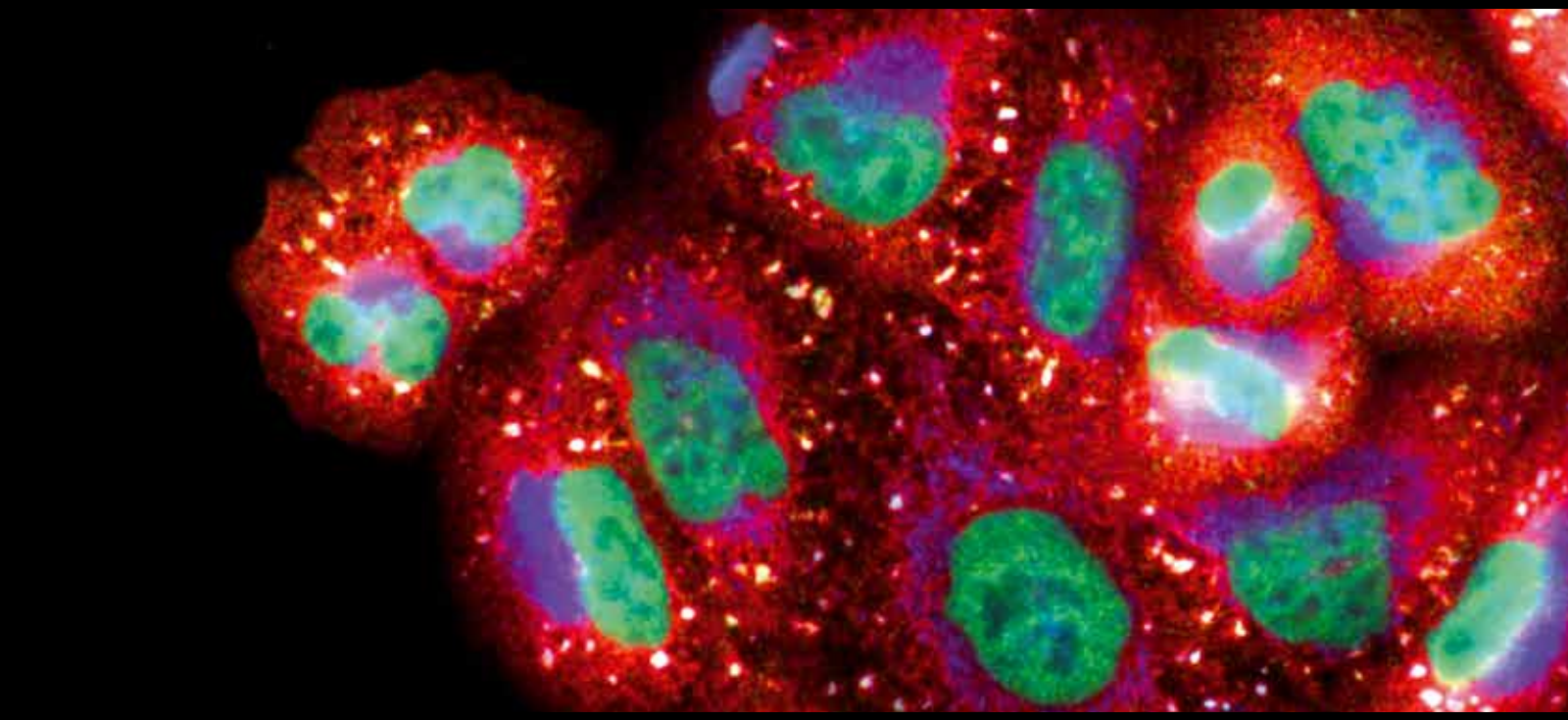
Oxidative Medicine and Cellular Longevity

Nucleic Acid Oxidation in Human Health and Disease

Guest Editors: Mu-Rong Chao, Pavel Rossner Jr., Siamak Haghdoost, Hueiwang Anna Jeng, and Chiung-Wen Hu



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Editorial Board

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Editorial

Nucleic Acid Oxidation in Human Health and Disease

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Growing scientific evidence suggests that oxidative stress plays an important role in human health and disease. Under oxidative stress, the excess levels of reactive oxygen species (ROS) may lead to modification of cellular nucleic acids. Oxidatively damaged DNA has been recognized in association with the development of aging, cancer, and some degenerative diseases. The topics covered in this special edition give some insight into how oxidatively damaged DNA is involved in human disease and its health impacts. This special issue contains five papers. Two papers are related to human disease, including Keshan disease and chronic kidney disease, while two papers cover the underlying mechanisms of inflammation-related carcinogenesis and the association between DNA damage and antioxidant capacity in humans. Finally, a paper summarizes the literature regarding the effect of phytoagents on nucleic acid oxidation in cancer cells.

Keshan disease (KD) is an endemic cardiomyopathy of unknown etiology affecting inhabitants of a narrow belt between Northeast China and Southwest China. This region is known for low levels of selenium in the environment as well as in food. In an original research article entitled "*Oxidative stress is involved in the pathogenesis of Keshan disease (an endemic dilated cardiomyopathy) in China,*" J. Pei et al. demonstrate that oxidative stress in the myocardium may play a crucial role in KD. The authors found elevated 8-oxodG levels in myocardial nuclei of the KD patients. Moreover, the expression of glutathione peroxidase 1 and thioredoxin reductases 1 (selenoproteins) was important in the antioxidant system of the organism and higher in the control group than in patients suffering from KD.

In a review article entitled "*Oxidative stress and nucleic acid oxidation in patients with chronic kidney disease,*" C.-C. Sung et al. provide a systematic review of the role of oxidative stress in chronic kidney disease (CKD). It begins with the mechanisms of radical production, antioxidant defense, pathogenesis, and recent biomarkers of oxidative stress in CKD patients. The authors also summarized and evaluated the potential benefit of antioxidant therapies in CKD patients, although their value as useful therapeutic tools is being tested and future studies are necessary to validate their prospective beneficial effects on CKD.

Infectious agents (e.g., parasites and viruses) have been identified as carcinogenic to humans (IARC, group 1). The underlying mechanism of their carcinogenicity includes inflammation accompanied by generation of reactive oxygen (ROS) and nitrogen species (RNS). In a review article entitled "*DNA damage in inflammation-related carcinogenesis and cancer stem cells*," S. Ohnishi et al. propose a model by which

chronic inflammation by infectious agents induces generation of cancer stem cells. The authors suggest that tissue injury caused by ROS/RNS may activate progenitor/stem cells for regeneration. Oxidative stress may then cause multiple mutations which may result in the formation of mutant stem cells and cancer stem cells thus leading to carcinogenesis.

In an original research article entitled "*Is the oxidative DNA damage level of human lymphocyte correlated with the antioxidant capacity of serum or the base excision repair activity of lymphocyte?*," Y.-C. Tsai et al. investigate the individual levels of oxidative DNA base damage in the human lymphocyte from different donors (healthy and infected patients) and its correlation with total antioxidant levels in the serum and the DNA base excision repair capacity. The most important finding is that antioxidant serum levels may not change the steady state levels of the oxidative base damage in the DNA. One should also consider that antioxidants in our body protect us in acute and highly stressful situations when we have high levels of ROS in a short time period rather than decrease the steady state levels.

In a review article entitled "*Phytoagents for cancer management: regulation of nucleic acid oxidation, ROS, and related mechanisms*," W.-L. Lee et al. present a detailed review of the role of phytoagents as redox regulators of nucleic acid oxidation in carcinogenesis. On the concept of genetic heterogeneity caused by nucleic acid oxidation as a major driving force of cancer progression, the authors highlight how and why phytoagents induce or prevent oxidative stress and their potential use in cancer prevention or therapy.

