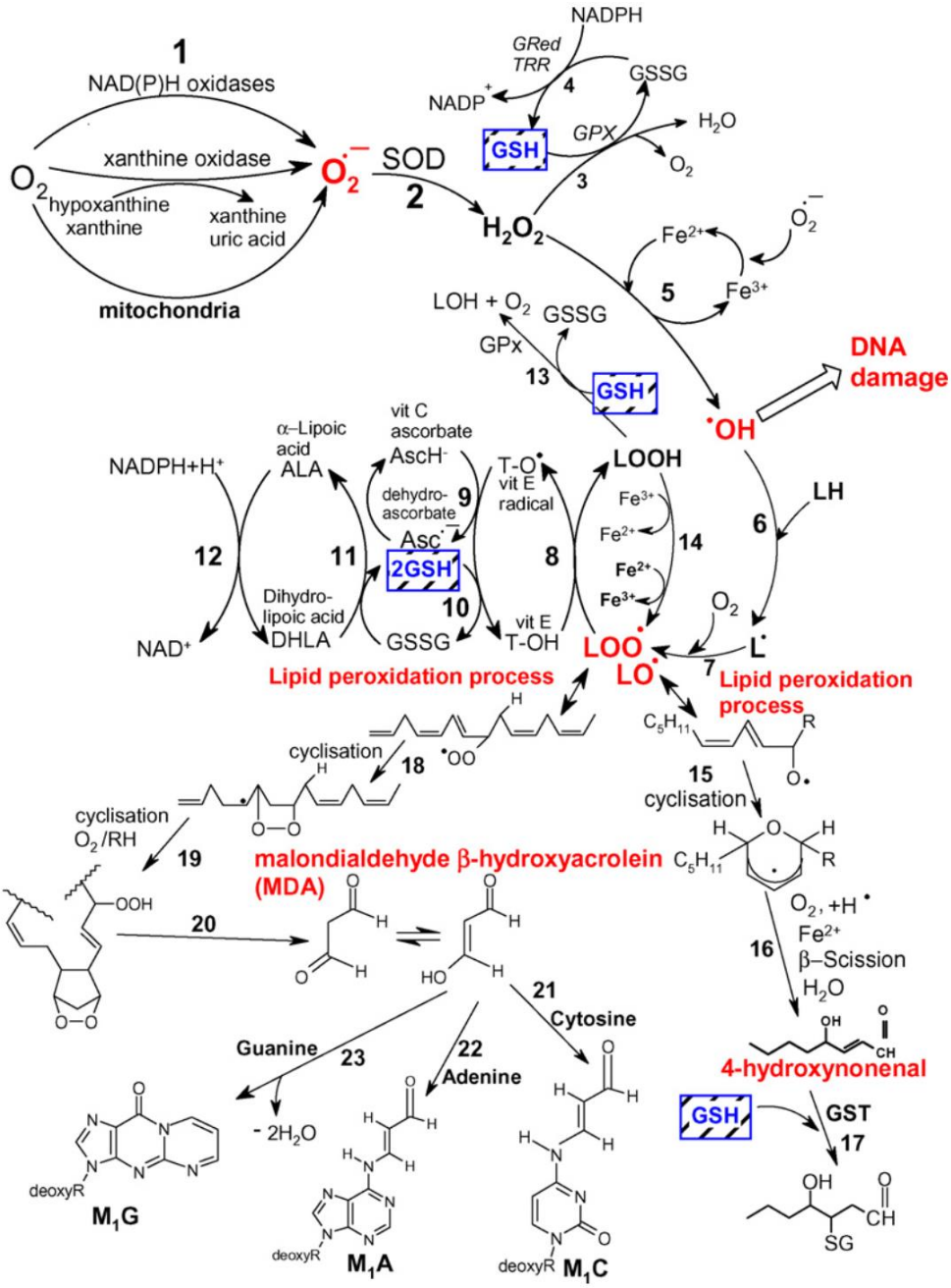

Fig. 2. Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (equations are not balanced). *Reproduced with permission from the International Journal of Biochemistry & Cell Biology 39(2007) 44–84*

To measure oxidative stress levels, many biomarkers has been used, such as 8-isoprostane, malondialdehyde (MDA), nitrotyrosine levels, and serum antioxidant

capacity. Each of them has distinct characteristics[23], and their advantages and disadvantages are shown in Table 1. To overcome these problems, the levels of the oxidized form of albumin was investigated as a marker of oxidative stress [1,24–26].

Table 1. Advantages and disadvantages of various biomarkers of oxidative stress. *Reproduced from Redox Biology 1 (2013) 483–491*

Biomarkers	Advantages	Disadvantages	Comments
Isoprostane	can be detected in various of samples (urine, serum) and has been shown to be elevated in the presence of a range of CV risk factors.	Current methods of quantification are impractical for large-scale screening or requires further validation.	No evidence linking this biomarker to clinical outcomes yet.
MDA	Technically easy to quantify spectrophotometrically using the TBARS assay. ELISA kits to detect MDA also have good performance. Studies show MDA can predict progression of CAD and carotid atherosclerosis at 3 years.	TBARS assay is non-specific (can detect aldehydes other than MDA) and sample preparation can influence results.	Shows promise as a clinical biomarker, however does not have a functional impact on the pathophysiology of CVD.
Nitrotyrosine	Human studies have demonstrated association with CAD independent of traditional risk factors.	Circulating levels are not equivalent to tissue levels. Current detection methods are expensive and impractical for scaling up.	Nitrotyrosine formation on particular cadiovascualr proteins have direct effect on function.
Serum antioxidant capacity	GPX-1 demonstrated to be inversely proportional to CAD. Commercial kits available to measure antioxidant capacity. Reproducibility quantified despite frozen sample storage.	Antioxidant activity in serum may not reflect that of cellular microdomains that are important to the pathogenesis of CVD.	Clinical relevance of antioxidant quantification to CVD risk need further investigation
MPO	Commercial assays available. Strong evidence shows that MPO correlates with CVD risk.	Influenced by sample storage and time to analysis.	MPO is a promising marker for CVD risk prediction.

1.4 Serum albumin

Serum albumin is the most abundant protein in serum, at a concentration of 3.5g/dl to 4.5g/dl and amount between 47% and 57% of total serum proteins throughout the neonatal period. In Sparague-Dawley rats, the absolute value of albumin increases while the albumin/globulin percentage decreases and become stable at about the age of 45 days[27]. (Table 2)

Table 2. Electrophoretic reference protein values for Spraque Dawley rats. *Reproduced from Journal of the American Association for Laboratory Animal Science,Vol 48, No 4 July 2009 Pages 387–390.*

Table 2. Electrophoretic reference values (g/dl and %) for Spraque Dawley rats

	7 d (n = 10)	14 d (n = 10)	21 d (n = 10)	28 d (n = 9)	45 d (n = 8)	53 d (n = 18)	60 d (n = 11)
Total protein (g/dL)*	$3.0 \pm 0^{a,e,h}$	$4.1 \pm 0.04^{b,f,i}$	$4.1 \pm 0.06^{c,g,j}$	4.6 ± 0.07^{d}	$5.5 \pm 0.20^{e,f,g}$	$5.4 \pm 0.10^{h,i,j}$	$6.5 \pm 0.20^{a,b,c,d}$
Albumin (g/dL)*	$1.6 \pm 0.03^{a,b,c,d}$	2.2 ± 0.03^{e}	2.2 ± 0.05^{f}	2.6 ± 0.04^{a}	2.6 ± 0.10^{b}	2.6 ± 0.0^{c}	$3.0 \pm 0.10^{d,e,f}$
Albumin (%)*	$52.2 \pm 1.0^{a,b,c,j}$	$54.7 \pm 0.6^{d,e,f}$	$53.4 \pm 1.2^{g,h,i}$	$56.9 \pm 0.5^{j,k,l,m}$	$46.6 \pm 0.9^{a,d,g,k}$	$48.6 \pm 0.8^{b,e,h,l}$	$47.0 \pm 0.9^{c,f,i,\ m}$
α1 Globulin (g/dL)*	$0.31 \pm 0.01^{a,b,c}$	$0.38 \pm 0.01^{d,e,f}$	$0.35 \pm 0.02^{g,h,i}$	0.71 ± 0.02	$1.10 \pm 0.05^{a,d,g}$	$1.1 \pm 0.04^{b,e,h}$	$1.01 \pm 0.08^{c,f,i}$
α1 Globulin (%)*	$10.2 \pm 0.4^{a,d}$	$9.4 \pm 0.3^{b,e,i}$	$8.4 \pm 0.5^{c,f,j}$	15.5 ± 0.4	$19.8 \pm 0.5^{a,b,c}$	$20.4 \pm 0.4^{d,e,f}$	$16.6 \pm 1.4^{i,j}$
α2 Globulin (g/dL)*	$0.24 \pm 0.004^{a,b,c}$	$0.32 \pm 0.01^{d,e,f}$	$0.31 \pm 0.01^{h,i,j}$	$0.30 \pm 0.02^{k,l,m}$	$0.50 \pm 0.02^{a,d,h,k}$	$0.50 \pm 0.02^{b,e,i,l}$	$0.51 \pm 0.03^{c,f,j,m}$
α2 Globulin (%)*	8.1 ± 0.1^{a}	7.9 ± 0.3^{d}	$7.4 \pm 0.2^{b,c}$	$6.6 \pm 0.4^{a,e,f}$	$9.1 \pm 0.3^{b,e,g}$	$9.0 \pm 0.2^{c,d,f}$	7.8 ± 0.3^{g}
β Globulin (g/dL)*	$0.72 \pm 0.02^{a,b,c,d}$	0.97 ± 0.02^{e}	1.10 ± 0.04^{a}	0.89 ± 0.05^{f}	1.20 ± 0.04^{b}	$1.10 \pm 0.03^{c,g}$	$1.65 \pm 0.11^{c,e,f,g}$
β Globulin (%)	$24.1 \pm 0.6^{a,b}$	23.8 ± 0.5^{c}	$26.5 \pm 0.6^{d,e,f}$	$19.3 \pm 1.0^{a,d,h}$	21.4 ± 0.8^{e}	$19.9 \pm 0.3^{b,c,f,i}$	$25.3 \pm 1.1^{h,i}$
γ Globulin (g/dL)*	0.17 ± 0.01^{a}	$0.22 \pm 0.02^{b,d}$	0.18 ± 0.02^{c}	$0.08 \pm 0.01^{a,b,c,e}$	0.20 ± 0.04	$0.10 \pm 0.01^{d,f}$	$0.25 \pm 0.03^{e,f}$
γ Globulin (%)*	$5.5 \pm 0.3^{a,b}$	$5.3 \pm 0.3^{c,d}$	$4.4 \pm 0.4^{e,f}$	$1.7 \pm 0.1^{a,c,e,h}$	3.2 ± 0.7	$2.1 \pm 0.1^{b,d,f}$	3.8 ± 0.4^{h}
Albumin: globulin ratio*	$1.1 \pm 0.04^{a,b,c,d}$	$1.2 \pm 0.03^{e,f,g,h}$	$1.2 \pm 0.06^{i,j,k,l}$	$1.3 \pm 0.03^{a,e,i,m,n,o}$	$0.90 \pm 0.03^{b,f,j,m}$	$1.0 \pm 0.03^{c,g,k,n}$	$0.88 \pm 0.03^{d,h,l,o}$

