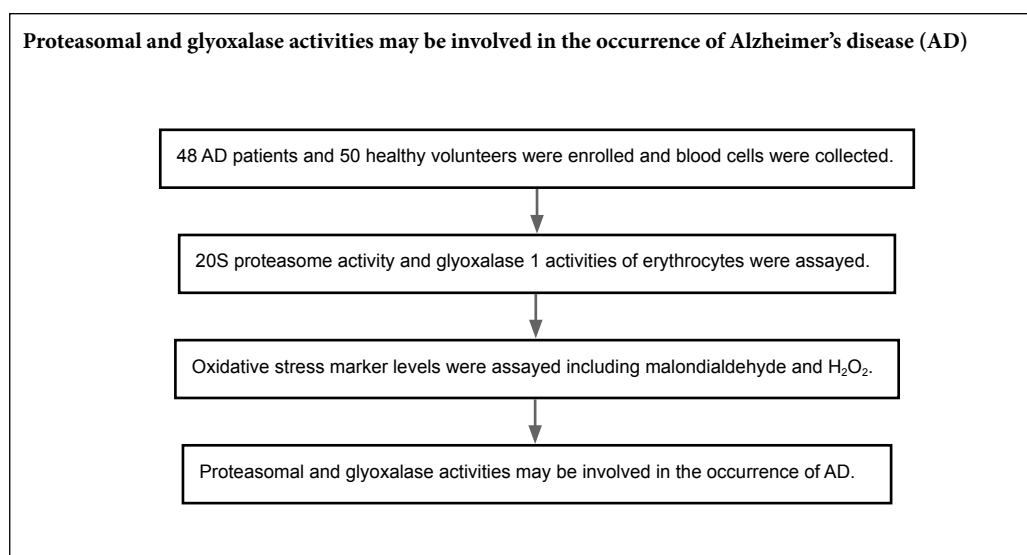


20S proteasome and glyoxalase 1 activities decrease in erythrocytes derived from Alzheimer's disease patients

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Graphical Abstract



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Abstract

As a result of accumulating methylglyoxal and advanced glycation end products in the brains of patients with Alzheimer's disease, it is considered a protein precipitation disease. The ubiquitin proteasome system is one of the most important mechanisms for cells to degrade proteins, and thus is very important for maintaining normal physiological function of the nervous system. This study recruited 48 individuals with Alzheimer's disease (20 males and 28 females aged 75 ± 6 years) and 50 healthy volunteers (21 males and 29 females aged 72 ± 7 years) from the Affiliated Hospital of Youjiang Medical University for Nationalities (Baise, China) between 2014 and 2017. Plasma levels of malondialdehyde and H_2O_2 were measured by colorimetry, while glyoxalase 1 activity was detected by spectrophotometry. In addition, 20S proteasome activity in erythrocytes was measured with a fluorescent substrate method. Ubiquitin and glyoxalase 1 protein expression in erythrocyte membranes was detected by western blot assay. The results demonstrated that compared with the control group, patients with Alzheimer's disease exhibited increased plasma malondialdehyde and H_2O_2 levels, and decreased glyoxalase 1 activity; however, expression level of glyoxalase 1 protein remained unchanged. Moreover, activity of the 20S proteasome was decreased and expression of ubiquitin protein was increased in erythrocytes. These findings indicate that proteasomal and glyoxalase activities may be involved in the occurrence of Alzheimer's disease, and erythrocytes may be a suitable tissue for Alzheimer's disease studies. This study was approved by the Ethics Committee of Youjiang Medical University for Nationalities (approval No. YJ12017013) on May 3, 2017.

Key Words: 20S proteasomal activity; Alzheimer's disease; erythrocytes; glyoxalase 1; H_2O_2 ; malondialdehyde; nerve regeneration; total ubiquitin

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Introduction

Alzheimer's disease (AD), which is accompanied by progressive loss of memory and other important mental functions, is the most common neurodegenerative disorder of the central nervous system (Lin et al., 2018; Das et al., 2019; Zhang et al., 2019). AD is now considered a protein precipitation disease because accumulations of methylglyoxal and advanced glycated end products have been observed in AD brains (Frandsen and Narayanasamy, 2018). Indeed, large amounts of advanced glycated end products, oxidized lipids, and proteins have been found in AD brain tissues (Buée et al., 2000).