Acknowledgments

We would like to thank the authors for their excellent contributions and patience in assisting us. The fundamental work of all reviewers on these papers is also very warmly acknowledged.

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Review Article

Phytoagents for Cancer Management: Regulation of Nucleic Acid Oxidation, ROS, and Related Mechanisms

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Accumulation of oxidized nucleic acids causes genomic instability leading to senescence, apoptosis, and tumorigenesis. Phytoagents are known to reduce the risk of cancer development; whether such effects are through regulating the extent of nucleic acid oxidation remains unclear. Here, we outlined the role of reactive oxygen species in nucleic acid oxidation as a driving force in cancer progression. The consequential relationship between genome instability and cancer progression highlights the importance of modulation of cellular redox level in cancer management. Current epidemiological and experimental evidence demonstrate the effects and modes of action of phytoagents in nucleic acid oxidation and provide rationales for the use of phytoagents as chemopreventive or therapeutic agents. Vitamins and various phytoagents antagonize carcinogen-triggered oxidative stress by scavenging free radicals and/or activating endogenous defence systems such as Nrf2-regulated antioxidant genes or pathways. Moreover, metal ion chelation by phytoagents helps to attenuate oxidative DNA damage caused by transition metal ions. Besides, the prooxidant effects of some phytoagents pose selective cytotoxicity on cancer cells and shed light on a new strategy of cancer therapy. The "double-edged sword" role of phytoagents as redox regulators in nucleic acid oxidation and their possible roles in cancer prevention or therapy are discussed in this review.

1. Nucleic Acid Oxidation as a Marker of Oxidative Insult by Reactive Oxygen Species and the Driving Force in Cancer Progression

The integrity of the genome is of crucial importance for proper gene expression and DNA replication. Loss of genome integrity jeopardizes normal cellular physiological activities and leads to cellular pathological events such as senescence, apoptosis, and tumorigenesis [1]. Under oxidative stress, the level of genotoxic reactive oxygen species (ROS) is abnormally elevated. ROS interact with and modify the chemical properties of biomolecules inside the cell, which causes oxidative insults such as oxidation of nucleic acids, peroxidation of lipids [2], and denaturation of proteins [3]. Oxidative modification to DNA structure mainly occurs in the form of base oxidation. Guanine, which possesses the lowest oxidation potential of the DNA bases, is the most frequent target of ROS. ROS-elicited changes in biomolecules can be used as biomarkers to indicate the presence and extent of oxidative insult. 8-Oxo-7,8-dihydroguanine (8-oxoG), the oxidation product of the DNA base guanine is a well-characterized marker for oxidative stress-induced DNA damage [4]. Following the oxidation of a DNA base, genome integrity is at increased risk because the DNA repair process, base excision repair (BER), can increase the level of interrupted DNA strands resulting in indirect single-strand break (SSB) [5], subsequently leading to introduction of mismatched base pairing during translesion DNA repair [6]. As a consequence, genome instability and accumulation of mutations lead to genetic heterogeneity in cancer cells that drive the adaptive evolution of cancer colonies with survival/expansion advantages [7]. Figure 1 shows the genetic

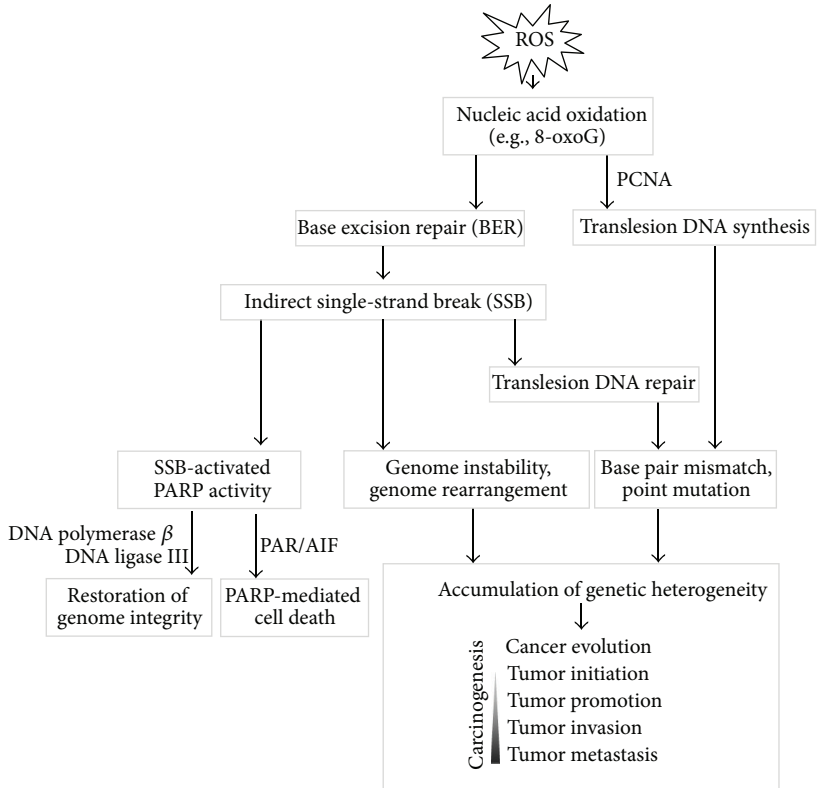

Figure 1: Genetic heterogeneity following nucleic acid oxidation is a major driving force of cancer progression. ROS causes the oxidation of DNA bases. Subsequent base excision repair (BER) introduces genetic errors during the repair process, and the accumulation of these errors drives cancer progression.

instability and heterogeneity caused by nucleic acid oxidation in cancer cells which lead to carcinogenesis and cancer evolution. During BER, indirect SSB are produced as intermediates after the removal of oxidized bases and their corresponding nucleotides. If SSB takes place at adjacent regions on both strands of the same chromosome, genome instability can ensue. Meanwhile, poly (ADP-ribose) polymerase (PARP) is activated after binding to SSB and consumes NAD+ to synthesize polyA chains which then recruit important DNA repair enzymes, such as DNA polymerase β and DNA ligase III. PARP also induces apoptosis through increased poly (ADP-ribose) (PAR) levels that facilitate the release of apoptosis-inducing factor (AIF) from mitochondria and elicit apoptosis. Otherwise, depletion of NAD due to excessive PARP activity will further deplete the ATP pool and lead to cell lysis (necrosis). Proliferating cell nuclear antigen (PCNA) promotes the switch to a specialized DNA polymerase with a larger active site that tolerates damaged bases at the expense of sacrificing fidelity during translesion synthesis/repair. Lower fidelity increases the chance of mismatch which gives rise to point mutations. The accumulation of genome instability and point mutations results in genome heterogeneity among cells and, chronologically, within cells. Tumor initiation is triggered by mutations that can activate oncogenes or silence tumor suppressor genes. Further mutations that give rise to gain/loss of function of genes then grant tumor cells the ability to resist growth control. Further gain/loss of function continues to drive cancer progression enabling tumor cells to escape layers of control and become capable of invasion and metastasis.

Elevated levels of oxidative DNA lesions (8-oxoG) have been noted in various tumors, supporting the argument that such damage contributes to the etiology of cancer. Therefore, 8-oxoG has been established as an important biomarker which is widely used to measure oxidative stress and assess risk of tumor initiation after exposure to various carcinogenic substances and pollutants [8]. In a cohort study involving esophageal cancer patients, more extensive oxidative damage to DNA as indicated by 8-oxoG levels was detected in cancer patients, in comparison to a healthy control group. Smoking habits and alcohol consumption, risk factors for esophageal cancer, were also correlated with the observed levels of oxidative DNA damage [9].