Data are presented as mean ± 1 SE. Asterisks indicate significant (Kruskal–Wallis 1-way ANOVA, $P < 0.05$) differences with age. For each parameter, values indicated with the same superscript letters are significantly different (Dunn pairwise multiple comparisons, $P < 0.05$).

Serum albumin has been intensively studied since 1960s. Albumin is the most abundant protein in plasma[28,29]. Albumin is not only found in the blood, but also other extracellular fluid such as lymph and chyle[30]. Albumin maintains oncotic pressure in the vascular system, whereby plasma albumin reduction leads to retention of water in the extravascular tissues and causes edema[31]. Albumin is a protein that contains one peptide chain of 580 to 585 amino acid residues. The general structure is characterized by several long α helices, with its secondary structure being a unique pattern of double flexible loops held together by disulfide bridges[32] with a molecular weight around 66 kDa[28,33]. Albumin is synthesized in the liver in the form of pro-albumin[34,35,36]. Albumin is synthesized but not stored in the hepatocyte and quickly secreted (about 20 minutes)[37]. The functional structure of rat serum albumin and gene structure of rat serum albumin are shown as Fig 3 and Fig 4 respectively. Albumin is a mixture of reduced albumin (mercaptalbumin) and oxidized albumin (non-mercaptalbumin) in extracellular fluid such as serum. Reduced

albumin has one free sulfhydryl group in Cys-34, while oxidized albumin has a ligand

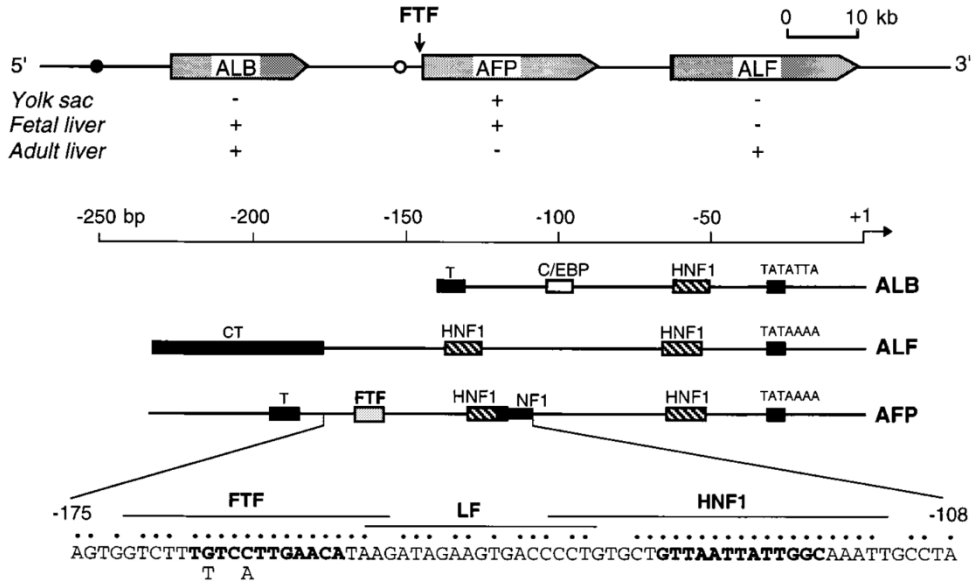

Fig. 3. Functional structure of the rat albumin (ALB), AFP, and a-albumin (ALF) genes. *Reproduced with permission from Molecular and Cellular Biology, Vol. 16, No. 7,p3853-3865,1996*

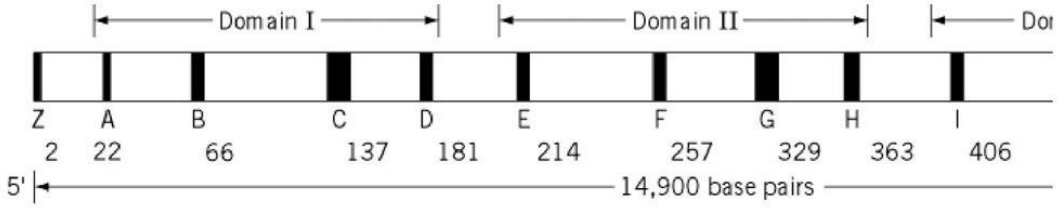

Fig. 4. The gene structure of rat serum albumin. The solid bars labeled with capital letters are exons, and the 14 intror correspond to the terminal amino acid residue in each exon. *Reproduced from http://what-when-how.com/molecular-biology/serum-albumin-molecular-biology/*

bound to the sulfhydryl group in Cys-34[26]. Reduced albumin is bound with its mixed disulphide with cysteine or glutathione[25]. Oxidized albumin has more oxidized products such as sulfenic (-SOH), sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) states [26](Fig 5).

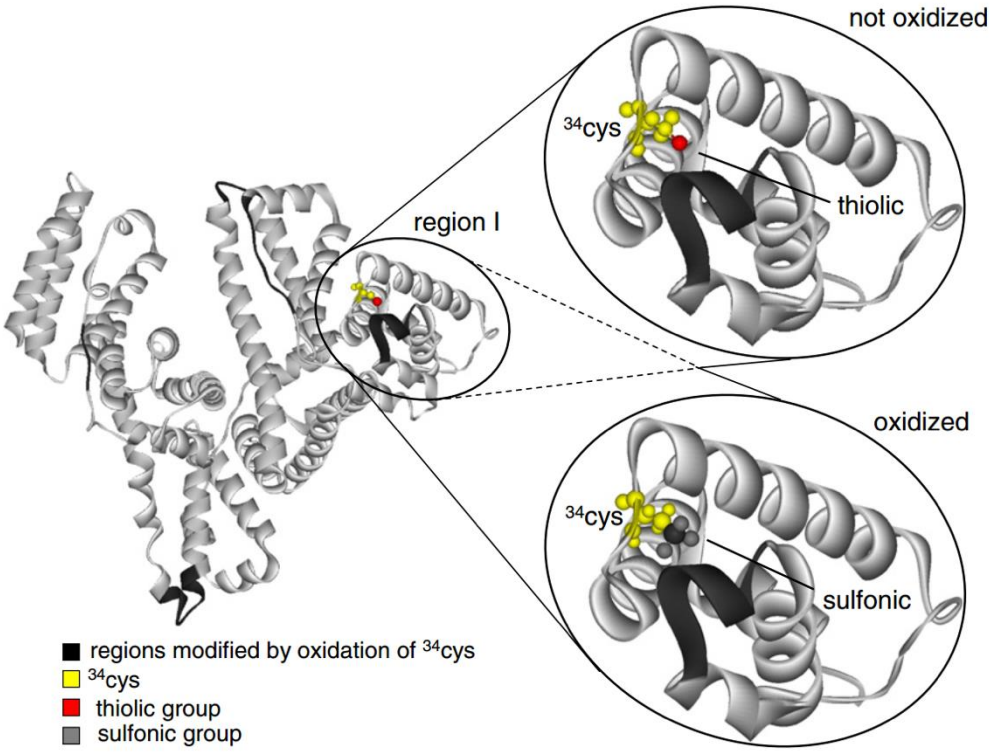

Fig. 5. Structure of oxidized albumin. (Oxidized albumin. The long way of a protein of uncertain function). *Reproduced with permission from Biochimica et Biophysica Acta, Volume 1830, Issue 12, December 2013, Pages 5473-5479*

1.5 Methods of serum albumin estimation

Several methods has been developed to measure biological proteins since 1960s. Those methods have advantages and disadvantages and the best methods are applied to clinical science according to their property.

