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Correlations between Serum Hydrogen Peroxide Level, Oxidative Damage Indices and Biochemical Markers in End Stage Renal Disease Patients

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ABSTRACT

Introduction: Hydrogen peroxide is an important intermediate of endogenous free radical activity which could lead to the generation of more potent free radicals that cause macromolecular damage. An imbalance between the excessive generation of pro-oxidant (free radicals) and insufficient antioxidant defence leads to oxidative stress and this event is marked in end stage renal disease (ESRD) patients. The objective of this study is to assess the correlation between serum hydrogen peroxide level and oxidative damage indices as well as several biochemical markers in ESRD patients. **Methods**: Fasting blood samples were collected from patients (n=106) attending the University Malaya Medical Centre, Kuala Lumpur. The control subjects mainly comprised healthy blood donors with no known clinical/biochemical abnormality. The serum and plasma were separated and the levels of hydrogen peroxide, oxidative damage indices (pentosidine, advanced oxidation protein products, malonaldehyde) and biochemical parameters (albumin, creatinine, ferritin, triglyceride, low density lipoproteins and total cholesterol) were estimated according to established methods. **Results**: Levels of hydrogen peroxide and the oxidative damage indices were significantly higher in the patients compared to healthy subjects. A strong positive correlation was present between hydrogen peroxide and the oxidative indices ($r>0.80$, $p<0.001$). Hydrogen peroxide was positively (weak) associated with the biochemical parameters except for albumin levels which showed a negative correlation with hydrogen peroxide levels in these patients ($r = -0.40$, $p< 0.01$). **Conclusion**: Serum hydrogen peroxide strongly correlates with oxidative damage indices and could serve as an additional marker of oxidative stress in end stage renal disease.

Keywords: ESRD, hydrogen peroxide, oxidative indices

INTRODUCTION

An imbalance between the excessive generation of pro-oxidant and insufficient antioxidant defence mechanism leads to oxidative stress.[1] In several disease conditions like hyperglycemia and uremia, the increase in oxidative stress is partly due to reactive oxygen species (ROS) and this may lead to more severe complications such as atherosclerosis and hypertension which increase the mortality and morbidity rate in these patients.[2]

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Physiologically, a small amount of ROS is required in signaling pathways to activate the cells. Over production of ROS can cause significant cellular damage through the alteration of protein structure of the signal molecules and thus destroys its function. [1]

Increased ROS-mediated oxidative stress has been observed in end stage renal disease (ESRD) patients and it results in the oxidation of important macro-molecules such as carbohydrates, proteins, lipids and nucleic acids.[3] The direct measurement of ROS *in vivo* is extremely difficult as these molecules are produced in minute quantities and are highly reactive. However, to elucidate the role of ROS in contributing to many pathological states including uremia, numerous stable end products of oxidant stress have been identified and these include malonaldehyde (MDA), pentosidine and advanced oxidation protein products (AOPP) for the oxidation products of lipids, carbohydrates and protein respectively. These biomarkers of oxidative stress have been reported to be elevated in ESRD patients.[2]

Hydrogen Peroxide (H_2O_2) is one of the ROS which serves as a key intermediate for a number of oxidative stress conditions including atherosclerosis and diabetic vasculopathy. It has a very short half life in the circulation and reacts with the hemeprotein, ascorbate and protein-thiol groups. The *in vivo* plasma H_2O_2 could diffuse into the erythrocyte, white blood cells, endothelial cells and platelet for metabolism. Although H_2O_2 is involved in the regulation of renal function and can act as an antibacterial agent in the urine, it is toxic to cells when in excess. Its levels are minimised by the action of antioxidant defence mechanisms especially catalase and glutathione peroxidase (GPx). [4]

Since, H_2O_2 has recently been acknowledged as an important messenger in cellular regulation, in the present study, the measurement of serum H_2O_2 level was carried out (within one hour of sampling) and the correlation between H_2O_2 and the major oxidative stress markers in ESRD patients were assessed. In addition, the levels of creatinine, cholesterol, LDL, triglyceride, ferritin and albumin in relation to the level of H_2O_2 were also evaluated.