Oxidative stress-induced lipid peroxidation is also associated with the early stages of carcinogenesis [10]. Malondialdehyde (MDA), the product of lipid peroxidation, can induce the formation of DNA adducts which leads to mutagenesis. In an epidemiological study of breast cancer, the level of the malondialdehyde-DNA adduct, 3-(2-deoxy-β-D-erythro-pentofuranosyl) pyrimido [1,2-α]purin-10(3H) one (M1dG), was significantly higher in breast tissue specimens from cancer patients than in those from healthy individuals [11]. Therefore, other than 8-oxoG, the level of M1dG has been employed as an indicator of cancer-associated oxidative DNA

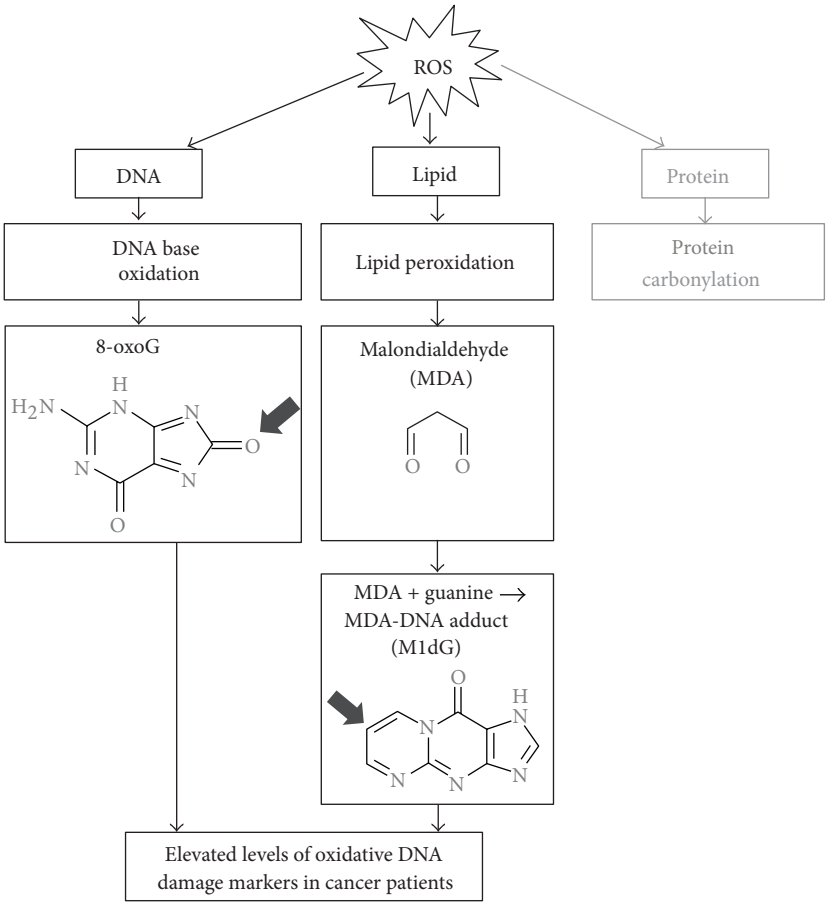

Figure 2: Markers of oxidative DNA damage are elevated in cancer patients. ROS causes oxidative damage to biomolecules such as DNA, lipids, and proteins, and the resulting end products are often detrimental to normal cell physiological functions. As the result of DNA base oxidation, 8-oxo-guanine (8-oxoG) can serve as a biomarker of primary oxidative DNA damage. When lipids are attacked by ROS, secondary DNA damage arises due to malondialdehyde (MDA), the end production of lipid peroxidation that can covalently bind to guanine and form MDA-DNA adduct (M1dG). In human cancer patients, both 8-oxoG and M1dG are found to be elevated, suggesting a correlation between higher oxidative stress and cancer.

damage. These markers are used as measures of antioxidant activity in studies that assess the chemopreventive efficacy of anticancer agents including phytochemicals [9, 12, 13] (Figure 2).

2. Sources of ROS and Cellular Antioxidant Defense

ROS are genotoxic and ubiquitous. They include the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), and the nitric oxide radical ($NO^{\bullet}$) [14]. For maintenance of genome integrity and normal cell physiological function, cells have developed strategies to control ROS levels. Such control is known as antioxidant defense [14]. Cellular redox status, the level of ROS, is the net result of ROS arising from various origins and the capacity of the cell to remove it by antioxidant defense. Many preventive/therapeutic regimens, including those phytoagent-based, intervene in disease progression by fine-tuning the level of ROS and the corresponding antioxidant responses in the cell [15], and thus shifting the redox balance in favor of human health. Introductions of the various origins of ROS and cellular antioxidant defense mechanisms are outlined below.

2.1. Origins of ROS. Sources of ROS can be divided into three major categories: exogenous, endogenous, metal-catalyzed (Figure 3(a)). Exogenously, ROS levels are mainly increased by environmental and dietary factors. These factors may serve as prooxidants that elicit ROS directly through chemical reactions or through the inhibition of cellular antioxidant defense or as substrates or stimulators of ROS-producing enzymes. Environmental factors that increase ROS production include ultraviolet light, ionizing radiation, air pollutants, cigarette smoke, pesticides, and industrial solvents or chemicals. Dietary factors that induce ROS include food containing peroxidized lipids (from rotten oil), polycyclic aromatic hydrocarbons (PAH, from high-temperature processed hydrocarbon-based food), and food additives (preservatives).

