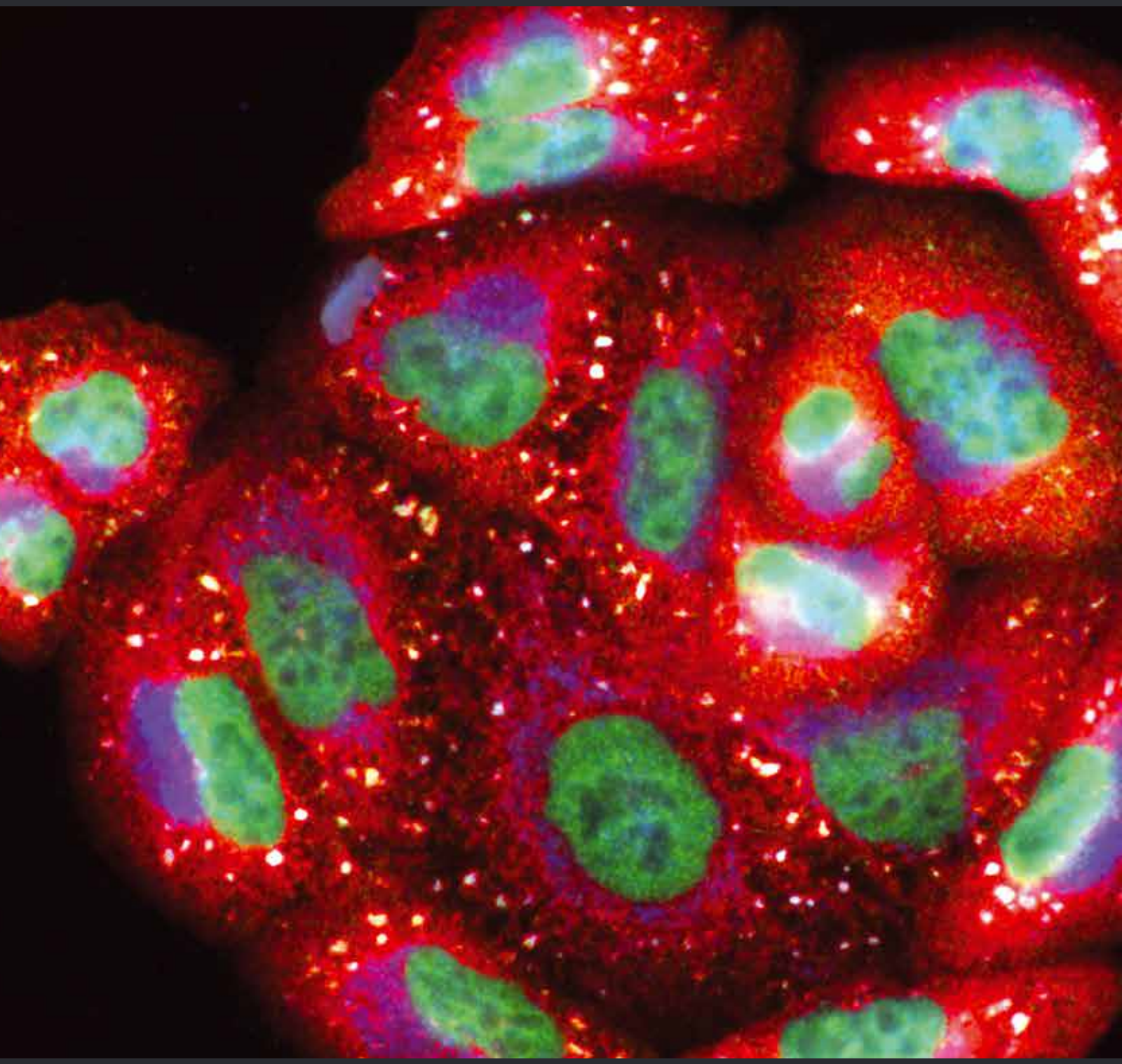
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Aging Diseases

Guest Editors: Peter X. Shaw, Geoff Werstuck, and Yan Chen



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Editorial Board

Contents

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Editorial

Oxidative Stress and Aging Diseases

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Many aging disorders, including atherosclerosis, Alzheimer's disease, diabetes, and age-related macular degeneration (AMD), result from years of combinatory impact of environmental assaults and genetic susceptibility. As a major environmental risk factor, oxidative stress, which can be enhanced by smoking and dietary habits, is known to increase the risk for many diseases associated with aging. Systemic oxidative stress not only results in accumulation of reactive oxygen species (ROS), but also damages DNA or modifies DNA structures at an epigenetic level, altering expression of genes that associate with the aging process. Local oxidative stress and ROS can affect cell signaling as well as trigger apoptosis, cellular senescence, inflammation, and even necrosis. Elucidation of mechanisms and pathways, through which oxidative stress modulates these cellular and molecular processes and affects age-related disease pathologies, is the major focus of this special issue. To respond to our call, authors presented their up-to-date studies and results in basic and translational studies of oxidative stress on diseases of aging.

ROS and derivatives of lipid peroxidation have been used as biomarkers to diagnose many aging diseases and monitor their progressions. In this issue, O. O. Erejuwa et al. reviewed the physiological and pathophysiological roles of ROS in cancer patients and highlighted evidence demonstrating other potential applications of lipid peroxidation products in cancer treatment, including the use of lipid peroxidation products as a biomarker for a diagnostic tool to predict the chances of cancer recurrence and to monitor treatment progress or how well cancer patients respond to therapy. A. Anand et al. estimated the levels of superoxide dismutase1 (SOD1) in patients with age-related macular degeneration (AMD) and examined the roles of oxidative stress, smoking, hypertension, and other factors involved in the pathogenesis of AMD. X. Xiao et al. observed the relationship between changes in renin-angiotensin-aldosterone system (RAAS) activity and blood plasma glucose after an administration of hydrochlorothiazide (HCTZ) for one year in patients with hypertension. Changes in RAAS activity were correlated with changes in plasma glucose levels after one year of HCTZ therapy.

Cataract is a common age-related eye disorder. D. Chang et al. investigated the activity of antioxidative enzymes and the products of oxidative stress in patients with age-related cataracts and compared the findings with those in healthy control subjects. They found that oxidative stress is an important risk factor in the development of age-related cataract, and augmentation of the antioxidant defence systems may be beneficial in preventing or delaying cataractogenesis.

To respond to our call for advances in genetics/epigenetics of aging related diseases, A. A. Rahman et al. determined the differential gene expression profile in genetically related individuals but at different age groups using genome-wide microarray analysis. Their results suggested that systemic telomere maintenance, metabolism, cell signalling, and redox regulation may be important for individuals to maintain their healthy state with age progression and that these processes play an important role in the determination of a healthy life span.

In other exciting papers, E. Palli et al. assessed the incidence of contrast-induced nephropathy (CIN) in critically ill patients with stable renal function who underwent computed

tomography with intravenous contrast media and found that older critically ill patients are more prone to develop renal dysfunction after the intravenous infusion of contrast agents in relation to their younger counterparts. M. Arciello et al. investigated the roles of mitochondria and metals in hepatitis C virus- (HCV-)related oxidative stress, highlighting the need to reconsider their deregulation in the HCV-related liver damage and in the antiviral management of patients.

In summary, this special issue covers a wide range of topics addressing the problems linking oxidative stress to age-related disorders and their potential treatments. These researchers not only enrich our understanding of how oxidative stress plays an important role in the initiation and progression of these diseases, but also provide evidence on antioxidant therapies for aging diseases in both experimental and clinical settings.

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Yan Chen

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Clinical Study

Contrast-Induced Nephropathy in Aged Critically Ill Patients

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Background. Aging is associated with renal structural changes and functional decline. The attributable risk for renal dysfunction from radiocontrast agents in critically ill older patients has not been well established. *Methods.* In this prospective study, we assessed the incidence of contrast-induced nephropathy (CIN) in critically ill patients with stable renal function who underwent computed tomography with intravenous contrast media. Patients were categorized into two age groups: <65 (YG) or ≥65 years old (OG). CIN was defined as 25% or greater increase from baseline of serum creatinine or as an absolute increase by 0.5 mg/dL until the 5th day after the infusion of contrast agent. We also evaluated the alterations in oxidative stress by assessing serum 8-isoprostane. *Results.* CIN occurred in 5 of 13 OG patients (38.46%) whereas no YG patient presented CIN ($P = 0.015$). Serum creatinine kinetics in older patients demonstrated a rise over five days following contrast infusion time while a decline was observed in the YG ($P = 0.005$). *Conclusions.* Older critically ill patients are more prone to develop renal dysfunction after the intravenous infusion of contrast agent in relation to their younger counterparts.

1. Introduction

Contrast-induced nephropathy (CIN) is a well-established complication of the use of the intravenous iodine contrast media representing the third most common cause of acute kidney injury in hospitalized patients [1]. The reported incidence ranges from below 5% in unselected populations to 50% in high-risk populations [2–8]. The development of CIN is associated with increase morbidity, length of hospitalization, chronic renal impairment, and higher mortality [9, 10].

Several risk factors have been related to CIN like decreased baseline renal function, heart failure, diabetes, dehydration, hypotension, older age, and the type and the amount of contrast agent applied [11–13]. Most of the studies mainly have been carried out in cardiological patients with unstable renal function who have undergone hemodynamic interventions and secondarily in other patient's groups. Critically ill is a group of patients who shared many predisposing factors for CIN which have been studied during the last few years with various results in respect to the incidence of CIN [14].

Aged critically ill patients represent a group with more compromised clinical status since kidney dysfunction is common among older people. Data from the National Health and Nutrition Examination Survey (NHANES III), a US study of community-dwelling adults, estimated that nearly 35% of the general population aged 70 years and older have moderate stage 3 chronic kidney disease [15]. Studies in Europe have also shown that there is an exponential rise in chronic kidney disease in the elderly [16–19].

In this study, we aimed to investigate whether the risk for critically ill patients >65 years old to develop CIN after exposure to intravenous contrast media is higher compared to critical care patients aged less than 65 years old. In addition, since it has been suggested that oxidative stress play a significant role in the pathogenesis mechanism of CIN, we assessed 8-isoprostane serum levels as a biomarker of oxidative stress changes following contrast infusion [20].

2. Material and Methods

This was a prospective observational study. Consecutive sampling was used to recruit patients from a general ICU, between 2011 and 2012.

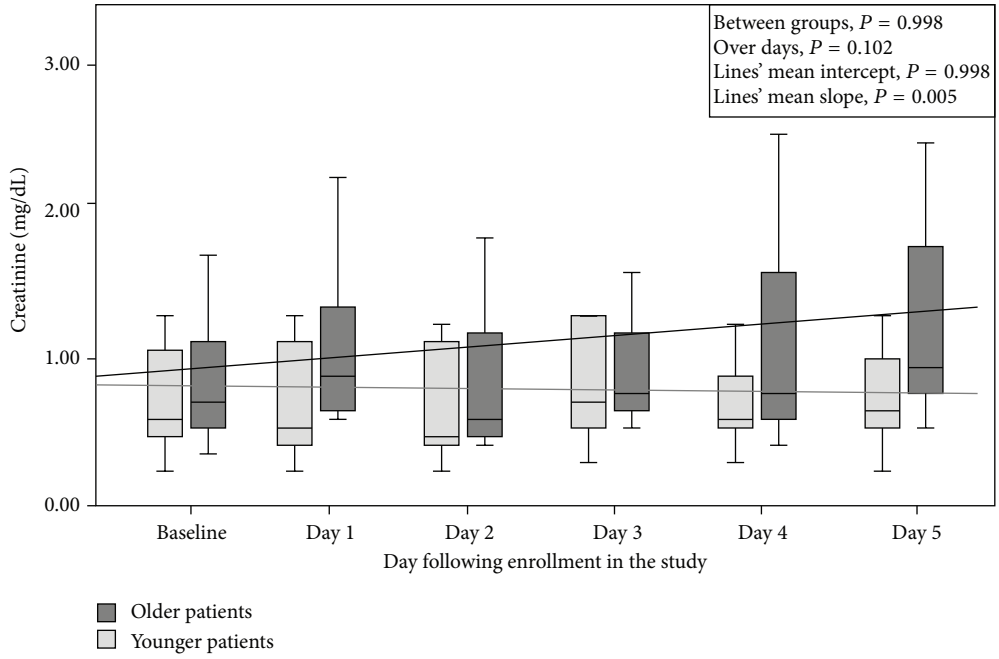

Figure 1: Serum creatinine (mg/dL) concentration in patients ≥65 years old and in patients <65 years old.

We included consecutive critically ill patients with stable renal function who needed computed tomography (C/T) imaging with the use of intravenous contrast media during a six-month period. Exclusion criteria were unstable renal function, defined as a change in serum creatinine values greater than 0.15 mg/dL between 2 consecutive days, patients under renal replacement therapy, and history of intravascular administration of contrast agent during the 5 days before to CT scan.

The patients were divided into two groups in respect to their age: YG group included patients <65 years old and OG group which included patients ≥65 years old. Patients in the two groups were matched for APACHE II score.

2.1. Outcome Measures. The primary end point of the study was the incidence of CIN. Secondarily, we evaluated the alterations in oxidative stress via changes of serum levels of 8-isoprostane and the need for renal replacement therapy among the examined groups, two weeks following the infusion of the contrast agent.

2.2. Clinical Assessment-Definitions. We aimed to keep all participants well hydrated before and after the infusion of contrast agents; thus, 1000 mL of fluids was infused in addition to the scheduled daily requirements of each patient. The CT scans were performed with the use of low osmolarity contrast agent, Iopamiro 370, Bracco.

Serum creatinine and urea levels were measured before the administration of radiocontrast agent and thereafter once daily until the fifth day following radiocontrast infusion.

2.3. 8-Isoprostane Assay. Serum was sampled before the infusion of contrast agents and 24 hours later. The measurement of 8-isoprostane was performed with a commercial enzyme-linked immunoassay Kit (Cayman CC, USA).

2.4. Statistical Analysis. Results are expressed as means ± standard error (SE). Kolmogorov-Smirnov test was used for normality assessment. Chi-square or Fisher's exact test was used to compare categorical variables and t-test or Man-Whitney U test to compare continuous variables as appropriate. To compare serum urea and creatinine differences between subgroups over time, linear mixed model analysis was performed. Linear regression analyses were used to determine associations among continuous variables. P values of <0.05 were considered to be statistically significant. Statistical analysis and graphs were performed with statistical software GraphPad version 5 and the statistical package SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Twenty-six critically ill patients were included in the study; 13 were <65 years old (YG) and 13 were ≥65 years old (OG). Table 1 shows the baseline characteristics of participants; no statistically significant differences were found between groups. Two patients in the OG presented renal failure in their past medical history; both had normal diuresis and baseline serum creatinine was mildly abnormal in each one of them.

Five patients in the OG fulfilled the criteria for CIN 38.46%, while no one did in YG ($P = 0.015$). Serum creatinine concentration in patients of YG presented a decline over time whereas in OG there was a mild rise in serum creatinine ($P = 0.005$ for mean slope) (Figure 1). Thus, there was an indication towards higher creatinine values in the

Table 1: Baseline characteristics.

Characteristic	Younger patients ($n = 13$)	Older patients ($n = 13$)	P value
Age	44.31 ± 12.28	72.62 ± 5.18	0.0001
Male gender	13 (100%)	10 (76.92%)	0.22
Body mass index (Kg/m^2)	24.4 ± 3.7	27.2 ± 4.1	0.59
Current smoking	5 (38.46%)	3 (23.07%)	0.67
APACHE II score in the day of CT scan	14.08 ± 4.46	17 ± 7.15	0.22
Baseline creatinine mg/dL	0.90 ± 0.66	0.89 ± 0.33	0.95
Diabetes mellitus	0	2 (15.38%)	0.48
Arterial hypertension	3 (23.07%)	6 (46.15%)	0.41
Dyslipidemia	1 (7.69%)	3 (23.07%)	0.59
Ischemic cardiac disease	1 (7.69%)	2 (15.38%)	1.0
Hepatic insufficiency	0	0	
COPD	1 (7.69%)	4 (30.76%)	0.32
Renal failure	0	2 (15.38%)	0.48
Noradrenaline μg/Kg/min (γ)			
Low dose until ≤5γ	6 (46.15%)	1 (7.69%)	0.073
Medium dose until 5–20γ	2 (15.38%)	3 (23.76%)	1.0
High dose >20γ	0	1 (7.69%)	1.0
Sepsis in the last 24 h before CT scan	2 (15.38%)	3 (23.07%)	1.0
Nephrotoxic medications			
Aminoglycosides	1 (7.69%)	2 (15.38%)	1.0
Colimycin	6 (46.15%)	7 (53.84)	1.0
Teicoplanin	2 (15.38%)	3 (23.07%)	1.0
Amphotericin B	1 (7.69%)	0	1.0
ACEI	3 (23.07%)	2 (15.38%)	1.0
Diuretic	4 (30.76%)	3 (23.07%)	1.0
NSAID	4 (30.76%)	3 (23.07%)	1.0
Volume of contrast medium (mL)	100	100	1.0

COPD: chronic obstructive pulmonary disease, ACEI: angiotensin-converting-enzyme inhibitor, and NSAID: nonsteroidal antiinflammatory drugs.

OG compared to YG at the 5th day following radiocontrast infusion ($P = 0.07$).

Furthermore, serum urea concentration in patients of YG presented a mild increase over time, whereas in patients of OG was detected a greater increase ($P < 0.001$ for mean slope) without significant differences between groups ($P = 0.546$) (Figure 2).

8-Isoprostane levels presented a peak at 24 hours after the infusion of contrasts in the OG; however, the difference compared to YG was not significant $P = 0.49$ (Figure 3).

All OG patients who developed CIN ($n = 5$) received one at least nephrotoxic medication, four of them colimycin and one of them amikacin, while in the group of OG patients who did not develop CIN ($n = 8$), only three received a nephrotoxic medication, colimycin, $P = 0.0754$ (Figure 4). We should point out that from two patients in the OG presented renal failure in their past medical history, one of them who had also received a nephrotoxic medication developed CIN.

No difference was found between groups, in the number of patient who underwent renal replacement therapy during the two weeks following the infusion; there were 4 patients in the OG (30.8%) and one patient in the YG (7.7%) ($P = 0.32$).

4. Discussion

The findings of the present study suggest that critically ill patients aged 65 or more years old are more prone to present renal injury after the intravenous infusion of radiocontrast media compared to patients aged less than 65 years old. Notably, 38.46% of older patients developed CIN, but no one of the younger patients in this cohort did ($P = 0.015$). The mean serum creatinine concentration of older patients presented an estimated rise during the examined period by 0.025 mg/dL every day after the infusion of contrast agent. In contrast, serum creatinine concentration of younger patients

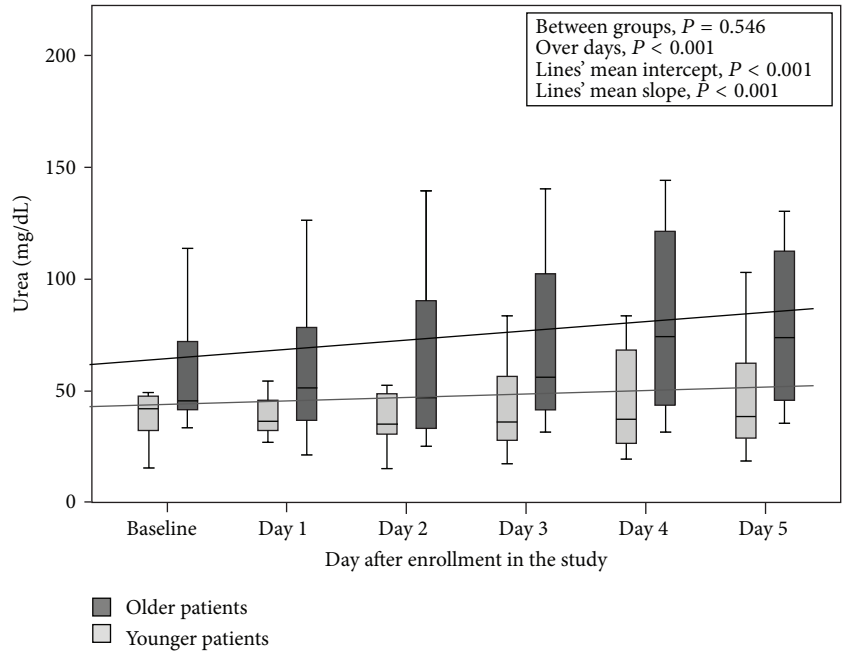

Figure 2: Serum urea concentrations (mg/dL) concentration in patients ≥65 years old and in patients <65 years old.

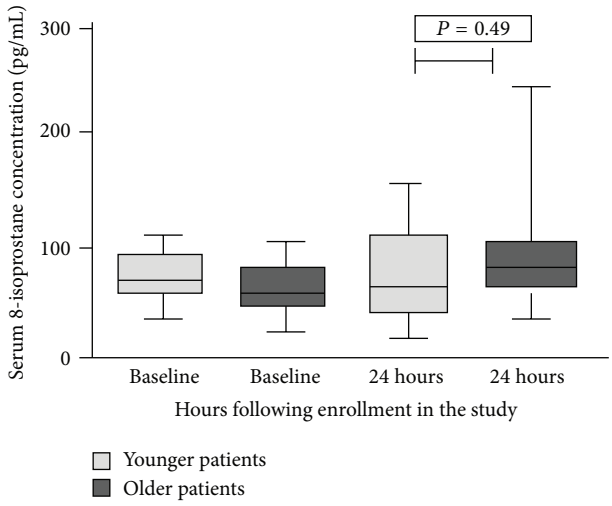

Figure 3: Serum levels of 8-isoprostane (pg/mL) in patients ≥65 years old and in patients <65 years old.

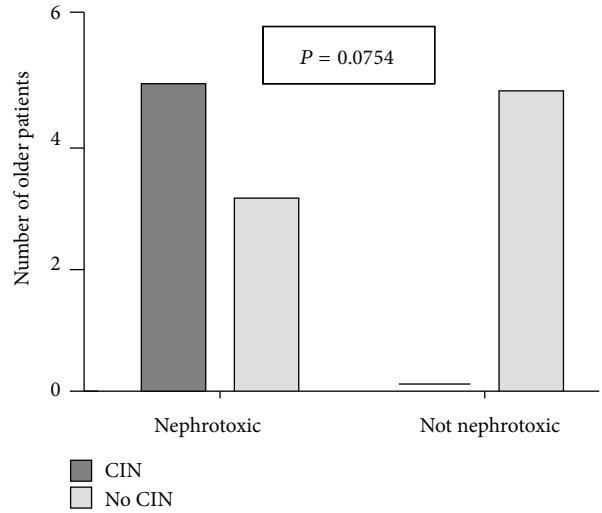

Figure 4: Contrast-induced nephrotoxicity (CIN) in patients ≥65 years old who developed CIN or not according to the use of nephrotoxic medications.

demonstrated a slight decline during the time by 0.007 mg/dL per day after the infusion of contrast agent (Figure 1).

One might argue that older patients present significant comorbidities that may predispose to CIN. Renal function is also known to decline with age and morphological changes, such as decrease of kidney weight, appearance of sclerotic glomeruli [21], and intimal proliferation in the renal artery, are some of the causes of renal dysfunction [22]. According to the recent literature, it is not yet clear how much of the functional loss in older people is due to physiologic consequence of aging [23–25] and how much is related to associate cardiovascular disease and life course exposure to CKD risk factors such as hypertension, diabetes, and smoking [26].

A plausible hypothesis therefore for the difference in CIN between YG and OG might be the longer prevalence of chronic kidney disease, and the presence of risk factors such as hypertension, diabetes and smoking in older patients. In this cohort, however, there was no significant difference regarding several potential predisposing factors which were assessed between two groups (Table 1). Nevertheless, those factors could not be assessed precisely in our patients.