1.5.1 Immunochemical methods

Radical immunodiffusions assays is an early technique which has been described in 1960s[38, 39] and has made appreciable progression in the evaluation of antigens. The antigen solution is added to the well created in the the agar gel containing anti-albumin

antibody. The antigen diffuses into the agar and at a certain radius, insoluble precipitation complex forms and represent the boundary between the antigen and the antibody. The square of the circle diameter is linearly related to the concentration of the antigen in the sample. This method is simple and reliable, but it needs long reaction time, large amount of anti-albumin antibody and lacks automation.

Enzyme-Linked Immuno Sorbent Assay (ELISA) test can provide faster results and requires less amount of anti-albumin antibodies. Based on the different modifications to the basic procedures, ELISA are mainly divided into four categories: direct, indirect, sandwich, and competitive. Among them, sandwich ELISA is the most widely used one. Anti-albumin antibody is coated on the surface of a multi-well plate. The function of the antibody is to capture the antigen in the sample. After the antigen is traped, enzyme labelled detection antibody is added and binds to the antigen. Finally, substrate is added and is converted to detectable signal by the enzyme[40]. Although the commercial ELISA kits are expensive, ELISA has become a widely accepted technique due to its high specificity and sensitivity and user-friendly.

Immunoturbidimetric assay has been used for measuring albumin in blood, urine and cerebrospinal fluid[41–43], and could detect urine albumin at concentration as low as 1 mg/ml[44]. Compared to radical immunodiffusions, immunoturbidimetric assays also use the interaction between anti-albumin antibody and albumin, not in a agar gel but in a solution. The interaction results in insoluble complex and causes turbidity in the solution, which can be analyzed by evaluating absorbance in spectrophotometer. Though the

method is simple and rapid, it needs a large amount of anti-sera antibodies and is unable to detect samples in fragments[45, 46]. High concentration of albumin samples can also cause error in valuation thus leading to "hooks effect"[31].

1.5.2 Spectroscopy based methods

Spectrophotometry-based methods are used for evaluating human serum albumin and glycated albumin. Near infrared spectroscopy is applied to human serum albumin evaluation[47, 48] and Raman spectroscopy can be used for measuring glycated albumin[49]. The principle of near infrared spectroscopy is as follows. Absorptions in the Near infrared part (around 780-2500 nm) of the electromagnetic spectrum are related to the molecular overtone and combination vibrations. The strength of different chemical bonds (e.g. O–H, C–H and N–H) varies, so the energy needed for the vibration jumping from one level to another is different. The energy gap is connected to the absorption peaks in a spectrum. By analyzing the spectrum, the existing chemical bonds and hence the corresponding molecules can be determined. With the help of multivariate analysis, the blood components can be quantitively determined. Recently, various studies described the application of near infrared spectroscopy in the study of glycated albumin.[50,51] Near infrared spectroscopy can simultaneously estimate the amount of serum total proteins, albumin, globulins without reagents, however, the quantative determination is highly depends on the selected near infrared region and multivariate analysis method used, lessening the certainty of the results[52].

1.5.3 Chromatography methods

The chromatography method is claimed to be more precise than other methods[31], and it is used for measuring glycated albumin in serum and urine[53–55]. However, the equipment is expensive thus limiting its widely use. Previous methods for analysing glycated serum albumin has become more accurate than before with coefficient of variation from 4.33±2.0% to 2.02±0.65%, including ion-exchange and boronate affinity chromatography for separating glycated albumin from other serum proteins. Affinity chromatography analysing process can be influenced by temperature, but other analysing factors such as column pressure and flow rate can be stabilized to accurately separate the samples[55–59].

The measurement for using high performance liquid chromatography to analysis human serum albumin were developed from 1980s[60]. Sogami et al. used a 2-meter column and took about 120 minutes to analyze human serum albumin from one sample, making the analysis process complicated and time-consuming. Era et al. improved the analysis condition by shortening the measurement time to about 60 minutes but the method is hard to analyze large quantity of samples[61]. The method also takes about 1 hour to balance the column plus measure one sample and a linear gradient condition for 3 steps-1%, 3% and 10% ethanol concentrations for analysis buffers[61,62]. Later the analysis method was revised by using bromocresol green（BCG）reagent prepared with citric acid. The revised method is relatively complicated but shortened the analysis time to 30 minutes[63]. In 2017,

a paper reported using HPLC to analyse human serum albumin within 10 minutes with a CV value less than 1%, simplifying the analysis method whilst making it fast and precise[24].

The measurement for using high performance liquid chromatography to analyse rat serum albumin have also been studied. An early study reported that rat serum albumin cannot be clearly separated[25]. Hayashi T et al. has since shown that HPLC method can separate both reduced and oxidize albumin in rat serum[26]. However, Hayashi's method takes about one hour to measure one sample under room temperature. In my study, data have shown that auto-oxidation occurs as quickly as 20 minutes, making it necessary to measure the samples over a short period of time. In this study I developed a novel method to measure non-mercaptalbumin in short time and can obtain stable and reliable data.

2. Materials and Methods

2.1. Animals

Three-week-old male Sprague Dawley (SD) rats (body weights ranging from 40 g to 50 g) were subjected to left uninephrectomy and then randomly divided into the following 3 groups: the normal salt diet (UNx+NS, 0.3% NaCl, n=20); high salt diet (UNx+HS, 8% NaCl, n=18) and high salt diet treated with a superoxide-dismutase mimetic, namely, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl, also known as Tempol (UNx+HST, 8% NaCl, 1 mmol/kg/day in drinking water, n=17). After 4 weeks of treatment, blood samples were collected by cardiac puncture. (Fig 6) The serum samples was centrifuged at 15,000 rpm for 5 minutes and then stored in -80℃ until the assay.

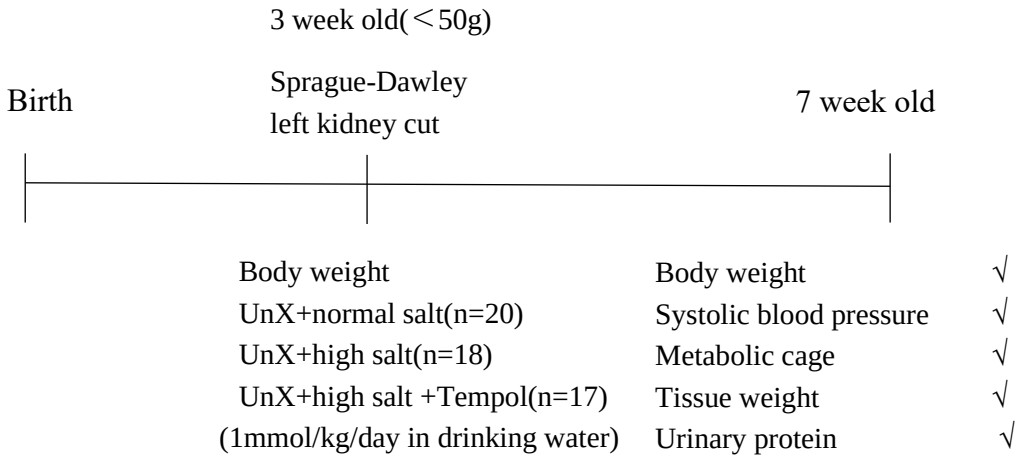

Fig. 6. Hypertension and proteinuria animal model protocol.

Rats were maintained in a humidity- (60±5%), temperature- (23±1.5°C), and light

cycle-regulated (0700–1900) room and had free access to food and drinking water. Reasearch Ethics Committee of the University of Tokyo approved this investigation (No:P14-145), which was conducted according to the guidelines for the care and use of laboratory animals of the University of Tokyo, Graduate School of Medicine.

Body weight and systolic blood pressure (SBP) were measured prior to sacrifice. SBP was measured using the tail-cuff method (P-98A; Softron, Tokyo, Japan). Twenty-four-hour urine samples were collected at the end of the 4-week treatment using metabolic cages to measure both the urinary protein (BCA assay) and the 8-isoprostane levels.

2.2. Establishing optimal conditions for HPLC

The HPLC system (LabSolutions system; Shimazu Co., Ltd, Kyoto, Japan) comprised of a degasser (DGU20A3R), two pumps (LC-20AT), an autosampler (SIL30AC), a thermostatic oven (CTO-20AC), a fluorescence detector (RF-20Axs) and a system controller (CBM-20 A).

The polyvinyl alcohol cross-linked gel (9 μm in diameter) (Asahipak GS-520; AsahiKasei Co., Ltd, Tokyo, Japan) which was dried in a vacuum for more than 16 hours was then suspended in 10 ml of dimethyl sulfoxide (DMSO; Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) per gram of the dried gel. Next, to suspend the gel, 20 mmol of epichlorohydrin were added to per gram of the dried gel for 20 hours at 30ºC. The activated gel was then filtrated, followed by an addition of 10% aqueous solution of diethyl amine (Wako Pure Chemical Industries, Ltd, Osaka, Japan) for another 20 hours.

Finally, the gel was packed in a stainless column (50 x 7.6 mm I.D.). By using the above prepared column which is of high resolution and anion exchanged, the analysis condition to separate rat reduced albumin and oxidized albumin was screened by optimizing the factors—buffers, pH value, ethanol concentration, flow rate, the linear gradient of magnesium concentration, oven temperature and sample volume. The pH value of solution I is adjusted by mixing solution A (pH = 4.56), solution B (pH = 9.06) and DDW with different volume ratio. Solution A is made of Sodium Dihydrogen Phosphate Dode-cahydrate (3.9 g) and Sodium Sulfate (8.52 g) with 500ml DDW, Solution B is made of Disodium Phosphate Dihydrate (8.9 g) and Sodium Sulfate (8.52 g) with 1L DDW.