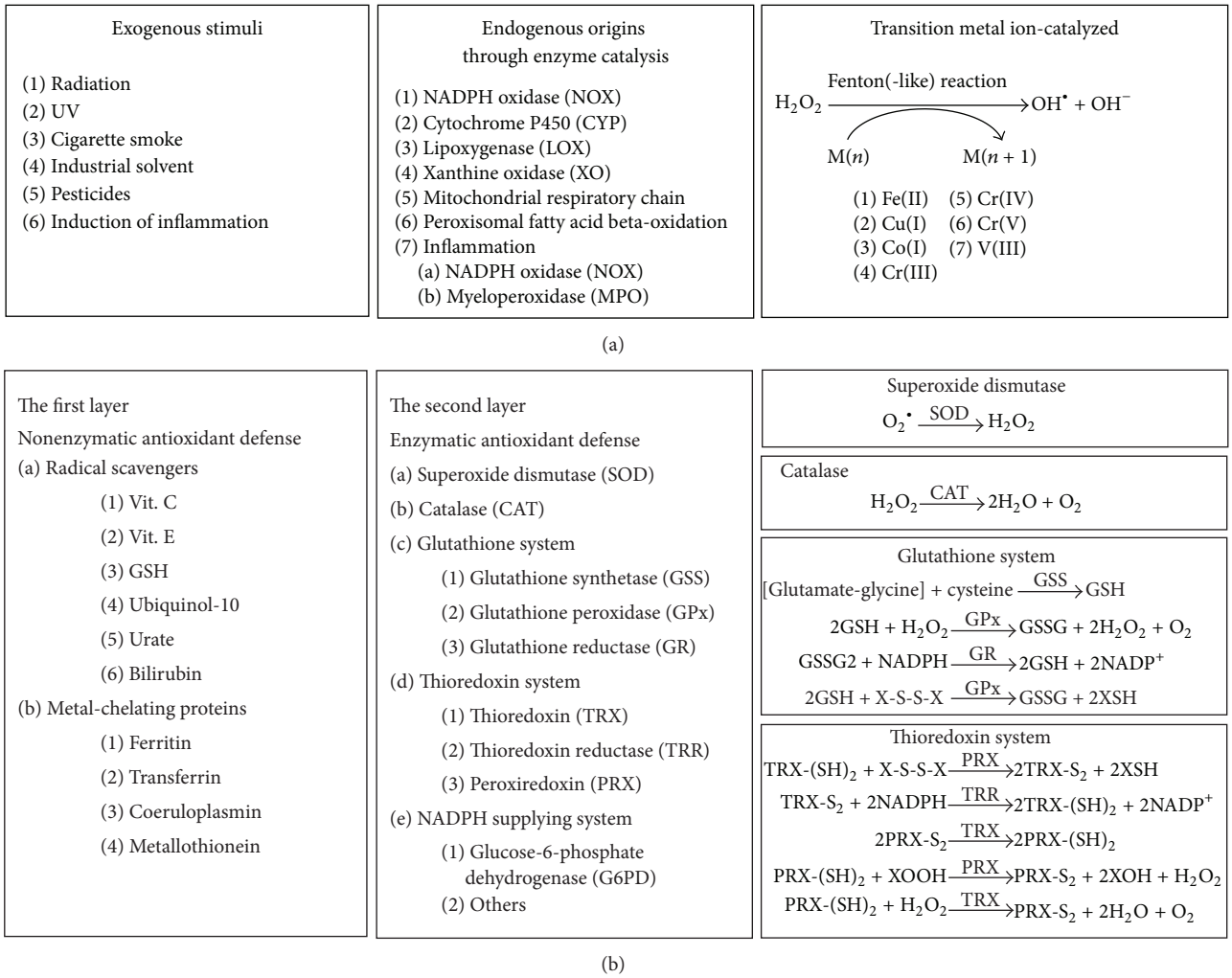

Figure 3: The source and clearance of ROS. (a) Three major origins of ROS. The sources of ROS can be roughly classified into three major categories: exogenous, endogenous, and transition metal ion-catalyzed. Exogenous sources of ROS can elicit radical chain reactions, contain/produce ROS, or stimulate enzymatic ROS production. Endogenous sources of ROS include the various enzymes that produce ROS as by-products or as signaling mediators or as antimicrobial agents during inflammation. Many of these enzymes can be activated by stimulation by cytokines and growth factors, such as NOX, LOX, XO, and MPO. Some CYPs are inducible and can be upregulated by environmental pollutants, dietary phytocompounds, or drugs. The transition metal ion-catalyzed Fenton-reaction produces highly reactive hydroxyl radical from hydrogen peroxide. (b) Layers of antioxidant defense. There are several layers of antioxidant defense. Basal level antioxidant defenses provide buffering capacity upon ROS challenge. Radical scavengers can directly quench ROS, and metal-chelating proteins can block ROS generation catalyzed by the Fenton or Fenton-like reactions. Further antioxidant capacity is provided by inducible antioxidant enzymes that are mostly under the regulation of Nrf2/ARE signaling (see Figure 4). ROS can oxidize the thiol group of amino acid residues leading to intermolecular or intramolecular disulfide bond formation. These disulfide bonds that are caused by oxidation can lead to structural/functional alteration of proteins. These disulfide bonds can be reduced by the glutathione system and the thioredoxin system allowing resumption of protein function. NADPH plays an indispensable role in the recycling of glutathione and thioredoxin, and therefore metabolic enzymes that are involved in NADPH generation also account for antioxidant defense.

Endogenously, ROS are generated during metabolic processes, such as mitochondrial oxidative phosphorylation, peroxisomal fatty acid beta-oxidation [16], catabolism of xenobiotics by cytochrome P450 monooxygenase (CYP) [17], purine by xanthine oxidase (XO) [18, 19], and lipid/fatty acid by cyclooxygenase (COX) [20, 21] or lipoxygenase (LOX) [22, 23]. Inflammation is another important endogenous source of ROS. During inflammation, ROS are generated via NADPH oxidase and myeloperoxidase which can protect against microbe or virus invasion; however, they might also be injurious to adjacent cells [24–27]. The positive feedback loop between oxidative insult, inflammation, and carcinogenesis is well recognized and appreciated as one of the hallmarks of cancer [28]. In metal-catalyzed generation of ROS, transition metal ions such as iron, copper, and chromium catalyze Fenton or Fenton-like reactions [29] that donate electrons and thus promote the production of hydroxyl radicals from hydrogen peroxide [30].

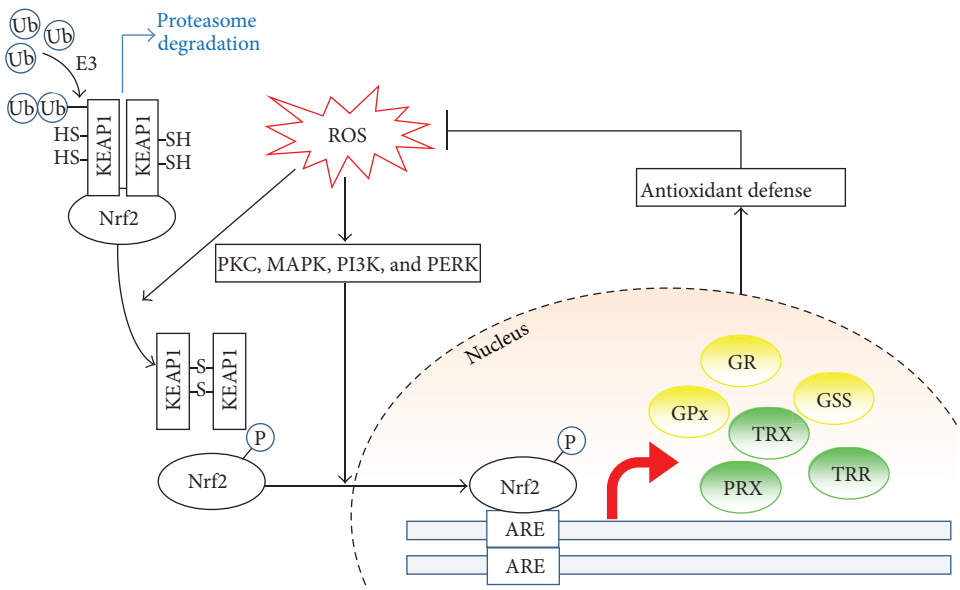

Figure 4: Inducible antioxidant defense regulated by Nrf2/Keap1 and the antioxidant response element. Under normal physiological conditions, the transcription factor Nrf2 is sequestered in the cytosol by Keap1. Keap1 recruits ubiquitin ligase E3 which then ubiquitinates Nrf2 and directs it to the proteasome degradation pathway. The increased level of ROS promotes the dissociation of Nrf2 and Keap1, either via activation of kinases that phosphorylate Nrf2 or by oxidization of key cysteine residues that govern Keap1 activity. The dissociated Nrf2 is then translocated into the nucleus and binds to the antioxidant response element (ARE). ARE-regulated genes are then transcriptionally activated, including a panel of antioxidant enzymes or proteins, such as glutathione synthetase (GSS), glutathione reductase (GR), glutathione peroxidase (GPx), thioredoxin (TRX), thioredoxin reductase (TRR), and peroxiredoxin (PRX). These inducible antioxidant enzymes then provide further ROS clearance capacity and antioxidant defense mechanism to exert a cytoprotective effect.