The ubiquitin proteasome system is the most important cellular pathway to degrade proteins (80–90%), including many regulated, short-lived, abnormal, denatured, and damaged proteins (Rock et al., 1994). Moreover, ubiquitination-dependent degradation by proteasomal machinery is involved in the regulation of several processes such as maintenance of cellular survival, transcription, stress responses, and apoptosis. The 20S proteasome is a 700-kDa particle with several peptidase activities including chymotryptic, tryptic, and peptidylglutamyl-like activities (Orlowski, 1990; Coux et al., 1996). Several reports have demonstrated the importance of the ubiquitin proteasome system in maintaining normal physiological function in the nervous system, especially in the context of neurodegenerative disorders such as AD (Ciechanover and Brundin, 2003).

Methylglyoxal is an endogenous byproduct of normal carbohydrate metabolism. Accumulation of methylglyoxal readily reacts *in vivo* with lysine and arginine residues of proteins, leading to the formation of advanced glycated end products. The glyoxalase system, which consists of glyoxalase 1 and 2 (Glo1/2) enzymes, is the main enzymatic route for methylglyoxal elimination and prevents the formation of advanced glycated end products. Impairment of the glyoxalase system can have a direct impact on the severity of AD (Frandsen and Narayanasamy, 2018). Thus, proteasome and Glo1 activities are important to eliminate advanced glycated end products and methylglyoxal, and may be associated with the incidence of AD. However, it remains unclear how these activities change in the blood cells of AD patients. As such, this study analyzed 20S proteasome and Glo1 activities in erythrocytes of AD patients and control subjects.

Participants and Methods

Study population

The case-control study was approved by the Ethics Committee of Youjiang Medical University for Nationalities (Baise, China; Approval No. YJ12017013) on May 3, 2017 (**Additional file 1**). Written informed consent (**Additional file 2**) was obtained from all AD patients and age-matched non-AD subjects, as well as their legal guardians, before enrollment. This study enrolled two groups of subjects: one composed of 48 AD patients (aged between 70 and 85 years), and the other comprised 50 age-matched healthy volunteers without AD. Clinical criteria for the diagnosis of AD includ-

ed insidious onset, progressive impairment of memory, and more than two other cognitive function deficits (Albert et al., 2011). All healthy volunteers had no other neurodegenerative diseases. All AD patients and healthy subjects were recruited from the Affiliated Hospital of Youjiang Medical University for Nationalities between 2014 and 2017. Diagnostic guidelines for AD were in accordance with previously reported criteria (Jack et al., 2011).

Blood sample isolation

Human blood was collected by venipuncture of the arm in vacutainer tubes containing heparin anticoagulant. Erythrocytes were collected by centrifugation of venous blood samples at $800 \times g$ for 10 minutes, followed by washing twice with phosphate-buffered saline. Erythrocyte pellets were lysed with a $5 \times$ volume of distilled water, followed by centrifugation at $1500 \times g$ for 10 minutes to remove membrane fragments. Lysates were assayed for glyoxalase activity.

Circulating oxidative stress markers

Plasma malondialdehyde (MDA) and H_2O_2 levels were measured with Cayman Chemical kits (Ann Arbor, MI, USA) according to the manufacturer's instructions. MDA (Sigma-Aldrich, Saint Louis, MO, USA) was used to generate a standard curve. MDA and H_2O_2 were normalized to serum protein concentrations, which were assayed using a Bio-Rad protein reagent (Hercules, CA, USA).

Glo1 activity assay

Glo1 activity was evaluated according to a previous method (Li et al., 2018) with some modifications. Briefly, the assay was carried out in Corning UV Transparent 96-well microplates with a microplate spectrophotometer. The reaction mixture (100 μ L/well) contained 50 mM sodium-phosphate buffer (pH 6.6), 2 mM methylglyoxal, and 2 mM GSH (pre-incubated for 30 minutes at room temperature). Erythrocyte lysates from samples (30 μ g per well) were added to the buffer. Linear formation of S-(D)-lactoylglutathione was monitored at 240 nm for 20 minutes at room temperature. One unit of Glo1 activity was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of S-(D)-lactoylglutathione per minute.