Nephrotoxic medications like nonsteroidal anti-inflammatory drugs which might have been used more in the past by older people could be also another potential explanation.

It should be also noted that there was a mild decline in serum creatinine in YG group over time which may have contributed to the differences found between YG and OG. A possible explanation for this decline in YG could be the influence of periprocedural hydration in relation to a greater reserve in the renal function of younger patients.

Regarding the special features of older patients who developed CIN in this study, an interesting observation is that all of them ($n = 5$) received one at least nephrotoxic medication, 4 of them colimycin and one of them amikacin, while in the group of older patients who did not develop CIN ($n = 8$), only 3 received a nephrotoxic medication, colimycin ($P = 0.0754$, Figure 4). Therefore, this difference could be better depicted in a larger cohort than ours and this is a limitation in our study.

In the present investigation, we used as criterion for CIN the increase in baseline serum creatinine by 25%, or the absolute increase of at least 0.5 mg/dL beyond 48 hours after the infusion of contrast agent.

This criterion is widely accepted in the literature [27]. Furthermore, the monitoring period for CIN assessment is important. In a short monitoring period, the incidence of CIN may be underestimated. Recent studies adopted longer time-periods of assessing of renal function in order to detect more cases of renal injury [28]. Our investigation is in line with this. We followed our patients for 5 days after the infusion of contrast agent. Notably, 3 patients in our study developed CIN before the 3rd day after the infusion of contrast agent while the other 2 patients at the 4th and 5ths days after, so a longer period of monitoring of renal function is considered advantageous for the diagnosis of CIN and therefore for better estimation of CIN incidence.

In the present study, we used serum creatinine as an index of renal injury. Serum creatinine concentration despite the problems related with the decreased muscle mass which is seen in older patients remains the most widely used index of renal function in clinical practice since it is a relatively inexpensive, standardized parameter which is available around the clock at almost any clinical chemistry laboratory whereas many of the new glomerular filtration rate biomarkers lack one or more of these essential features [29]. Other studies have used different indexes of renal injury such as serum cystatin C. However, results varied regarding its superiority as an indicator of renal impairment in relation to serum creatinine [29–31].

The exact pathophysiological mechanisms responsible for the development of CIN are complex and poorly understood [28]. Experimental studies suggest that the pathogenesis involves a combination of vasoconstrive free oxygen radicals generation, renal medullary ischemia, and direct tubular epithelial cell toxicity [32–34]. In this respect, we aimed to assess serum levels of 8-isoprostane as a surrogate of oxidative stress. A peak was detected 24 hours after the infusion of contrast media in OG while in YG the progress of levels represented a more constant course. 8-Isoprostane serum levels were not correlated with the alterations of serum creatinine in the same group. Between the two groups, no significant differences were found. A possible explanation is that in critically ill patients multiple medical entities which contribute to oxidative stress production may coexist; thus, their contribution might obscure the impact of radiocontrast used in CT on the oxidative burden of the critically ill patients. Certainly, one might argue that performing an additional assay like DCFH-DA might have provided further insight into the alterations of renal function due to increased oxidative stress burden. Unfortunately, we have not included an additional method for oxidative stress assessment in our study; we certainly acknowledge this limitation.

5. Conclusion

Older critically ill patients seem to be more susceptible in developing renal injury after the intravenous infusion of contrast agents in relation to their younger counterparts, so additional protective measures, beyond the well hydration which is the cornerstone, may have a role in prevention of CIN.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Evidence in Support of Potential Applications of Lipid Peroxidation Products in Cancer Treatment

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Cancer cells generate reactive oxygen species (ROS) resulting from mitochondrial dysfunction, stimulation of oncogenes, abnormal metabolism, and aggravated inflammatory activities. Available evidence also suggests that cancer cells depend on intrinsic ROS level for proliferation and survival. Both physiological and pathophysiological roles have been ascribed to ROS which cause lipid peroxidation. In spite of their injurious effects, the ROS and the resulting lipid peroxidation products could be beneficial in cancer treatment. This review presents research findings suggesting that ROS and the resulting lipid peroxidation products could be utilized to inhibit cancer growth or induce cancer cell death. It also underscores the potential of lipid peroxidation products to potentiate the antitumor effect of other anticancer agents. The review also highlights evidence demonstrating other potential applications of lipid peroxidation products in cancer treatment. These include the prospect of lipid peroxidation products as a diagnostic tool to predict the chances of cancer recurrence, to monitor treatment progress or how well cancer patients respond to therapy. Further and detailed research is required on how best to successfully, effectively, and selectively target cancer cells in humans using lipid peroxidation products. This may prove to be an important strategy to complement current treatment regimens for cancer patients.

1. Introduction

The physiological and pathophysiological roles of ROS have been a subject of research interests in the last few decades. Due to their short half-lives, quantification of ROS (either *in vivo* or *in vitro*) is a very challenging task. Consequently, the oxidation products of ROS in biological samples are of great interest because they are more stable and reflect the magnitude of oxidative stress [1]. One of such oxidation products is lipid peroxidation products which are formed when ROS attack polyunsaturated fatty acids (PUFAs) leading to membrane structural and/or functional damage [2]. Lipid peroxidation gives rise to the formation of highly reactive aldehydes which are extremely diffusible and attack or form covalent links with distant cellular components/targets [3]. Once ensued, lipid peroxidation is capable of self-propagating and initiating chain reactions [4]. In most cases, the reactions continue except they are terminated (e.g., by intervention with antioxidants such as vitamin E) or there is complete substrate utilization. Among the common lipid peroxidation products are malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein [5]. Others include isoprostanes (IsoPs) and neuroprostanes (neuroPs), which are oxidized products of arachidonic acid and docosahexaenoic acid, respectively [6]. These lipid peroxidation products covalently bind to histidine, cysteine, or lysine residues of protein through Michael addition resulting in structural alteration and impaired functions of these protein residues [7]. These aldehydes in turn propagate further attack on cellular membrane constituents, primarily lipids and proteins, to generate other end products of lipid peroxidation commonly known as lipofuscin-like pigments (LFPs) [8]. The role of lipid peroxidation is implicated in the pathophysiology of or associated with many chronic diseases such as Alzheimer's disease, diabetes mellitus, hypertension, and cancers in both animals and humans [9–12]. In spite of the detrimental biological effects of lipid peroxidation, this review presents evidence that suggests lipid peroxidation products could be utilized in the treatment of cancer.

2. Lipid Peroxidation Products

2.1. Malondialdehyde. Malondialdehyde is a highly reactive and toxic aldehyde formed as a consequence of peroxidation of PUFAs. MDA can also be produced from the breakdown of prostaglandin via the action of cyclooxygenase [13]. Other nonlipid precursors including carbohydrates and amino acids can also generate MDA [14]. Accumulation of MDA can alter the membrane permeability as well as impair fluidity of the membrane lipid bilayer [15]. MDA remains one of the most mutagenic lipid peroxidation products. It can react with deoxyadenosine and deoxyguanosine in DNA leading to the development of DNA adducts which are mutagenic [16, 17]. Malondialdehyde is a commonly used biomarker for the assessment of lipid peroxidation [18].

2.2. 4-Hydroxy-2-nonenal. 4-Hydroxy-2-nonenal is a product generated following peroxidation of n-6 polyunsaturated fatty acids such as linoleic acid. Of all the aldehydes formed from the lipid peroxidation of PUFAs, HNE is considered the most important [19]. HNE, via Michael addition, is able to bind to cysteine, lysine, and histidine residues of proteins. These HNE-bound protein residues can impair normal protein structure and function [20]. HNE also reacts with many important cellular constituents such as nucleic acids, lipids, and vitamins as well as signaling molecules [21]. Such HNE interactions can interfere with normal cellular functions such as impaired glucose uptake at synapses and contributes to synaptic degeneration [22]. It can also cause loss of organelle functions such as microtubule dysfunction [23]. With these effects, accumulation of HNE can exert harmful effects on cellular functions and signaling and thereby elicit a number of pathological states including neurodegenerative disorders and cancers.

2.3. Acrolein. Acrolein is also one of the lipid peroxidation products of PUFAs. It is the most reactive of all the lipid peroxidation products [21]. In addition to PUFAs, it can be produced from partial burning of organic materials or fuel such as coal, petrol, and wood. It is also present in most types of smokes including cigarette smoke. Other sources of acrolein include cyclophosphamide bioactivation, threonine metabolism by myeloperoxidase of activated phagocytes, and overheated frying oils [5, 24]. Like HNE and MDA, acrolein is an electrophilic compound which rapidly interacts with or binds to key cellular nucleophiles and enzymes resulting in their depletion or inactivation [25]. It also reacts with nucleophilic sites in proteins and DNA, an important mechanism by which it exerts its cytotoxicity. Acrolein induces cell death, deterioration of cognitive function, and degeneration of hippocampal neurons, suggestive of its significant role in the pathophysiology of neurodegenerative diseases [26].

2.4. Isoprostanes. The isoprostanes, discovered in 1990, constitute a family of prostaglandin-like compounds formed as a consequence of free radical-induced oxidation of arachidonic acid (independently of cyclooxygenase) and later liberated from membrane phospholipids by phospholipases [27]. Compared to other lipid peroxidation products, IsoPs are more easily detected and chemically and metabolically stable in several biological samples such as urine, plasma, and tissues [28]. Accumulation of IsoPs can impair integrity, fluidity, and normal functions of membrane and is also associated with many pathophysiological disorders [29]. F2-IsoPs are among the well-researched IsoPs and considered better biomarkers of endogenous lipid peroxidation [30]. The measurement of F2-IsoPs is considered one of the most precise methods to evaluate oxidative stress and lipid peroxidative damage *in vivo* [28, 31]. Conversely, its use may be restricted in certain conditions with elevated concentrations of oxygen which suppress the formation of IsoPs [32].

2.5. Isofurans. Isofurans (IsoFs) belong to a class of compounds formed as a result of free radical-induced peroxidation of arachidonic acid. Similar to IsoPs, IsoFs are stable products, both chemically and metabolically [33]. However, unlike the IsoPs which are generated under low oxygen tension, the formation of IsoFs occurs or is favored at high oxygen concentrations [32, 34]. Therefore, isoFs may likely be important markers of oxidative stress in the brain as a result of its high oxygen consumption potential. As a result of the differences in oxygen tension required for the generation of IsoPs and IsoFs, concurrent quantification of both IsoPs and IsoFs is considered a better approach to evaluate oxidative damage in disorders or conditions with varying concentrations of oxygen such as anesthesia or surgery [35].

2.6. Neuroprostanes. The neuroprostanes (NeuroPs), also known as F4-isoprostanes, are products of free radical-catalyzed oxidation of docosahexaenoic acid (DHA). As a result of the elevated concentrations of DHA in neuronal membranes, neuroPs are regarded as important biomarkers for quantitative assessment of oxidative damage in neurons, cerebrospinal fluid, and brain tissues [36].

3. Cancer and Lipid Peroxidation Products

The incidence of and deaths due to cancers have risen vastly in the past years. These have been attributed to several factors. For instance, the global prevalence of lung cancer, which is the most predominant type of cancer, has been on the rise following increased worldwide cigarette smoking [37]. Other common factors linked to the increased cancer incidence and deaths include lifestyle patterns (such as physical inactivity, obesity, and alcohol consumption) and genetic disposition [38]. Research has linked ROS to the pathogenesis of many chronic diseases including cancers. While elevated levels are detrimental, moderate levels of ROS can be beneficial by serving as a second messenger in cell signaling [39]. It is, therefore, important to maintain redox homeostasis. The mechanisms of increased ROS formation in cancer remain poorly understood. Mitochondrial mutations or dysfunction, which are frequently observed in cancer patients, may play a role [40, 41]. Increased ROS formation due to mitochondrial mutations is caused by impaired electron transfer which results in leakage of electrons and subsequent generation of superoxide radical and other ROS [42]. Other factors implicated in the increased formation of ROS in cancer

cells include oncogenic initiation, abnormal metabolism, and enhanced activity of inflammatory cytokines [43, 44].

Many forms of cancers are associated with enhanced ROS production resulting in lipid peroxidation. Plasma MDA levels have been found to be considerably higher in patients with lung cancer [45], breast cancer [46], colorectal cancer [47], and prostate cancer [48] than in healthy controls. Increased MDA levels have also been reported in patients with other forms of cancers such as laryngeal, oral cavity, and gastrointestinal tract cancers [49, 50]. Besides MDA, elevated levels of urinary IsoPs have been associated with breast or lung cancer risk [51]. Urinary IsoPs excretion was observed to be markedly higher in patients with prostate cancer than in the controls [52]. A similar increase in urinary IsoPs excretion was reported in gastric cancer patients [53]. These data clearly indicate that lipid peroxidation products are elevated in most forms of cancers and implicate the role of lipid peroxidation products in the etiology or progression of cancers.

4. Lipid Peroxidation Products and Cancer Growth Inhibition

Polyunsaturated fatty acids (PUFAs) of cell membrane are highly susceptible to lipid peroxidation. Hence, incorporating or increasing the PUFAs content of cancer cells may predispose these cells to enhanced lipid peroxidation. Besides, PUFAs in combination with anticancer agents such as doxorubicin and arsenic trioxide have been shown to elicit synergistic cytotoxic effect in various cancer cells such as breast, colon, cervical, pancreatic, and renal cancer cells [54–56]. A recent study showed that PUFAs augmented the sensitivity and cell death of leukemic cells to anticancer agents such as doxorubicin, vincristine, and fludarabine [57]. In addition to enhancing the antitumor effect of anticancer drugs, lipid peroxidation products may also be used as an adjunct in radiotherapy. A study found that omega-3 PUFAs enhanced the sensitivity of human colorectal adenocarcinoma cells to radiation in a dose-dependent manner [58]. In all these studies, increased cytotoxicity was mediated via enhanced formation of lipid peroxidation products such as MDA and ROS or via markedly reduced intracellular concentrations of glutathione and activity of glutathione S-transferase (an important enzyme that scavenges lipid peroxidation products) in cancer cells [57, 58]. This synergistic effect was markedly inhibited by antioxidants or lipid peroxidation inhibitors, such as N-acetylcysteine, lipoic acid, and α-tocopherol, and thereby corroborate the role of lipid peroxidation products [54–56].

Of late, reports indicate that lipid peroxidation products may serve as a therapeutic tool to induce death of proliferating tumor cells. In one of such studies, acrolein was found to exert cytotoxic effect in lung carcinoma and glioblastoma cells. This cytotoxicity was suppressed by lipid peroxidation inhibitors or antioxidants such as vitamin E, ebselen (a glutathione peroxidase mimic), and selenite [59]. In addition to its inhibiting effect on cancer growth, lipid peroxidation products in combination with antitumor agents or cancer therapy may exert additive or synergistic effect. Acrolein combined with TNF-related apoptosis-inducing ligand (TRAIL) potentiates TRAIL-induced apoptosis via downregulation of Bcl-2 expression and ROS-dependent upregulation of TRAIL death receptor 5 in human renal cancer cells [60]. Similarly, HNE in combination with panobinostat, a histone deacetylase inhibitor (HDACI), exerts greater inhibition of PC3 prostate cancer cell proliferation. The combination also induces greater G2/M arrest, DNA damage, and cell death in prostate cancer cells [61]. These findings reveal that lipid peroxidation products have antitumor activity and can also potentiate the cytotoxicity of anticancer agents and radiotherapy. Recent studies indicating that many investigational antitumor agents (such as elesclomol, costunolide, and deltonin) exert their anticancer effect via selective mitochondrial ROS induction in cancer cells provide additional evidence in support of the potential role of lipid peroxidation products in cancer growth inhibition [62–64].

The potential role of lipid peroxidation products in cancer growth inhibition has also been demonstrated in rodents. A study which investigated the effect of diets comprising varied amounts and types of fat in female athymic nude mice implanted with human breast carcinoma cells found that the mice fed fish oil diets had markedly lower mean volume of human breast carcinoma than those fed corn oil diets [65]. It was also observed that tumor lipid peroxidation product levels were significantly increased only in the fish oil diets-fed mice [65]. These data suggest that the reduced breast carcinoma volume observed in mice fed fish oil diets could be attributed to elevated levels of tumor lipid peroxidation products in the same mice. Addition of antioxidants to the fish oil diets significantly decreased the level of tumor peroxidation products and increased tumor volume. On the other hand, addition of ferric citrate (a potent inducer of lipid peroxidation) to the fish oil diets resulted in markedly increased levels of tumor lipid peroxidation products as well as diminished tumor volume [65]. Similarly, dietary fish oil supplementation in athymic mice implanted with MX-1 human mammary carcinoma was accompanied by tumor growth inhibition as well as increased tumor lipid peroxidation and protein oxidation products [66]. These data convincingly indicate that dietary fish oil inhibits the growth of breast carcinoma in nude mice via increased formation of tumor lipid peroxidation products.

In nude mice implanted with DLD-1 human colon cancer cells, α-eleostearic acid (α-ESA) supplementation induced apoptosis (enhanced DNA fragmentation) via increased formation of lipid peroxidation products [67]. The addition of an antioxidant led to suppression of lipid peroxidation products and apoptosis, indicating that the antitumor effect of α-ESA is mediated via accumulation of lipid peroxidation products [67]. A recent study also provides further evidence in support of the antitumor effect of lipid peroxidation product (HNE) in rodents. The authors found that blockade of mercapturic acid pathway-mediated elimination of HNE resulted in total remission of human cancer xenografts in nude mice [68].

Table 1: A summary of the effect of lipid peroxidation products (alone or in combination with cancer therapy) on cancer cells.

Type of cancer or tumor cells	Lipid peroxidation products	Summary of key findings	Reference
Leukemic cells	MDA	Enhanced the cytotoxicity of doxorubicin, vincristine, and fludarabine on leukemic cells	[57]
Colorectal adenocarcinoma cells	MDA	Enhanced the sensitivity of colorectal adenocarcinoma cells to radiotherapy	[58]
Lung carcinoma and glioblastoma cells	Acrolein	Inhibition of tumor growth	[59]
Renal cancer cells	Acrolein	Potentiation of TRAIL-induced apoptosis; downregulated expression of Bcl-2; ROS dependent upregulation of TRAIL death receptor 5	[60]
Prostate cancer cells	HNE	Potentiation of inhibiting effect of panobinostat; augmented G2/M arrest; enhanced DNA damage and cell death	[61]
Breast and mammary carcinoma cells	MDA	Inhibition of tumor growth	[65, 66]
Colon cancer cells	MDA	Increased DNA fragmentation; induction of apoptosis	[67]
Neuroblastoma cells	HNE	Inhibition of cell proliferation; reduction of S-phase cells; induction of apoptosis; upregulated expression of p53 tumor suppressor and target proteins	[78]
Leukemic and colon carcinoma cells	HNE	Inhibition of cell proliferation; downregulation of TERT expression and telomerase activity; inhibition of c-Myc expression; activation of Mad-1 expression; interference with DNA binding activity of c-Myc and Mad-1 to TERT promoter	[80, 81]

MDA: malondialdehyde; HNE: 4-hydroxynonenal; ROS: reactive oxygen species; TERT: telomerase reverse transcriptase; TRAIL: TNF-related apoptosis-inducing ligand.

5. Other Potential Applications of Lipid Peroxidation Products in Cancer Treatment

In addition to its inhibitory effect on cancer growth, available evidence suggests that the levels of lipid peroxidation products may reflect cancer severity. In patients with carcinoma of the oral cavity and oropharynx, it was found that MDA levels in patients with T3-4 tumors were markedly higher than in those with T1-2 cancers [69]. The authors also found that patients who exhibited recurrence had significantly higher MDA levels than those with complete remission [69]. Similarly, Chole and colleagues found that serum MDA levels in patients with oral precancer were much lower than in those with oral cancer, and it was observed to be the lowest in the controls [70]. Increasing levels of lipid peroxidation biomarkers including MDA and HNE have also been observed in patients suffering from other types of cancers (such as myeloid leukaemia, gastric, and breast cancers) as the disease progressed [71–73]. Lipid peroxidation products, therefore, could serve as a marker of initial stages of tumor development, progression, and metastasis as well as predicting the chances of cancer relapse.

The levels of oxidative stress markers such as lipid peroxidation products may also reveal the effectiveness of cancer therapy or surgical intervention. Reduced MDA levels have been demonstrated postoperatively in lung cancer patients following removal of cancer-associated parenchyma [74]. A similar finding (significantly reduced MDA levels) has also been reported in surgically treated colorectal cancer patients (both early and advanced stages) [75]. Hence, these findings suggest that, by comparing data obtained before and after cancer treatment, the levels of lipid peroxidation products could be used to monitor the progress and/or evaluate the effectiveness of therapy or surgery in patients with cancers.

6. Potential Mechanisms of Cancer Growth Inhibition by Lipid Peroxidation Products

In the last few years, the roles of several transcription factors have been implicated in the development of cancers. The p53 is a transcription factor which prevents free radical-induced gene mutations by detecting and getting rid of oxidatively damaged DNA [76]. Upon stimulation, p53 also induces a host of other genes which in turn cause cell cycle arrest and apoptosis [76, 77]. The loss of function of p53 family is known to contribute to cancer progression. In human neuroblastoma cell lines, HNE has been shown to inhibit proliferation, reduce S-phase cells, and induce apoptosis via upregulated expression of p53 tumor suppressor and target proteins [78].

The telomerase and telomerase reverse transcriptase (TERT) constitute an essential component of the telomerase complex which role is also implicated in the etiology and progression of cancer. Telomerase catalyzes the elongation of telomeres in DNA strands with the aid of telomerase complex [79]. This enzyme, therefore, plays an essential role

in the transformation of senescent cells to potentially nonsenescent or immortal cells, which is of significant importance in tumorigenesis. HNE has been found to downregulate the expression and activity of TERT and telomerase, respectively, in human leukemic cell lines [80]. Similar to inhibitory effect of HNE on telomerase activity, downregulation of TERT expression and inhibition of cell proliferation have also been observed in colon carcinoma cells [81].

Besides inhibiting telomerase complex, lipid peroxidation products may modulate the expression of c-Myc/Mad/Max network. The c-Myc/Mad/Max network consists of transcription factors which interactions elicit activation or suppression of specific or target genes involved in cell-cycle progression, genomic instability, and tumor progression and expansion [82, 83]. For instance, the c-Myc which can activate telomerase complex (telomerase and TERT) is overexpressed in many forms of cancers [84]. The c-Myc also induces DNA damage and attenuates p53 function [85]. HNE has been shown to inhibit c-Myc expression while it upregulates Mad-1 expression. HNE also interferes with the DNA binding activity of c-Myc and Mad-1 to the hTERT promoter [80]. The ability of lipid peroxidation products such as HNE to modulate a number of transcription factors known to play vital roles in the pathophysiology or progression of cancer may be one of the mechanisms of its cytotoxicity in cancer cells. The summary of the effect of lipid peroxidation products on cancer cells is presented in Table 1.

7. Conclusions

There is no doubt that ROS and lipid peroxidation play a role in cancer development. However, evidence also indicates that cancer cells require certain amounts of ROS for proliferation and survival. This, therefore, suggests strategies aimed at further increasing the levels of ROS and oxidative damage such as lipid peroxidation products may be deleterious to cancer cells and thus beneficial in cancer management. As highlighted in this review, compelling evidence shows that lipid peroxidation products exert antitumor effect and also potentiate the cytotoxicity of anticancer drugs and radiotherapy. Lipid peroxidation biomarkers could also serve as a diagnostic tool to predict the chances of cancer recurrence and be used to monitor the progress or effectiveness of therapy in cancer patients. The findings also reveal insights into the possible molecular mechanisms (via modulation of key cancer-related transcription factors) by which lipid peroxidation products may inhibit or suppress the growth of cancers. Selectively enhanced ROS formation and its suppressed elimination remain key viable options in exploring lipid peroxidation-mediated cancer cell death. At the moment, as a result of the double-edged sword property of ROS, one of the main challenges is how best to successfully, effectively, and selectively target cancer cells in humans using lipid peroxidation products. This is an area that requires further and detailed research as it may prove to be an important strategy to complement current treatment regimens for cancer patients.