2.2. Cellular Antioxidant Defense Mechanisms: Control of ROS Levels and Repair of Oxidized DNA Bases. Proper control of ROS is critical for the maintenance of redox balance and genome integrity. Otherwise, excessive levels of ROS would overwrite the roles of ROS as signaling mediators and jeopardize the normal physiological processes inside the cell. Several layers of antioxidant defense have been proposed as preventive strategies against nucleic acid oxidation, including nonenzymatic removal of ROS by scavenger molecules, chelation of metals that catalyze ROS formation, inducible enzymatic removal of ROS, and the DNA repair system responsible for oxidative DNA lesion. Cellular molecules that can serve as radical scavengers form a first line of defense in the control of ROS levels (Figure 3(b)). These molecules include metabolites such as vitamin C, vitamin E, ubiquinol-10, and urate, as well as the tripeptide glutathione (GSH) and the thioredoxin (TRX) system [31]. Meanwhile, cellular metal-chelating proteins play key roles in controlling the level of free metal ions and thus enhance or prevent ROS generation by metal-catalyzed Fenton of Fenton-like reactions. These proteins include ferritin [32, 33], transferrin [34], coeruloplasmin [35], and metallothionein [36].

ROS scavengers and metal-binding proteins do not provide complete protection from ROS damage. Therefore, another layer of protection is provided in the form of enzymatic removal of ROS. Superoxide dismutase (SOD) is responsible for the transformation of superoxide anions into hydrogen peroxide, which is subsequently transformed into oxygen and water by catalase (CAT) or into water by glutathione peroxidase (GPx) [14]. The removal of hydrogen peroxide by GPx consumes the reduced form of glutathione (GSH) and generates the oxidized form (GSSG). GSSG can later be recycled by glutathione reductase (GR) and so replenish the GSH pool. Notably, metabolic enzymes responsible for NADPH production are critical factors in maintaining cellular redox balance, because NADPH is an indispensable factor responsible for the recycling of GSH and TRX by GR and thioredoxin reductase (TRR). Defects in NADPH supplying enzymes, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency in humans, compromise recycling of glutathione and thioredoxin and so weaken antioxidant capacity and confer susceptibility toward oxidative insult [37]. SOD, CAT, GPx, GR, TRR, and NADPH producing enzymes together, therefore, increase the capacity of the cell to remove ROS through enzymatic means (Figure 3(b)).

Cellular antioxidant defense is inducible and often up-regulated in response to oxidative stress or plant antioxidants. Cells sense and respond to changes in redox status by nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/kelch-like ECH-associated protein 1 (Keap1) complex [38, 39], which when dissociated allows Nrf2 nuclear translation and binding to the antioxidant response element (ARE) to transactivate antioxidant enzymes and thus further elevate antioxidant capacity [40] (Figure 4). Under normal physiological conditions, transcription factor Nrf2 is sequestered in the cytosol by Keap1, which recruits ubiquitin ligase E3 that ubiquitinates

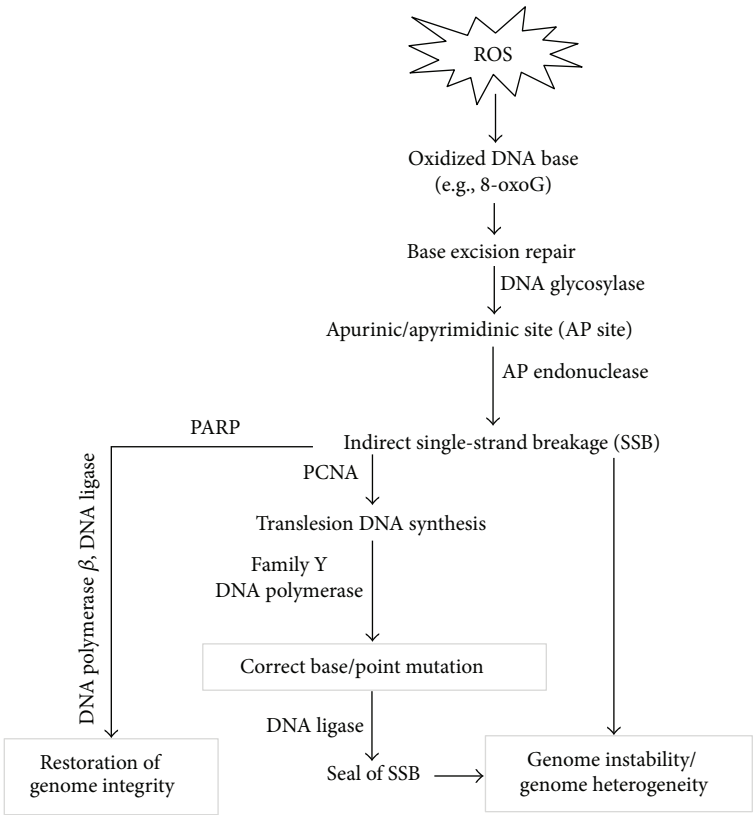

Figure 5: Repair of oxidative DNA damage introduces genome heterogeneity and instability. ROS causes oxidation of DNA bases which then elicit base excision repair machineries. First, the oxidized base is cleaved by glycosylase leaving an apurinic/apyrimidinic site (AP site). Second, the AP site is recognized by AP endonuclease that cleaves the phosphodiester bonds to remove the AP nucleotide and create the single-strand break (SSB) intermediate. DNA polymerase then resynthesizes the missing part of the DNA and later DNA ligase seals the nick. The low fidelity of the translesion DNA polymerase increases the chance of mismatched base-pairing and thus, leads to accumulation of point mutations which creates genome heterogeneity.

Nrf2 and directs it to the proteasome degradation pathway. Increased levels of ROS promote the dissociation of Nrf2 and Keap1, either by the oxidization of key cysteine residues that govern Keap1 activity or via the activation of kinases (e.g., protein kinase C (PKC), mitogen activated protein-kinase (MAPK), phosphatidylinositide 3-kinases (PI3K) [41], and protein kinase (PKR-) like endoplasmic reticulum kinase (PERK) that phosphorylate Nrf2 [42]. The dissociated Nrf2 then translocates into the nucleus and binds to the ARE. ARE-regulated genes, such as glutathione synthetase (GSS), GR, GPx, TRX, TRR, and peroxiredoxin (PRX) are then transcriptionally activated [40]. These inducible antioxidant enzymes provide further ROS clearance capacity and thus confer cytoprotective effects ensuing Nrf2 activation in response to oxidative stress stimulation during inflammation [43] or in the presence of redox-modulating phytoagents [44, 45] (Figure 4).

As nonenzymatic and enzymatic control of ROS levels cannot guarantee perfect/complete protection against ROS damage, oxidative damage continues to occur and accumulate in cells. To alleviate the negative effects elicited by oxidized biomolecules, especially DNA, cells have evolved sophisticated specific enzymatic repair systems. One such system, base excision repair (BER), repairs oxidized DNA bases (Figure 5) [5]. During BER, the oxidized base is first recognized and removed by DNA glycosylase leaving an apurinic/apyrimidinic (AP) site which is later recognized and cleaved by AP endonuclease on the phosphodiester backbone and leaves a DNA single-strand break (SSB) intermediate with a free $3'$-OH end. Subsequently, PPAR binds to the SSB and recruits DNA polymerase β and DNA ligase which synthesizes the missing nucleic acid and seals the SSB to restore genome integrity. Nonetheless, PCNA, a DNA clamp protein that associates with and coordinates the DNA repair pathway, facilitates a DNA polymerase switch to the specialized Family Y DNA polymerase and increases the potential of generating point mutation. Family Y DNA polymerase carries out translesion DNA synthesis. The low fidelity of Family Y DNA polymerase introduces a higher frequency of mismatched base pairing than in regular DNA synthesis and therefore increases the incidence of point mutations [46, 47]. In the last step, DNA ligase seals the nick between the *de novo* synthesized nucleotide and adjacent nucleotides and completes the base excision repair process. The point mutations

introduced during translesion DNA repair lead to genome heterogeneity between different cells and, chronologically, within the same cell (Figure 5).