20S proteasome activity assay

Erythrocytes were lysed in 20S lysis buffer [20 mM Tris (pH 7.8), 0.2% NP-40]. After removing cell debris by centrifugation, protein concentrations were determined with a bicinchoninic acid assay kit. For proteasome activity assays, 30 μ g of protein was used. Substrate concentrations were 50 μ M, prepared in $5 \times$ 20S assay buffer [125 mM Tris HCl (pH 7.5), 25 mM ATP (pH 7.5), 2.5 mM DTT, 0.1% sodium dodecyl sulfate]. Chemotryptic activity was assayed by hydrolysis of the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC (Bachem, Torrance, CA, USA). Trypsin activity was assayed with substrate Z-Leu-Leu-Glu-AMC (Bachem). Proteasome specific activity units are nmoles AMC per mg protein.

Western blot assay

Thirty micrograms of total erythrocyte membrane protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels, then transferred onto polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, membranes were incubated with polyclonal rabbit anti-ubiquitin (1:1000; Abcam, Cambridge, UK) and polyclonal rabbit anti-Glo1 (1:1000; Abcam) antibodies for two hours at room temperature. Subsequently, membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:1000 (Santa Cruz Biotechnology, Dallas, TX, USA) for 2 hours at room temperature. Immunoreactivity against total ubiquitin and Glo1 proteins was detected with super signal reagents (Thermo Scientific, Rockford, IL, USA). A housekeeping protein was also assayed using a mouse anti-GAPDH antibody (1:1000; Santa Cruz Biotechnology). Bands were scanned and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data, expressed as mean \pm standard error, were statistically analyzed by independent samples *t*-test. All analyses were carried out using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Basic characteristics of AD patients and healthy control volunteers

Table 1 shows basic characteristics of AD patients and healthy control volunteers. There were no significant differences between the two groups with regard to age, sex, body mass index, erythrocyte quality, or glucose level ($P > 0.05$).

Increased serum MDA and H₂O₂ levels in AD patients

Oxidative stress is always an important risk factor for neurodegenerative diseases (Uttara et al., 2009). To evaluate oxidative stress levels in AD patients, average serum MDA and H₂O₂ levels were assayed. The results indicated significantly increased MDA levels in AD patients compared with healthy control subjects ($P < 0.05$). As another oxidative stress marker, serum H₂O₂ levels in AD patients were also increased compared with healthy control subjects ($P < 0.05$; **Figure 1**).

Table 1 Demographic data of AD patients and healthy control volunteers

	AD group (n = 48)	Healthy control group (n = 50)	P-value
Sex (male/female)	20/28	21/29	> 0.05
Age (years)	75 \pm 6	72 \pm 7	> 0.05
Body mass index (kg/m ²)	23.5 \pm 2.8	21.8 \pm 2.5	> 0.05
Erythrocyte count ($\times 10^{12}$ /L)	4.6 \pm 0.3	4.8 \pm 0.5	> 0.05
Hemoglobin (g/dL)	14.6 \pm 2.3	15.6 \pm 3.2	> 0.05
Glucose (mM)	5.2 \pm 0.6	5.0 \pm 0.8	> 0.05

Data are expressed as the mean \pm SEM except the gender (independent samples *t*-test). AD: Alzheimer's disease.

Decreased Glo1 activity, but not protein levels, in erythrocytes of AD patients

To evaluate Glo1 activity in AD patients, we assayed Glo1 activity in erythrocytes. The results indicated significantly reduced Glo1 activity in erythrocytes of AD patients compared with healthy subjects ($P < 0.05$; **Figure 2**). However, upon measuring Glo1 protein levels in patients and controls, we could not find any significant difference between the two groups ($P \geq 0.05$, average density of Glo1/GAPDH bands, ratio of density is 0.062 \pm 0.018 vs. 0.069 \pm 0.021).