All of the reagents were either HPLC grade or a special grade (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

To confirm oxidized albumin peak, commercially available standard albumin which contains almost 100% oxidized albumin (Sigma-Aldrich, production No.A6272, Japan) was diluted. Retention time of control rat oxidized albumin is identical to standard albumin, and the oxidized albumin peak can be identified (Fig 7).

The rat serum albumin analysis condition factors are summarized as below: The fluorescence detection wavelengths were 280 nm and 340 nm. Two eluent buffers contained 60 mM sodium sulfate and 25 mM sodium phosphate. Three microliters of serum or plasma samples was directly injected into the system. The flow rate was 1 ml/min, and the analytical temperature was 40ºC (Fig 8). Based on the peak areas, the results were expressed as Oxidized albumin% = Oxidized albumin / (Reduced albumin +

Oxidized albumin)×100% [24,60,64](Fig 8).

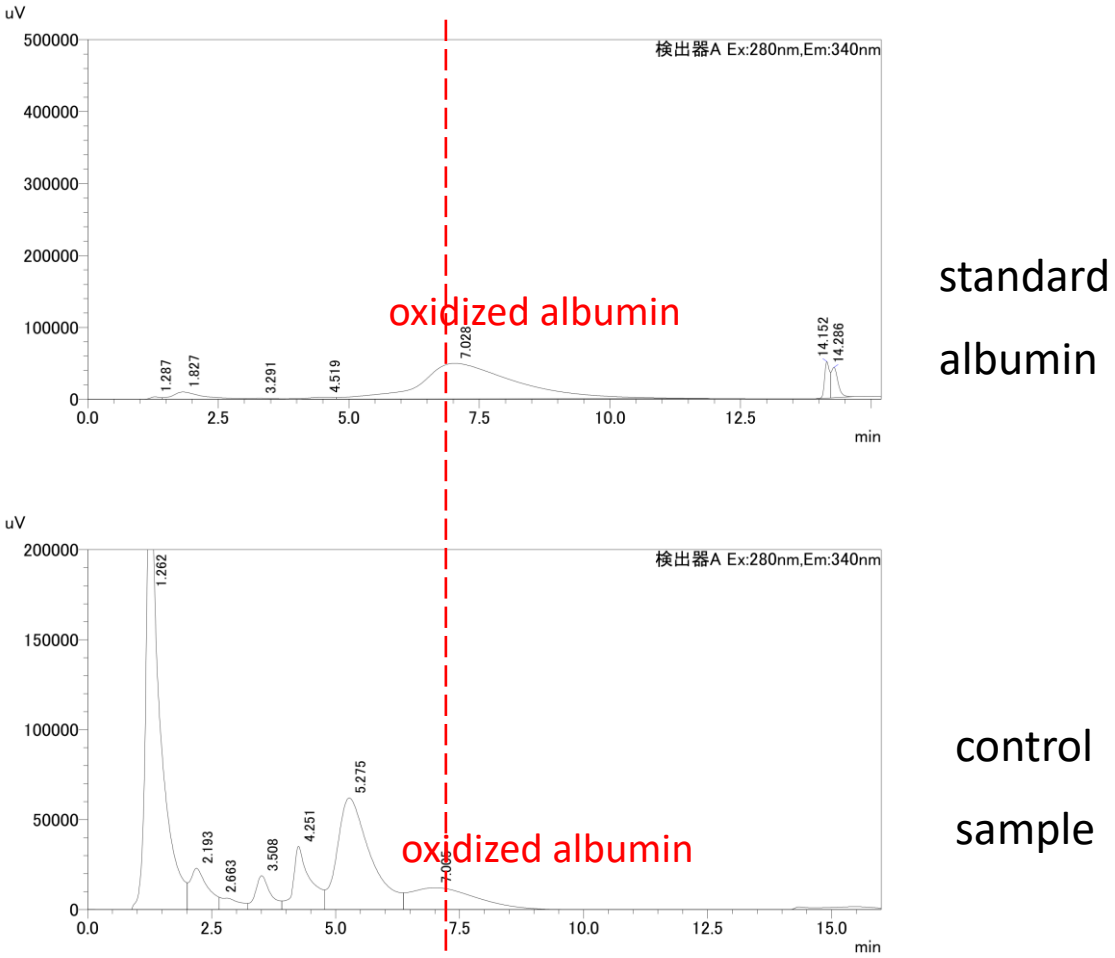

Fig. 7. To confirm oxidized albumin peak, we analyzed commercially available standard albumin which is almost 100% oxidized with rat serum albumin condition we have established. Retention time of control rat oxidized albumin is corresponding and identical to standard albumin, we can confirm the oxidized albumin peak.

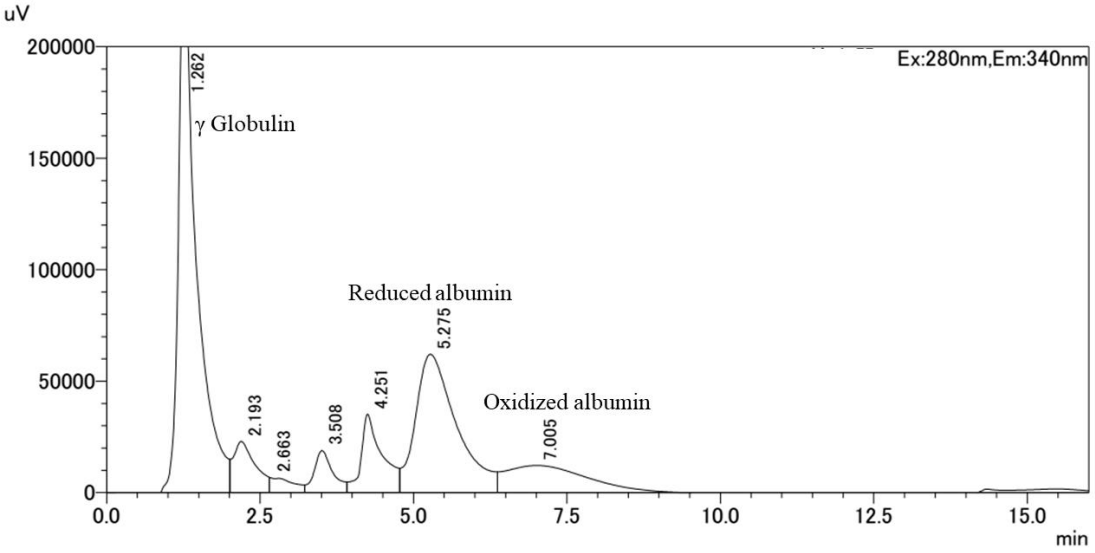

Fig. 8. Chromatograph of reduced albumin and oxidized albumin from a healthy rat. Oxidized albumin% was calculated from this formula: Oxidized albumin%=oxidized albumin / (oxidized albumin + reduced albumin) *100%.

2.3. Evaluation of method performance

After centrifuging, serum samples were sepaprated into 50µl/tube and stored in -80℃ refrigerator, samples were thawed at room temperature. Intra-day reproducibility was repeated 10 times continuously in one day by using one sample, inter-day reproducibility was repeated for 10 consecutive days, using High Performance Liquid Chromatography System (LabSolutions system; Shimazu Co., Ltd, Kyoto, Japan).

2.4. Sensitivity of the method

To study the detection limit of rat oxidized albumin in the method, serum samples from healthy rats was used and diluted by using PBS. Oxidized albumin level was

measured as described in section 2.2.

2.5. Evaluation of auto-oxidation

To investigate the time dependent auto-oxidation of albumin at room temperature, control samples of serum albumin was measured every 20 minutes for a total 120 minutes.

2.6. Interference study

Standard albumin (100% oxidized albumin) was added to the rat serum samples following a linear concentration gradient from 0% to 48% (the sample concentration gradient increased by 8%) and then measured oxidized albumin level.

2.7. Method for evaluation of the 8-isoprostane concentration

Urine samples of the rats were centrifuged at 10,000 rpm for 5 minutes. The urine samples from rats fed normal salt diets were diluted 5 times, whilst samples from rats fed a high salt diet or high salt diet plus Tempol were not diluted.

According to the manufacturer's instructions, 8-isoprostane level was measured by using the 8-iso-PGF2α Elisa kit (Enzo Life Sciences, Inc. Farmingdale, New York, USA). Briefly, the plate was incubated with the sample at room temperature on a plate shaker at 120 rpm for 120 minutes and then washed 5 times using washing solution. After incubating at room temperature with a solution of p-nitrophenyl phosphate in buffer for 45 minutes without shaking and adding stop solution, the optical density was measured

at 420 nm. The concentration of 8-isoprostane was calculated. Urine samples and standard samples were all run in duplicate.

2.8. Statistical analysis

Data was expressed as the mean ± SEM. All urinary protein levels were logarithmically transformed prior to statistical analysis by ANOVA with Tukey's post hoc test because they had a heteroscedastic distribution. Statistical analysis among the 3 groups was performed by one-way ANOVA followed by Tukey's test. The receiver operating characteristic (ROC) curve for oxidized albumin and 8-isoprostane was drawn to compare the diagnostic value. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Separation of rat oxidized and reduced albumin by using HPLC

Previous reports studied the analysis conditions by using buffers whose pH between 6.5-7.0, but cannot achieve the separation of reduced albumin and oxidized albumin (data not shown). The hypothesis is that on the one hand, negative charged albumin is deficient for column retention if pH belows 5.0 and the retention time can be prolonged when the analysis solution pH becomes alkaline,on the other hand, separation of reduced albumin and oxidized albumin is poor when the analysis solution pH becomes too alkaline.[25] Therefore, an optimum pH range should exist. I tried pH 6.0, pH 5.57, pH 5.0 and pH4.54. all of which are analysed at an oven temperature of 40℃. Clear separation can be attained at pH 5.57 and pH5.0 (Fig 9).