3. "Double-Edged Sword" Role of Phytoagents as Redox Regulators in Cancer Management

3.1. Phytoagents in Cancer Management. Plants produce a remarkably diverse array of secondary metabolites (phytochemicals), many of which have evolved to combat microbial attack, resist environmental stress, or function as signaling molecules in interplant communication [48]. Human civilizations have used botanical preparations for treating and preventing various human diseases throughout history. Today, more than half of the anticancer drugs in clinical use are natural products or their derivatives and many are plant-derived phytochemicals [49, 50]. As cancer remains a major threat to health worldwide, there is global demand for more affordable and effective therapeutic alternatives. Moreover, concerns about drug resistance and the side effects of conventional therapeutic regimens currently used for cancer have renewed interest in phytochemicals derived from dietary foods and traditional medicines [51–55].

The US National Cancer Institute (NCI) has identified more than 1,000 different phytoextracts or phytochemicals that possess cancer-preventive activity [15] and the components responsible for many of the cancer chemopreventive effects of various edible plants have been determined. For example, the cancer preventive effects of allium species (e.g., garlic) and cruciferous vegetables (e.g., broccoli and watercress) are attributed to organosulfur compounds (e.g., diallyl trisulfide) and isothiocyanates (e.g., sulforaphane (SFN) and phenethyl isothiocyanate (PEITC)), respectively [56]. Other naturally occurring phytochemicals found in fruits, vegetables, spices, herbs, beverages, and medicinal plants, such as resveratrol [57], genistein [58], curcumin [59], (−)-epigallocatechin gallate (EGCG) [60], and sesquiterpene lactones (e.g., deoxyelephantopin [61–63], artemisinin [64], and parthenolide) [65–67] have been reported to modulate multiple signaling cascades that are known to deregulate cancer cell activities [68]. Interestingly, these representative phytocompounds (Figure 6) exert their anticancer cell effects through modulating ROS activity and oxidative stress in cancer cells by antioxidant, pro-oxidant, or a dual as antioxidant and prooxidant under certain physiological or pathological conditions. The important dual, seemingly oppositional role of phytoagents as redox regulators involved in nucleic acid oxidation in cancer cells, is discussed below.

3.2. Phytoagents as Antioxidants for Cancer Prevention. In general, phytoagents with antioxidant properties are potentially useful in cancer prevention because they can protect healthy cells from oxidative DNA damage through direct radical scavenging, upregulation of antioxidant defense system, metal ion chelation, and/or additional anti-inflammatory activity. The latest developments in the evaluation of the antioxidant effects and related defense systems or molecular mechanisms of phytocompounds, with focus on oxidative DNA damage as a biomarker in cancer prevention, are discussed below.

3.2.1. Major Antioxidant Mechanisms of Action of Phytoagents

(a) Direct ROS Scavenging. Phytoagents can attenuate ROS insults on biomolecules through direct scavenging of ROS. "Scavenging" refers to direct chemical modification of ROS and their stabilization by chemical reduction or electron-donation. In this way, the reduced form of a phytoagent molecule is consumed to buffer injurious ROS that might otherwise cause DNA damage. Phytoagents might have different scavenging capacity for different ROS and free radical species. For example, vitamin E and the carotenes have long polyunsaturated fatty acid chains, while vitamin C, flavonoids, and polyphenols have ring structures. They all share one structural commonality: conjugated systems, characterized by intermittent single bonds and double bonds which together form aligned p orbitals where pi electrons can move freely. The conjugated system can, therefore, donate electrons more easily and thus have high reducing capacity. This property gives these phytoagents ROS buffering capacity that protects important biomolecules from ROS attack.

(b) Attenuation of the Fenton(-Like) Reaction by Direct Metal Ion Chelation. Oxidative damage is one of the main forms of toxicity conferred by transition metal ions. In the Fenton(-like) reaction, the reduced form of a transition metal ion catalyzes the generation of the highly reactive hydroxyl free radical from hydrogen peroxide. Therefore, the more free form transition metal ions there are, the more hydroxyl free radical formation occurs by the Fenton(-like) reaction, and the more serious the oxidative damage to biomolecules including DNA. Will be Phytoagents can attenuate Fenton(-like) reaction by reducing the level of transition metal ion. Through direct chelation by phytocompounds containing a catechol or galloyl structure, transition metal ions are sequestered from solution and therefore prevented from participating the Fenton(-like) reaction [69, 70]. This is another indirect way by which phytoagents exert antioxidant effects.

(c) Induction of Antioxidant Response Element-Controlled Genes through Nrf2 Activation. Dietary levels of phytochemicals have been suggested to trigger induction of low levels of oxidative stress that may "prime" cellular antioxidant defense systems to resist higher levels of oxidative insults thus offering protection against carcinogenic insult [60]. These types of phytochemicals might have little antioxidant effect *in vitro* in terms of ROS scavenging capacity; nonetheless, in some cases, they activate the master transcription factor Nrf2 which governs the expression of a set of antioxidant-related genes. Therefore, through activation of Nrf2 and the subsequent upregulation of endogenous antioxidant defense, these phytochemicals confer antioxidant effects in an indirect way.

Phytoagents from various structural categories have been shown to activate Nrf2 with varied potency [71]. In general, phytoagents with electrophilic groups that are thiol-reactive induce the most potent Nrf2 activation when compared based on fold of induction of Nrf2-regulated