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Clinical Study

Superoxide Dismutase1 Levels in North Indian Population with Age-Related Macular Degeneration

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Aim. The aim of the study was to estimate the levels of superoxide dismutase1 (SOD1) in patients of age-related macular degeneration (AMD) and examine the role of oxidative stress, smoking, hypertension, and other factors involved in the pathogenesis of AMD. *Methods.* 115 AMD patients and 61 healthy controls were recruited for this study. Serum SOD1 levels were determined by ELISA and were correlated to various risk factors. Logistic regression model of authenticity, by considering SOD1 as independent variable, has been developed along with ROC curve. *Results.* The SOD1 levels were significantly higher in AMD patients as compared to those of the controls. The difference was not significant for wet and dry AMD. However, the difference was significant between wet AMD subtypes. Nonsignificance of the Hosmer-Lemeshow goodness of fit statistic ($\chi^2 = 10.516$, df = 8, $P = 0.231$) indicates the appropriateness of logistic regression model to predict AMD. *Conclusion.* Oxidative stress in AMD patients may mount compensatory response resulting in increased levels of SOD1 in AMD patients. To predict the risk of AMD on the basis of SOD1, a logistic regression model shows authenticity of 78%, and area under the ROC curve (0.827, $P = .0001$) with less standard error of 0.033 coupled with 95% confidence interval of 0.762–0.891 further validates the model.

1. Introduction

AMD is the most important cause of blindness which is characterized by progressive degeneration of macula leading to severe irreversible loss in vision [1]. The vision loss results either from retinal degeneration (dry AMD) or from the choroidal neovascularization (wet AMD). The clinical manifestation of AMD includes drusen, geographic atrophy, hyperplasia of the retinal pigment epithelium (RPE), and angiogenesis of choroidal vessels (CNV) [2].

Smoking, alcohol, oxidative stress, and genetic factors are implicated in the pathogenesis of AMD [3], but the exact cause of AMD remains complex. It has been reported that aging is associated with pathological and biochemical changes in the eye. In general, aging and AMD are believed to result from cumulative and increased oxidative damage [4]. Oxidative stress can exert molecular or cellular damage mediated by reactive oxygen species (ROS) which has been earlier shown to be implicated with diseases of ageing [5]. The elevated levels of endogenously synthesized ROS are known to be regulated by various antioxidant, enzymatic, and nonenzymatic protective biochemical mechanisms like Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) [6]. ROS which includes free radicals, nascent oxygen, hydrogen peroxide and the by-products of oxygen metabolism are deleterious for eye pathophysiology. Due to the high consumption of O_2, the high concentration of polyunsaturated fatty acid and direct exposure of light render retina susceptible to oxidative stress [7]. Various factors are responsible for oxidative stress generated from aging; these include decreased levels of vitamin C and vitamin E in plasma [8]. It has also been shown that oxidized glutathione levels

increase in plasma and that glutathione levels decrease with the age [9]. Increased lipid peroxidation is also reported in aging [10], and the consequences of these imbalanced biochemical changes lead to increased susceptibility of retinal pigment epithelium cells (RPE) to oxidative damage with aging. Even catalase activity and vitamin E levels have been reported to decrease with aging in RPE cells [11]. There are several pathological features which accompany aging. These include increased volume of lipofuscin contents (increased lipid and protein contents) which enhance the oxidative damage susceptibility and decreased optical density of macular pigment [12] which results in membrane blebbing of RPE cells, a phenomenon observed in AMD eyes and aging [13].

We hypothesized that oxidoreduction alteration in the eye might result from deranged SOD1 levels. We, therefore, analysed the expression of superoxide dismutase1 in patients of AMD as compared to controls. The major antioxidant system in the retina consists of three superoxide dismutase (SOD) isoenzymes that catalyse dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2) [14]. SOD is an antioxidant enzyme useful in the defense system against ROS. Superoxide dismutase catalyzes the dismutation reaction of O_2^- (superoxide radical anion) to H_2O_2, which is then catalyzed to H_2O and O_2 by catalase and glutathione peroxidase. There are three major families of superoxide dismutase, depending on metal cofactors: Cu-Zn SOD (SOD1), present in cytosol, Mn (Fe)-SOD (SOD2) present in mitochondrial matrix, and the extracellular SOD (SOD3) interstitium of the tissues as a secretary form [15].

The amount and activity of the Cu-Zn SOD (SOD1) are the highest among the three isoenzymes in human retina, so it seems reasonable to screen SOD1 for possible role in accelerating age-linked changes in the retina [15].

Currently, there is no study examining the role of SOD1 in Indian AMD patients, and this investigation will likely provide the substrate for future therapies in AMD.

2. Methodology

2.1. Study Participants. This study was approved by Institute Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India (letter no. Micro/10/1411). Patients and controls were first informed about the study and thereafter enrolled in patient/control group after obtaining written proforma from all participants. All enrolled participants were recruited from the Department of Ophthalmology, PGIMER, Chandigarh, India, in which phenotypic criteria were strictly followed. A retina specialist carried out ophthalmic examination of all AMD patients for best corrected visual acuity, dilated fundus examination, and slit lamp biomicroscopy of anterior segment. All patients underwent optical coherence tomography (OCT) and fluorescein fundus angiography (FFA). AMD diagnosis was based on FFA and ophthalmoscopic findings.

We included a total of 176 case-control samples consisting of 115 AMD patients from PGIMER, Chandigarh, India, with 61 genetically distinct healthy controls as per inclusion and exclusion criteria. However, some demographic details were not available for some subjects.

2.2. Inclusion and Exclusion Criteria. 50 years or older AMD patients with more than five drusen in case of dry AMD in at least one eye and/or choroidal neovascularization in case of wet AMD were incorporated in the study [16, 17]. The controls in the study included those with age 50 years or more with the absence of other diagnostic criteria for AMD.

The exclusion criteria excluded the retinal diseases involving the outer retinal layers and/or photoreceptors other than AMD loss, such as central serous retinopathy, high myopia, diabetic retinopathy, retinal dystrophies, uveitis, and vein occlusion, or similar outer retinal diseases that have been present earlier to the age of 50 and opacities of the ocular media, or other problems enough to preclude satisfactory stereo fundus photography. These situations contain occluded pupils due to cataracts and opacities and synechiae due to ocular diseases.

2.3. Baseline Examination. A trained interviewer collected the information about medical history, demographic characteristics, and lifestyle risk factors like smoking, alcohol, and so forth, using a standard risk factor questionnaire [18, 19]. Smokers were defined as those having smoked at least three cigarettes per day or 54 boxes for at least 6 months and were segregated further into smokers and nonsmokers. Nonvegetarian patients were defined as those having chicken, meat, or fish for at least 6 months, and alcohol consumers were defined as those having whiskey, rum, wine, or homemade alcohol for at least 6 months. Hypertension was defined as diastolic blood pressure ≥90 mm Hg and systolic blood pressure ≥140 mm Hg at the time of examination and for this condition whether they had ever taken medications. Similar practices have been used in previous studies [20]. Participants were also asked to report any previous diagnosis of migraine, use of antihypertensive medications, stroke, diabetes, or history of heart diseases.

2.4. Collection of Blood and Serum Separation. 4.0 mL of blood was collected in serum separator tube (BD Biosciences, USA) from both AMD and controls and left for 30 minutes at 37°C to allow it to clot according to the standard procedures. Serum was subsequently separated by centrifugation for 30 minutes at 3000 rpm. The separated serum was frozen at −80°C until analysis [21, 22].

2.5. Total Protein Estimation. The Bradford assay was used to estimate the total serum proteins for normalization of SOD1 levels analysed by ELISA as per the manufacturer's recommendations [23, 24].

2.6. SOD1 Expression. Serum from AMD patients and controls was used to carry out the quantitative detection of SOD1 using commercially available enzyme-linked immunosorbant assay (ELISA) (AB Frontier; Catalog no. LF-EK0101) as per the instructions from manufacturer, and absorbance was taken at 450 nm using 680XR Microplate reader (Biorad,

Hercules, USA). Sample assays were carried out in duplicate. The procedure to analyse the SOD1 levels was provided by manufacturer of the kit. This assay recognizes native and recombinant human SOD1 with the detection of more than 12.5 pg/mL. The standard curve was generated by linear regression analysis for SOD1 in both controls and patients. All the values were normalized with total serum protein.

2.7. Statistical Analysis. All statistical calculations were carried out by statistical product and service solutions SPSS (IBM SPSS Statistics 20.0, Chicago, IL, USA) software. The assumption of normality was tested with the help of Normal Quantile plot (Q-Q plot), and it was observed that data were not normally distributed. Therefore, the Mann-Whitney U-test was applied to compare the two groups. For comparing more than two groups, the Kruskal-Wallis oneway analysis of variance (ANOVA) followed by post hoc for multiple comparisons was applied. The $P \leq 0.05$ was considered significant. The measure R^2 (coefficient of determination) was used to determine the goodness of standard curve fit for ELISA and total protein. The linear and quadratic regressions with $R^2 > 0.80$ were considered to be of a good fit. In order to identify the risk factors associated with AMD, a logistic regression was carried out, and adjusted odds ratios were also obtained. ROC (receiver operating characteristics) curve defines the sensitivity/specificity of the experiment. The ROC curve is basically important for the evaluation of diagnostic tests. The true positive rate (sensitivity) is plotted as the function of the false positive rate (100-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal). ROC curve for predicted model was mapped [25, 26].

Table 1: Clinical and demographic details of subjects.

Variables	AMD	Controls
Total	115	61
Wet AMD	84 (73.04%)	—
Dry AMD	31 (26.96%)	—
Minimal classic	7 (11.9%)	—
Predominant classic	16 (27.1%)	—
Occult	36 (61.0%)	—
Avastin treated	55 (65.5%)	—
Not treated with Avastin	29 (34.5%)	—
Duration of disease[¥]	23 ± 2.6 (M)	—
Smokers	50 (43.5%)	11 (20%)
Nonsmokers	65 (56.5%)	44 (80%)
Alcoholic	37 (32.2%)	17 (30.9%)
Nonalcoholic	78 (67.8%)	38 (69.1%)
Vegetarian	61 (53%)	31 (56.4%)
Nonvegetarian	54 (47%)	24 (43.6%)
Hypertension	52 (45.2%)	10 (16.4%)
Nonhypertensive	61 (53%)	45 (73.8%)
Heart disease	16 (13.9%)	—
No heart disease	60 (52.2%)	55 (100%)
Age	64.97 ± 7.1	60.38 ± 13.2
Male	75 (65.2%)	40 (65.6%)
Female	40 (34.8%)	21 (34.4%)

AMD: age-related macular degeneration; M: months; Age: age of onset. Values are mean ± SD or percentage, [¥]Duration of disease is the interval between appearance of the first symptom of AMD and collection of sample. AMD subjects were asked to provide all clinical and demographic details at the age of disease onset.

3. Results

Summary statistics of important variables have been shown in Table 1. 115 AMD patients were recruited for the study with average age of 64.97 ± 7.1, whereas 61 controls were recruited with an average age of 60.38 ± 13.2. The AMD population was divided according to presence of clinical features and Avastin treatment. The recruited patients and controls were further classified based on their food habits and smoking as well as alcohol consumption and the presence of other associated diseases like hypertension, heart disease, and so forth in order to estimate the levels of SOD1 among these groups. The serum SOD1 level was found to be significantly elevated in AMD subjects as compared to normal controls (Figure 1, Table 2, $P = 0.0001$). However, there was no significant difference between the levels of dry and wet AMD (Table 2, $P = 0.117$). Moreover, in the wet AMD subgroups, significant difference was found. The levels of SOD1 in predominantly classic ($P = 0.022$) and occult AMD patients ($P = 0.023$) were significantly higher as compared to those of minimal classic (Figure 2(a)). An independent analysis was carried out while adjusting the risk factors to AMD. Important risk factors like smoking, alcohol, food habits, gender, hypertension, and heart diseases were analyzed to examine their association with SOD1. The SOD1 levels were found to be higher among hypertensive patients (Figure 2(b), Table 2, $P = 0.015$), those with heart disease (Figure 2(c), Table 2, $P = 0.002$) and male AMD patients (Figure 2(d), Table 2, $P = 0.035$), as compared to nonhypertensive patients or those without heart disease and female AMD patients, respectively. However, the difference was not significant between AMD smokers and AMD nonsmokers, alcohol consumers and alcohol nonconsumers, and vegetarian and nonvegetarians (Table 2). The levels were not found to be significant when compared among Avastin treated AMD patients versus untreated AMD patients (Table 2). It has been observed that there was a significant association of levels of SOD1 with AMD subtypes ($\chi^2 = 6.326$, $P = .042$), gender ($\chi^2 = 6.860$, $P = .032$), and smoking ($\chi^2 = 6.291$, $P = .043$). The prediction equation for AMD, by considering SOD1 as independent variable, shows that 78% of the cases have been correctly classified (model authenticity 78%) with attending confidence intervals for ROC curve. The area under ROC was 0.827 ($P = .0001$) with standard error of 0.033 and confidence interval of 0.762–0.891 (Figure 3).

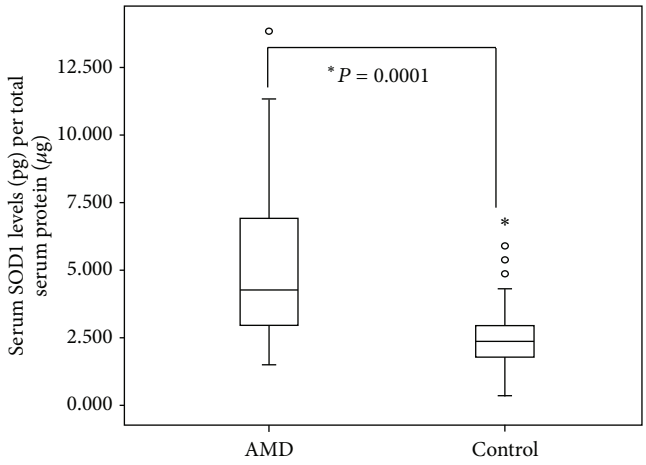

Figure 1: Serum levels of SOD1 in AMD and normal controls. Boxes include values from the first quartile (25th percentile) to third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisk, respectively. Levels of SOD1 were normalized to total protein. Data was analyzed by using the Mann-Whitney U test.

Table 2: SOD1 levels according to different subtypes. ELISA levels were compared by applying the nonparametric Kruskal-Wallis H test followed by the Mann-Whitney U test.

Subjects	Mean rank	Z value	P value
AMD	107.61	7.08	0.0001*
Control	50.42		
Dry	49.97	1.56	0.117
Wet	60.96		
Minimal classic	15.29		
Predominant classic	35.75	2.272	0.022*
Occult	30.31	2.270	0.023*
Avastin treated	43.71	0.626	0.532
Not treated	40.21		
Alcoholic	60.57	0.569	0.570
Nonalcoholic	56.78		
Smokers	59.26	0.355	0.722
Nonsmokers	57.03		
Vegetarian	60.13	0.729	0.466
Nonvegetarian	55.59		
Hypertensive	65.13	2.437	0.015*
Nonhypertensive	50.07		
Heart disease	93.62	3.107	0.002*
No heart disease	62.16		
Male	62.80	2.114	0.035*
Female	49.00		

*Significant.

4. Discussion

The major reason for vision loss in elderly population is accounted for by AMD [27]. To understand the mechanism of AMD pathogenesis, several studies have attempted to correlate various targets and biomarkers with conflicting reports and unverified data from the Caucasian population. Facts suggest that oxidative stress plays a major role in the pathogenesis of AMD [28, 29].

This study was carried out to determine whether the serum SOD1 levels are altered in AMD patients as compared to normal controls as this region is characterized by unique dietary habits. Results from this study indicate that the SOD1 levels were elevated significantly in AMD as compared to normal controls. To our knowledge, this non-Caucasian study is first to demonstrate elevated SOD1 serum levels in Indian AMD patients. However, several other studies have been carried out to estimate the activity (not levels) of SOD along with other biomarkers associated with oxidative stress in various population [30–32].

Retina is very susceptible for lipid peroxidation [11, 12] which increases with age in macular region [11]. This is associated with cellular damage which involves decreased cellular antioxidants [13]. In our results, the high levels of SOD1 indicate that lipid peroxidation and oxidative stress are involved in tissue damage in AMD patients. Whether increased SOD1 levels in our study are indeed due to compensatory regulation or causative of AMD can be determined by conducting longitudinal study performed on intermediate or early AMD patients.

SOD1 levels of occult and predominantly classic AMD patients were found to be higher as compared to those of minimally classic AMD patients. This corresponds to disease severity whether induced by SOD1 or resulting from disease. Interestingly, it has been shown previously that the protein content of SOD1 and SOD2 in RPE homogenates increases in the later stages of AMD [33].

The fact that SOD1 levels were found to be higher among male, hypertensive, and heart disease patients could be ascribed to high oxidative stress in these patients. It was shown that the oxidative stress could be involved in the cardiovascular diseases and hypertensive patients [34, 35]. It is pertinent to point out that although SOD1 is an antioxidant, its over expression can lead to increased oxidative stress. Studies on transgenic animals have shown that increased levels of SOD1 lead to more hypersensitivity to oxidative stress [36, 37]. It is possible that the negative effects seen with high levels of SOD1 are caused by an increased level of the product of the dismutation reaction which yields hydrogen peroxide [38]. Kowald et al. have defined such situation by deriving mathematical equations and proposed the three alternative mechanisms driven by SOD1: (i) reaction of hydrogen peroxide with CuZnSOD that leads to formation of hydroxyl radicals, (ii) superoxide radicals acting as chain breaker, and (iii) interchange between oxidized and reduced form of SOD while detoxifying superoxide radicals [38]. These studies do not demonstrate the dual function of SOD, but instead they indicate an alternative pathway which is driven by the free radical burden inside the cell. Recently, it has been found that peroxidase activity of superoxide dismutase depends on CO_2. The generation of free radicals by the peroxidase activity of superoxide dismutase was found to be higher in the presence of bicarbonate-carbon dioxide.

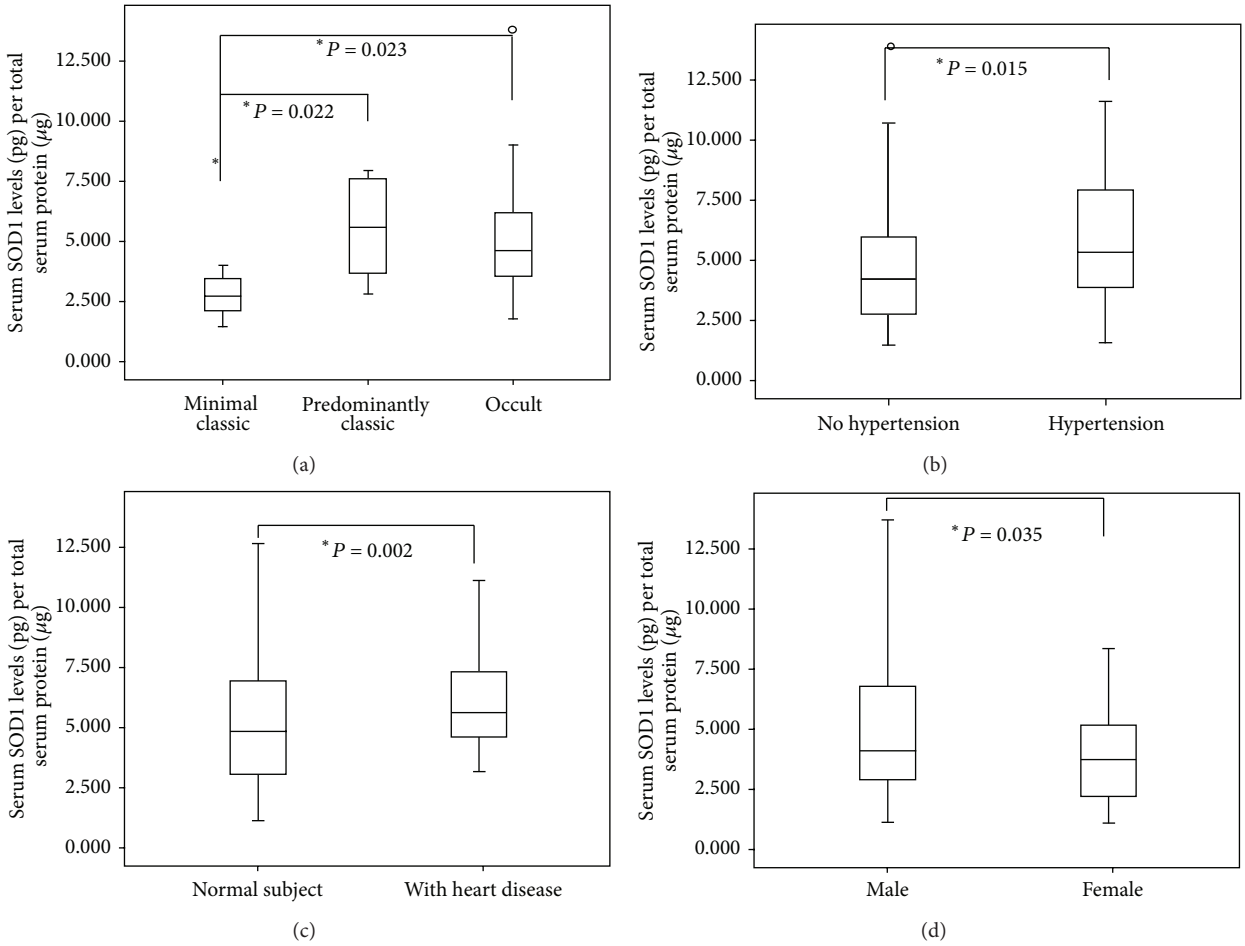

Figure 2: (a) Serum levels of SOD1 in minimal classic, predominant classic, and occult AMD. (b) Serum levels of SOD1 in hypertensive and nonhypertensive AMD patients. (c) Serum levels of SOD1 in heart disease and no heart disease patients. (d) Serum levels of SOD1 in male and female AMD patients. Boxes include values from the first quartile (25th percentile) to the third quartile (75th percentile). The thick horizontal line in the box represents median for each dataset. Outliers and extreme values are shown in circles and asterisks, respectively. Levels of SOD1 were normalized to total protein. Data was analyzed by using the Mann-Whitney *U* test.

This mechanism explains why strong oxidant is generated during peroxidase activity of SOD which is followed by CO_2 oxidation to carbonate radicals. These free carbonate radicals have tendency to oxidize various biomolecules inside the cell [39].

Moreover, we have earlier reported that the VEGFR2 levels increased significantly in the AMD patients as compared to those in normal control [40]. We hypothesized that there is positive correlation between the increased SOD1 and VEGFR2 levels because under *in vitro* conditions oxidative stress has been correlated previously with upregulation of VEGF and is thought to be involved in the increased expression of VEGF [41, 42]. In addition, several studies involving tissue culture and animal models have pointed out that oxidative stress is a critical moderator in the transduction of the mitogenic effects of VEGF [43, 44].

We have attempted to predict AMD based on SOD1 using logistic regression, which showed 78% model predictivity, and area under curve is 0.827. The high value of AUC may be used to diagnose AMD patients with very less standard error. The association with gender, smoking, and AMD types means that the increased levels of SOD1 are associated with these factors.

Therefore, the oxidative stress is considered as an important causative factor for AMD, which can lead to induced apoptosis of RPE and result in impairment of RPE function [45–47], and, hence, its analysis in larger AMD cohort is imperative.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Akshay Anand and Neel K. Sharma contributed equally to the paper.