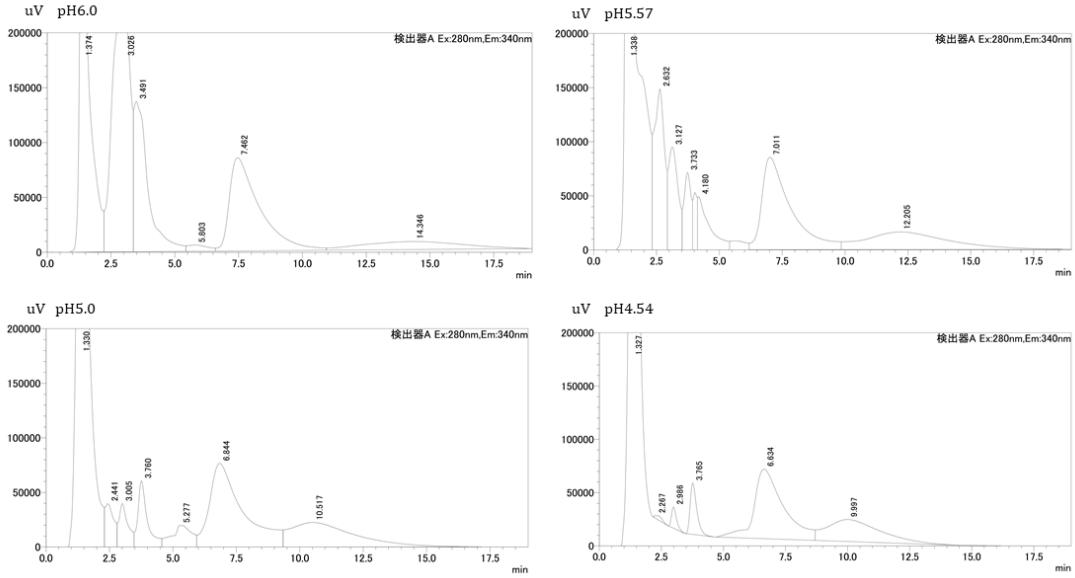

Fig. 9. Analysis condition of pH6.0, pH5.57, pH5.0, pH4.54. All of the above conditions are under 40℃ oven temperature, clearer separation can be attained at pH5.0.

The optimal condition for measurement of rat oxidized and reduced albumin are as follows: 25 mM phosphoric buffer with 60 mM sodium sulfate plus 1.5% ethanol (solution I, pH 5.3), and 1000 mM magnesium chloride (solution II). After balancing the column, the flow rate was set to 1 ml/min. The oven temperature was set to 40 ºC, and the samples volume was 3 microliters. The whole process of the serum analysis lasted for 12 minutes, and the linear gradient was 0-65% from solution I to solution II. Thus, only a total of 16 minutes was needed to analyze one sample (including the column balancing and sample analysis times). A representative chromatograph is shown in Fig 8.

3.2. Reproducibility of analysis method

The reproducibility of my analysis method is summarized in Table 3. The CV values

of reproducibility for inter-day and intra-day reproducibility were 0.77% and 0.81%, respectively. Several samples was diluted for several times, the minimal oxidized albumin concentration for detection by was 6.4 mg/ml (Fig 10).

Table 3. Inter-day and Intra-day reproducibility (%) for analysis conditions

	1	2	3	4	5	6	7	8	9	10	AVE.	SD.	CV.
Inter-day Reproducibility (%)	71.25	70.52	71.96	71.49	70.15	70.52	71.31	71.68	70.88	70.80	71.06	0.55	0.77
Intra-day Reproducibility (%)	72.83	72.46	72.36	71.90	71.79	71.79	71.43	71.48	71.16	70.83	71.80	0.58	0.81

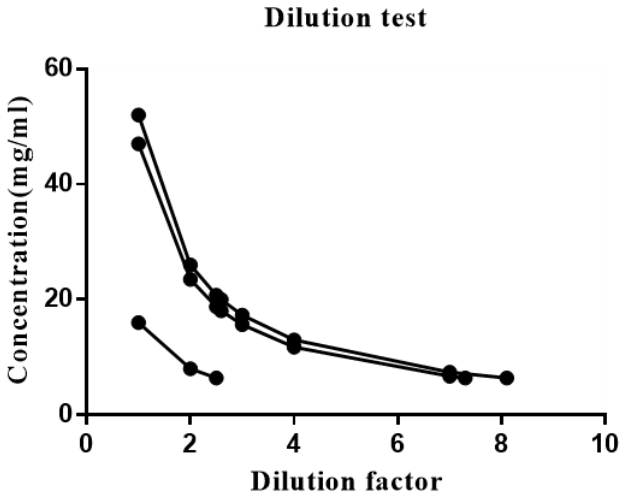

Fig. 10. Dilution test. X axis means dilution factor,Y axis stands for the concentration of serum albumin. Serum samples were diluted with PBS. The minimal concentration that can be measured is 6.4 mg/ml in serum.

The result showed that 20 minutes of incubation at room temperature caused a significant increase in the percentage of oxidized albumin in a sample, and that the oxidation of albumin is time-dependent. This process was named “auto-oxidation”.

Serum albumin could be auto-oxidized at room temperature after 20 minutes (Fig 11). The gradual increase in oxidized albumin in the intra-day reproducibility analysis may resulted from auto-oxidation.

An “interference evaluation” study was conducted. In brief, commercially available oxidized albumin standards was used to calculate the percentage of oxidized albumin, and demonstrated a positive correlation between standard albumin/total albumin and oxidized albumin (Fig 12).

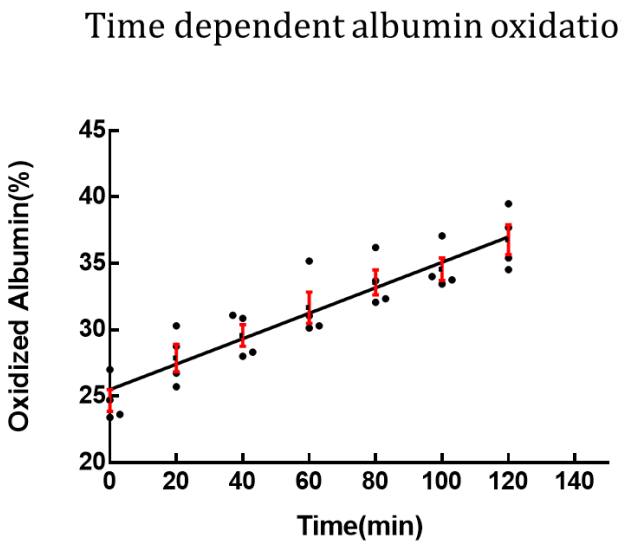

Fig. 11. Time-dependent oxidation of albumin. Samples were left at room temperature before analysis. The horizontal axis indicates the time that the sample was left at room temperature (0 min, 20 min, 40 min, 60 min, 80 min, 100 min, or 120 min). The vertical axis indicates the percentage of oxidized albumin of each sample. The samples used were from healthy rats.

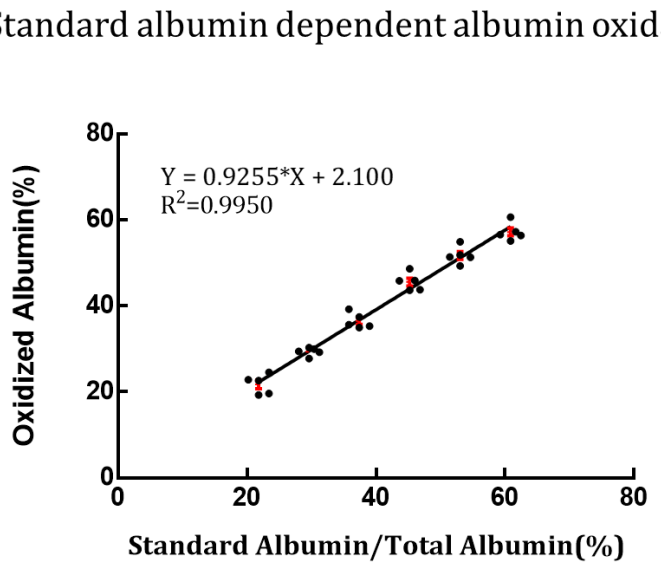

Fig. 12. Standard albumin/total albumin% dependence on oxidation of albumin. Standard albumin was added in 8% increments, and oxidized albumin% was increased linearly.

3.3. Validation of method in a rat model of hypertension and proteinuria

3.3.1. Effect of salt loading and uninephrectomy on blood pressure, renal function, cardiac and renal weight changes in young Sprague-Dawley rats

The method of measuring oxidized albumin was validated in an established rat model of hypertension and proteinuria[65](Fig 6).

High salt-loading resulted in significantly higher systolic blood pressure (176.1±34.6 mmHg) in rats than normal salt loading ones (126.4±9.4 mmHg) (Fig. 13). The urinary protein levels in the high salt diet group was also significantly higher (78.75±87.13 mg /day) than that of the normal salt diet group (8.59±8.95 mg/day). (Fig 14).

