論文の内容の要旨

論文題目　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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## *1. Introduction*

Oxidative stress has elicited high levels of interest in the field of biology for a long time. 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, but does not necessarily reflect a ubiquitous oxidative stress level. 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 bound to the sulfhydryl group in Cys-34[1]. Reduced albumin is bound with its mixed disulphide with cysteine or glutathione[2]. Oxidized albumin has more oxidized products such as sulfenic (-SOH), sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) states[1].

A former study reported that oxidized rat serum albumin cannot be clearly separated from reduced albumin by conventional HPLC method[2]. Hayashi T et al. has developed a method by HPLC which can separate both reduced and oxidize albumin in rat serum[1]. However, Hayashi's method takes about one hour to measure one sample in room temperature. Here, a simple and rapid method have been established for measuring oxidized albumin in rat serum. This method is validated by using an established rat model of high oxidative stress which demonstrated proteinuria and hypertension.

## *2. Results*

### *2.1 Separation of rat oxidized and reduced albumin by using HPLC*

By systematically screening of measurement conditions, I eventually determined the optimal condition for measuring rat oxidized and reduced albumin: 25 mM phosphoric acid 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 time of 16 minutes was needed to analyze one sample (including the column balancing and sample analysis times). A representative chromatograph is shown in Fig. 1.

### *2.2 Reproducibility of analysis method*

The CV values of reproducibility for inter-day and intra-day were 0.77% and 0.81%, respectively. Multiple dilutions have been performed, and the minimal detectable concentration of oxidized albumin was 6.4 mg/ml (Fig. 2a).

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 (Fig. 2b). This process was named as "auto-oxidation". Serum albumin could be auto-oxidized at room temperature after 20 minutes (Fig. 2b).

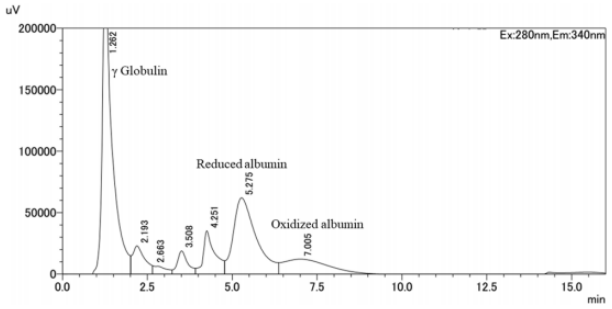


***Figure 1.*** **Chromatograph of reduced albumin and oxidized albumin from a healthy rat.**

The "interference evaluation" study has also been conducted. In brief, commercially available oxidized albumin standards were used to calculate the percentage of oxidized albumin, and demonstrated a positive correlation between standard albumin/total albumin and oxidized albumin (Fig. 2c).

### *2.3 Validation of method in a rat model of proteinuria and hypertension*

This method of measuring oxidized albumin was validated in an established rat model of proteinuria and hypertension[3]. As expected, high salt-loading resulted in significantly higher systolic blood pressure (176.1±34.6 mmHg) in rats with uninephrectomy (UNx) compared to normal salt treated UNx rats (126.4±9.4 mmHg), and additional Tempol in drinking water attenuated high salt loading-induced elevation in systolic blood pressure (140.4±15.0 mmHg) in rats (Fig. 3a).

The urinary protein level in the high salt diet group was significantly higher (78.75±87.13 mg /day) than that from normal salt diet group (8.59±8.95 mg/day), and after treatment with Tempol, the effect of high salt diet on urinary protein was abolished (4.82±5.29 mg /day) (Fig. 3b).

Serum oxidized albumin in high salt diet group (35.43%±4.39%) was significantly higher compared to that from normal salt diet group, and Tempol significantly reversed this effect of high salt loading (26.98%±2.19%) (Fig. 3c).

The 8-isoprostane level in high salt diet group (36.7±45.9 ng/day) was significantly higher than that in normal salt diet group (10.5±7.4 ng/day), and Tempol reversed this effect (11.0±16.6 ng/day) (Fig. 3d).

There are positive correlations between oxidized albumin% and both proteinuria (Fig. 4a) and 8-isoprostane (Fig. 4b). 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 is a new biomarker with greater sensitivity for evaluating oxidative stress, compared to a traditional marker, urinary 8-isoprostane ($p<0.01$) (Fig.

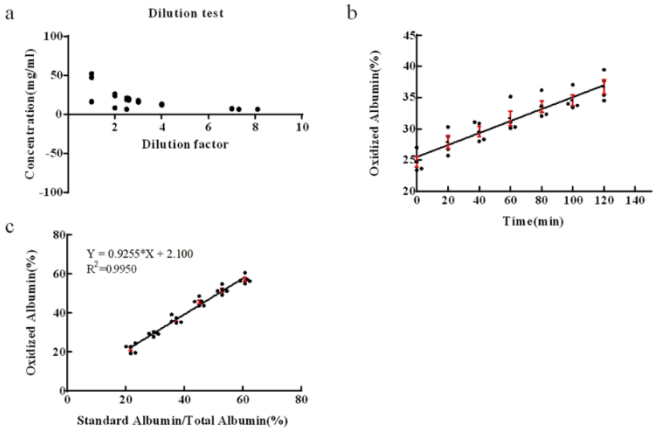


***Figure 2.*** **Effects of dilution factor, time changes and standard albumin concentration on oxidized albumin%. (a)** Dilution test. Serum samples were diluted with PBS. The minimal concentration that can be measured by our method is 6.4 mg/ml in serum. **(b)** Time-dependent oxidation of albumin. 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. **(c)** Standard albumin/total albumin% dependence on oxidation of albumin. Standard albumin was added in 8% increments, and oxidized albumin% was increased linearly.