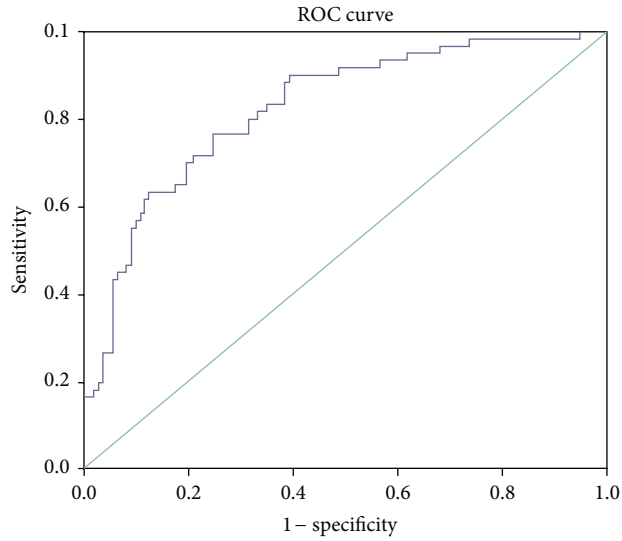

Figure 3: Receiver operating characteristic (ROC) obtained from binary logistic regression model which generates significant predictors of AMD. Area under the curve is reported to be 82.7%.

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Research Article

Senescence-Related Changes in Gene Expression of Peripheral Blood Mononuclear Cells from Octo/Nonagenarians Compared to Their Offspring

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Mechanisms determining both functional rate of decline and the time of onset in aging remain elusive. Studies of the aging process especially those involving the comparison of long-lived individuals and young controls are fairly limited. Therefore, this research aims to determine the differential gene expression profile in related individuals from villages in Pahang, Malaysia. Genome-wide microarray analysis of 18 samples of peripheral blood mononuclear cells (PBMCs) from two groups: octo/nonagenarians (80–99 years old) and their offspring (50.2 ± 4.0 years old) revealed that 477 transcripts were age-induced and 335 transcripts were age-repressed with fold changes ≥1.2 in octo/nonagenarians compared to offspring. Interestingly, changes in gene expression were associated with increased capacity for apoptosis (*BAK1*), cell cycle regulation (*CDKN1B*), metabolic process (*LRPAP1*), insulin action (*IGF2R*), and increased immune and inflammatory response (*IL27RA*), whereas response to stress (*HSPA8*), damage stimulus (*XRCC6*), and chromatin remodelling (*TINF2*) pathways were downregulated in octo/nonagenarians. These results suggested that systemic telomere maintenance, metabolism, cell signalling, and redox regulation may be important for individuals to maintain their healthy state with advancing age and that these processes play an important role in the determination of the healthy life-span.

1. Introduction

The aging process determined by genetic and environmental factors remains unchanged despite increasing the average life-span of the general population in recent decades [1]. The heritability component of human longevity ranged from 20% to 30% and can increase to about 50% after the age of 60 [2]. Thus, the search for genes affecting longevity in humans was mostly conducted on subjects at an advanced age involving centenarians. Families living such extremely long lives perhaps possess genetic variations that affect either the rate of aging or genes that result in decreased susceptibility to age-associated diseases [1]. Identification of innate genes that changes in expression with age, in a small population to minimize variations arising from environmental factors such as diet and lifestyle, can be useful as targets for intervention or can be used as biomarkers of aging.

Uncovering interactions of major regulatory pathways and targets is crucial to elucidate aging mechanisms. Some of the most promising candidate genes appear to be involved in regulatory pathways such as stress resistance, immune/inflammatory response, insulin signalling, or cardiovascular function. Such information could be extracted from transcriptomic studies [3]. However, reports on gene expression analysis of human aging are usually focused on determining small age changes and were based on candidate genes. Global changes in gene expression were investigated mainly as comparison between diseased and healthy subjects,

while changes in mRNA expression patterns with age in humans are limited. Moreover, compared to genetics studies, the relation in gene expression profiles associated with lifespan and longevity is less emphasized [4]. To this end, studies looking into longevity genes usually involved long-lived individuals and younger controls that are not related [1]. A comparison of long-lived individuals and their offspring could provide some insights of differentially expressed innate genes and provide insight into time dependent changes in expression of certain genes.

Various tissues across model organisms such as brain [5] were studied in relation to aging. Yet, the effect of aging is not limited to one specific tissue. The use of peripheral blood mononuclear cells (PBMC), which has been accepted to represent changes in the whole body, may provide a useful tool to study human aging [4]. It is suggested that PBMC may also represent the biological processes occurring in the body relevant to aging and longevity. Processes affecting the body system may be reflected in the blood where blood parameters have been successfully used for classification of disease subgroups and disease progression rates and monitoring of medical treatment [4]. Therefore, this study was conducted to determine the differential expression of human PBMC in healthy octo/nonagenarians in comparison with their offspring.

2. Experimental Procedures

2.1. Recruitment of Subjects. This study was approved by the Ethics Committee of Universiti Kebangsaan Malaysia (UKM). After obtaining written informed consent, recruitment of subjects with comparable lifestyle and diet was conducted at villages located in Pahang, Malaysia, to control for environmental and lifestyle factors. A total of 40 subjects were prescreened and 18 subjects of healthy octo/nonagenarians and offspring of octo/nonagenarians that best fit the inclusion criteria were included in the study. The age of the offspring volunteers ranged between 45 and 56 years old. As for octo/nonagenarians, participants recruited fulfilled the inclusion criteria of age between 80 and 99 years, not institutionalized, and have no evidence of medical conditions or chronic diseases in the previous month. Exclusion criteria were cancer or cancer history and alcohol drinking. Blood was drawn between 10 a.m. and 12 p.m.

2.2. Isolation of PBMC. PBMCs were isolated from 15 mL of whole blood by centrifugation through Lymphoprep gradient centrifugation (Axis-Shield PoC, Norway). Briefly, blood was centrifuged at 1500 ×g for 30 min, and the mononuclear cells at the interface were carefully removed and washed three times in phosphate buffer saline (PBS).

2.3. Total RNA Extraction and Purification. The cell pellet was lysed in TRI-Reagent solution (Molecular Research Center, OH, USA) and immediately stored at −80°C until further processing. Total RNA extraction was done according to the manufacturer's protocol. Air-dried RNA pellet was solubilized in RNase-free water and purified using RNeasy Mini Kit columns (QIAGEN, Canada) according to the kit's manual instructions. Nucleic acid concentrations were determined at 260 nm by NanoDrop 1000 A spectrophotometer (Thermo Scientific, USA), while the RNA quality was assessed by the RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

2.4. Gene Expression Profiling. Gene expression profiling of PBMC samples from nine octo/nonagenarians and nine offspring was performed using 18 Human Gene 1.0 ST Array chips. Each chip contains 764,885 25-mer probe sets corresponding to 28,132 unique transcripts. Labelled target for the microarray experiment was prepared using 150 ng of total RNA. cDNA was synthesized using the GeneChip WT (Whole Transcript) Sense Target Labelling and Control Reagents kit as described by the manufacturer's protocol (Affymetrix, Santa Clara, USA, http://www.affymetrix.com/support/technical/manuals.affx). The sense cDNA was then fragmented by UDG (uracil DNA glycosylase) and APE 1 (apurinic/apyrimidic endonuclease 1) and biotin-labelled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, USA). Hybridization was performed using ~25 ng/μL of biotinylated target, which was then incubated in the GeneChip 1.0 ST Array at 45°C for 17 hours. Following hybridization, nonspecifically bound material was removed by washing and detection of specifically bound target was performed using the GeneChip Hybridization, Wash and Stain kit via the GeneChip Fluidics Station 450/250 (Affymetrix, Santa Clara, USA). The arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, USA), where CEL file was produced by GeneChip operating software (GCOS) (Affymetrix). Finally, the raw data was extracted from the scanned images and analyzed with Expression Console software (Affymetrix, Santa Clara, USA). For each sample, Affymetrix default settings were used, and statistical parameters such as background, noise, and spike-in controls were within acceptable limits. All procedures were performed at UKM Medical Molecular Biology Institute (UMBI), UKM.

2.5. Data Analysis. Data acquisition and global background normalization were obtained using GeneSpring GX 10 software. Entities were filtered with the range of interest of 100 for upper percentile cutoff and 20 for lower percentile cutoff and retained with at least 100% of the values in any two out of two conditions within range. Genes that do not meet the criteria for differential expression were removed by computing t-test, followed by the Benjamini-Hochberg method for false discovery rate (FDR) control adjustment, where an FDR less than 5% was chosen with significance levels of $P < 0.05$ for the factor of age and their interaction for each gene. Annotation and ontology analyses were done using the Pathway Studio 7.0 software and Database for Annotation, Visualization and Integrated Discovery (DAVID, http://appsl.niaid.nih.gov/David/). The degree of enrichment for gene ontology and heat maps were also generated by Gene Set Enrichment Analysis (GSEA) using nonparametric Kolmogorov-Smirnov statistical test to calculate the P value indicating the significance of the expression changes,

Table 1

Accession number	Gene symbol	Forward (5′-3′)	Reverse (5′-3′)	Product size (bp)
NM_005938	*FOXO4*	ccagcttcagtcagcagttatg	agagaccactccgagatagcag	118
NM_153201	*HSPA8*	aggcccaaggtccaagtaga	agcatctttggtagcctgacg	186
NM_000074	*CD40LG*	agccagcctctgcctaaagt	gctcacttggcttggatcag	175
NM_003672	*CDC14A*	agcccgtttaccaacctctt	tggtgtgctcctctgtcttg	157
NM_001469	*XRCC6*	aagccgttggtactgctgaag	ctccagacacttgatgagcagag	123
NM_003326	*TNFSF4*	gctacttctcccaggaagtcaac	gtaagtcagagaggccaccatc	116
NM_006311	*NCOR1*	agcggctatgctctctaccag	gctgaaggacttcccactctc	163
NM_002737	*PRKCA*	aggatgatgacgtggagtgc	gacgaagtacagccgatcca	119
NM_004064	*CDKN1B*	tccggctaactctgaggacac	caggtcgcttccttattcctg	104
NM_032983	*CASP2*	agtggtgctagccaaacagc	aggcagcaagttgaggagttc	145
NM_001681	*ATP2A2*	ctacctcatctcgtccaacgtc	tcaccagattgacccagagc	110
NM_001188	*BAK1*	cggcagagaatgcctatgag	agtcaggccatgctggtaga	137
NM_001101	*ACTB*	cctggacttcgagcaagagat	aggaaggaaggctggaagagt	141

according to the ranking of the genes in our experimental dataset across every pathway in the database (enrichment score).

Highest fold change obtained was 2.63 and the lowest fold change was 2.47 for an unknown protein. Significant genes that changed by less than 1.2-fold with adjusted $P > 0.05$ were removed from subsequent analysis. Since the expected differences of transcriptomic expression due to aging are much smaller and difficult to detect [4], we have opted to use 1.2-fold as cutoff level as has been reported in various studies of aging [3, 7]. Hierarchical clustering was performed using differential distance metrics and centroid linkage rule of the replicates per condition. Analysis of overrepresentation of specific biological pathways by the resulting list of genes was conducted via Fisher's exact test. Pathway Studio 7.0 from Ariadne was mainly used for analysis and generating pathway figures. Functional attribution was made according to online databases such as SOURCE (http://source.stanford.edu/), GenAge (http://genomics.senescence.info/genes/) [8], and biological interpretation was derived from the literature search.

2.6. Real-Time RT-PCR. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to quantitate and verify expression changes resulting from the microarray experiments. Four upregulated and eight downregulated genes were selected for validation. Genes and forward/reverse primers used for RT-PCR were as in Table 1.

The same RNA samples used in the microarray experiment were subjected to two-step RT-PCR using iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad Laboratories, USA). Fluorescence was measured using iCycler iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Briefly, 500 ng of total RNA was reverse-transcribed according to manufacturer's instructions. Each 20 μL aliquot contained 4 μL of 5x iScript reaction mix, 1 μL of iScript reverse transcriptase, and 15 μL of total RNA or water as negative control. The reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C to obtain the cDNA template. The genes were amplified with a 25 μL of reaction mix consisted of 12.5 μL of iQ SYBR Green Supermix, cDNA template and primers were added at 200 nM final concentrations. Initial denaturation of DNA was carried out at 95°C for 3 minutes. Forty amplification cycles were performed, each cycle consisting of denaturation (95°C, 10 s) and annealing and extension (61°C, 30 s). The data collection and real-time analysis were performed at 95°C for 1 minute and 55°C for 1 minute, respectively. Each sample was amplified in duplicates, and the results were normalized with β-actin (ACTB) as reference gene. Fold change in octo/nonagenarians was determined by the Ct comparative method, using the average of Ct values after subtraction with the Ct value of ACTB from nine individuals of each octo/nonagenarians and offspring groups.

3. Results

3.1. Subject Demographics. A total of four male and fourteen female healthy octo/nonagenarians (86.1 ± 6.0 years old) and offspring (50.2 ± 4.0 years old) subjects were recruited from villages in Pahang with nine subjects in each group. No significant differences in body mass index (BMI) values were observed between the two groups (Table S1, Supplementary Material available online at http://dx.doi.org/10.1155/2013/189129). There were no significant differences in the total RNA recovered from PBMCs (data not shown); and the quality of the RNA was consistent between octo/nonagenarians and offspring with an average RNA integrity number (RIN) of 8.14 ± 0.38.

3.2. Gene List Selection. A total of 18 individual chips were analyzed by GeneSpring GX 10 software. P value computation was made with asymptotic assumptions and Benjamini-Hochberg multiple testing corrections estimates of the microarray dataset to generate a t-test statistic. Analysis revealed that 477 genes were significantly ($P < 0.05$) age-induced and 335 genes were significantly age-repressed with fold change ≥1.2.

The complete list of 812 differentially expressed genes is available in Table S2. At present, only selected differentially

expressed genes including forkhead box O4 (*FOXO4*), TERF1 (TRF1)-interacting nuclear factor 2 (*TINF2*), X-ray repair cross-complementing protein 6 (*XRCC6*), beclin 1 (*BECN1*), and upstream transcription factor 1 (*USF1*) that have attracted our interest will be discussed. The chosen candidates were selected based on current knowledge to aging in general (e.g., oxidative stress leading to DNA damage) or to immune system, a similar effect of age on expression of genes in the same functional group and/or comparable effect of aging on immune cells gene expression in individuals.

Gene set comparison was also conducted on a list of 1312 significantly expressed genes ($P < 0.05$) with fold change >1.0 (Table S2) using the Gene Set Enrichment Analysis (GSEA) method to allow smaller degree of changes to be identified as functional category of genes (gene sets) that are regulated together. Furthermore, a computation of *P* value to determine whether the overlapping observed between the entities and the pathway is due to chance was done by Fisher's exact test.

Gene sets that may be relevant to the regulation of age-related changes between octo/nonagenarians and offspring were identified. Seven gene sets including cell growth, response to stress, response to DNA damage stimulus, chromatin modification, and phospholipid biosynthetic process were found to be downregulated in octo/nonagenarians, while 12 gene sets such as inflammatory and immune response, insulin action, apoptosis, cellular metabolic process, and cell cycle regulation were shown to be upregulated (Table 2). Fisher's exact test revealed gene ontology, transcription and insulin signalling with the most overlapping entities with 113 and 70 entities, respectively. Other gene ontologies such as translation, metabolic process, and cell cycle were overlapped with more than 30 entities. The gene ontology was ranked based on the highest *P* value (Table 3).

The significant up- or downregulated genes in octo/nonagenarian were reported with their *P* values and fold changes and sorted by functional group (Table 4). Some gene expression changes less than 1.2-fold (fold change ≥1.16) were worth mentioning based on the potential role in aging. Hierarchical clustering of the dataset generated by GeneSpring GX 10 software revealed further differences between the octo/nonagenarian and offspring groups datasets denoted by the letters ON and OF, respectively. The octo/nonagenarians datasets were subgrouped accordingly from the offspring microarrays (Figure 1). The global background intensity of each microarray was normalized (as described in Section 2).

3.3. Microarray Results Validation. In order to validate the results in Table 3, mRNA transcript levels of four upregulated and eight downregulated genes were quantified by real-time RT-PCR using the 18 PBMCs samples from each subject. The genes were selected based on a chosen list of significant biological processes generated by GSEA data, Fisher's exact test for enriched entities and pathways (Tables 2 and 3), and were ranked as overrepresented in the particular chosen pathway built from the gene set by Pathway Studio 7.0 software.

The relative differences in gene expression of four upregulated genes generated by microarray data were comparable to those of RT-PCR evaluations. For example, the expression of *FOXO4* (FC = 1.27) appeared to be upregulated in octo/nonagenarian and, similarly, it was validated by RT-PCR (FC = 1.45) in octo/nonagenarian samples. *BAK1* which represents the biological process of regulation of apoptosis, with only 1.52-fold change in the octo/nonagenarian microarray, was validated by RT-PCR with a greater fold change of 1.91 (Table 5).

Furthermore, the *HSPA8* mRNA levels in octo/nonagenarian were found to be markedly downregulated (−4.01) as assessed by RT-PCR, compared to a lesser fold change (−1.47) in the microarray analysis. Overall, the fold changes of differentially expressed genes obtained by microarray analysis (*t*-test independent, GeneSpring GX 10 software) and RT-PCR were equivalent.

3.4. Functional Categorization. The functional categorizations of the chosen genes in octo/nonagenarian listed in Table S2 are summarized in Figure 2. Genes that were grouped under immune and related function (22%) and signalling and communication (18%) were shown to be most affected by age, while only 2% of the expression of genes contributing to translation changed with age. A rearrangement by functional categorization of the significant genes from the entire dataset is summarized in Table 3. Overall, the pattern of gene expression in octo/nonagenarian showed a decline in response to stress, chromatin modification, and low response to DNA damage stimulus. Upregulation of genes in octo/nonagenarian that code for cell cycle regulation, with enhanced expression of proapoptotic genes and downregulation of antiapoptotic genes, suggests increased apoptosis events in aging cells. An increase of metabolic process might indicate a possible rise in counterbalance of insulin signalling and cellular metabolic efficiency in the cells, while a decrease of positive regulation of inflammatory response may be a sign of decreased inflammatory response in octo/nonagenarian.

4. Discussion

4.1. Alterations in Gene Expression Patterns of Octo/Nonagenarians. Genes such as peroxiredoxin (*PRDX2*, *PRDX5*) or gene families such as FOXO transcription factors, insulin growth factors, autophagy-related genes beclin 1 (*BECN1*), and sirtuins (*SIRT7*) found to be differentially expressed in this study were similarly reported in an aging mouse study [9], favourable to successful aging. However, within these gene families, the upregulation of specific genes such as *FOXO4*, *SIRT7*, and *BECN1* and downregulation of *IGF2R* were seldom reported in human aging studies. In addition, specific genes such as S100 calcium binding protein A4 and A6 (*S100A4*, *S100A6*) were also differentially expressed, in agreement with the findings of other gene expression studies involving aging cells of mice, rats, and humans [10].

Gene expression changes in aging PBMC in the current study suggested an increase in immune response and apoptosis or cell death with age which were also similarly reported in the human brain [5]. Also a decrease of cellular stress response and an increase of DNA repair mechanism

Table 2: A list of statistically significant categories in octo/nonagenarians based on gene set enrichment analysis (GSEA) sorted according to the normalized enrichment score (NES).

Biological process (GSEA)	Normalized enrichment score (NES)[a]	Median change	P value[b]
Inflammatory response	2.07	1.01	$0.00E+00$
Immune response	1.93	1.02	$0.00E+00$
Insulin action	1.57	1.02	$0.00E+00$
Regulation of lipid metabolic process	1.50	1.02	$3.80E-02$
Cholesterol biosynthetic process	1.46	1.04	$2.10E-02$
Induction of apoptosis	1.42	1.02	$0.00E+00$
Cellular metabolic process	1.41	1.02	$3.80E-02$
Antiapoptosis	1.31	1.01	$3.40E-02$
Cell cycle regulation	1.30	1.01	$0.00E+00$
Cell proliferation	1.28	1.00	$0.00E+00$
Cell growth	−1.42	−1.03	$2.40E-02$
Response to stress	−1.46	−1.01	$0.00E+00$
Response to DNA damage stimulus	−1.49	−1.08	$3.80E-02$
Chromatin modification	−1.52	−1.02	$0.00E+00$
Positive regulation of cell proliferation	−1.63	1.01	$0.00E+00$
Phospholipid biosynthetic process	−1.56	−1.04	$0.00E+00$
Positive regulation of inflammatory response	−1.75	−1.03	$0.00E+00$

[a]Positive NES indicates an upregulation in octo/nonagenarians, whereas negative NES reflects an upregulation in offspring samples.
[b]P value estimates the statistical significance of the enrichment score for a single gene set using Kolmogorov-Smirnov statistical test.

Table 3: Analysis of gene list ($P < 0.05$, FDR) by Fisher's exact test.

Biological process	Source[a]	List hits[b]	Population hits[c]	P value[d]
Transcription	GO:0006355	113	2246	$5.63E-13$
Translation	GO:0006412	40	635	$1.08E-07$
Insulin action	GO:0046626	70	905	$2.45E-05$
Cell cycle	GO:0007049	30	539	$4.43E-05$
Cell division	GO:0051301	18	266	$1.42E-04$
Response to stress	GO:0006950	16	239	$3.67E-04$
Double-strand break repair	GO:0006302	5	30	$8.14E-04$
Cell cycle arrest	GO:0007050	5	115	$1.66E-03$
Regulation of apoptosis	GO:0006915	12	177	$1.72E-03$
Chromatin modification	GO:0016568	12	186	$2.60E-03$
Response to DNA damage stimulus	GO:0006974	14	236	$2.60E-03$
Metabolic process	GO:0008152	35	858	$3.38E-03$
Induction of apoptosis	GO:0006917	12	207	$6.10E-03$
Immune response	GO:0006955	18	604	$2.55E-01$
Inflammatory response	GO:0006954	10	293	$2.00E-01$

[a]Source is from the Gene Ontology, http://www.geneontology.org/ .
[b]Number of genes present in this set of 812 genes; $P < 0.05$, FDR (list hits) (see Supplementary Table S2).
[c]Number of genes in each category present in the entire array (population hits).
[d]The P value refers to Fisher's exact test (see experimental procedures).

(Table 2) may be critical factors that favour survival in octo/nonagenarians.

4.2. Functional Annotation Clustering and Genes Differentially Expressed with Age

4.2.1. Immune and Related Functions. Abrupt changes in the immune system were detected in healthy individuals aged over 75 years old [2]. Among the 812 genes differentially expressed with age in octo/nonagenarian PBMC, the top cluster were the immune-related functions, followed by signalling transduction, metabolism, and apoptotic pathway (Figure 2). A study by Vo et al. [3] supported these observations where aged lymphocytes are more responsive toward apoptotic stimuli. One of the major characteristics of the aging process, called inflammaging, arises from continuous antigenic challenge. The inflammatory genes such as tumor necrosis factor superfamily member 4 (*TNFSF4*), CD40

Table 4: Genes differentially expressed in PBMC of octo/nonagenarians[a] ($n = 9$) versus offspring ($n = 9$). Statistical analysis was carried out with t-test. Genes were classified based on functional category.