Decreased 20S proteasome activity in erythrocytes of AD patients

We measured 20S proteasome activity, as well as chymotryptic and trypsin activities of erythrocytes from AD and healthy subjects. Chymotryptic activity was decreased in AD patients compared with healthy volunteers ($P < 0.05$), as was trypsin activity ($P < 0.05$; **Figure 3**).

Increased total ubiquitin protein in erythrocyte membranes of AD patients

Western blot assay results indicated that total ubiquitin in AD patients was 1.7-fold higher than in healthy volunteers ($P < 0.05$; **Figure 4**).

Discussion

The present study provides evidence for decreased 20S proteasome and Glo1 activities in erythrocytes of AD patients. Protein homeostasis, also known as proteostasis, is regulated by a complex network of cellular mechanisms that monitor the folding and cellular location of proteins from synthesis through degradation (Tanaka, 2009). Accumulation of proteins is a recurring event in many neurodegenerative diseases, including AD (Gorman, 2008).

The ubiquitin proteasome system is the primary selective degradation system in eukaryotic cells. Declining proteasome activity has been observed in human tissues during aging, and is considered an important risk factor for AD (Selkoe, 2011). Indeed, AD is characterized by the deposition of two aggregates, amyloid and tau, that are proven to be degraded by the 20S proteasome (David et al. 2002). In this study, age-matched AD patients and healthy volunteers were used as subjects to minimize age-related effects. The results indicated reduced 20S proteasomal activity in erythrocytes of AD patients compared with healthy subjects. This is consistent with results from brain tissues of AD patients compared with age-matched controls (Keck et al., 2003). As degradation of tau protein by the 20S proteasome is at least partly ubiquitin-dependent, we measured total ubiquitin levels in erythrocytes. Our results demonstrated increased total ubiquitin levels in erythrocytes of AD patients compared with healthy volunteers. Total ubiquitin levels are always a negative indicator of proteasomal activity. Moreover, these results were consistent with previous studies indicating accumulation of ubiquitin in AD brains (Mori et al., 1987; Perry et al., 1987; Morishima-Kawashima et al., 1993; Tabaton and Piccini, 2005). As we do not have access to the brain tissues

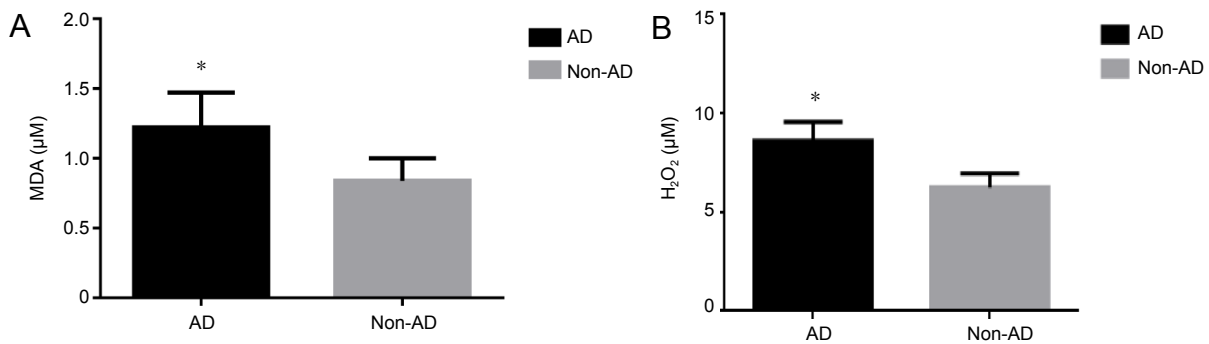


Figure 1 Serum oxidative stress markers MDA and H₂O₂ in AD patients and healthy control subjects.

Oxidative stress markers MDA (A) and H₂O₂ (B) in 20 μL serum of 48 AD patients and 50 healthy volunteers were assayed using kits from Cayman Chemical (Ann Arbor, MI, USA). Data are expressed as the mean ± SEM (independent samples *t*-test). **P* < 0.05, vs. healthy control (non-AD). All experiments were conducted in triplicate. AD: Alzheimer's disease; MDA: malondialdehyde; SEM: standard error of the mean.