METHODS

Subjects

The study subjects consisted of 106 ESRD patients (with or without type 2 diabetes mellitus, DM and/or hypertension) attending the Dialysis Clinic at University Malaya Medical Centre (UMMC), Kuala Lumpur. The subjects were recruited between the years 2005 and 2007. The main characteristics of the patients studied are presented in Table 1. All the subjects were on chronic hemodialysis treatment (blood flow: 250ml/min, dialysate flow: 500ml/min, bicarbonate buffer, substitution volume: 1.8/h), using Gambro AK 200 machines. Hepatitis C patients and those with any other active infections were excluded from the study. The control subjects (n = 100) mainly comprised healthy blood donors attending the UMMC with no known clinical/ biochemical abnormality.

This study was approved by the Medical Ethics Committee, UMMC in accordance with the declaration of Helsinki. Informed consent was obtained from all the participants of the study.

Table 1. Demographic and biochemical data of study subjects

Characteristics	Control	ESRD Patients
n	100	106
Age (mean ± SD)	51 ± 13	53 ± 17
Gender		
Male (n)	36	63
Female (n)	64	43
Ethnicity		
Malay (n)	75	56
Chinese (n)	11	31
Indian (n)	14	19
Hemodialysis treatment		
Number of sessions per week	-	3
Duration of dialysis(years)	-	1-6
Biochemical parameters		
Total cholesterol (mmol/L)	NA (3.6 – 5.2)	4.33 ± 1.4
HDL (mmol/L)	NA (1.1 – 2.2)	0.9 ± 0.4
Triglyceride (mmol/L)	NA (0.4 – 1.5)	2.4 ± 1.2
LDL (mmol/L)	NA (1.68 – 4.53)	2.4 ± 0.7
Creatinine (umol/L)	NA (62 – 115)	855.8 ± 359.7
Ferritin (ug/L)	NA (22 – 322)	656.1 ± 339.3
Albumin (g /L)	NA (35 – 50)	28.5 ± 9.2

Note: Data for the biochemical parameters for the ESRD is given as mean ± SD. Data not available (NA) for the control subjects thus, have been presented as the standard normal range.

Sample Preparation

The venous blood samples from the patients were drawn prior to the hemodialysis course. Blood samples (from patients and healthy volunteers) that were collected into 4 ml EDTA coated vials and 10 ml plain tube were centrifuged at 2500 xg for 10 min to obtain plasma and serum respectively. The plasma was used for the detection of Advanced Oxidation Protein Product, (AOPP) whilst serum was used for the detection of MDA, H_2O_2, and pentosidine. The MDA and H_2O_2 assays were run immediately (within an hour of sampling) to minimise auto-degradation or autooxidation. The remaining samples were stored at -80°C for the other assays.

Measurement of Hydrogen Peroxide

Serum H_2O_2 concentrations were measured using ferrous ion oxidative xylenol orange version-2 (FOX-2 assay).[5] The concentration of H_2O_2 in the serum was calculated based on a calibration curve obtained using H_2O_2 within a concentration range of 10-100µM.

Measurement of Pentosidine

Each of the 96-well immunoplate was coated with 100ul of Anti-Pentosidine Ab in 0.05M carbonate buffer, pH 9.6, covered with aluminum foil and incubated for an hour at room temperature. The plate was washed thrice with phosphate buffer saline containing 0.05% Tween-20 (Buffer A), and incubated with 100ul of serum sample or standard that had been constituted in buffer A for an hour at room temperature. The plate was washed thrice with buffer A to remove the unbound Ag (pentosidine). Then the plate was incubated with 100ul of Conjugate Antibody, Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP (horse radish peroxidase) for an hour at room temperature. After washing thrice with buffer A, the plate was further incubated with 100ul of Sodium Citrate Buffer pH5.2 containing 0.04% Ortho-phenylenediamine dihydrochloride and 0.09% H_2O_2 until an adequate colour developed. The enzyme reaction was terminated with the addition of 100µl of 0.5M sulphuric acid and the absorbance was measured at 492nm using a microplate reader. The amount of pentosidine was quantified based on a calibration curve with a concentration range of 10^{-2} to 10^4 nmol/ml.

Measurement of Plasma AOPP

AOPP was determined based on the spectrophotometric method by Witko-Sarsat *et al.* The concentrations are expressed as chloramine units (µmol/L).[6]

Measurement of Serum MDA

This assay was carried out using thiobarbituric acid method as described by Ratty *et al.* and the standard curve was prepared using 1,1,3,3-tetraethoxypropane. The concentrations are expressed as µmol/L.[7]

Biochemical Parameters

Blood creatinine, albumin, total cholesterol, low density lipoprotein (LDL), triglyceride and ferritin (only ESRD patient samples) were assayed using a Synchron CX7 (Beckman-Coulter CA, USA) by routine enzymatic methods.