Function	Gene symbol	Corrected P value[b]	P value[c]	Fold change[d]	Regulation	Gene name	Entrez ID[e]
Cell cycle	*BAK1*	3.88$E-04$	1.25$E-07$	1.52	Up	BCL2-antagonist/killer 1	NM_001188
	CDKN1B	4.92$E-03$	2.94$E-05$	1.43	Up	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	NM_004064
	CDK3	2.38$E-03$	3.62$E-06$	1.31	Up	Cyclin-dependent kinase 3	NM_001113324
	TRADD	4.60$E-03$	2.28$E-05$	1.48	Up	TNFRSF1A-associated via death domain	NM_003789
	CASP2	3.17$E-02$	1.35$E-03$	1.33	Down	Caspase 2, apoptosis-related cysteine peptidase	NM_032982
	CDC14A	4.41$E-03$	2.16$E-05$	1.53	Down	CDC14 cell division cycle 14 homolog A (*S. cerevisiae*)	NM_003672
Immune and related function	*CD40LG*	1.09$E-02$	1.81$E-04$	1.46	Down	CD40 ligand (TNF superfamily, member 5, hyper-I gM syndrome)	NM_000074
	CYSLTR2	9.86$E-03$	1.43$E-04$	1.62	Down	Cysteinyl leukotriene receptor 2	NM_020377
	IL6ST	1.31$E-02$	2.64$E-04$	1.46	Down	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	NM_002184
	TNFSF4	4.99$E-02$	3.02$E-03$	1.93	Down	Tumor necrosis factor (ligand) Superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34 kDa)	NM_003326
Metabolism							
Oxidative phosphorylation	*ATP5E*	3.78$E-02$	1.88$E-03$	1.30	Up	ATP synthase, H^+ transporting, mitochondrial F1 complex, epsilon subunit	NM_001001977
	ATP5I	1.39$E-02$	2.88$E-04$	1.26	Up	ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit E	NM_007100
	COX5B	6.46$E-03$	6.16$E-05$	1.24	Up	Cytochrome c oxidase subunit Vb	NM_001862
	COX6A1	9.84$E-03$	1.41$E-04$	1.47	Up	Cytochrome c oxidase subunit VIa polypeptide 1	NM_004373
	CYC1	4.91$E-02$	2.92$E-03$	1.19	Up	Cytochrome c-1	NM_001916
	NDUFA13	3.31$E-02$	1.49$E-03$	1.16	Up	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	NM_015965
	NDUFB10	3.34$E-02$	1.51$E-03$	1.16	Up	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22 kDa	NM_004548
	NDUFS6	2.75$E-02$	1.01$E-03$	1.21	Up	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13 kDa (NADH-coenzyme Q reductase)	NM_004553
	NDUFV1	9.27$E-03$	1.28$E-04$	1.16	Up	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51 kDa	NM_007103
	SDHA	4.92$E-03$	2.95$E-05$	1.21	Up	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_004168
	SDHB	4.92$E-02$	2.94$E-03$	1.19	Up	Succinate dehydrogenase complex, subunit B, iron sulfur [6]	NM_003000

Table 4: Continued.

Function	Gene symbol	Corrected P value[b]	P value[c]	Fold change[d]	Regulation	Gene name	Entrez ID[e]
	UQCR	$7.01E-03$	$7.07E-05$	1.21	Up	Ubiquinol-cytochrome c reductase, 6.4 kDa subunit	NM_006830
	UQCRFS1	$3.01E-02$	$1.24E-03$	1.39	Up	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	NM_006003
	ATP2A2	$1.31E-02$	$2.64E-04$	1.37	Down	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2	NM_170665
	ATP2C1	$1.07E-02$	$1.74E-04$	1.22	Down	ATPase, Ca^{++} transporting, type 2C, member 1	NM_014382
	COX2	$4.29E-02$	$2.32E-03$	1.19	Down	Cytochrome c oxidase II	NC_001807
	ISCA1	$2.78E-02$	$1.05E-03$	1.21	Down	Iron-sulfur cluster assembly 1 homolog (*S. cerevisiae*)	NM_030940
Insulin action	*IGF2R*	$4.85E-02$	$2.85E-03$	1.28	Down	Insulin-like growth factor 2 receptor	NM_000876
	PIK3R4	$4.26E-02$	$2.30E-03$	1.21	Down	phosphoinositide-3-kinase, regulatory subunit 4	NM_014602
	PIP3-E	$2.01E-02$	$5.62E-04$	1.34	Down		NM_015553
	PIP5K3	$4.54E-02$	$2.52E-03$	1.17	Down	Phosphatidylinositol-3-phosphate/phosphatidylinositol 5-kinase, type III	NM_015040
	PRKACB	$4.35E-02$	$2.36E-03$	1.44	Down	Protein kinase, cAMP-dependent, catalytic, beta	NM_182948
	PRKCA	$2.76E-02$	$1.02E-03$	1.38	Down	Protein kinase C, alpha	NM_002737
Cholesterol, lipid and, lipoprotein maintenance	*LRPAP1*	$3.04E-03$	$6.72E-06$	1.47	Up	Low density lipoprotein receptor-related protein associated protein 1	NM_002337
	SCD	$1.23E-02$	$2.34E-04$	1.23	Down	Stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063
	FOXO4	$3.07E-02$	$1.28E-03$	1.27	Up	Forkhead box O4	NM_005938
	HMOX2	$4.07E-02$	$2.13E-03$	1.19	Up	Heme oxygenase (decycling) 2	NM_002134
Response to stress	*PIN1*	$1.04E-02$	$1.63E-04$	1.24	Up	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	NM_006221
	PRDX2	$1.74E-02$	$4.36E-04$	1.15	Up	Peroxiredoxin 2	NM_005809
	PRDX5	$3.17E-02$	$1.35E-03$	1.27	Up	Peroxiredoxin 5	NM_012094
	HSPA8	$5.58E-03$	$4.12E-05$	1.47	Down	Heat shock 70 kDa protein 8	NM_006597

Table 4: Continued.

Function	Gene symbol	Corrected P value[b]	P value[c]	Fold change[d]	Regulation	Gene name	Entrez ID[e]
DNA repair and telomere maintenance	*CIB1*	$3.34E-02$	$1.51E-03$	1.20	Up	Calcium and integrin binding 1 (calmyrin)	NM_006384
	FEN1	$2.05E-02$	$6.00E-04$	1.24	Up	Flap structure-specific endonuclease 1	NM_004111
	TERF2IP	$1.46E-02$	$3.15E-04$	1.16	Up	Telomeric repeat binding factor 2, interacting protein	NM_018975
	TINF2	$2.73E-03$	$4.60E-06$	1.31	Up	TERF1 (TRF1)-interacting nuclear factor 2	NM_012461
	XRCC6	$2.26E-02$	$7.02E-04$	1.27	Up	X-ray repair complementing defective repair in Chinese hamster cells 6 (Ku autoantigen, 70 kDa)	NM_001469
	DICER1	$3.45E-02$	$1.60E-03$	1.28	Down	Dicer 1, ribonuclease type III	NM_177438
Translation and transcription	*USF1*	$5.58E-03$	$3.91E-05$	1.33	Up	Upstream transcription factor 1	NM_007122
	KEAP1	$1.18E-02$	$2.15E-04$	1.23	Up	Kelch-like ECH-associated protein 1	NM_203500
	NRF1	$4.87E-02$	$2.88E-03$	1.19	Up	Nuclear respiratory factor 1	NM_005011
	SIRT7	$3.55E-02$	$1.67E-03$	1.18	Up	Sirtuin (silent mating type information regulation 2 homolog) 7 (*S. cerevisiae*)	NM_016538
Autophagy	*BECN1*	$4.78E-03$	$2.40E-05$	1.32	Up	Beclin 1, autophagy related	NM_003766
	VPS18	$1.81E-02$	$4.74E-04$	1.26	Up	Vacuolar protein sorting 18 homolog (*S. cerevisiae*)	NM_020857

[a]The genes shown in this table are ordered according to the biological processes. For a complete list of all 812 genes significantly affected in octo/nonagenarians, see Table S2.

[b]Corrected P value refers to P value after multiple testing corrections by Benjamini-Hochberg method.

[c]P value refers to P value before multiple testing corrections by Benjamini-Hochberg method.

[d]The fold change refers to the ratio of the expression values of octo/nonagenarians over offspring.

[e]Source of entrez ID is from National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/gene/.

Table 5: Genes differentially expressed in PBMC of octo/nonagenarian versus offspring. Statistical analysis was carried out using independent *t*-test (RT-PCR) with a Benjamini-Hochberg false-discovery rate (microarray).

Biological process	Gene symbol	Gene name	RT-PCR		Microarray data	
			Fold change	*P* value[a]	Fold change	Corrected *P* value
Cell cycle	*CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.48	$9.46E-03$	1.43	$4.90E-03$
Double-strand break repair	*XRCC6*	X-ray repair cross-complementing protein 6 (Ku autoantigen, 70 kDa)	1.49	$1.34E-02$	1.27	$2.30E-02$
Cell cycle arrest	*FOXO4*	Forkhead box O4	1.45	$3.49E-03$	1.27	$3.00E-02$
Regulation of apoptosis	*BAK1*	BCL2-antagonist/killer 1	1.91	$3.80E-02$	1.52	$3.90E-04$
Response to stress	*HSPA8*	Heat shock 70 kDa protein 8	−4.01	$1.30E-04$	−1.47	$5.60E-03$
Immune response	*CD40LG*	CD40 ligand	−2.71	$1.32E-04$	−1.46	$1.10E-02$
Cell division	*CDC14A*	CDC14 cell division cycle 14 homolog A	−2.04	$4.21E-04$	−1.53	$4.40E-03$
Inflammatory response	*TNFSF4*	Tumor necrosis factor (ligand) superfamily, member 4	−2.57	$4.02E-05$	−1.93	$4.90E-02$
Chromatin modification	*NCOR1*	Nuclear receptor corepressor 1	−1.69	$7.86E-03$	−1.43	$4.40E-02$
Insulin action	*PRKCA*	Protein kinase C, alpha	−1.64	$3.07E-02$	−1.38	$2.70E-02$
Induction of apoptosis	*CASP2*	Caspase 2	−2.08	$3.66E-03$	−1.33	$3.10E-02$
Metabolic process	*ATP2A2*	ATPase, Ca^{++} transporting	−1.61	$5.49E-03$	−1.37	$1.30E-02$
Housekeeping gene (cytoskeleton)	*ACTB*	Beta Actin				

[a]The *P* values for the real-time reverse transcription PCR (RT-PCR) refer to a two-tailed *t*-test for the differences in means between the normalized Ct values in octo/nonagenarians versus offspring.

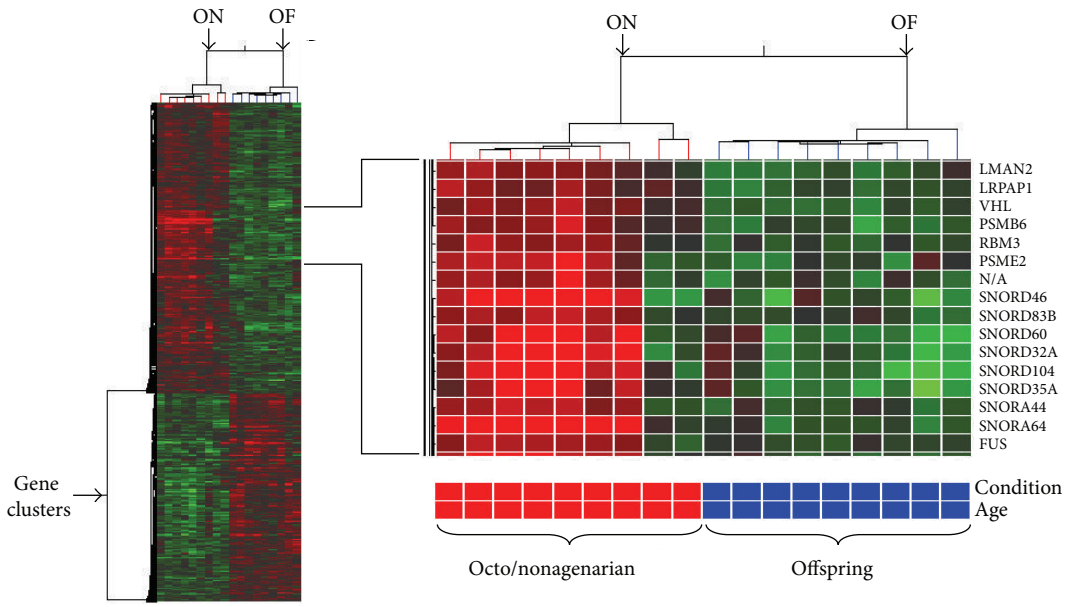

Figure 1: Hierarchical clustering of octo/nonagenarian (ON) versus offspring (OF). Most similar expression profiles are joined together to form a group. The expression profiles analyzed in this figure corresponded to the 812 genes that were found to be changed significantly (fold change ≥1.2; $P < 0.05$) in octo/nonagenarians. Bright red and green indicate high and low expression, respectively.

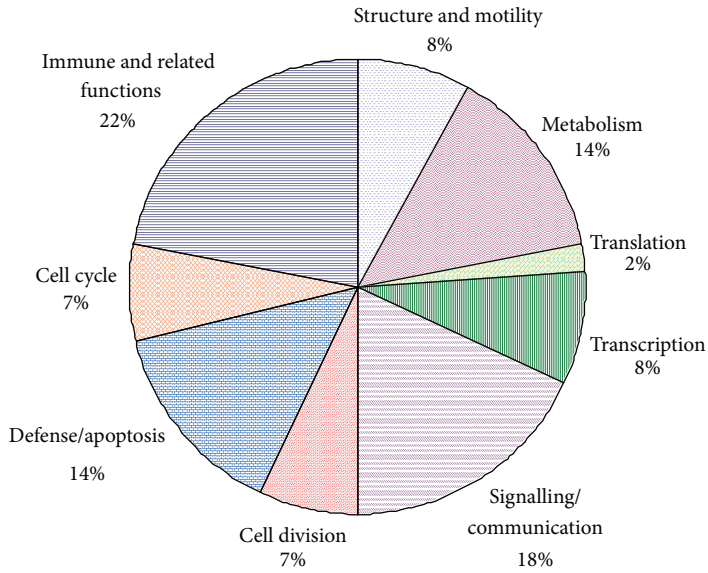

Figure 2: Summary of the functional attribution of significant differential expressed genes listed in Table S2, with percentage repartition of differentially regulated genes of octo/nonagenarians compared to offspring.

ligand (*CD40LG*), interleukin 6 signal transducer (*IL6ST*), and cysteinyl leukotriene receptor 2 (*CYSLTR2*) were found to be downregulated in octo/nonagenarians.

A study conducted by Zuliani et al. [11] reported an increase of plasma soluble gp130 (IL6ST) levels, which plays an important role in response to environmental stress, in 997 older subjects with metabolic syndrome mediated by insulin resistance. In this context, changes in inflammation and the proinflammatory genes observed in aged subjects in this study may be the result of a favourable reaction helping older people to cope with chronic antigenic stressors. However, the association between *IL6R* genotype and longevity has not been reported despite strong evidence for association of cardiovascular disease with IL-6 and sIL-6R phenotypes [12]. In agreement with Jylhävä et al. [13], reduced expression of *CD40LG* in octo/nonagenarians (this study) which functions in full T-cell and B-cell signalling and activation suggests that the aged immune system failed to respond adequately to antigen stimuli.

Lipid mediators, cysteinyl leukotrienes (CysLTs), were downregulated in octo/nonagenarians, which are known to possess potent proinflammatory action. A decline of

CysLT(2)R in transgenic mice overexpressed with human *CysLT(2)R* significantly reduces myocardial infarction damage [14]. It is possible that the decrease of *CysLT(2)R* offers benefit to the aged system, although the role of *CysLT(2)R* needs to be further elucidated.

4.2.2. Cell Cycle and Apoptosis. The increased expression of proapoptotic genes such as BCL2-antagonist/killer 1 (*BAK1*) and TNFRSF1A-associated via death domain (*TRADD*) in aged PBMC in this study further emphasized that multiple cell types are poised for apoptosis in aging phenotype. *BAK1* is a crucial mediator of B-cell death and plays a role in the prevention of autoimmune disease. Elevated mRNA and protein expression of *TRADD* have been associated with increased apoptosis in lymphocytes from aged subjects [15]. This may imply that when encountering an oxidative challenge, PBMCs readily undergo apoptosis, whereby they more efficiently eliminate damaged cells, thus extending the life-span of the healthy cells.

4.2.3. Response to Stress and Oxidative Damage. Major downregulation of genes reported in microarray studies of aged model organisms [10] and PBMC observed in current study may be linked to increased oxidative stress and damage, where over 41% of the differentially expressed genes were age-repressed in octo/nonagenarian PBMC. Also, heat shock 70 kDa protein 8 (*HSPA8*), heat shock 27 kDa protein 1 (*HSPB1*), heat shock 90 kDa protein 1, and beta (*HSP90AB1*) mRNA were found to be decreased, while heme oxygenase 2 (*HMOX2*), *PRDX2*, *PRDX5,* and *FOXO4* from octo/nonagenarians were found to be increased.

Aged individuals develop a constitutively low level of several chaperones including HSPA8, HSP27, and HSP90 [16]. HMOX2, PRDX2, and PRDX5 are essential enzymes associated with oxidative stress in many cell types and organisms such as human endothelial cells [17] and *Drosophila* [18]. Overexpression of *PRDX2* in *C. elegans* [19] and *PRDX5* in *Drosophila* [18] increased resistance to oxidative stress and extended their life-span. *HMOX2* plays a critical role in cell defence against oxidative stress [17], while FOXO4 transcription factor possesses antioxidative protection through a mechanism of *O*-GlcNAcylation and improves cell survival in response to oxidative stress [20]. FOXO4 is also able to modulate the expression of genes involved in oxidative stress dependent apoptosis, cell cycle arrest, DNA damage repair, and other cellular functions. Genes involved in insulin signalling including FOXO transcription factors integrate longevity pathways and metabolic signals in a complex interaction which affects the life-span determinant pathways, such as the beneficial effects of caloric restriction which may be modulated by deacetylation of FOXO4 by SIRT1 [20]. The findings that these transcripts increased in octo/nonagenarians may be explained by compensatory induction of these genes to cope with oxidative stress and damage in aging. Moreover, Terry et al. [21] suggest that low serum Hsp70 might be a marker for health given that long-lived individuals might have less cellular stress to respond to.

4.2.4. DNA Repair and Telomere Maintenance. Defects in DNA repair mechanisms and telomere maintenance processes may accelerate the accumulation of oxidatively modified proteins and nucleic acids, promote the deposition of toxic protein aggregates, mitochondrial dysfunction, and aberrant gene transcription and, in turn, may lead to mutations in cancer [22]. Our results suggest an overexpression of genes crucial for genomic stability such as *XRCC6, TINF2,* TRF2-interacting telomeric protein 1 (*TERF2IP*), flap endonuclease 1 (*FEN1*), and calcium and integrin binding 1 (*CIB1*).

The ability to retain a high level of XRCC6, a DNA repair gene and part of the DNA-dependent protein kinase (DNA-PK) complex, may partially contribute to the long life-span in a longevity community in Seoul, Korea [22]. TINF2 and TERF2IP are a part of the shelterin complex which functions to prevent the activation of a DNA damage response at chromosome ends. TINF2 is essential for maintaining a functional telomere capped structure, while the role of TERF2IP is associated with telomere protection [23]. Furthermore, FEN1 is involved in regulating telomerase activity at telomeres, where mammalian cells expressing low levels of FEN1 showed increased telomere instability [24]. Finally, CIB1 is a DNA-PKcs-interacting protein and plays a positive role in telomere length maintenance [25]. Increased levels of these genes may provide proper protection, maintenance of mammalian telomeres, and DNA stability during aging. A recent study by Dekker et al. [26] reported that fibroblast strains obtained from nonagenarians of the Leiden 85-plus study with a high maximum proliferation capacity are less likely to go into stress-induced cellular senescence and have longer telomeres. On the other hand, there is a possibility that some of the age-related changes of gene expression might reflect adaptive changes; for example, the genes known to regulate specific pathways such as the DNA repair enzymes and the cell cycle regulators may function as a part of other signalling pathways [10].

4.2.5. Metabolism-Related Function. Gene set for metabolism-related function including energy production by mitochondria oxidative phosphorylation (OXPHOS), insulin/IGF-1 signalling, maintenance of cholesterol levels, and lipid and lipoprotein was shown to be upregulated in octo/nonagenarian.

Age-related decline in OXPHOS enzyme activities in humans may be due to the accumulation of mutations in mtDNA. Yet, energy metabolism was shown to be generally preserved in long-lived subjects and centenarians [27]. Transcript components of OXPHOS complex I, mitochondrial respiratory chain (*NDUFV1, NDUFA13, NDUB10, and NDUFS6)*, II succinate dehydrogenase (*SDHA, SDHB*), III ubiquinol-cytochrome-c reductase (*CYC1, UQCRFS1, and UQCR*), IV cytochrome c oxidase (*COX5B, COX6A1, and COX8A*), and surfeit locus protein 1 (*SURF1*) were found to be abundant in octo/nonagenarians. Studies on long-living Ames dwarf mice showed that several components of OXPHOS system were increased relative to wild-type mice, which suggests enhanced mitochondrial function and efficiency [9]. Moreover, the expression of four genes that are

involved in ATP production in complex V (ATP synthase) was altered in octo/nonagenarians where *ATP5E* and *ATP5I* were increased, while *ATP2A2* and *ATP2CI* were decreased. Decline of complex V protein activities with age may be caused by oxidative protein modification.

It is hypothesized that the genes involved in insulin/IGF-1 signalling might also be important for human longevity, based on the reported association between decreased insulin/IGF-1 signalling and longevity in worms, flies, and mice [28]. Expression of genes involved in insulin/IGF (IIS) pathway such as insulin-like growth factor 2 receptor (*IGF2R*), phosphoinositide-3-kinase, subunit 4 (*PIK3R4*), phosphoinositide-binding protein *PIP3-E* (*IPCEF1*), protein kinase C alpha (*PRKCA*), protein kinase A beta (*PRKACB*), and phosphatidylinositol-3-phosphate 5-kinase (*PIP5K3*) was declined in octo/nonagenarians. Organisms with reduced IIS activity are resistant to a variety of cellular stresses, suggesting that mechanisms counteracting stress are enhanced [28]. Moreover, extreme human longevity seen in centenarians correlated with a low degree of insulin resistance [29]. It is expected that long-lived people are insulin sensitive all through their life-span which may protect them from age-related decline of insulin action and its associated diseases. Nevertheless, results from human studies have been inconsistent, where disruptions in insulin signalling have been linked with age-related diseases such as insulin resistance and diabetes [30].

Low-density lipoprotein related-receptor associated protein-1 (*LRPAP-1*) gene which functions as a transporter of amyloid beta protein (AβP) was upregulated in octo/nonagenarians. According to Zhang et al. [31], an increase of *LRPAP-1* expression resulting from supplementation of Chinese traditional medicine "Bushen Yinao Pian" on senescence-prone mouse 8/Ta (SAMP/Ta) may delay age-related cognitive defects. Finally, downregulation of stearoyl-CoA desaturase (*SCD1*) was observed in octo/nonagenarians. SCD1 catalyses the biosynthesis of mono-unsaturated fatty acids from palmitoyl-CoA and stearoyl-CoA, reported to be important in the maintenance of phospholipid membrane fluidity for normal cellular function. Decreasing SCD1 expression might protect against obesity and insulin resistance, where mice deficient in SCD1 are resistant to metabolic syndrome and are insulin sensitive [32].

4.2.6. Autophagy and Vesicular Trafficking. Autophagy functions in housekeeping and quality control that contribute to health and longevity. In some cases, autophagy can protect cells against intracellular pathogens. Beclin 1 (*BECN1*), the central protein regulator of autophagy, was overexpressed in octo/nonagenarians. Deletion of *bec-1* inverts longevity in worms and a recent study demonstrated *Becn1* as potential therapeutic target in Alzheimer's disease, where lack of Becn1 modulates amyloid precursor protein metabolism and promotes neurodegeneration in mice [8].