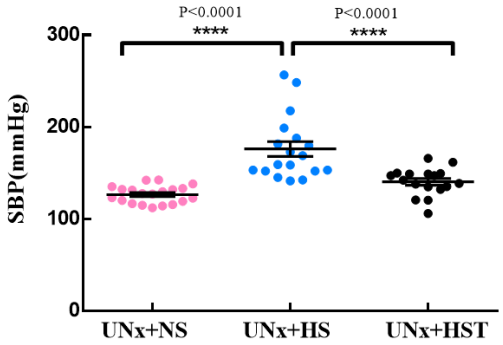

Fig. 13. The systolic blood pressure of the UNx+NS group (n=20), UNx+HS group (n=18), and UNx+HST (n=17) for 4 weeks. The values are shown as the mean±SEM.

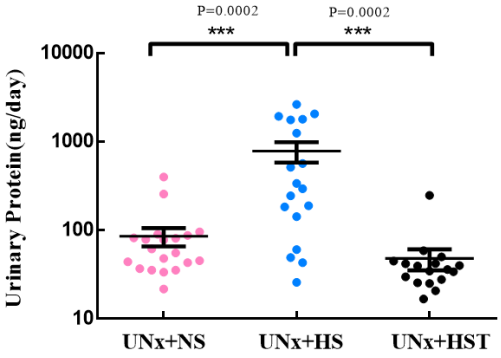

Fig. 14. The urinary protein level of the proteinuria and hypertension model. The UNx+NS group (n=20), UNx+HS group (n=18), and UNx+HST group (n=17) were evaluated. The values are shown as the mean±SEM. (All urinary protein levels were logarithmically transformed prior to statistical analysis by ANOVA with Tukey's post hoc test because they had a heteroscedastic distribution).

Urinary sodium level was significantly increased by high salt loading for 4 weeks than normal salt loading group, the same effect cannot be seen in urinary potassium level (Fig. 15).

Left ventricular weight to body weight (HW/BW) ratio was significantly increased in high salt loading rats than normal salt loading ones (Fig. 16). Kidney weight to body weight (KW/BW) ratio was also significantly higher than in normal salt diet group (Fig. 17).

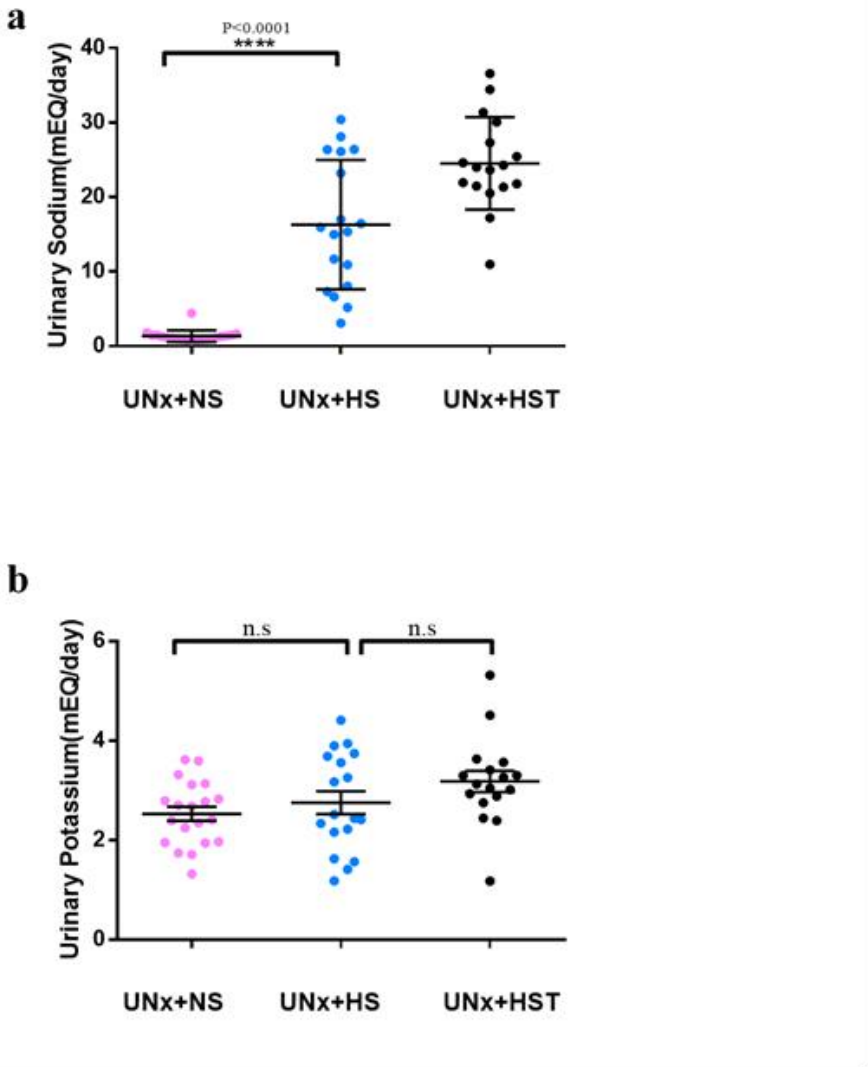

Fig. 15. The effect of 4 weeks salt loading with Tempol. (a)24 hours sodium excretion and (b) 24 hours potassium excretion. Data is presented in mean±SEM.

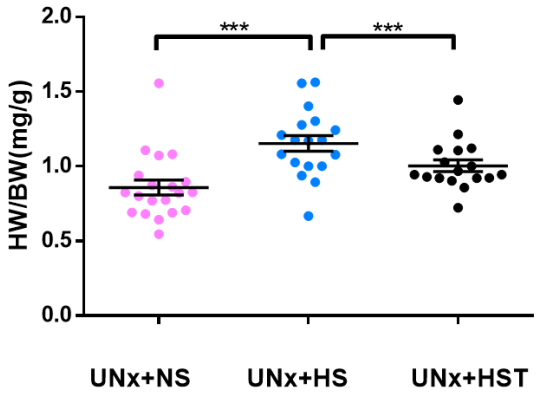

Fig. 16. The effect of salt loading with Tempol on Heart weight/Body weight (HW/BW). Data is presented in mean± SEM, ***P<0.001.

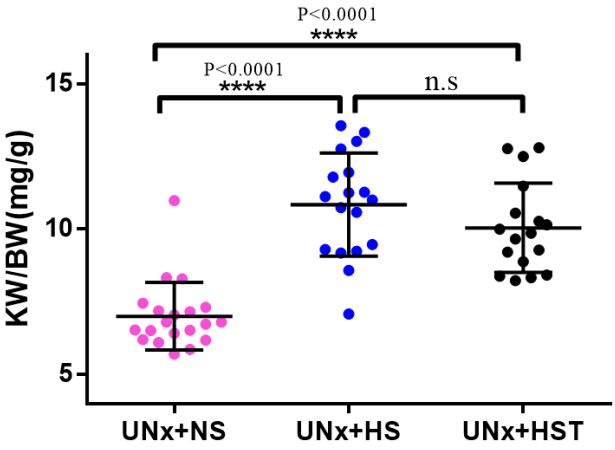

Fig. 17. The effect of salt loading with Tempol on Kidney weight / Body weight (KW/BW). Data is presented in mean± SEM, ****P<0.0001.

3.3.2. Effect of tempol on blood pressure, cardiac function and renal weight changes of uninephrectomized Sprague-Dawley rats loading with high salt diet

At 4 weeks of treatment, Tempol significantly lowered systolic blood pressure (140.4±15.0 mmHg) compared with rats loading with high salt (126.4±9.4 mmHg). Urinary protein level was significantly reduced in the presence of Tempol (4.82±5.29 mg /day) than that of the high salt diet loading group (78.75±87.13 mg /day). These data suggested that Tempol can protect kidney function and have blood pressure lowering effect.

There was no significant changes in urinary sodium and potassium level at 4 weeks of treatment with Tempol. There was also no significant difference between left ventricular weight to body weight (HW/BW) ratio and kidney weight to body weight (KW/BW) ratio in rats treated with Tempol.

3.3.3. Oxidized albumin level changes in hypertension and proteinuria model

Oxidized albumin was measured by High Performance Liquid Chromatography. Oxidized albumin levels are higher in the high salt group (35.43%±4.39%) compared to the normal salt group (29.55%±2.40%). and Tempol significantly reversed the effect of high salt (26.98%±2.19%) (Fig 18). These result suggested that oxidative stress may play an important role in hypertension and proteinuria, and that oxidized albumin maybe an useful marker in a rat model of hypertension and proteinuria.

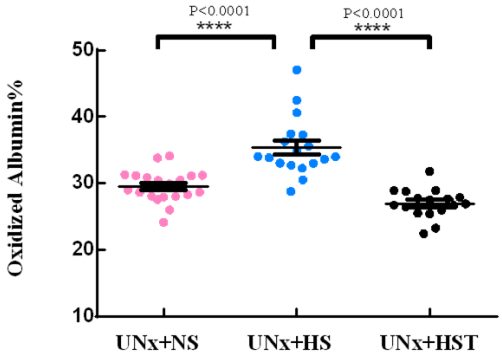

Fig. 18. Oxidized albumin% of the proteinuria and hypertension model. The UNx+NS group (n=20), UNx+HS group (n=18), and UNx+HST group (n=17) were evaluated. The values are shown as the mean±SEM.

3.3.4. Oxidative stress marker isoprostane expression changes in hypertension and proteinuria model

Isoprostane is produced from arachidonic acid of membrane phospholipids and is a reliable and sensitive marker of free radical formation[66]. Consistent with previous studies [67–69] 24-hour urine samples were collected. After 4 weeks of high salt loading, the 8-isoprostane level in the high salt diet group (36.7±45.9 ng/day) was significantly higher than that in the normal salt diet group (10.5±7.4 ng/day), and Tempol significantly reversed the effect of high salt (11.0±16.6 ng/day) (Fig 19). The data suggested that 4 weeks high salt loading may be a reason for oxidative stress and Tempol can reversed this effect.