Statistical Analysis

All data were analysed using Statistical Package for Social Science (SPSS 12 for Windows). Data are expressed as mean ± SD. Statistical significance between control and ESRD patients were analysed by analysis of variance (ANOVA) and independent sample *t*-test. Correlations between variables were studied by Pearson's correlation coefficients and differences were considered significant when $p< 0.05$.

RESULTS

All ESRD patients (with or without type 2 DM and/or hypertension) had significantly higher levels of H_2O_2, pentosidine, MDA and AOPPs compared to normal healthy subjects (Table 2). Interestingly, the serological levels of H_2O_2, MDA, AOPP and pentosidine in the ESRD patients were not confounded by age, ethnicity, gender and the presence of co-morbidities such as type 2 DM or hypertension (results not shown).

Table 2. Comparison of oxidative indices between ESRD and healthy subjects

	ESRD patients	Healthy subjects
Pentosidine (nmol/ml)	17.9 ± 11.6**	ND
MDA (μmol/L)	1.84 ± 0.7**	0.16 ± 0.4
AOPP (μmol/L)	448.6 ± 168.8**	133.0 ± 47.3
H_2O_2 (μM)	29.5 ± 7.9**	15.5 ±1.8

Note : Data is expressed as mean ± SD. **p< 0.01 compared to control (Student's *t*-test).
ND: Not detectable

Table 3. Correlation between H_2O_2 oxidative indices and biochemical parameters in ESRD patients

Clinical parameters	r
H_2O_2 / Pentosidine	0.93**
H_2O_2 / MDA	0.80**
H_2O_2 / AOPP	0.84**
Triglyceride / H_2O_2	0.3**
Total Cholesterol / H_2O_2	0.2*
LDL / H_2O_2	0.3**
Creatinine / H_2O_2	0.30**
Ferritin / H_2O_2	0.30**
Albumin / H_2O_2	-0.40**
Albumin / pentosidine	-0.40**
Albumin / AOPP	-0.40**
Albumin / MDA	-0.30**

Note: The analyses were carried out using Pearson's Correlation Analysis, SPSS version 12.0 for Windows. The data represent Pearson's Correlation analysis, r values. Significance of correlation; *p<0.05, **p<0.01.

A significant strong positive correlation was present between H_2O_2 and the oxidative indices (MDA, AOPP and pentosidine) (p<0.01) in the ESRD patients (Table 3). Increasing levels of H_2O_2 were also positively (weakly) associated with the increasing levels of triglyceride, ferritin, creatinine, LDL and total cholesterol (Table 3). A negative correlation was observed between albumin and the levels of H_2O_2 as well as the oxidative indices in these patients (Table 3).

DISCUSSION

Serum levels of H_2O_2 were found to be significantly higher in ESRD patients compared to the healthy group. This is in agreement with the *in vitro* study by Tepel *et al.* which showed

an increased H_2O_2 production in the lymphocytes of ESRD patients.[8] The net concentration of H_2O_2 not only depends on the numerous activities within the cell, but also several other factors such as site and source of H_2O_2 production, the spontaneous and enzymatic dismutation of H_2O_2 and also the concentration of the protective agents.[9] Although the half-life of H_2O_2 is short, we have demonstrated that when measured within one hour of blood sampling, the serum level in ESRD patients is significantly high.

Superoxide dismutase (SOD) levels in ESRD patients have been shown to increase with the progression of renal insufficiency.[10,11] The elevated SOD activity is believed to increase the conversion rate of $O_2^{\cdot-}$ radicals to H_2O_2. Interestingly, several reports have shown that the activities of H_2O_2 inactivating antioxidant enzymes such as catalase and GPx are reduced in these patients.[2,11,12] Thus, it is possible to speculate that the increased SOD activity and the decreased catalase and GPx activities result in a net increase in H_2O_2 level, observed in these ESRD patients.

The levels of oxidative stress biomarkers including pentosidine, AOPP, and MDA were higher in ESRD subjects compared to normal subjects. This finding is in agreement with our previous report[13] and numerous other reports.[2,6,12] The imbalance in the activities of extracellular antioxidant enzymes in the ESRD patients has been suggested to result in the accumulation of free radical species which in turn could lead to unscheduled oxidation of susceptible molecules.[14] The reduced levels of detoxifying agents such as reduced glutathione and catalase may give rise to augmented levels of a wide range of oxidatively damaged compounds.