4.2.7. Transcription and Protein Synthesis. Increased mRNA abundance of *SIRT7, USF1, NRF,* and *KEAP1* was shown in octo/nonagenarian group. According to Ford et al. [33], SIRT7 is a positive regulator of RNA polymerase I transcription and is essential for cell survival in mammals. USF1 regulates genes involved in inflammation, lipid, and glucose metabolism. A study by [34] reported the association of USF1 haplotype with lower cholesterol levels and decreased risk of early-onset coronary atherosclerosis in young adults and suggests the involvement of USF1 in the regulation of human longevity. Nuclear respiratory factor 1 (*NRF1*), a DNA-binding transcription factor, plays a role in nuclear-mitochondrial interactions and other cellular functions such as protein synthesis, DNA repair, and cell proliferation. As *NRF1* gene knockout mice results in mtDNA instability and embryonic lethality, thus, regulation of *NRF1* is crucial in order to balance the cell energy demands [35]. Besides acting as a repressor protein that binds NRF2 to promote its proteasomal degradation, kelch-like ECH-associated protein 1 (KEAP1) also plays a central role in regulating the protective response [36]. Perhaps the induction of transcription in older cells relative to younger cells indicates that the aging profile shows an active response to damage and genome instability.

5. Conclusion

The current findings suggest that long-lived phenotype has cellular survival mechanisms that may guard against oxidative stress and DNA damage by activating gene sets common to DNA repair, telomere maintenance, antioxidant response, autophagy pathway, and metabolism-related genes. This implies that a boost of key DNA repair elements and efficient cellular trafficking may promote lower ROS production or oxidative substrate damage [18, 22]. Importantly, the unique regulation of possible "innate" genes in octo/nonagenarians' PBMC may shed some light on the pathophysiology of the long-lived phenotype. This study has also revealed potential candidate genes such as *XRCC6, FOXO4, IGF2R, SIRT7,* and *KEAP1* which may impact the aging process and survival and henceforth contribute to human longevity.

Conflict of Interests

There is no conflict of interests involved in this study.

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Review Article

Mitochondrial Dysfunctions and Altered Metals Homeostasis: New Weapons to Counteract HCV-Related Oxidative Stress

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The hepatitis C virus (HCV) infection produces several pathological effects in host organism through a wide number of molecular/metabolic pathways. Today it is worldwide accepted that oxidative stress actively participates in HCV pathology, even if the antioxidant therapies adopted until now were scarcely effective. HCV causes oxidative stress by a variety of processes, such as activation of prooxidant enzymes, weakening of antioxidant defenses, organelle damage, and metals unbalance. A focal point, in HCV-related oxidative stress onset, is the mitochondrial failure. These organelles, known to be the "power plants" of cells, have a central role in energy production, metabolism, and metals homeostasis, mainly copper and iron. Furthermore, mitochondria are direct viral targets, because many HCV proteins associate with them. They are the main intracellular free radicals producers and targets. Mitochondrial dysfunctions play a key role in the metal imbalance. This event, today overlooked, is involved in oxidative stress exacerbation and may play a role in HCV life cycle. In this review, we summarize the role of mitochondria and metals in HCV-related oxidative stress, highlighting the need to consider their deregulation in the HCV-related liver damage and in the antiviral management of patients.

1. Introduction

Hepatitis C virus (HCV) is a human pathogen affecting about 4 million new subjects every year [1]. Approximately 3% of the world's population is estimated to be chronically infected by HCV [2]. Differently from the other hepatitis viruses (A, B, and E), more than 80% of HCV patients become chronic [3].

HCV is a member of the genus *Hepacivirus* of Flaviviridae family. It is a single-stranded RNA virus with positive polarity. The genome of HCV encodes a polyprotein of about 3000 amino acids that is expressed from a single long open reading frame (ORF). This polyprotein is cleaved into ten different products: the core protein (Core) and the envelope glycoproteins 1 and 2 (E1 and E2, resp.), which are constituents of the HCV particles, p7 and nonstructural protein 2 (NS2), primarily involved in HCV assembly, NS3, NS4A, NS4B, NS5A, and NS5B nonstructural proteins with important roles in the polyprotein processing and HCV replication [4]. HCV infection frequently leads to severe liver diseases, including liver cirrhosis and HCC [5]. Chronic HCV infected patients are commonly characterized by metabolic derangements, such as steatosis, insulin resistance (IR), and altered homeostasis of trace metals [6–8]. Many works suggest that oxidative stress (OS) plays a pivotal role in the occurrence of all these pathological features. OS is the condition occurring when the cellular or systemic redox balance is altered, as a consequence of unusual exposure to prooxidant molecules, like reactive oxygen species (ROS) or reactive nitrogen species (RNS) [9], which in turn can be either associated with an inadequate antioxidant response or not. The overproduction of ROS and RNS can be caused either by endogenous or exogenous sources [9]. OS produces oxidative damage to proteins, lipids, and nucleic acids, thus altering their physiological functions.

Mitochondria are the main source of ROS production through the electron transport chain (ETC) complexes and the mitochondrial dehydrogenases [10] and, at the same time, they are the main targets of reactive molecules. Mitochondria

are well-known targets of HCV protein actions; however, also extramitochondrial sources of ROS are involved in HCV-related OS onset: ER, peroxisomes and other cell compartments [11, 12], xanthine oxidase or NADPH oxidases [13], cytochromes P450, and resident immune cell populations in the liver (e.g., Kupffer cells). To avoid the deleterious effects of ROS, biological systems have developed several mechanisms of detoxification that use a wide number of small molecules, peptides, and enzymes, like glutathione (GSH) or superoxide dismutases (SODs), respectively.

However, it should not be forgotten that ROS are also potent second messengers in a plethora of cellular functions; they are involved in modulating key physiopathological processes [14, 15], such as those mediated by the signal transducer and activator of transcription (STAT) and nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) [16].

This review will summarize some relevant mechanisms through which HCV may promote OS onset, focusing the attention on virus-related mitochondrial OS, trace elements derangement, and novel therapeutic opportunities to counteract these viral actions.

2. Oxidative Stress during HCV Infection

The OS occurrence, as a consequence of HCV infection, is well established [17]. Several reports put in evidence a deep redox imbalance in infected patients, focusing on its potential correlation with the course of the liver disease.

Increased markers of oxidative-damaged DNA (8-hydroxyguanosine, 8-OHdG) and lipid peroxidation products (8-isoprostane) were detected in serum, peripheral blood mononuclear cells (PBMC), and liver specimens of infected patients [5, 18, 19].

Accordingly, we recently demonstrated that HCV patients are characterized by increased plasma levels of 7-ketocholesterol (7K) and 7-β-hydroxycholesterol (7βOH) [20], two of the well-known peroxidation products of cholesterol, called oxysterols. Oxysterols are extremely interesting, not only because they can modulate the mitogen-activated protein kinases (MAPKs), thus affecting cell growth and promoting cell transformation [21], but also because they were detected in oxidized-low density lipoprotein (oxLDL) [22]. Oxysterols are the specific ligands of liver X receptors (LXRs), nuclear receptors deeply involved in several pathophysiological processes, for example, lipid metabolism and inflammation [23]; through oxysterols HCV might profoundly modulate host metabolism. Accordingly, steatosis is a common feature in HCV-infected hepatocytes [24] and it is characterized by high oxidation rates with a consequent increase of electron delivery to the ETC that might cause ROS overproduction. Infected patients show a correlation between OS markers and inflammation, grade of fibrosis, and hepatic iron storage [25, 26]. However, the increased levels of oxysterols give rise to the question whether the OS is directly due to HCV-host cell interactions or if host immune response and iron overload are main reasons for OS onset [27–29]. Surely, a synergistic mechanism exists.

Moreover, HCV patients are deeply characterized by a reduction of their antioxidant defense. Glutathione (GSH), a key player of the first line of antioxidant defense, produced by all cells and especially concentrated in liver [30], is commonly decreased in chronic HCV subjects [31–33]. Accordingly, the ratio between oxidized (GSSG) and reduced (GSH) forms, a well-accepted parameter representative of the oxidative status, is increased [32]. As well as GSH, glutathione reductase, glutathione peroxidase, and Cu/Zn containing SOD are often found decreased in PBMC of infected patients too [34].

All reported data demonstrate that OS occurrence is due to HCV, but some discrepancies exist about the entity of the induction of liver damage linked to this pathological mechanism. To note, OS markers are found in HCV patients with mild, moderate, or no liver disease [35]. However, proteomic analysis revealed an upregulation of antioxidant enzymes at early (F1 to F3) but not at late stages of fibrosis [36].

On the contrary, some works described an enhanced expression of thioredoxin (Trx) [37] or heme oxygenase (HO-1) [38]. The latter enzyme, in particular, is a known target gene of the nuclear factor erythroid2-like 2 (Nrf2) protein. This data seems in contrast with what we have previously described, but, in our opinion, the activation of Nrf2 could be functional to HCV virus. In fact, Nrf2 is a transcription factor which recognizes a common conservative sequence, called antioxidant response element (ARE), in the promoter regions of many antioxidant enzymes [39, 40], and is crucial to preserve the mitochondrial activities and to enhance cell survival of infected cells [41]. Thus is essential for viral life cycle.

3. Mitochondria: In the Center of the Viewfinder of HCV

As mentioned above, the main parts of ROS produced in the cells are generated by mitochondria. These organelles are the "cellular power plants," because they are mainly responsible for cell supply of adenosine triphosphate (ATP). By sensing the energy status they may decide the cell fate [42]. The relevance of mitochondria to metabolism, energy production, and cell fate was brought to light by several reports in a plethora of human diseases, from neurodegeneration to metabolic disorders [43].

Several lines of evidence describe, without any doubt, mitochondria as a main target of the HCV virus. It is well known that HCV produces ultrastructural alteration of these organelles and causes oxidative damage and a reduction in mitochondrial DNA copy number, in both hepatocytes and lymphocytes of infected patients [44, 45].

Mitochondria possess a complex architecture; they comprise an outer membrane (OMM) that encloses the entire organelle and has a structure similar to that of plasma membrane. The OMM contains proteins and complexes that allow diffusion of small proteins or factors characterized by a specific signaling sequence at their N-terminus. The OMM can associate with the ER through a structure called mitochondria-associated ER membrane (MAM) [46]. MAMs are zones of junctions where the inner mitochondrial membrane (IMM) meets the OMM and allows exchanges of Ca^{2+} and lipid between ER and mitochondria [46]. Thus, MAMs are key elements in the maintenance of mitochondrial

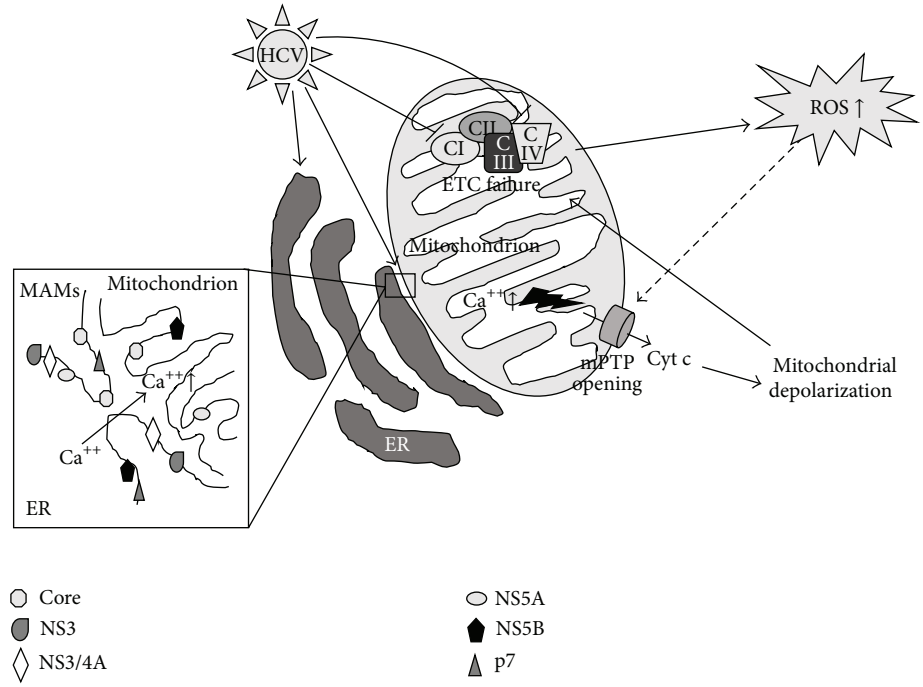

Figure 1: Molecular mechanisms through which HCV induces mitochondrial damage and the consequent increased ROS production. Several HCV proteins associate with both endoplasmic reticulum (ER) and mitochondria. In particular, Core localizes also on MAMs and may play a role in the increase of mitochondrial Ca^{++} pool, which in turn is involved in mitochondrial permeability transition pore (mPTP) opening, release of cytochrome c (Cyt C), and consequent mitochondrial depolarization. Organelle depolarization may be responsible for electron transport chain (ETC) failure and reactive oxygen species (ROS) overproduction. Enhanced ROS, in turn, may cause a further mPTP opening (dashed line), worsening the mitochondrial depolarization, thus leading to the onset of a vicious deleterious circle that aggravates the mitochondrial damage. Furthermore, some reports indicate that Complexes I and IV (CI and CIV, resp.) of the ETC are main targets of HCV actions. The fall of their activities plays key role in ETC failure and increased ROS production.

homeostasis of lipids and Ca^{2+} [46] and are crucial to modulating the opening of the mitochondrial permeability transition pore (mPTP). The latter is a critical event in the decision of cell fate [47]. Furthermore, MAMs have a key role in the cellular pathways leading to very-low-density lipoprotein (VLDL) assembly and secretion [48]. These cellular activities are also essential for the formation and maturation of HCV particles [49].

Several HCV proteins have been shown to directly associate with mitochondria [50–52] (Figure 1). In particular, Core is able to associate with OMM through a specific sequence at its C-terminal region [53, 54]. It was detected in MAMs on the mitochondrial surface [53] and, by electronic microscopy; it was also localized in the IMM [52] (Figure 1). Biochemical studies revealed that the interaction of HCV with mitochondria, through the Core protein, plays a key role in organelles sensitization to Ca^{2+} influx, with the consequent opening of mPTP, release of cytochrome c [55], mitochondrial depolarization, and ETC failure. This cascade of events leads to an increased ROS generation (Figure 1).

Other viral proteins reinforce the Core action. Indeed, in experimental models, the proteins p7 and NS4A, the NS3/4A complex, and the proteins NS5A and NS5B were found to be localized to mitochondria and ER by subcellular-fractionation and confocal and electron microscopy [55–59] (Figure 1). Furthermore, both NS5A and NS5B were also localized in the IMM and in the mitochondrial matrix [55, 58] (Figure 1).

These data clearly demonstrate the intimate connection of HCV to mitochondria.

The mitochondrial sensitization to Ca^{2+} along with the mPTP opening, caused by viral proteins, lead to building up a vicious circle characterized by the inhibition of the ETC, with the consequent increase in ROS production. This, in turn, induces mPTP opening [60, 61] and further worsening of mitochondrial failure (Figure 1).

One of the main mitochondrial targets of HCV is Complex I of ETC (also known as NADH:ubiquinone oxidoreductase) [62]. Complex I activity, being the first step of ETC, is crucial for the aerobic respiration. Mitochondria isolated from transgenic mice expressing Core, E1, and E2 glycoproteins are characterized by an increased ROS production from Complex I substrates and reduced Complex I activity [17, 50] (Figure 1). The Complex IV (cytochrome c oxidase) is the other mitochondrial protein complex affected by HCV (Figure 1). On the contrary, Complexes II and III are not altered during HCV infection [63]. It is interesting to note that the inhibition of viral replication, *via* IFN treatment, can fully restore the activities of both Complexes I and IV in hepatic cells [63].

Increased oxidation of the GSH and thioredoxin pools further provides a demonstration of the HCV-related mitochondrial redox imbalance [64, 65]. Core has also been shown to induce the expression of mitochondrial but not cytoplasmic SOD [65], suggesting that HCV generates ROS at mitochondrial level and, at the same time, strengthens the cellular antioxidant system against OS. The virus, to avoid excessive cytotoxic effects produced by a massive increase of ROS, might activate this behavior.

4. HCV and Metals Homeostasis Derangement

HCV infection may prompt OS onset by deregulation of homeostasis of trace metals, like zinc (Zn), iron (Fe), and copper (Cu) [8]. Zn, Fe, and Cu are essential trace elements that play important roles in various biological processes.

HCV patients show low plasma concentrations of Zn, whereas Cu and Fe concentrations were high [8, 66]. Notably, higher amounts of both Fe and Cu can interfere with Zn homeostasis, worsening the Zn deficiency [67].

Zn is largely present in the cells [68] and has several relevant biological functions: it is involved in insulin management [69] and in the maintenance of immune system [70]. Zinc is a redox inert metal; thus, it does not directly participate in cellular reactions of reduction and oxidation. The antioxidant potential of Zn is exerted through different mechanisms: (a) it is able to bind the sulfhydryl groups of proteins avoiding their oxidation by free radicals; (b) it participates in the antioxidant response through the modulation of metallothioneins, GSH, and Nrf2; (c) it may antagonize redox-active transition metals, such as Fe and Cu [68]. Zn has also a structural relevance to HCV because some of its proteins, that is, NS3 and NS5A, are zinc metalloproteins [71]. At hepatic level, Zn is known to promote antioxidant and anti-inflammatory effects that result in reduced hepatocyte injury, in chronic HCV infected patients [72, 73]; moreover, Zn is able to inhibit NFκB activation, thus counteracting the production of inflammatory cytokines [74]. HCV replication, instead, enhances the NFκB pathway activation triggered by tumor necrosis factor-alpha (TNFα) [75]; thus the low Zn plasma levels may prompt the onset of an inflammatory environment known to play a key role in virus-related liver disease progression. Accordingly, some studies suggest that zinc administration, through a drug called polaprezinc, may improve the outcome in HCV and HCV-related cirrhotic patients [76, 77]. The idea that low levels of Zn may be functional to HCV-related liver damage seems further supported by some studies that have suggested its potential in inhibiting HCV replication [78]; however, the mechanism is still not clear.

As well as in other pathologies, such as cancer or diabetes [70, 79, 80], in the HCV infection a Zn deficiency occurs parallel to a Cu increase [8], particularly in those patients affected by NAFLD as well [81], thus paving the way for a redox imbalance. The increase in Cu serum levels correlates with viral load [82]. Since Cu overload may lead to several deleterious effects, it is possible to speculate that the Zn therapy is beneficial for HCV patients because it also provokes a reduction in Cu levels, as already described in other pathologies like Wilson's disease [83].

Cu, in fact, is a transition metal extremely harmful because, as well as Fe, it is characterized by an elevated redox potential; thus it may participate in redox reactions, like Fenton's reaction, promoting ROS generation and consequently OS, as already reported in other tissues and pathologies [84]. To avoid its unhindered reactivity, biological systems developed an intricate network of proteins that prevents the existence of free copper. GSH, a key antioxidant, able to suppress Cu toxicity through its binding to this metal, maintains it in a reduced state and avoids its redox cycling [85]. GSH decrease, associated with Cu deregulation, may play a key role in the HCV-related OS onset. The role of Cu in HCV infection and the related OS is probably underestimated. Oxidative potential of Cu on low-density lipoprotein (LDL), in fact, is known for a long time [86]. This event not only has a clinical relevance in cardiovascular risk enhancement, but also may have a deep impact in HCV-related damage, even if some apparent contrasting data are reported. Several studies, in fact, describe the presence of a high level of oxLDLs in HCV infected patients [20, 87], according to the plasma Cu elevation, suggesting a possible role in disease pathogenesis; on the contrary, other papers suggest their potential role in inhibiting the HCV entry in the cells [88, 89]. Nevertheless, why should the virus produce something that could be able to inhibit its entry into cells? Currently, there is not an explanation. It could be a protective response of the organism; the oxidation of LDL, in fact, as a consequence of the inflammatory response activation, could be an attempt to counteract viral infection. Alternatively, it may only be a secondary phenomenon occurring only in a late phase as a result of the prooxidant environment generated by the HCV, also through the imbalance of transition metals homeostasis, such as copper. This latter hypothesis implies that LDL oxidation has actually a low impact in countering the infectivity of HCV, since it occurs only when the virus has already activated its pathological mechanisms. Although the oxidation of LDL is an event well established in HCV patients, the mechanisms underlying it are not well clarified. The topic is intriguing, and the role of Cu needs to be better explained, not only because Cu and oxLDL evaluations may have a deep diagnostic and prognostic impact, but also because a deep comprehension of their involvement in HCV-related disease could open the way to new antiviral approaches.

Altered Cu homeostasis is associated with a reduction of ceruloplasmin [90–92]. This protein is important for both the trafficking of copper, binding 95% of circulating copper [70], and Fe, because, at hepatic level, it is the main ferroxidase enzyme, which is crucial for a proper Fe transport [93]. Thus, its deregulation may also represent a sign of systemic Fe perturbation.

As a matter of fact, patients with chronic HCV infection are commonly characterized by elevated levels of serum ferritin and hepatic iron [8]. Hepatic iron increase could be functional to viral cell cycle; in fact, a recent paper indicates that transferrin receptor 1 (Tfr1) may be important for the cell entry of viral particle [94]. The relevance of this

metal is further highlighted by several studies and clinical trials that demonstrate how the hepatic iron levels influence hepatic injury and response to therapy in chronic HCV patients [95–98]. Furthermore, patients with a virus-induced severe grade of hepatitis show higher levels of serum iron with respect to patients characterized by a lower grade of hepatitis. Note that serum iron is positively correlated with intense steatosis, fibrosis, and biochemical and histological parameters, indicating liver inflammation [99].

The mechanism underlying iron deregulation is still unclear; however, in such a context, hepcidin seems to be a key player. Hepcidin is a protein synthesized in the liver where iron, inflammation, and OS promote its expression [100]. This protein, through the modulation of ferroportin, is crucial for the maintenance of systemic iron homeostasis.

Through the use of a mouse model of HCV infection, it was suggested that the hepatic decrease of hepcidin causes an increased intracellular iron storage and, at the same time, OS. This, in turn, may promote the expression of ferroportin in the duodenum and in the macrophages, thus leading to increased iron serum levels [101]. This mechanism could explain the iron derangement in HCV patients. Furthermore, it was recently proposed that the HCV-related downregulation of hepcidin may also exert a deep impact on the virus life cycle, because it seems to possess an antiviral activity [102]. Therefore, through the hepcidin inhibition HCV reaches two goals to save its viral cycle and to promote derangement of iron homeostasis.

The latter event may be crucial in OS-mediated liver injury [103]. In fact, Fe is an extremely reactive transition metal, and an excessive presence may induce mitochondrial injury increasing the risk of HCC development [104]. Accordingly, a Fe reduction therapy has been shown to counteract hepatocyte injury in patients with HCV infection [97], confirming an important role of iron in HCV-related liver injury.

So, the OS induced by HCV promotes the onset of a dangerous loop involving, once again, mitochondrial damage; hence, the deranged homeostasis of metals may enhance ROS production and mitochondrial failure, which may participate in the alteration of metal homeostasis. Mitochondria, in fact, play a key role in the maintenance of Cu and Fe homeostasis [105, 106].