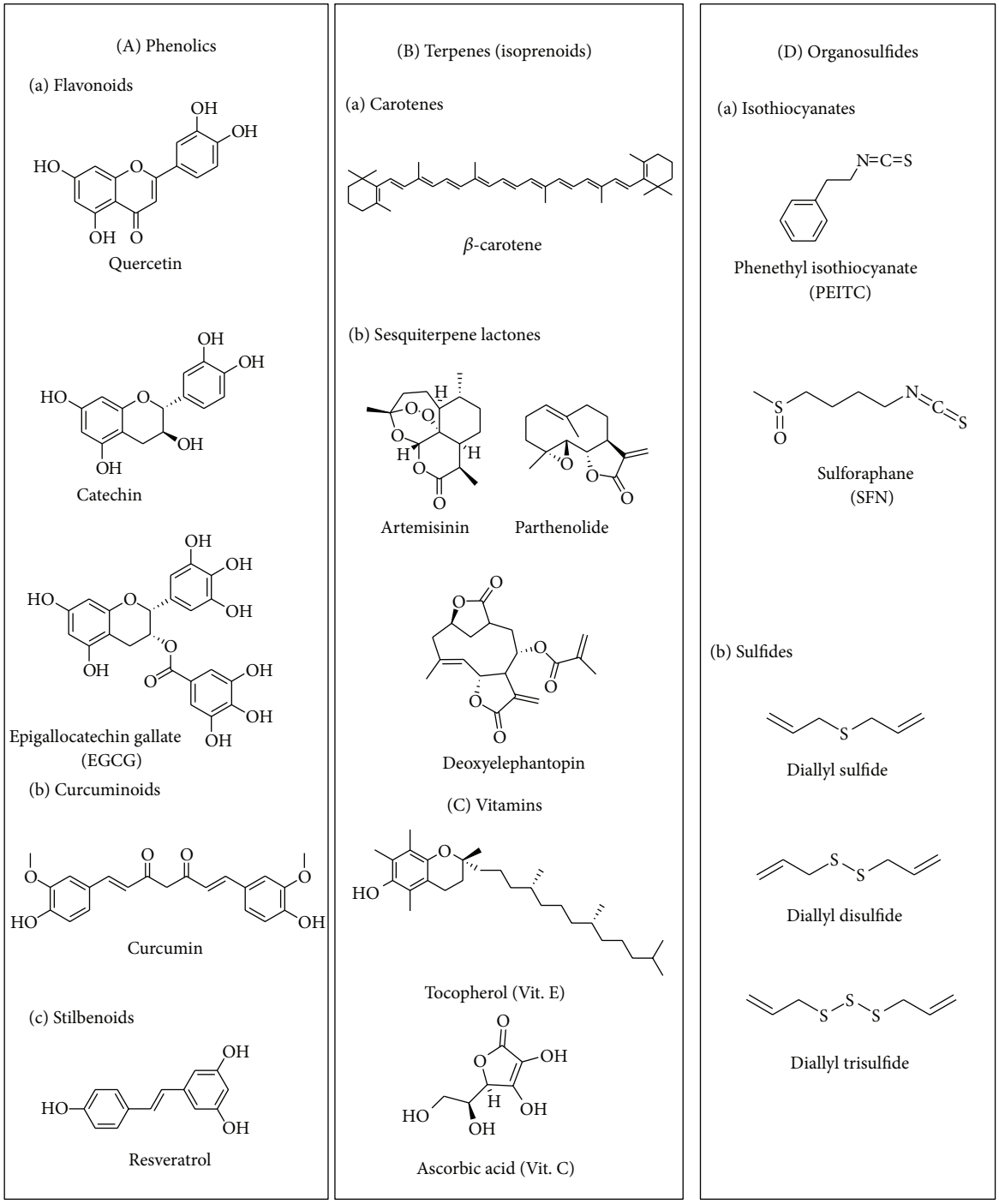

Figure 6: Representative phytocompounds with redox regulation capability. There are four major types of phytocompounds that can modulate intracellular redox status: (A) phenolics, (B) terpenes, (C) vitamins, and (D) organosulfides. They show free radical scavenging, Nrf2/ARE activation, and/or facilitation of ROS production in cancer cells.

NADPH: quinone reductase [72]. Some phytoagents without electrophilic groups could also induce Nrf2, though to a lesser extent. These types of phytoagents might activate Nrf2 indirectly through modulating signaling pathways whereas thiol-reactive electrophiles can directly modify the redox-sensitive cysteine residues in the Nrf2/Keap1 complex thereby promoting the dissociation of the complex and the nuclear translocation of Nrf2.

(d) Attenuation of Inflammation through Inactivation. NF-κB is the master transcription factor that governs the expression of many inflammation-related genes. Notably, the activation

of NF-κB is redox-sensitive. High endogenous ROS level stimulates NF-κB activation, which then leads to a pro-inflammatory response and further exacerbates the intracellular redox status [73–77]. Such a feedback loop mediated by redox-sensitive NF-κB activation often leads to chronic inflammation, one of the hallmarks of cancer. Many phytoagents exhibiting an anti-inflammatory effect have been shown to efficiently suppress NF-κB activation. Suppression of NF-κB can be achieved by either the aforementioned antioxidant actions or through direct chemical modification of NF-κB redox-sensitive cysteine residues by phytoagents with electrophilic groups, such as C=O, N=C=S or organosulfide groups to compromise its ability to translocate to the nucleus and bind DNA.

3.2.2. Antioxidant Effects and Defense Systems of Selected Phytoagents. Vitamins and phenolics (two well-known groups of antioxidants), as well as electrophilic phytocompounds, are used below to exemplify the latest developments in the evaluation of the antioxidant effects and related defense systems of phytocompounds with a focus on oxidative DNA damage as a biomarker in cancer prevention.

(a) Vitamins. The ability of macronutrients and micronutrients present in fruits and vegetables to reduce the risk of cancer is well known. Among these compounds, the antioxidant vitamins and their precursors have been extensively studied [15]. Vitamin C (ascorbic acid), vitamin E, and β-carotene are often referred to as "antioxidant vitamins." Vitamin C cooperates with vitamin E to generate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins. Through working along with other antioxidant enzymes, these antioxidants have been suggested to reduce oxidative damage in humans [78], and thereby minimizing the risk of certain chronic diseases [79–81]. However, early epidemiological studies and clinical trials investigating the efficacy of these vitamins in affecting disease outcome concluded that there was insufficient evidence to link supplementation of humans with vitamin C, vitamin E, or β-carotene with a reduction in *in vivo* oxidative damage to lipids, proteins, or DNA based on the measurement of oxidative biomarkers [82]. More recent clinical trials also suggest no correlatable effect between individual vitamins and chemoprevention [83, 84]. Further, anticancer properties reported for different vitamins have been discrepant. The history of the most well-known antioxidant, vitamin C, in cancer treatment is controversial while vitamins A and E only showed dispensable effects in tumor elimination [85]. However, the role of vitamin D in cancer treatment and prevention is promising [86, 87]. Interestingly, a large-scale, randomized, double-blind, placebo-controlled trial in male physicians showed that, compared with placebo, men taking a daily multivitamin had a statistically significant reduction in the incidence of total cancer; however, there was no significant effect on some specific cancer types, such as prostate cancer and colorectal cancer. It was therefore concluded that "daily multivitamin supplementation modestly but significantly reduced the risk of total cancer [88]."

Recently, in a large cohort study with 356 healthy subjects, dietary intake of vitamins was demonstrated to be associated with reduced levels of markers of DNA damage and oxidation (M1dG and 8-oxoG) measured in peripheral white blood cells. Notably, the associations were stronger in nonsmokers than in smokers [89]. It is important to keep in mind that several environmental factors can affect the antioxidant capacity of these vitamins. Environmental factors such as smoking and metal intoxication that causes excessive ROS burden to the body should be avoided, because antioxidant phytoagents can prevent *de novo* oxidation to nucleic acid but are not able to rescue or reverse oxidized nucleic acid caused by persistent oxidative insults from environmental stimulation. In another study, the protective effects of vitamin C and a natural phenol resveratrol on ethanol-induced oxidative DNA damage in human peripheral lymphocytes were investigated. Resveratrol showed significant DNA protection in a 24 h experiment, while the protective effect of vitamin C was seen in only 1 h. Both compounds were shown to directly scavenge hydroxyl radicals produced during ethanol metabolism. In addition, resveratrol inhibited dehydrogenase gene expression and activated the base excision repair (BER) system, mechanisms which may underlie its substantial effect on DNA protection. Vitamin C, however, showed no effect on the ethanol metabolic pathway or the BER system [90]. The antioxidant properties of vitamins in comparison to whole fruits and vegetables as anticancer agents are also of interest. The effectiveness of kiwifruit in decreasing oxidative DNA damage was assessed using comet assay (single-cell gel electrophoresis) to measure damage to lymphocytes collected from a human trial in which subjects drank kiwifruit juice. It was observed that a simple extract of kiwifruit was more effective than a solution of vitamin C in protecting DNA from damage *in vitro* [91]. This study demonstrated that the significant antioxidant activity of kiwifruit *ex vivo* and *in vitro* is not attributable entirely to vitamin C contained in the fruit. Instead, other components like phenolics and vitamin E may also contribute to the antioxidant effect of kiwi fruit extract [92]. These studies suggest an undetermined role of vitamin C present in fresh fruits, although different vitamin C content present in kiwifruit extract might result in different protective effects.