This study revealed the existence of strong positive correlations between the levels of H_2O_2 and the levels of pentosidine, MDA and AOPP indicating that these oxidative stress biomarkers may be produced via interdependent reactions.[2,6] H_2O_2 is a well known substrate for the myeloperoxidase enzyme reaction. This reaction yields a very potent oxidising agent, hypochlorous (HOCl) which has the ability to oxidise lipid, proteoglycans and thiol groups. HOCl may also react with the endogenous amines to form protein oxidation products such as 3-chlorotyrosine, dityrosine [2,12] and chloramine which were measured as AOPP in this study. Uremic state is also in favour of the high level of pentosidine, a marker for advanced glycation end product (AGE). It is formed by non-enzymatic glycation reactions, which in turn leads to the formation of shift bases and Amadori products.[15] It is noted that $O_2^{\cdot-}$ can be generated in the pathway for Amadori product formation. Meanwhile, in the process of Amadori product degradation, H_2O_2 can be formed via both 1,2- and 2,3-enolisation and the oxidation of enolate anion.[16] The generation of $O_2^{\cdot-}$ by AGE and AOPP could trigger monocyte NADPH oxidase activation and further lead to the production of H_2O_2.[1,8,13] Schmidt *et al.* showed that H_2O_2 plays a central role in exacerbating cellular perturbation through AGE-RAGE interaction via NFκB activation.[17] Such evidence and the strong positive correlation observed in this study support the speculation that AGE and H_2O_2 may be produced concomitantly.

The present study also showed that H_2O_2 levels were significantly (weakly) correlated with the level of total cholesterol, LDL and triglyceride in ESRD patients. The elevated levels of triglyceride in these patients could increase the risk of the lipid peroxidation and ROS formation.[18] A previous study has reported that the unsaturated double bonds of fatty acids, which are present in high concentrations in the cell membrane, are susceptible

to attack by ROS.[19] Chronic renal failure patients have been shown to possess younger and more flexible erythrocytes which increases their susceptibility to oxidative damage.[20] The membrane polyunsaturated fatty acids undergo $O_2^{\cdot-}$ and H_2O_2 mediated lipid peroxidation process, through several pathways including NADPH-dependent oxidation of microsomal lipid and xanthine oxidase system. [17,21]

This study also reveals a significant relationship between H_2O_2 and ferritin in the ESRD patients group. It is noteworthy that ferritin plays an important role in the regulation of cellular iron homeostasis through the binding of iron regulatory protein and iron responsive elements which are involved in the production of $O_2^{\cdot-}$ and H_2O_2.[23,24] More specifically, the combined reaction between H_2O_2 (physiological concentrations) and the heme component of hemoglobin has been shown to induce rapid peroxidation of LDL *in vitro*, liberating free iron.[24] The liberation of iron by ferritin is known to stimulate the formation of OH through $O_2^{\cdot-}$ and H_2O_2 [24], which culminates in more macromolecular damage.

Hypoalbunemia is associated with increased mortality in patients with kidney disease. [26] Interestingly, a negative correlation between serum levels of albumin and H_2O_2 as well as the oxidative indices was observed in the ESRD patients. Albumin offers its cycteine sites for the reduction of slower-reacting, diffusible free radical compounds such as H_2O_2 and HOCl. [25] Brown *et al.* showed that albumin decreases H_2O_2 concentration *in vitro*. [27] Though the relationship between albumin and H_2O_2 levels is clearly demonstrated in the present study, the exact role and mechanisms involved require further investigation.

Previously, hydrogen peroxide has been shown to act as a first messenger in its ability to transmit pro-inflammatory signals from one cell to another [28] and most disease conditions have been shown to be in a pro-inflammatory state. The present study implicates a possible central role played by H_2O_2 in oxidative damages in ESRD patients which ultimately could manifest in disease complication and mortality.

CONCLUSION

The present study demonstrates the strong, positive relationship between the levels of serum H_2O_2 and oxidative stress/damage indices namely pentosidine, MDA and AOPPs in ESRD patients. Serum H_2O_2 levels also showed a weaker but significant positive correlation with several biochemical parameters and a significant negative correlation with albumin which implicates the important role of albumin in reducing the H_2O_2 mediated oxidative damage. Serum H_2O_2 may also serve as an additional marker that reflects oxidative stress in ESRD patients.

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