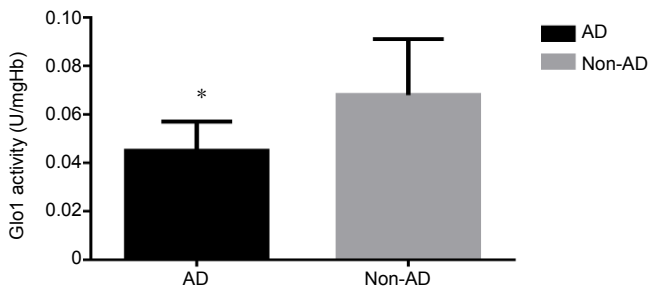


Figure 2 Glo1 activity in erythrocytes from AD patients and healthy control subjects.

Glo1 activity was assayed in erythrocyte lysates from 48 AD patients and 50 healthy volunteers. Data are expressed as the mean ± SEM (Student's *t*-test). **P* < 0.05, vs. healthy control (non-AD). All experiments were conducted in triplicate. AD: Alzheimer's disease; SEM: standard error of the mean.

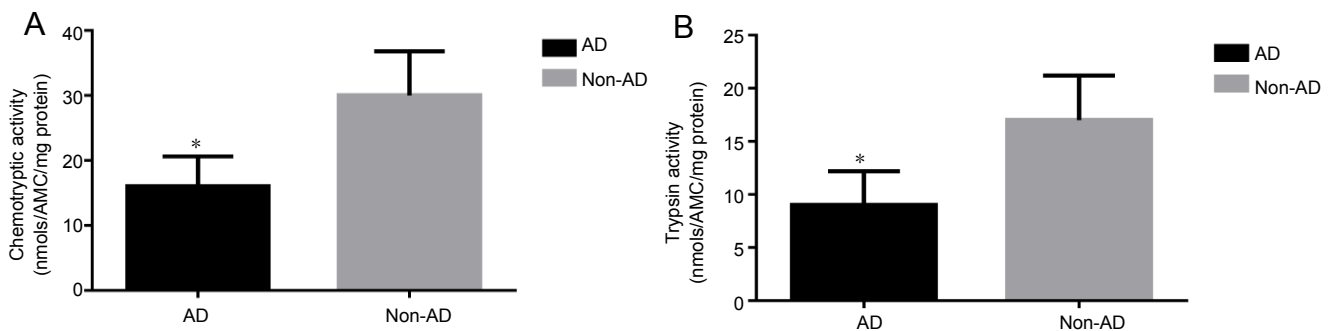


Figure 3 Decreased proteasome activity in AD patients and healthy control subjects.

Erythrocyte lysates of 48 AD patients and 50 healthy volunteers were used for a proteasomal activity assay. Both chemotryptic and trypsin activities in AD blood cells were significantly lower than in healthy volunteers (*P* < 0.05). Data are expressed as the mean ± SEM (independent samples *t*-test). **P* < 0.05, vs. healthy control (non-AD). All experiments were conducted in triplicate. AD: Alzheimer's disease; SEM: standard error of the mean.

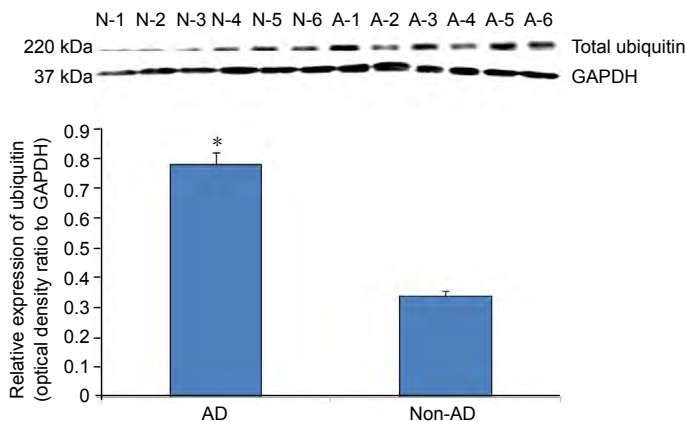


Figure 4 Total ubiquitin levels in AD patients and healthy control subjects.