5. New Potential HCV Therapeutical Approaches

In the last decade, the gold standard in the HCV treatment was represented by the combination of pegylated interferon (IFN)-α and ribavirin. This therapy, administered for 24 or 48 weeks, produced viral suppression in approximately 40–50% of patients infected by HCV genotype 1 and in 80% of those infected by HCV genotypes 2 and 3 [107]. Today, the new therapeutic approach contemplates the use of a triple therapy, (IFN)-α and ribavirin plus telaprevir or boceprevir, two direct-acting antiviral (DAA) agents known to be NS3/NS4A protease inhibitors [107]. Unfortunately, although these new treatments reach a sustained viral response (SVR) in 63–75% HCV genotype 1 patients and a reduction in therapy length, persistent limitations to treatment still exist. In particular, many new side effects have been encountered which are in need of adequate management strategies as well as drug interactions, other than the persistence of virus resistance and interferon intolerance [108]. To overcome these problems today new therapeutic treatments are under investigation, such as DAA of second generation, targeting NS5B, or host targeting molecules, like cyclophilin inhibitors, and the use of IFN-free therapy, to reduce intolerance to treatment and to enlarge the potential patients cohorts [107].

The use of molecules counteracting oxidative stress at mitochondrial levels or treatments able to restore a proper metal homeostasis could be really helpful in HCV therapy. Accordingly, in fact, it was recently demonstrated that the use of a cell-permeant iron chelator and GSH ethyl ester decreased oxidative RNA damage, positive selection, and the nucleotide and amino acid substitution rates of HCV [109]; all these events are involved in the virus resistance to the antiviral therapy. Unfortunately, antioxidants and other strategies decreasing ROS/RNS in HCV patients achieved poor effects. In fact, when being used alone, antioxidants like N-acetylcysteine (NAC), vitamin E, or ascorbic acid ameliorate liver damage but did not affect HCV titer [107]; currently, only seldom, the outcome of antiviral therapy was improved when they were used in combination with IFN [107].

The above-mentioned scarce efficacy could be justified by the fact that the canonical antioxidants are not taken up into mitochondria, the major cellular source of damaging free radicals within cells [61]. To decrease specifically mitochondrial oxidative damage, mitochondria-targeted antioxidants have been developed like mitoquinone (MitoQ) [110, 111]. This novel class of compounds combines the antioxidant potential of ubiquinone with a lipophilic triphenylphosphonium cation, which facilitates the mitochondrial storage [111]. Some studies, both *in vitro* and *in vivo*, have shown that the selective mitochondrial accumulation of MitoQ enhances its antioxidant potential if compared to untargeted antioxidants [112]. This molecule, which accumulates in the liver after oral administration [110], was employed in phase II trials against HCV [113]. Despite its scarce impact on the viral load, it was able to decrease the liver damage. It is conceivable to imagine that the great reduction of OS and inflammation, due to its mitochondrial specificity, may be highly detrimental for the virus persistence.

Another class of molecules that gained great interest as antioxidant and mitochondrially targeted antiviral agents is the analogues of cyclosporine A (CsA), for example, the cyclophilin (Cyp) inhibitors [114]. They are nonimmunosuppressive molecules and have a great anti-HCV potential, as demonstrated by *in vivo* and *in vitro* studies [115–117]. The prototype of this class of molecules is Alisporivir (also known as Debio-025 or DEB025) [118]. This drug, besides its ability to counteract viral replication, was demonstrated to prevent the HCV-related mitochondrial respiration dysfunctions, the collapse of mitochondrial membrane potential, the consequent ROS overproduction, and the mitochondrial calcium overload [119]. Cyp inhibitors are host oriented therapeutic, and for this reason, their use in the common clinical practice

is difficult because of the risk of cell toxicity; even clinical and experimental data push towards this direction.

Another mechanism through which it is possible to counteract the HCV-related OS is the maintenance or the rescue of a correct metals homeostasis. In fact, the positive effects reached through a Zn supplementation on the HCV-related liver damage were described, although viral titer was not affected [120]. Other reports, indeed, describe that phlebotomy treatment, done to reduce the circulating iron content, produces a 2.95 odds ratio of response to IFN therapy [121]. To date, the potential effect of copper reduction in HCV treatment was not yet described, but we need to keep in mind an interesting issue. Recently, the ability of a wide range of natural polyphenols to counteract various steps of HCV life cycle, like cell entry, replication, or spreading was deeply reviewed [122]. Of particular interest among these molecules are quercetin, (−)-Epigallocatechin-3-gallate (EGCG), and Silymarin/Silibinin [122], because they are known to be powerful antioxidants. However, a property of these compounds, too often underestimated, is their ability to bind reactive metals like Fe and Cu [123, 124]. This behavior is extremely interesting, but to date its relevance in HCV treatment has not been yet investigated. Through the metal binding, in fact, polyphenols could exert their antiviral actions via different mechanisms, because not only they can control metals redox reactivity, counteracting the OS occurrence, but also through the modulation of intracellular metal content, they could hinder the activity of some viral proteins, for example, NS3 or NS5A. It could be imagined that polyphenols, reaching and penetrating the hepatocytes, once bound to Fe or Cu, can cause an intracellular rise of metal levels, which can, in turn, counteract the viral replication. Accordingly, it has been reported that increased intracellular levels of Fe and Cu can create a hostile environment for the life cycle of the virus [125, 126].

On these bases, the use of molecules with an antioxidant potential, targeting mitochondria, or being able to bind metals, could be really helpful to eradicate HCV infection, at least as treatments complementary to gold standard therapies. On the other hand, a deeper knowledge of the mechanisms of action of such compounds can reveal new interesting abilities that could lead to the formulation of new efficient therapies with less side effects and major tolerance.

6. Discussion

The hepatitis C virus promotes a prooxidant cellular status through several molecular mechanisms. While at cellular level mitochondria appear to be the most affected organelles, at systemic level the deregulation of trace metals homeostasis is associated with pathological mechanisms involving OS and inflammation, which in turn may be once more correlated with mitochondrial failure. Furthermore, if we consider the fact that HCV infection is associated with a decrease of antioxidant defenses, it leaps to the eyes that the increase of ROS is not efficiently counteracted. Thus, the virus establishes a vicious circle in which the molecules can suffer oxidative damage with the consequent alteration of their physiological functions.

To this regard, an antioxidant therapy could be useful to counteract, at least in part, the pathological consequences caused by HCV-related OS. Unfortunately, until now, antioxidant therapy had scarce effectiveness, either if used alone or combined with interferon (IFN) antiviral treatment [127]. In fact, as previously reported, the main goal reached by antioxidant supplementation is the reduction of OS and inflammatory state caused by HCV, which causes, in turn, a reduction of virus-related liver damage.

On the other hand, we need to keep in mind that HCV induces OS through numerous molecular pathways, for example, mitochondrial damage (Figure 1) and altered metal homeostasis; thus it is difficult to imagine an antioxidant approach with a such wide range of action. Furthermore, it should always be remembered that HCV produces several other effects besides the OS; thus, we think that the antioxidant effects may be helpful to counteract HCV-related damage, but it is likely that they are not so effective if used alone. Probably, the use of antioxidants able to selectively target mitochondria can more efficiently counteract the OS related mitochondrial dysfunctions, thus leading to more powerful healthy effects than those obtained through the use of canonical untargeted antioxidants.

Another potentially effective approach is the use of natural polyphenols to counteract the OS related to HCV infection. Several natural compounds have shown antiviral effects counteracting viral entry (e.g., Honokiol), replication (e.g., Quercetin), or spreading (e.g., Silymarin) [122]. Natural compounds are known to be powerful antioxidants, and some of them are also able to bind Cu and Fe, controlling, in this way, their redox potential [128]. If we consider their abilities, it could be conceivable to imagine that the use of "natural cocktails," obtained by mixing different compounds, each of which is able to counteract a specific aspect of HCV infection (entry, replication, and OS generation), could be effective.

In conclusion, we believe that despite the poor results obtained so far by using antioxidant therapies, this antiviral therapeutic strategy should not to be set aside.

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Clinical Study

The Influence of Long Term Hydrochlorothiazide Administration on the Relationship between Renin-Angiotensin-Aldosterone System Activity and Plasma Glucose in Patients with Hypertension

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Objective. To observe the relationship between changes in renin-angiotensin-aldosterone system (RAAS) activity and blood plasma glucose after administration of hydrochlorothiazide (HCTZ) for one year in patients with hypertension. *Methods*. 108 hypertensive patients were given 12.5 mg HCTZ per day for one year. RAAS activity, plasma glucose levels, and other biochemical parameters, as well as plasma oxidized low density lipoprotein (oxLDL) levels, were measured and analyzed at baseline, six weeks, and one year after treatment. *Results*. After one year of treatment, the reduction in plasma glucose observed between the elevated plasma renin activity (PRA) group (-0.26 ± 0.26 mmol/L) and the nonelevated PRA group (-1.36 ± 0.23 mmol/L) was statistically significant ($P < 0.05$). The decrease of plasma glucose in the elevated Ang II group (-0.17 ± 0.18 mmol/L) compared to the nonelevated Ang II group (-1.07 ± 0.21 mmol/L) was statistically significant ($P < 0.05$). The proportion of patients with elevated plasma glucose in the elevated Ang II group (40.5%) was significantly higher than those in the nonelevated Ang II group (16.3%) ($P < 0.05$). The relative oxLDL level was not affected by the treatment. *Conclusions*. Changes in RAAS activity were correlated with changes in plasma glucose levels after one year of HCTZ therapy.

1. Introduction

The renin-angiotensin-aldosterone system (RAAS) is composed of a series of hormones and corresponding enzymes. By controlling the blood volume and peripheral resistance, RAAS helps maintain the balance between human blood pressure, water and electrolytes, and thus homeostasis. Currently, the levels of plasma renin activity (PRA), angiotensin II (Ang II), and aldosterone (ALD) have become the key indicators for diagnosis, treatment, and clinical research about both primary and secondary types of hypertension. Research has demonstrated that RAAS activation not only was an important mechanism for the development of hypertension, but also could modulate insulin resistance [1]. Patients with hypertension usually exhibit insulin resistance and the risk of diabetes is elevated compared to nonhypertensive patients [2]. Consequently, as first-line antihypertensive medications, diuretics may influence RAAS [3]. However, little research has examined the relationship between changes in RAAS and changes in plasma glucose level. Thus, we examined changes in RAAS activity and plasma glucose in primary hypertensive patients taking HCTZ for one year, with the hope of providing new insight into the study of hypertension and its treatment.

2. Materials and Methods

2.1. Patient Enrollment. From November 2007 to October 2008, 108 patients diagnosed with primary hypertension in Liangshan First People's Hospital in China were recruited. Inclusion criteria are (1) older than 18 years, either sex; (2) Han ethnicity; (3) diagnosis of hypertension based on

World Health Organization and the International Union of Hypertension (WHO/ISH) diagnosis and grading standards issued in 1999: mild to moderate hypertension refers to those who had 3 consecutive systolic blood pressures of 140 to 179 mmHg (1 mmHg = 133.32 Pa) and/or diastolic blood pressures of 90 to 109 mmHg in the sitting position measured on different days. Exclusion criteria are (1) secondary hypertension; (2) severe renal and hepatic dysfunction; (3) severe valvular heart disease, cardiomyopathy, and unstable angina or undergoing coronary artery bypass surgery in 6 months; (4) gout or diabetes mellitus; (5) never taken antihypertensive medications. The ethics committee at each participating center approved the study. All eligible subjects participated voluntarily and written informed consent was obtained from all patients.

2.2. Medication Administration. All 108 patients were given 12.5 mg HCTZ (Southwest Pharmaceutical Co., Ltd., batch number: 0707002 Chongqing, China) by mouth once daily for one year and the follow-up interval was one month. Dosage was increased to 25 mg in patients with inadequate blood pressure control. A variety of biochemical indicators were tested at baseline, six weeks and one year after beginning treatment.

2.3. Clinical Observations

2.3.1. General Data Collection. General information that was collected or calculated included gender, age, height, weight, body mass index (BMI), and previous medical history. Standard program was used to calculate height and body mass index.

2.3.2. Blood Pressure Measurement. Blood pressure was measured while the patient was seated after at least 5 min and had not smoked for 15 min before each measurement. Calibrated mercury sphygmomanometer was used to measure blood pressure. Two weeks before drug administration, three measurements were taken and the mean pressure levels were considered as the baseline pressure. All measurements were standardized: the same time (8:00 a.m. to 9:00 a.m.), arm, sphygmomanometers, and doctor.

2.3.3. Blood Sample Collection and Biochemical Indicators Measurement. Patients avoided vigorous activity and maintained normal eating habits for 3 days prior to blood draws. Blood samples were drawn at 8 am after overnight fasting to measure laboratory biochemical parameters, including blood urea nitrogen (BUN), creatinine (Cr), plasma glucose (GLU), blood potassium (K^+), triglyceride (TG), total cholesterol (CHO), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and very low density lipoprotein cholesterol (VLDL-C). The measurement was performed in the biochemistry laboratory of Sichuan Provincial People's Hospital using automated biochemical analyzer (OLYMPUS AU5400). The blood drawings were conducted at the time of enrollment for baseline, six weeks, and one year after beginning HCTZ treatment.

2.3.4. ELISA. Sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure the plasma level of oxLDL as previously described [4]. Goat anti-human apoB (Sigma, St. Louis, MO, USA) was coated on a 96-well microtiter plate as the capturing antigen. A 1 : 100 dilution of plasma was added to the plate and followed by monoclonal anti-oxPL antibody TEPC-15 (Sigma, St. Louis, MO, USA). The amount of bound oxLDL was detected with biotinylated anti-mouse IgA followed by neutral avidin-alkaline phosphatase (AP). The light emission substrate Lumi-Phos 530 was added and the chemiluminescence was measured by GloMax Luminometer (Promega) and expressed as relative light units (RLU).

2.3.5. Detection of RAAS Activity. For the RAAS activity determinations, venous blood was taken from a vein in the antecubital fossa in the presence of the anticoagulant heparin. Serum angiotensin converting enzyme (ACE) levels and ALD levels were measured in the dual-points ending-point determination method. Diagnostic kits were purchased from Beijing Shizhen Zhongtuo Biotechnology Co., Ltd., or provided by Northern Biotechnology Research Institute. PRA and Ang II levels were measured by radioimmunoassay in venous blood after adding enzyme inhibitors (included in the kit). Diagnostic kits were also provided by Northern Biotechnology Research Institute. All measurements were performed according to the manufacturers' protocols.

2.4. Statistical Analysis. SPSS 13.0 software was used to establish and analyze the database. Numeric variable data is expressed as mean ± standard deviation. Paired t-test was used to compare the values at baseline and those after one year of treatment. Levels of plasma GLU and blood K^+ in different RAAS groups were compared with Two-Sample Comparison t-test. Chi-square test and multivariate linear regression analysis were used for multifactorial variables. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Patients. All 108 patients (68 males, 40 females) completed this study during their one-year drug administration and followups. The average age was 57.0 ± 8.1 years, and mean BMI was 27.2 kg/m^2 ± 3.4 kg/m^2. After treatment with HCTZ for one year, GLU, CHO, LDL-C, PRA, and Ang II levels in all patients were significantly decreased and HDL-C levels were increased significantly. However, there were no significant changes in other indexes (Table 1).

3.2. Relationship between GLU Concentration and RAAS Activity. According to the changes in RAAS activity after one year of medication, patients were divided into elevated and nonelevated RAAS groups. Glucose concentrations and changes were compared between these two groups (Table 2).

There were no statistically significant differences ($P > 0.05$) between the GLU concentrations of patients with elevated PRA and Ang II levels and those in the nonelevated patient groups (Table 2), despite the increasing tendency.

Table 1: Clinical characteristics of patients after treatment for one year (mean ± sd).

Indexes	Baseline level	Level after one year of medication	t	P
BUN/mmol/L	5.60 ± 1.67	5.58 ± 1.38	0.111	0.912
Cr/μmol/L	90.68 ± 16.61	91.56 ± 16.51	−0.603	0.548
GLU/mmol/L	6.21 ± 2.37	5.28 ± 2.51	6.053	0
K^+/mmol/L	4.62 ± 0.60	4.53 ± 0.49	1.398	0.165
TG/mmol/L	1.84 ± 0.10	1.78 ± 0.12	0.543	0.588
TC/mmol/L	6.04 ± 1.08	5.35 ± 1.11	6.537	0
HDL-C/mmol/L	1.49 ± 0.39	1.89 ± 0.72	−6.132	0
LDL-C/mmol/L	3.69 ± 1.09	2.26 ± 0.78	10.783	0
VLDL-C/mmol/L	0.37 ± 0.21	0.41 ± 0.57	−0.895	0.372
oxLDL (RLU)	6591 ± 1592	6075 ± 2223	−1.677	0.187
PRA/ng/mL·h	1.89 ± 1.50	1.46 ± 1.31	2.538	0.013
ACE/U/L	39.60 ± 17.52	41.25 ± 21.81	−0.951	0.344
AngII/pg/mL	67.58 ± 32.17	58.29 ± 44.59	2.482	0.015
ALD/ng/L	152.06 ± 53.14	144.78 ± 68.49	0.802	0.424

Table 2: Comparison of GLU concentrations and changes between paired RAAS activity groups after one year of medication (mean ± sd).

RAAS activity after 1 year of medication	Groups	GLU baseline (mmol·L^{-1})	ΔGLU* (mmol·L^{-1})
PRA	Elevated	5.71 ± 3.05	−0.26 ± 0.26
	Nonelevated	5.12 ± 2.44	−1.36 ± 0.23
	t	−0.981	−3.502
	P	0.329	0.003
ACE	Elevated	5.24 ± 2.39	−0.76 ± 0.23
	Nonelevated	5.29 ± 2.63	−1.09 ± 0.19
	t	0.116	−1.083
	P	0.908	0.281
Ang II	Elevated	5.58 ± 2.91	−0.17 ± 0.18
	Nonelevated	5.23 ± 2.59	−1.07 ± 0.21
	t	−0.576	−2.865
	P	0.566	0.005
ALD	Elevated	5.41 ± 2.80	−0.67 ± 0.28
	Nonelevated	5.18 ± 2.44	−1.12 ± 0.19
	t	−0.441	−1.338
	P	0.66	0.184

*Δ refers to mean changes when compared with baseline level after one year of hydrochlorothiazide.

However, the GLU concentration reductions in patients with elevated PRA and Ang II levels were statistically significantly lower ($P < 0.05$) than those in the nonelevated patient groups. The reductions of GLU concentration in patients with elevated ACE and ALD concentration were lower than those in nonelevated patients; however, the differences were not statistically significant ($P > 0.05$).

3.3. Relationship between Changes in GLU and RAAS Activity after Medication. According to changes in RAAS and GLU levels after 1 year of medication, patients were divided into either elevated or nonelevated groups. The proportions of patients with both elevated RAAS activity and GLU concentrations were determined. Results are shown in Table 3, in which we demonstrated that there was a statistically significantly higher ($P < 0.05$) proportion of patients with a higher GLU in the Ang II elevated group compared with those in the Ang II nonelevated group.

3.4. Multivariate Analysis of GLU Concentration after Treatment. After one year of medication, multivariate analysis was performed using the change of GLU levels as dependent variable against factors that may affect the GLU changes resulting from medication, including gender, age, BMI, baseline GLU level, RAAS changes, and changes in serum K^+, into the linear regression equation. The results showed that after adjustment for other factors, the serum Ang II levels were independently

Table 3: Relationship between changes in RAAS and changes in plasma glucose after one year of HCTZ therapy.

RAAS activity		After one year of medication		χ^2	P
		GLU elevated patients	GLU nonelevated patients		
PRA	Elevated group	13	32		
	Nonelevated group	17	58	0.696	0.404
ACE	Elevated group	19	43		
	Nonelevated group	12	45	1.226	0.268
Ang II	Elevated group	17	25		
	Nonelevated group	13	67	8.023	0.005
ALD	Elevated group	16	34		
	Nonelevated group	14	55	2.109	0.146

Data in Table 3 demonstrates that there was a statistically significantly higher ($P < 0.05$) proportion of patients with a higher GLU in the Ang II elevated group compared with the Ang II nonelevated group.

Table 4: Multivariate analysis of the change* in plasma GLU level after treatment.

Variable	β	SE	t	P
Gender	0.094	0.346	0.877	0.383
Age	−0.052	0.021	−0.054	0.616
BMI	−0.115	0.05	−1.083	0.282
Baseline GLU	−0.037	0.07	−0.347	0.729
Blood K^+ change	−0.178	0.23	−1.757	0.083
PRA change	0.079	0.139	0.597	0.552
ACE change	0.181	0.009	1.705	0.092
Ang II change	0.283	0.005	2.616	0.011
ALD change	0.194	0.002	1.744	0.085

*The change of GLU was used as the dependent variable for the analyses.

associated with GLU level after taking HCTZ for one year (Table 4).

4. Discussion

RAAS is one of the main mechanisms through which the body regulates water and salt metabolism. Its activation not only plays an important role in the pathogenesis of hypertension [5], but also can affect insulin resistance. Studies conducted by Scheen [6] have shown that excessive RAAS activity, acting synergistically with microcirculatory changes, can affect pancreas, the major insulin secreting organs, and insulin sensitivity [7] and impair cellular responses to insulin signaling, thereby affecting GLU metabolism. The inhibition of RAAS can increase the adiponectin concentration [8], thus improving B cell function [9] and insulin sensitivity. Studies have also shown that the prevalence of diabetes in hypertensive patients is about 4% to 36% [10], more than in normal patients (3.62%). The prevalence rate of hypertension in patients with impaired glucose tolerance or diabetes was 2 to 3 times that in nondiabetic patients. These facts suggested that a relationship between RAAS activation and glucose metabolism existed and prompted increasing attention drawn to cardiovascular drugs which could affect RAAS activity. As a common diuretic, thiazides can lower blood pressure by reducing blood volume; however, they may also activate RAAS through negative feedback.

Our study showed that there was less reduction in GLU concentrations in patients with elevated Ang II, and the proportions of patients with elevated GLU were higher than those in patients in whom Ang II was not elevated. Multivariate analysis showed that changes in Ang II concentrations were positively correlated with changes in GLU concentrations; that is, there was a statistically significantly smaller decrease in GLU concentrations in patients who had a smaller reduction in Ang II after taking HCTZ for one year ($P < 0.05$). Our results also provided other evidence to confirm that changes in RAAS activity were positively correlated with change in GLU concentration: the higher the RAAS activity, the higher the GLU concentration. These results also suggest that when providing treatment to diabetic patients with hypertension, in order to achieve the desired therapeutic effects, lowering both the GLU concentration and RAAS activity should be considered simultaneously.

We did not observe a relationship between RAAS activity and blood K^+ concentrations (data not shown) after administering HCTZ for one year, while previous publications showed that the influence of thiazide diuretics on glucose metabolism was related to reductions in blood K^+ concentration. We think that the reasons for this inconsistency may include that (1) thiazide diuretics may exert their effects on glucose metabolism through mechanisms unrelated to RAAS activity; (2) effects of diuretics are dosage-related, and the dosage of HCTZ in this study was 12.5 mg daily, a relatively low dosage. This dosage may not have been high enough to influence blood K^+ concentration; (3) a differential response to HCTZ between the domestic population and patients from other countries could not be ruled out.

We did not observe significant effect on plasma oxLDL level before and after one-year HCTZ treatment. Although the trend indicates a slight reduction of oxLDL, this may be due to the more consciousness of the patients during the treatment towards the more healthy diet.

5. Conclusions

There was a correlation between changes in blood Ang II levels and changes in blood GLU concentrations in patients with hypertension after one year of hydrochlorothiazide

administration. This result suggests that, when treating patients with hypertension with thiazide diuretics, attention must also be paid to controlling RAAS activity to avoid the negative impact of the drug on GLU metabolism. As there was a one-year follow-up period in this study, longer-term follow-up may be necessary to confirm this conclusion.

6. Summary

What is known about this topic is the following.

(i) RAAS is one of the main mechanisms through which the body regulates water and salt metabolism. Its activation not only plays an important role in the pathogenesis of hypertension [5], but also can affect insulin resistance.

(ii) Excessive RAAS activity, acting synergistically with microcirculatory changes, can affect pancreas, the major insulin secreting organs, and insulin sensitivity [7] and impair cellular responses to insulin signaling, thereby affecting GLU metabolism.