4c).

### 3. *Discussions*

In the present study, I have described a simple, adapted method for the measurement of rat oxidized albumin. The total time taken to measure oxidized albumin with this method was only a short 16 minutes, with an intra-day and inter-day deviation within 1% and a detection limit at a concentration of 6.4 mg/ml. 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.

The gradual increase in oxidized albumin in the intra-day reproducibility analysis may resulted from auto-oxidation. My experiments have 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 using this method is as short as 16 minutes (column equilibrating time included). This method is rapid and capable of separating peaks clearer than former reports[1,2].

For the peaks to be sharp and be clearly separated, optimal concentrations of magnesium chloride and ethanol was needed. The acidity of the solutions is 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. 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. It is also noteworthy that there are also limitations of this method. It is reported that both in human and rat cases, oxidized albumin is the mixture of NA-1(Non-mercaptalbumin-1) and NA-2(Non-mercaptalbumin-2)[4–6].These two states of oxidized albumin may represent different pathophysiology[7]. In this model, the new method has not shown separation of these two peaks. Further research in different model should be conducted.

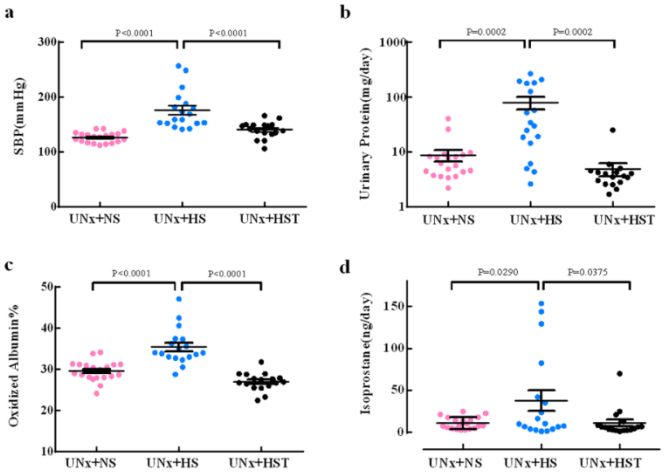


***Figure 3*. Validation study of oxidized albumin using a rat model of proteinuria and hypertension. (a)** The systolic blood pressure of the UNx +NS group (n =20), UNx+HS group (n =18), and UNx +HST(n=17) for 4 weeks. **(b)** The urinary protein level of the proteinuria and hypertension model. The UNx +NS group (n = 20), UNx +HS group (n =18), and UNx +HST group (n =17) were evaluated. **(c)** 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. **(d)** 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.

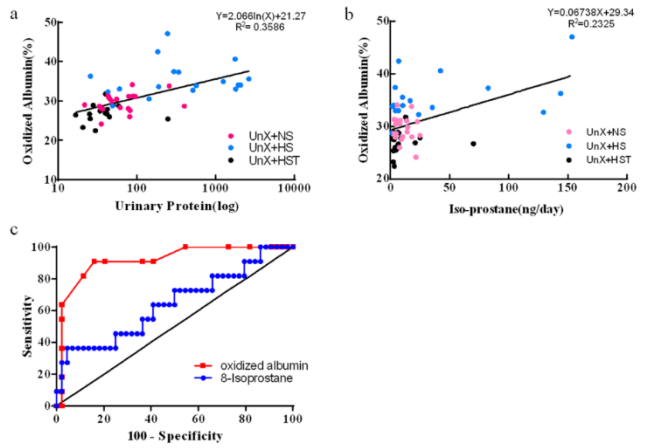


***Figure 4*. Correlation with conventional method. (a)** Correlation between oxidized albumin% and urinary protein. The UNx + NS group (n =20), UNx+HS group (n =18), and UNx + HST group (n =17) were evaluated. **(b)** Correlation between oxidized albumin% and 8-isoprostane. The UNx +NS group (n =20), UNx+HS group (n =18), UNx+HST group (n =17) were evaluated. **(c)** ROC curves for markers. 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, which was an advanced marker compared to 8-isoprostane.

For the interference study, I did an “interference evaluation”. As standard oxidized albumin (100% oxidized) increased by 8%, the percentage of oxidized albumin increased by 7.42% (AVE). There is a positive correlation between the percentage of standard albumin/total albumin and oxidation albumin. My 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, the method was sensitive enough to measure rat oxidized albumin at a concentration as low as 6.4 mg/ml.

To validate the method, I utilized an established rat model of high in oxidative stress which is associated with proteinuria and hypertension[3]. The results from my study showed that the percentage of oxidized albumin was significantly higher in the disease model than in the control group and that this change was reversed by the anti-oxidant drug Tempol. To further confirm my results, I have compared serum oxidized albumin with urinary 8-isoprostane levels, which is a commonly used marker to indicate the oxidative stress. The high salt group also showed a significantly higher 8-isoprostane level than the other two groups, and it positively correlated with the measurement of serum oxidized albumin. These findings suggest that the method of measuring oxidized albumin is in accordance with the currently using 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.

8-Isoprostane is found in cell membrane phospholipids. Oxidized albumin is a useful marker for oxidative stress-induced kidney disease, and appear to be an advanced marker compared to 8-isoprostane. A reduction in the percentage of oxidized albumin may also be a target for the prevention of renal diseases.

### *4. Conclusion*

In conclusion, a simple and rapid method for measuring oxidative stress levels with oxidized albumin has been established. This method was validated by using an established rat model of proteinuria and hypertension which demonstrate high levels of oxidized albumin and 8-isoprostane. The 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.

**References**


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