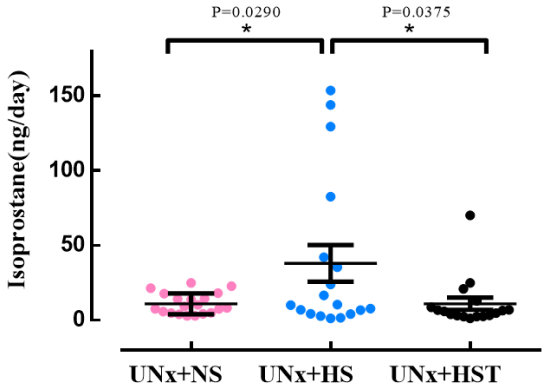

Fig. 19. The 8-isoprostane level of the proteinuria and hypertension model. The UNx+NS group (n=20), UNx+HS group (n=18), and UNx+HST group (n=17) were evaluated. The values are shown as mean±SEM.

3.4. Correlations among urinary protein, 8-isoprostane and oxidized albumin

Urinary protein levels is a marker for renal damage [65,70] and reflects glomerular damage. A previous report shows that 8-isoprostane is not a prerequisite for renal damage [71]. This data shows there is a positive relationship between oxidized albumin and urinary protein (Fig 20), positive correlation between 8-isoprostane and oxidized albumin is also shown as Fig 21.

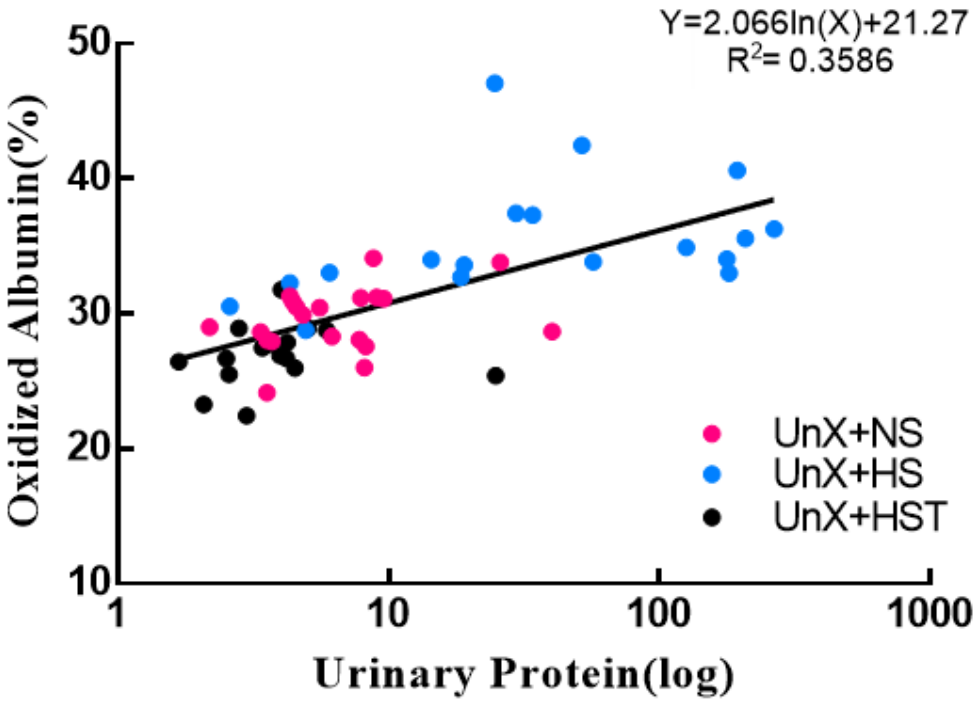

Fig. 20. Correlation between oxidized albumin% and urinary protein. The UNx+NS group (n=20), UNx+HS group (n=18), and UNx+HST group (n=17) were evaluated. The values are shown as the mean±SEM.

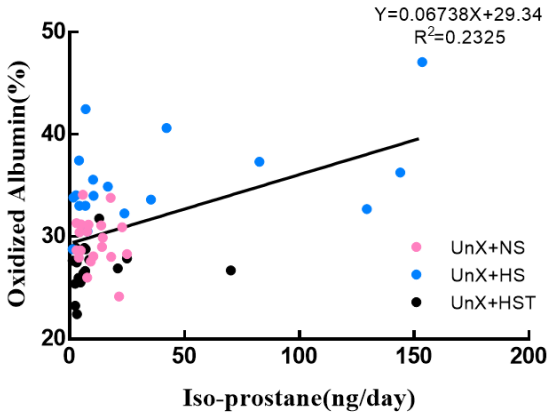

Fig. 21. Correlation between oxidized albumin% and 8-isoprostane.The UNx+NS group (n=20), UNx+HS group (n=18), UNx+HST group (n=17) were evaluated.

3.5. ROC curve of isoprostane and oxidized albumin

Based on the ROC curve, the areas under the curve (AUCs) were 0.643 for urinary 8-isoprostane and 0.917 for oxidized albumin, indicating that oxidized albumin was a superior marker to urinary 8-isoprostane ($p<0.01$) (Fig. 22) for oxidative stress-induced organ damage. The correlation data between oxidized albumin and urinary protein also suggests that measured oxidized albumin may be a better marker for oxidative stress-related organ damage, but there's no evidence of superiority of this method to estimate oxidative stress in other disease models.

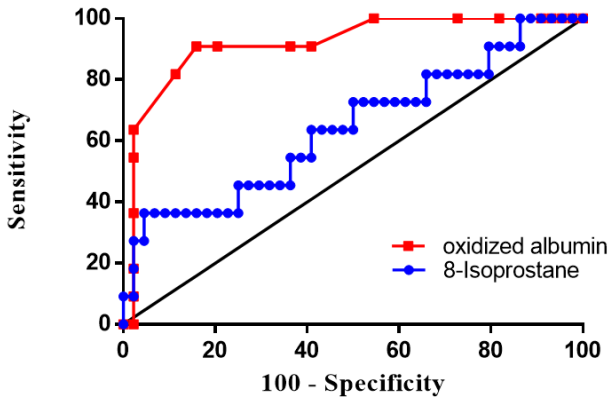

Fig. 22. ROC curves for markers. This curve is for proteinuria. Receiver operating characteristic (ROC) curves were drawn for oxidized albumin and 8-isoprostane. The area under the curve (AUC) for 8-isoprostane was 0.643, and the AUC curve for oxidized albumin was 0.917.

4. Discussion

In the present study, a revised method for the measurement of rat oxidized albumin was established. The total time taken to measure oxidized albumin with the method was as short as 16 minutes, with an intra-day and inter-day deviation within 1%. This method is sensitive and rapid, and has an advantage over conventional methods and may be useful for future studies of animal models of oxidative stress.

Human and rat albumin differs in various aspects such as molecular weight, amino acid residues and iso-electric point. Therefore, the method to measure human oxidized albumin was not applicable to rat and resulted in poor peak separation. In the current study, we modified the method for human to optimize the measurement conditions of rat albumin. The final measurement conditions of rat serum albumin are dramatically different from human serum in the respects of buffer pH value, ethanol concentration and the linear gradient time of magnesium concentration. In order to clarify the importance of this study, a comparison table was added about the comparison to measurement conditions of human serum albumin as follows. The present analysis method for human serum albumin will not only be a useful tool to evaluate liver disease, chronic kidney disease and aging but also providing an useful method to clarify the role of oxidized albumin throughout the human body.

Table 4. Species difference of properties of rat and human serum albumin

Measurement conditions	Rat	Human[1]
Buffer	25mM phosphoric buffer with 60mM sodium sulfate. High conc. Magnesium chloride	25mM phosphoric buffer with 60mM sodium sulfate. High conc. Magnesium chloride
Flow rate	1ml/min	1ml/min
Oven temperature	40°C	40°C
Sample volume	3μl	3μl
Ethanol	1.5%	No use of Ethanol
pH Value	pH 5.3	pH 6.0
Linear gradient time	12min	7.5min

This study is not the first method by which HPLC was used to evaluate rat serum albumin, and former studies shows either difficulty in separating serum albumin to oxidized albumin and reduced albumin or no reproducibility for the analysis condition which maybe difficulties in widely use[25,26]. Previous study reported that rat serum albumin cannot be clearly separated. Hayashi T et al. have since shown that HPLC method can separate both reduced and oxidize albumin in rat serum. However, their method takes about one hour to measure one sample in room temperature. This data has shown that auto-oxidation occurs as quickly as 20 minutes, making it necessary to measure the samples over a short period of time. The total time taken to analyze a sample is as short as 16 minutes (column equilibrating time included). This method is rapid and can separate peaks clearer than former reports[25,26]. The strongest advantage of this method is that this method is rapid, continuous and can separate peaks clearer than former reports, moreover, this method can be widely used owing to its accuracy and reproducibility.

For the peaks to be sharp and be clearly separated, the optimal concentrations of magnesium chloride and ethanol was applied. The acidity of the solutions are also

important when combining solutions I and II, whereby the acidic II solution was added gradually to solution I to obtain an optimized linear gradient. The pH values of solution I are 6.0 and 5.3 in the human and rat, respectively, which is due to the isoelectric point difference between humans and rats. Ethanol was used for separation of the peak and shortening the retention time as ethanol is an organic solvent for lowering the the interaction of hydrophobic[26]. Both the ion-exchange and hydrophilic interaction of the resin can contribute to the separation of oxidized albumin and reduced albumin by the column, which is further enhanced by varying magnesium concentration and the pH of solution I.