What this study adds is the following.

(i) We found a positive correlation between changes in blood Ang II levels and changes in blood GLU concentrations in patients with hypertension after one year of hydrochlorothiazide administration. When treating patients with hypertension with thiazide diuretics, attention must also be paid to controlling RAAS activity to avoid the negative impact of the drug on GLU metabolism.

Conflict of Interests

The authors declare that no conflict of interests exists in this study.

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Clinical Study

Serum Antioxidative Enzymes Levels and Oxidative Stress Products in Age-Related Cataract Patients

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Purpose. To investigate the activity of antioxidative enzymes and the products of oxidative stress in patients with age-related cataracts and compare the findings with those in healthy control subjects. *Method*. Sixty patients with age-related cataract and sixty healthy controls of matched age and gender were included in this study. Serum samples were obtained to detect the antioxidative enzymes of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and oxidation degradation products of malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), conjugated diene (CD), advanced oxidation protein products (AOPP), protein carbonyl (PC), and 8-hydroxydeoxyguanosine (8-OHdG). *Results*. Serum SOD, GSH-Px, and CAT activities in cataract group were significantly decreased as compared to the control subjects ($P < 0.05$). The levels of MDA, 4-HNE, and CD in cataract patients were significantly higher than those in the control subjects ($P < 0.05$, $P < 0.01$). Cataract patients had higher levels of 8-OHdG, AOPP, and PC with respect to the comparative group of normal subjects ($P < 0.01$). And there was no statistical significance in concentration of antioxidative enzymes and oxidative stress products in patients with different subtype cataract. *Conclusions*. Oxidative stress is an important risk factor in the development of age-related cataract, and augmentation of the antioxidant defence systems may be of benefit to prevent or delay cataractogenesis.

1. Introduction

The age-related cataract is an ever-increasing visual problem that accounts for approximately 50% of blindness worldwide [1]. Epidemiologic studies have indicated that half of the general population older than 65 has cataract [2]. In developing countries, 50–90% of all blindness is caused by cataracts [3]. In the United States more than two million lens extractions are performed annually with the attendant significant health care costs, consuming 12% of the Medicare budget, and a steady increase is projected [4, 5]. Over 50 million people worldwide suffer from cataracts and the number will increase as individuals in the current generation grow older [6, 7]. Currently, there is no effective medical treatment for cataract except surgery. For these reasons, there is much interest in the prevention of cataract as an alternative to surgery. The development of age-related cataract is a slow process; the exact mechanism of cataract formation has not been clearly defined [3]. Multiple mechanisms have been implicated in the development of cataract formation such as excessive tissue sorbitol concentrations, abnormal glycosylation of lens proteins, and increased free-radical production in the intraocular region. They may result in an increasing clouding of the lens until the whole lens loses its normal transparency and becomes white and opaque [8]. There is increasing evidence that oxidative stress has been implicated in the development

of age-related cataract. Biochemical evidence demonstrates that the oxidative damage of the lens proteins is involved in the genesis of age-related cataract. In particular, the lens proteins are subjected to extensive oxidative modifications [9–14].

The pathogenesis of cataract is known to be influenced by a number of factors including oxidative stress. Oxidative stress is essentially an imbalance between the production of various reactive species and the ability of the organism's natural protective mechanisms to cope with these reactive compounds and prevent adverse effects. The reactive oxygen species (ROS), which consist principally of molecules like the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals, are detoxified by enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). The antioxidant system and the amount of ROS are kept in a certain state of homeostasis. When exogenous or endogenous factors increase oxidative stress, homeostasis is disturbed and the ROS denature many basic intracellular molecules like nucleic acids, proteins, and lipids. Oxidative stress is widely acknowledged to be a major initiating factor in the development of age-related cataracts [9–14].

Taking these studies into account, this study is to evaluate the levels of antioxidative enzymes and oxidative stress products in age-related cataract patients and investigate the relationship between oxidative stress and age-related cataract.

2. Materials and Methods

2.1. Subjects. Sixty patients with newly diagnosed senile nonpathologic cataract are recruited for the experiment group, among whom 25 patients have cortical cataract, 21 patients have nuclear cataract, and 14 patients have posterior subcapsular cataract. All patients with cataract had severe visual disturbances, and their corrected visual acuities were under 0.3 and had an age-related cataract in at least one eye;they also had no other eye abnormalities that could explain the vision loss. These patients were recruited from the First Affiliated Hospital of the Harbin Medical University and the Eye Hospital of Heilongjiang Province. We excluded patients with secondary cataract due to diabetes, trauma, steroid administration, and other causes. Both groups belonged to the same ethnic group. Sixty healthy, age- and sex-matched subjects were also included for the control. The control subjects were recruited from subjects who came to the same hospital for an annual refractive checkup. The control subjects were in good health, as determined by a medical history questionnaire, physical examination, and normal results of clinical laboratory tests, such as glucose, cholesterol, triglycerides, and blood pressure. None of the study subjects had a history of cardiovascular, hepatic, gastrointestinal, or renal dysfunction; none were alcoholic, smokers; and none used exogenous hormones. Subjects were not permitted to take any supplemental vitamin or carotenoid for more than 6-week before the start of the study and were limited to drinking less than two cups of tea per day during this 6-week period.

All the subjects underwent a complete ophthalmologic evaluation that included medical history, slit lamp biomicroscopy, Goldmann applanation tonometry, and funduscopy. Ethics Committee of Harbin Medical University has already approved the study protocol. Written informed consent was obtained from each study subject, and all subjects consented to giving blood samples.

2.2. Blood Sampling. Venous peripheral blood samples (10 mL) were collected after 12 h overnight fasting from each subject. The samples were placed on ice and centrifuged for an hour at 3500 rpm, 4°C for 15 min, and the supernatants were stored at −80°C, and determination of the samples occurred within 3 months. All samples from each patient were run in the same assay.

3. Laboratory Analysis

3.1. Measurement of Activity of Antioxidative Enzymes. SOD activity was determined using the method of Sun et al. [15]. Glutathione peroxidase (GSH-Px) activity was measured by the method ofPaglia and Valentine [16]. Catalase (CAT) activity was assayed based on the procedure ofAebi [17].

3.2. Measurement of Lipid Peroxidation Products. Quantitative estimation of products of lipid peroxidation included assays for conjugated dienes (CD) and malondialdehyde (MDA). CD were extracted from plasma using a 2 : 1 (vol/vol) mixture of chloroform and methanol. Four mL of the chloroform-methanol mixture, preheated to 45°C, was added to 0.1 mL of serum. The mixture was then vigorously mixed (with a vortex machine) for 2 min, then mixed with 2.0 mL of distilled water acidified with 0.1 M HCl to a pH of 2.5. After agitation that used a vortex instrument, the material was subjected to centrifugation (2,000 g for 5 min), and 1.5 mL of the lower layer was aspirated, transferred to a test tube, and dried under a flow of nitrogen gas. The residue was reconstituted with 1.0 mL of heptane and measured spectrophotometrically at 233 nm.

MDA was measured as thiobarbituric-acid-reacting substance (TBARS) production in the following manner. 0.1 mL of sample was added to a 1 : 1 : 1 (vol/vol/vol) solution of trichloroacetic acid (15%, wt/vol), thiobarbituric acid (0.375%, wt/vol), and hydrochloric acid (0.25 M). The mixture was heated at 100°C for 30 min. The mixture was immediately cooled and then centrifuged (3,500 g for 5 min) to remove undissolved materials. Then the absorbance at 532 nm was determined. The amount of TBARS was calculated from comparison with authentic malondialdehyde.

3.3. Measurement of Protein Damage Products. Products of protein damage included advanced oxidation protein products (AOPP) and protein carbonyl content. AOPP were quantified as described byWitko-Sarsat et al. [18]. We placed 200 μL of serum diluted 1 : 5 in phosphate-buffered saline into each well of a 96-well microtitre plate and added 20 μL of acetic acid to each well. For the standards, we added 10 μL of 1.16 M potassium iodide (Sigma, St Louis, MO, USA)

to 200 μL of chloramine-T solution (0 to 100 μmol/L) (Sigma, St Louis, MO, USA) in a well and then added 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank consisting of 200 μL of phosphate-buffered saline, 10 μL of 1.16 M potassium iodide, and 20 μL of acetic acid. AOPP concentrations are expressed as μmol/L of chloramine-T equivalents.

Protein carbonyl (PC) concentrations in plasma were measured by the spectrophotometric assay described by Reznick and Packer [19]. Briefly, to 200 μL plasma, 4 mL of 10 mmol 2,4-dinitrophenylhydrazine (DNPH)/L in 2 mol HCl/L was added. In another tube, 4 mL of 2 mol HCl/L was added to 200 μL plasma (of the same patient). The tubes were left in the dark for 1 h at room temperature and were mixed by vortex every 15 min. Five milliliters of 20% trichloroacetic acid solution was then added to both tubes for a 10 min incubation on ice, after which the tubes were centrifuged (3000 ×g, 5 min, 4°C). The supernatant fluid was discarded and another wash was performed by using 4 mL 10% trichloroacetic acid. The protein pellets were broken mechanically and washed 3 times with ethanol-ethyl acetate to remove free DNPH and lipid contaminants. The final precipitates were dissolved in 2 mL of 6 mol guanidine hydrochloride/L, and the UV absorbance at $\lambda = 370$ nm was measured spectrophotometrically. The carbonyl content (nmol/mL) was calculated using $\varepsilon_M = 22,000$.

3.4. Measurement of Serum 8-OHdG and 4-Hydroxynonenal. Serum 8-OHdG and 4-HNE were measured with enzyme-linked immunosorbent assay (ELISA) method following the maker's instructions. Quantification of the 8-OHdG and 4-HNE was achieved by comparing the optical densities of each sample to that of an internal standard of known 8-OHdG and 4-HNE at various concentrations.

The assay variances of all methods described above were <10%.

4. Other Biochemical Parameters

Blood glucose, triglycerides, and cholesterol were determined using routine clinical chemical assays.

Statistical Analysis. Data are presented as mean ± SD. All experimental data in this study were statistically analyzed with SAS 9.13. The statistical significance was evaluated using unpaired Student's t-test. Results were considered significant at $P < 0.05$.

5. Results

5.1. Baseline Characteristics. The clinical characteristics of the age-related cataract patients and control subjects are shown in Table 1. Age and gender of the patients were not significantly different from those of the controls. Initial clinical laboratory test values, such as glucose, cholesterol, triglycerides, and blood pressure, were also not significantly different between the two groups. Furthermore, the test values were all in normal range.

Table 1: Clinical characteristics of age-related cataract patients and control.

Demographics	Health group	Cataract patients
N	60	60
Age (years)	58.9 ± 8.4	61.0 ± 10.3
Sex (F/M)	30/30	32/28
Cholesterol (mmol/L)	5.01 ± 0.69	4.97 ± 0.63
Triglycerides (mmol/L)	1.16 ± 0.38	1.14 ± 0.32
Glucose (mmol/L)	4.65 ± 0.53	4.68 ± 0.70
Systolic blood pressure (mmHg)	126.4 ± 6.6	125.5 ± 6.7
Diastolic blood pressure (mmHg)	78.9 ± 5.1	78.2 ± 6.4

Data are means ± SD.

5.2. Results of Activity of Antioxidative Enzymes in Serum. As shown in Table 2, we found the depression of antioxidative enzymes in serum of patients. Compared with the control group, the activities of SOD, GSH-Px, and CAT in cataract group were lower than those in the control group ($P < 0.05$, $P < 0.01$).

5.3. Results of Products of Lipid Peroxidation in Serum. As shown in Table 3, there was a significant increase in serum MDA in cataract patients (3.75 ± 1.11 nmol/mL) compared with normal subjects (3.28 ± 1.03 nmol/mL, $P < 0.05$). Serum conjugated dienes in cataract patients have also significantly increased than those in control (0.488 ± 0.125 versus 0.418 ± 0.122, $P < 0.01$). And the concentration of 4-HNE significantly increased in patients' serum compared with normal subjects (15.33 ± 4.63 versus 13.41 ± 3.48, $P < 0.05$).

5.4. Results of Protein Oxidative Damage Products in Serum. As shown in Table 4, the increased levels of AOPP as well as protein carbonyl in cataract patients were also observed. The concentration of AOPP in cataract patients was increased significantly than that in control subjects (20.83 ± 5.38 versus 17.81 ± 6.07; $P < 0.01$). Cataract patients had significantly higher protein carbonyl compared with healthy subjects (3.37 ± 1.04 versus 2.90 ± 0.68; $P < 0.01$).

5.5. Results of DNA Damage Products in Serum. There was a significant increase in serum 8-OHdG in cataract patients (22.13 ± 4.80 ng/mL) compared with age-matched normal subjects (6.33 ± 1.73 ng/mL, $P < 0.01$).

5.6. Results of Antioxidative Enzymes in Subtype Cataract Patients. We divided 60 patients with age-related cataract into 3 groups, depending on a type of cataract. The results of antioxidative enzymes in subtype cataract patients were shown in Table 5. There were no statistically significant differences among patients with different subtype of age-related cataract ($P > 0.05$).

Table 2: Serum activity of SOD, GSH-Px, and CAT.

Group	SOD (U/mL)	GSH-Px (μmol/L)	CAT (U/mL)
Control	103.47 ± 18.97	147.90 ± 20.21	5.75 ± 1.30
Cataract	97.26 ± 13.56*	135.75 ± 32.45*	5.05 ± 1.46**

Data are means ± SD. *$P < 0.05$ versus healthy subject, **$P < 0.01$ versus healthy subject.

Table 3: Serum levels of MDA and conjugated dienes.

Group	MDA (nmol/mL)	4-HNE (nmol/mL)	Conjugated dienes (OD, 233 nm)
Control	3.28 ± 1.03	13.41 ± 3.48	0.418 ± 0.122
Cataract	3.75 ± 1.11*	15.33 ± 4.63*	0.488 ± 0.125**

Data are means ± SD. *$P < 0.05$ versus healthy subject, **$P < 0.01$ versus healthy subject.

Table 4: Serum levels of AOPP and protein carbonyl.

Group	AOPP (μmol/mL)	Protein carbonyl (nmol/mL)
Control	17.81 ± 6.07	2.90 ± 0.68
Cataract	20.83 ± 5.38**	3.37 ± 1.04**

Data are means ± SD. **$P < 0.01$ versus healthy subject.

Table 5: The results of antioxidative enzymes in subtype cataract patients.

	Cortical cataract	Nuclear cataract	Posterior subcapsular cataract
SOD (U/mL)	98.05 ± 12.35	96.31 ± 15.65	97.26 ± 13.17
GSH-Px (U/mL)	130.52 ± 33.60	140.57 ± 30.97	137.88 ± 33.54
CAT (U/mL)	4.93 ± 1.43	5.15 ± 1.50	5.09 ± 1.56

Data are means ± SD.

5.7. Results of Oxidative Stress Products in Subtype Cataract Patients. We divided 60 patients with age-related cataract into 3 groups, depending on a type of cataract. As shown in Table 6, the results of oxidative stress products in subtype cataract patients showed no statistically significant differences between the three groups ($P > 0.05$).

6. Discussion

Cataract is the major cause of blindness and visual impairment worldwide. According to the anatomical location within the lens, cataract can be subdivided into three subtypes: cortical cataract, nuclear cataract, and posterior subcapsular cataract. The prevalence of the 3 main types of age-related cataracts differs in different regions of the world and in different racial groups. In most tropical areas, nuclear cataracts are the most common. In northern China, cortical cataracts are more prevalent. The incidence of posterior subcapsular cataract is lower than that of nuclear and cortical cataract [20, 21]. Whether these differences are related to differences in genetics, environment, diet, or other factors is difficult to discern; oxidative damage has been implicated as a major contributor to the pathogenesis of age-related cataracts.

Numerous scientific investigations have confirmed the presence of oxidative stress in ocular diseases, and ROS may play a significant role for the pathophysiology in cataracts. ROS have physiological functions at low levels but are toxic to the cell at high levels. To protect against toxic effects of ROS and to modulate physiological effects of ROS, the cell has developed antioxidant defence systems. The systems are very complex, being composed of antioxidative enzymes (such as SOD, GSH-Px, and CAT) and antioxidant compounds (vitamins A, C, E, and so on). SOD decomposes superoxide into hydrogen peroxide. CAT reduces H_2O_2 to water. GSH-Px reduces all organic lipid peroxides. They protect cells against ROS produced during normal metabolism and after an oxidative insult. Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defence systems [22]. ROS is mostly generated within the mitochondria in lens epithelium cells and the superficial fiber cells, which are highly reactive and can damage macromolecules in living cells, such as lipids, proteins, and nucleic acids, causing mutagenesis and cell death [23–25].

The lens epithelium cell (LEC) is the center of metabolic activities in lenses, and oxidative damage to LECs plays a significant role in the pathogenesis of many forms of cataracts [14, 25]. At normal conditions, LECs use several strategies to maintain ROS at low levels to protect lipids, proteins, and nucleic acids. These strategies include activation of the ROS scavenger enzymes such as SOD, CAT, GSH-Px, and DNA repair enzymes. These antioxidative enzymes are present in all parts of the lens, which protect the lens from oxidative stress and maintain lens clarity [26, 27]. However, there is a diminution of these ROS scavenger enzymes and decreased DNA repair capability, placing the lens at risk for oxidative damage and cataract [28]. It has been demonstrated that antioxidant enzymes levels are altered in cataracts. Some reports showed that the activity of SOD, GSH-Px, and CAT decreased in cataract [29, 30], but there were other reports with conflicting results [31]. In the present study, we found that there was a significant decrease in the activity of SOD, GSH-Px, and CAT in serum of cataract patients, compared with normal control. But there is no significant difference in subtype cataracts. This result leads us to think that oxidative stress is an important mechanism in the development of cataracts.

Oxidants are highly reactive compounds with a half-life of only seconds. Therefore,their in vivo determination is generally not feasible. In contrast, lipids, proteins, carbohydrates, and DNA, after being modified by oxyradicals,having lifetimes ranging from hours to weeks, can be measured with biochemical assays, which makes them ideal markers of oxidative stress. Many biomarkers have been developed to evaluate oxidative stress. These markers include lipid peroxidation products (such as acrolein, MDA, CD, and 4-hydroxynonenal), protein oxidation products (such as AOPP, PC), and DNA oxidation products 8-hydroxy-2-deoxyguanosine (8-OHdG). Oxidation of proteins, lipids, and DNA has been observed in cataractous lens [32–34].

Oxidation of lipids has been widely studied, and there are more biomarkers available for assessing oxidation of this substrate than for protein and DNA combined. Lipid

Table 6: The results of oxidative stress products in subtype cataract patients.

	Cortical cataract	Nuclear cataract	Posterior subcapsular cataract
MDA (U/mL)	3.57 ± 1.22	3.75 ± 1.12	4.06 ± 0.89
CD (OD, 233 nm)	0.496 ± 0.127	0.511 ± 0.119	0.440 ± 0.129
4-HNE (nmol/mL)	15.20 ± 5.00	15.18 ± 3.47	15.78 ± 5.68
AOPP (U/mL)	20.66 ± 6.51	21.27 ± 4.69	20.50 ± 4.33
PC (mg/L)	3.40 ± 1.11	3.14 ± 0.93	3.63 ± 1.06
8-OHdG (ng/mL)	21.88 ± 5.09	23.04 ± 4.17	21.22 ± 5.24

Data are means ± SD.

peroxidation is initiated by free-radical attack of membrane lipids, generating large amounts of reactive products, which have been strongly implicated in the mechanisms of cataractogenesis [35–37]. MDA, 4-HNE, and CD are three markers of lipid peroxidation, widely used in large studies. MDA is a product of the breakdown of mainly unsaturated fatty acids into their essential chains through the oxidation mechanism. 4-HNE derives from ω-6 polyunsaturated fatty acids like linoleic and arachidonic acids whose conjugated double bonds are an easy target for species that can extract a hydrogen atom or add to a double bond. CD is the initial formation of a lipid peroxide. Some studies have revealed the increased lipid peroxidation products in human cataractous lenses. Micelli-Ferrari et al. [36] reported that increased levels of MDA were observed in cataractous lenses of cataract patients compared with the control. Babizhayev [37] reported that CD was distinctly accumulated in cataractous lenses of cataract patients compared with the control. In this study, we have demonstrated that the products of lipid peroxidation, MDA, 4-HNE, and CD, increased significantly in serum of cataract patients compare with that of control. Even though the certain mechanisms responsible for lipid peroxidation-mediated cataractogenesis are not clear, the mechanisms for defence against lipid peroxidation can be perceived as one of the major deterrents of cataract caused by the oxidative stress.

Biomarkers of protein oxidation are often applied when a battery of markers of oxidative stress status is being studied. Several protein modifications may result from oxidative stress and lead to formation of the high-molecular-weight insoluble aggregates that are common in cataractous lenses [38]. The biomarker that is generally used to estimate protein oxidation is protein carbonyl (PC), which is derived from amino acids during metal-catalyzed oxidation of proteins in vitro and in vivo, representing a direct measure of the oxidative injury to these molecules [39]. The concentration of PC was stable, yielded quantitative results, and appeared to reflect disease endpoints in a biologically significant way. Advanced oxidation protein products (AOPP), a new marker of protein oxidation, have begun to attract the attention of various investigators [18, 40, 41]. They are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by myeloperoxidase in activated neutrophils). They are elevated in patients with renal insufficiency and diabetes mellitus [42, 43]. In our previous study, we found elevated AOPP in diabetic retinopathy [44]. Elevated markers of protein oxidation have also been associated with cataractogenesis, [45]. In the present study, we have also determined the level of AOPP and protein carbonyl, two products of protein oxidation, which were increased, and demonstrated that there was protein oxidative damage in cataracts. To the best of our knowledge, no studies have identified the relationship between AOPP and cataracts. Our results show that AOPP is a biomarker available for assessing oxidative stress in age-related cataracts.

DNA bases are very susceptible to ROS oxidation; the most commonly used biomarker for assessing oxidative DNA damage is 8-hydroxy-2-deoxyguanosine (8-OHdG). DNA can be oxidized to produce many oxidative products; however, oxidation of the C-8 of guanine is one of the most common oxidative events and results in a mutagenic lesion that produces predominantly G-to-T transversion mutations. 8-OHdG was found to be increased in a normal human LEC culture after induced oxidative stress [46]. Ates et al. [47] confirmed a significantly higher level of leukocyte 8-OHdG in the patients with cataract than in the control patients. Some studies have showed association of DNA damage with human cataracts [25, 48]. One important study showed association of DNA damage with human cataracts [33]. In another study, Sorte et al. [49] found that there was significant DNA damage in LECs of senile cataract patients. Moreover, DNA damage in cortical cataracts was significant when compared to that of nuclear or posterior subcapsular cataracts, but the DNA damage between nuclear and posterior subcapsular cataracts was not significant. In our study, we found an overall elevation of serum 8-OHdG in patients with cataract, but there were no statistically significant differences among patients with different type of age-related cataract. These data suggest that 8-OHdG levels are a potentially useful marker of oxidative DNA damage in cataract patients.

7. Conclusions

In the present study, we found that there is a significant disequilibrium status of antioxidative systems in serum in the age-related cataract patients. Compared with the control group, the activities of SOD, GSH-Px, and CAT in cataract group were lower than those in the control group and the oxidative stress products MDA, 4-HNE, CD, AOPP, PC, and 8-OHdG were significantly increased in serum in cataract patients. But there was no statistically significant difference among patients with different subtype of age-related cataract. Our results obtained here confirm that oxidative stress,

present or initiating factor in all three types of cataract, is involved in the development of cataract, and augmentation of the antioxidant defences may be helpful to prevent or delay cataractogenesis.

Authors' Contribution

Dong Chang and Xuefei Zhang contributed equally to this work.

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