Erythrocyte membrane proteins were extracted from 6 AD patients and 6 healthy subjects. Total ubiquitin levels in AD patients were increased compared with healthy subjects. Data are expressed as mean ± SEM (independent samples *t*-test). **P* < 0.05, vs. healthy control (non-AD). All experiments were conducted in triplicate. A: AD patients; AD: Alzheimer's disease; N: Healthy subjects; SEM: standard error of the mean.

of AD patients, we could not determine the correlation between 20S proteasome or Glo1 activities in erythrocytes and brain tissues. However, if these activities have good correlation between erythrocytes and brain, erythrocytes could be a useful alternative to brain tissue for studying AD. Notably, reduction of both activities could be the result of increased oxidative stress levels, and may underlie the occurrence of AD, as they could induce increased tau protein accumulation in brain tissues.

Advanced glycated end products are toxic metabolites derived from reactions between dicarbonyl compounds and proteins (Li and Chen, 2014; Snelson and Coughlan, 2019). The Glo system is a detoxification system for endogenous acyclic dicarbonyl metabolites such as methylglyoxal and glyoxal. Induction of Glo1 expression has been observed in response to inflammatory stress by amyloidosis in AD patients (Chen et al., 2004). However, our results indicated decreased Glo1 activity in erythrocytes of AD patients compared with healthy control subjects. There are two potential explanations for this discrepancy. First, the tissues used for the two Glo1 assays were different: brain and erythrocytes. Second, the former study only measured Glo1 protein expression and not Glo1 activity, as was assayed in this study. We did not find any difference of Glo1 protein levels in erythrocytes between the two groups. Moreover, Glo1 protein level does not always correlate with Glo1 activity (More et al., 2013). Thus, Glo1 activity is a better indicator of Glo1 function than protein levels.

Several lines of evidence indicate that brain tissue of AD patients is exposed to oxidative stress, which is considered to play an important role in the pathogenesis of AD (Huang et al., 2016). Our results indicated increased levels of two serum oxidative stress markers, MDA and H₂O₂, in AD patients compared with healthy subjects. As previous studies demonstrated that oxidative stress could inhibit proteasomal activity (Fataccioli et al. 1999; Reinheckel et al. 2000), higher oxidative stress levels in AD patients could underlie observed reductions in proteasomal activity. As such, further studies must be performed to determine why proteasomal activity is decreased in AD patients. To this end, tools for enhancing proteasome, autophagy, and Glo1 activities could be useful for future treatment or prevention of AD.

The results of his study indicate clear reductions in 20S proteasome and Glo1 activity in erythrocytes of AD patients; however, the clinical significance of these results remains unclear and needs further investigation. The primary limitation of this study is that no brain tissues were available to evaluate proteasomal and Glo1 activities. It will be more significant to compare the two activities in brain and blood cells from the same patients.

In conclusion, proteasome and Glo1 activities were decreased in erythrocytes of AD patients compared with healthy volunteers. Moreover, oxidative stress indicators serum MDA and H₂O₂, as well as total ubiquitin levels of erythrocytes, were increased in AD patients compared with healthy subjects. Thus, erythrocytes may be a convenient alternative to brain tissue for AD studies.

Author contributions: Study design, paper writing: HL, CDJ; experimental implementation: GYW, CSG; data analysis: YFD, YMJ, CG. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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Institutional review board statement: This study was approved by the Ethics Committee of the Youjiang Medical University for Nationalities, China (approval No. YJ12017013) on May 3, 2017.

Informed consent statement: The authors certify that they have obtained all appropriate patient consent forms. In the form the patients or their legal guardians have given their consent for patients' images and other clinical information to be reported in the journal. The patients or their legal guardians understand that their names and initials will not be published and due efforts will be made to conceal their identity.

Reporting statement: This study followed the Strengthening of Reporting of Observational Studies in Epidemiology (STROBE) Statement.

Biostatistics statement: The statistical methods of this study were reviewed by the biostatistician of Youjiang Medical University for Nationalities, China.

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Additional files:

Additional file 1: Hospital's ethics approval form (Chinese).

Additional file 2: Informed consent form (Chinese).

Additional file 3: Open peer review report 1.

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