This optimized analysis condition may be useful for high reproducibility of this measurement (CV value of intra-day and inter-day are both less than 1%).

For the interference study, the “interference evaluation” was conducted. As standard oxidized albumin (100% oxidized) increased by 8%, the percentage of oxidized albumin increased by 7.42% in average (Fig 13). There is a positive correlation between the percentage of standard albumin/total albumin and oxidation albumin. The result showed that the method for measuring oxidized albumin closely reflect the actual amount of standard albumin. Based on the intra-day and inter-day reproducibility, this method was sensitive enough to measure rat oxidized albumin at a concentration as low as 6.4 mg/ml. The detect limitation of the following three techniques: (a) enzymelinked immunosorbent assay (ELISA) performed directly on intact albumin (direct ELISA); (b) ELISA performed on an albumin hydrolysate (hydrolysis ELISA); (c) high-performance liquid

Chromatographie fluorescence detection of AF-lysine adduct after albumin hydrolysis and immunoaffinity purification were ~100. 5.0, and 5.0 pg AF/mg respectively.

The term of oxidative stress was defined 1950s[72]. Later, the phrase oxidative damage to cells and organs was described since 1985[73]. The redox balance is important in keeping cellular functions[74]. Oxidative stress is the result of severe imbalance between the formulation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and antioxidant species such as biochemical process, damaging exposure or restricted ability to digest antioxidants[75–78]. There are many proteomic markers and corresponding techniques to evaluate oxidative stress level such as superoxide by EPR (electron paramagnetic resonance), thiobarbituric assay for MDA, whole organ extracts for total protein oxidation levels and so on, one of the advantages for evaluating oxidative stress level by proteins is that proteins preserve some dimensional stress information on the details of localization[79].

Oxidized species can combine biomolecules such as DNA and lipids, however, proteins are preferable due to their widely existance in cells, body fluids and cells[80]. Albumin is the most abundant protein in the plasma and synthesized in the liver, and it maintains the balance of metabolic function by adjusting osmotic pressure[81,82]. Different from human serum albumin which has 585 amino acids including a unique amino acids at the N-terminus, rat serum albumin is a peptide consisting of 608 amino acids with signal and pro-peptide sequences at the N-terminus[83]. The isoelectric point of rat serum albumin is pH5.7. Previous study showed that rat serum albumin contained more tyrosine

and less lysine, cysteine and leucine realtively compared with human serum albumin[84].

Former reports have shown that many diseases are associated with high oxidative stress levels in both humans [1–3] and animal models [4–8, 85–88] Oxidative stress in chronic kidney disease patients undergoing heamodialysis are more prevalent than general patients[89]. Factors that are involved in oxidative stress of human CKD including superoxides from nicotinamide adenine dinucleotide phosphate oxidation, nitric oxide, NO production, hypertension and angiotensin II activity [90–93] , antioxidants involved in the kidney injury including niacin, melatonin, and omega-3 fatty acids which can protect kidney damage[94–96].

Previous animal study has also shown oxidative changes in the blood and serum albumin in rats with monoarthritis and polyarthritis [97]. There are also reports on uninephrectomy rat model showing kidney relationship with oxidative stress[85–87]. To validate the method, an established rat model of hypertension and proteinuria associated with high oxidative stress levels was utilized. In this study, left uninephrectomy plus high salt diet treating for four weeks caused hypertension and proteinuria . After using Tempol, a superoxide dismutase (SOD) mimetic, systolic blood pressure and urinary protein level are significantly decreased. Increase in urinary protein excretion is a novel marker for hypertension and proteinuria and its progression, suggesting that the positive change may result from a decrease in oxidative stress levels in the rats.

The correlationship data of proteinuria and oxidized albumin suggest that oxidative stress may play an important role in rat model of proteinuria and hypertension, and that

oxidized albumin may be a useful marker in this model. High salt group significantly increase the level of proteinuria and this effect was significantly decreased in Tempol group. High salt increase urinary protein level may result from inflammatory process and high salt is harmful to the selective permeability of the glomerular basement membrane and worsen the excretion of albumin, both high salt and inflammatory process are associated with oxidative stress[98]. Tempol is an SOD mimetic thus it can preserve the glomerular capillary permeability barrier to protein[99]. Therefore, the reason of good correlation between serum albumin and proteinuria is that oxidative stress may plays an important role in proteinuria and oxidized albumin maybe a good marker for oxidative stress.

This study also demonstrated that the percentage of oxidized albumin was significantly higher in the disease model than in the control group and that this change was also reversed by the anti-oxidant drug Tempol. The result was consistent with both human and rat chronic kidney diseases data[24, 100]. In fact, oxidative stress has been shown to play an important role on the modulation of cell activity, works as a second messenger and regulates gene expression via reactive oxygen such as NF-kB[101]. Many studies studying the mechanism of oxidative stress in rat chronic kidney disease model including activated macrophages, vascular and glomerular cells[102]. The mechanism of CKD model hypertension result from the progressive losing of renal function of excreting sodium which is the reason for overloaded volume[103]. Besides, after uninephrectomy and hypertension and proteinuria development, proteinuria is the predictor for glomerular

filtration[86, 104]. According to former reports, the oxidative stress level lies in the disease progression of the uninephrectomy model, whereby oxidative stress related damage is time-dependent in models of chronic kidney disease[105, 106]. Therefore, uninephrectomy rat feeding with high salt diet gradually developed severe kidney damage with degeneration of remnant glomeruli, inflammation effects maybe also added are the reason for oxidative stress level increasing.

In hypertension and proteinuria model , kidney weight / body weight ratio was significantly increased compared with normal salt loading group. According to former study, this morphorlogical change was followed by renal macrophage infiltration and interstitial substances which is also marker for inflammation process[106]. Kidney weight / body weight increased after glomerular expansion followed by higher systolic blood pressure of uninephrectomized rats of young age after treating with 4 weeks high salt diet. This finding can be seen in early stage of CKD, and is in accordance with former studies[103, 106].

8-Isoprostanes can be found in the urine and plasma, and are usually determined from the urine because the method of collection is non-invasive and it is stable in the urine [107]. 8-Isoprostane is formed by the peroxidation of arachidonic acid [108], which is found in cell membrane phospholipids and is a target of reactive oxygen species [109], whilst urinary protein is a representative marker to evaluate renal damage [68, 73] and reflects glomerular damage. A previous report showed that 8-isoprostane was not a prerequisite for renal damage [71]. Taken together, oxidized albumin is a useful marker for oxidative

stress-induced kidney disease, and appear to be a superior marker to 8-isoprostane. A reduction in the percentage of oxidized albumin may also be a target for the prevention of renal diseases. To further confirm the result, oxidized albumin was compared with 8-isoprostane levels, which is a commonly used marker to indicate the oxidative stress level. The high salt group also showed a significantly higher 8-isoprostane level than the other two groups, and it correlated with the oxidized albumin levels. This finding indicates that my method of measuring oxidized albumin is in accordance with the currently used marker 8-isoprostane and has the potential to be applied to other disease models. The correlation data between oxidized albumin and urinary protein also suggests that measured oxidized albumin may be a better marker for oxidative stress-related organ damage.

For high salt induces oxidative stress and Tempol decreases oxidative stress level in the moel of hypertension and proteinuria, high salt reduces the nitric oxide synthase activity, thus NO production was reduced[110,111], and also, high salt can increase superoxide poduction which is the reason for decreased NO bioavailability[112]. The reduction amount of bioactive NO can form toxic peroxynitrite which can in turn contribute to oxidative stress[113]. Tempol is stable and membrane permeable, metal-independent superoxide dismutase mimetic which can inhibit reaction between superoxide and NO and thus release the inactivated NO. Tempol reduce oxidative stress by enhance the activity of NO system, which is an oxidative stress scarvenger[114,115].

The limitation is that this method was developed to analyse oxidative stress in a rat

model of hypertnesion and proteinuria, but not to discover the underlying molecular mechanism and progression of inflammation. Further studies should focus on other models and molecular mechanisms involved in this process to better clarify oxidative stress induced kidney damage.

5. Conclusion

A simple and rapid method for measuring oxidative stress levels with oxidized albumin was established. This method was validated by using an established rat model of hypertension and proteinuria which demonstrate high levels of oxidized albumin and 8-isoprostane. This method is sensitive and rapid, and has an advantage over conventional methods and may be useful for future studies of animal models of oxidative stress. Further study is also needed to be done to elucidate the mechanisms.

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Acknowledgement

My deepest gratitude goes first and foremost to Prof. Yutaka Yatomi and Prof. Tatsuo Shimosawa, my supervisors, for their warm support, constant encouragement and guidance throughout my whole Ph.D study, for giving me opportunities to attend international conferences to share my work with other researchers. I learned not only knowledge but also correct research attitude, paper writing and presentation skills, all of which are helpful for my future.

I would like to express my deep gratitude to Dr. Keiko Yasukawa for her help on discussions and comments during my study. Especially I would like to express my gratitude for her care both in my personal life and in my research career.

I would like to thank all the members in Shimosawa lab. Thanks for their kindness and for all the fun we have had in the past five years. I sincerely acknowledge Dr. Suang Suang Koid who taught me how to conduct experiments, write and think logically.

Finally, I am deeply thankful to my family, my husband Dr. Jie Chen Lu and my new born baby, XingYuan Lu, for their endless support and encouragement during my Ph.D study.