(b) Phenolics. Phenolic compounds are present in high concentrations in many components of the so-called "Mediterranean diet," including fruit and vegetables. These compounds seem to scavenge ROS, resulting in protection against oxidative DNA. This assumption was verified by testing the effect of Mediterranean plant extracts (*Crepis vesicaria* L, *Origanum heracleoticum*, *Scandix australis* L, *Amaranthus* sp., *Scolymus hispanicus* L, and *Thymus piperella* L) on oxidative DNA damage induced in lymphocytes by H_2O_2 in relation to their polyphenolic content using comet assay [93]. This study revealed that the protection of DNA by phenols present in Mediterranean plants is only partly due to ROS scavenging properties. Phenols can also attenuate Fenton(-like) reactions through metal ion chelation and induce endogenous antioxidant defense through Nrf2 activation. Apparently, ROS scavenging only partially contributes to antioxidant

activity of Mediterranean diet-derived phenolics or other phytochemicals. Their protection against oxidative DNA may involve other redox regulation such as upregulation of antioxidant enzymes in cells and attenuation of Fenton(-like) reaction by metal ion chelation.

In the carcinogenesis of hepatocellular carcinoma (HCC), oxidative stress is a major predisposing condition which is relevant to the development and progression of the cancer. In search for a dietary chemopreventive approach for the lethal HCC, pomegranate, an ancient fruit has gained attention owing to its significant antioxidant properties mainly contributed by the anthocyanins and ellagic acid derivatives [94, 95]. Pomegranate emulsion, a proprietary combination of aqueous phase extract and pomegranate seed oil containing several polyphenolic compounds, mixed with octadecatrienoic acids, sterols, steroids, and γ-tocopherol, was found to prevent hepatocarcinogenesis through induction of Nrf2-regulated phase II xenobiotic-metabolizing genes such as several GST isozymes that are involved in antagonizing oxidative stress [96]. A similar Nrf2-mediated antioxidant effect was also observed in HCC rats treated with blackcurrant anthocyanins [97].

Flavonoids are naturally occurring diphenylpropanoids that appear in animal and human cells following consumption of vegetables, fruits, and beverages such as tea and wine. Flavonoids can be classified into six major subgroups: flavonols (e.g., quercetin, kaempferol), flavones (e.g., apigenin, luteolin), flavanones (e.g., hesperidin, naringenin), flavan-3-ols (e.g., catechin, theaflavin, and gallic esters of catechin and theaflavins), anthocyanidins (e.g., pelargonidin, cyanidin), and isoflavones (e.g., genistein, daidzein). Epidemiological studies suggest that dietary intake of flavonoids may reduce the risk of tumors of the breast, colon, lung, prostate, and pancreas. However, the generalizability of these anticancer effects remains a subject of study [98].

(c) Electrophilic Phytochemicals. Electrophilic phytochemicals, such as phenethylisothiocyanate (PEITC), sulforaphane (SFN), turmeric, curcumin, and EGCG, prevent oxidative modification and mutation of genes through activation of the Nrf2/Keap1 complex [45, 99–101]. These phytochemicals modulate Keap1-associated transcriptional regulation which results in up-regulation of ARE-bearing genes encoding phase II detoxifying enzymes and transporters that protect normal cells from ROS, reactive nitrogen species (RNS) or reactive metabolites of carcinogenic species [71]. Such responses are thought to represent a form of cellular adaptation to chemicals and oxidative stress that maintains cellular redox homeostasis [15, 99]. Therefore, the use of dietary phytochemicals to regulate Nrf2-dependent antioxidant response to counter oxidative DNA damage has emerged as a promising strategy for cancer prevention.

Hormonal factors, especially 17ß-estradiol (E2), play a major role in the etiology of breast cancer where the circulating levels of E2 itself are an independent risk factor. E2 can cause both oxidative DNA damage and attenuate DNA repair leading to oncogenic mutagenesis [102]. In the liver, the metabolism of E2 to its various phase I metabolites, such as the carcinogenic 4-hydroxy estradiol (4E2), primarily involves the cytochrome P450 enzymes CYP1A2 and CYP3A4 [103]. Dietary berries and their chemical constituents are known for their cancer preventive potential, which were recently shown to affect the enzymes involved in carcinogen metabolism in mouse liver [104] and significantly reduced hepatic oxidative DNA damage, indicated by the level of 8-oxoG and other polar adducts validated by P^{32}-postlabeling experiments. Compared to crude berry juices, ellagic acid, one of the bioactive components found in berries, showed more elimination of oxidative DNA adducts induced by redox cycling of 4E2 catalyzed by copper chloride *in vitro* [105].

3.3. Phytocompounds as Prooxidative Agents for Cancer Therapy. Prooxidant phytoagents, on the other hand, are particularly effective in treating aggressive tumors with abnormally radical-reactive cellular environments. They act by tipping the limit of oxidative stress that can be tolerated by tumor cells over a limit, thus triggering apoptosis and cell death [106]. Although pro-oxidant effects are observed after treatment with certain phytoagents, generally, phytoagents do not produce ROS directly. Instead, their prooxidant effect is highly dependent on the original redox status of the cell which determines sensitivity to cytotoxicity mediated by phytoagents. The basal redox levels of cancer cells are different from those of normal cells. Higher levels of free form metal ions and higher levels of endogenous ROS production in cancer cells sensitizes them to phytoagent-mediated prooxidant cytotoxicity [30, 107, 108]. In this section, we elaborate on how phytoagents act as prooxidants to selectively kill cancer cells and their effects in cancer chemotherapy.

3.3.1. Major Prooxidant Mechanisms of Action of Phytoagents

(a) Promotion of Fenton(-Like) Reactions by Catalyzing Redox-Cycling of Metal Ions. Phytoagents with strong reducing capacity can reduce not only ROS but also metal ions. Under normal physiological conditions, most metal ions are complexed with proteins and few exist in free form. However, in the presence of abundant free form metal ions, phytoagents catalyze Fenton(-like) reactions that produce injurious hydroxyl radicals [29, 109]. Notably, cancer cells develop abnormally high concentrations of metal ions due to overexpression of the transferrin receptor [110, 111]. When excessive concentrations of free form metal ions exist, classical antioxidant phytoagents catalyze the redox cycling of metal ions by reducing their oxidized form. As a result, a burst of hydroxyl free radical production ensues and the phytoagents become pro-oxidants.

(b) Basal ROS Generation through Glutathione Depletion by Electrophiles. Phytoagents with electrophilic groups can form covalent bonds with cysteine resides of proteins. Glutathione, the most abundant cysteine-containing peptide, thus can be rapidly depleted due to adduct formation with electrophilic phytoagents [112–115]. Upon glutathione depletion, the buffering capacity of ROS is attenuated so that the basal ROS production is revealed. Therefore, electrophilic

phytoagents exhibit pronounced pro-oxidant effect in cancer cells with high ROS production and push cancer cells over the tolerable limit of ROS. In contrast, the same dosage of phytoagents produces a negligible pro-oxidant effect in normal cells with low basal ROS production and boosts antioxidant response by Nrf2 activation [71, 100, 116–121].

3.3.2. Prooxidant Effects and Defense Systems of Selected Phytoagents. ROS and cellular oxidative stress have long been associated with cancer [122]. Hypoxic condition, that is, low ambient oxygen pressure, is well described in cancer cells, particularly in the central area of the tumor nodule or mass [123]. These cancer cells act more like anaerobic bacteria, showing low levels of mitochondrial oxidative phosphorylation, and generally survive through the generation of ATP in an oxygen-independent manner [124]. Many conventional anticancer drugs including vinblastine, doxorubicin, campthotecin, cisplatin, and inostamycin have been reported to activate a caspase-3(-like) protease causing generation of H_2O_2 presumably through the activation of NADPH oxidase that subsequently induces apoptosis in cancer cells [125]. Intriguingly, cancer cells are frequently deficient in crucial antioxidative enzymes, such as catalase, GPx, and SOD, and therefore demonstrate a high vulnerability to ROS. One antitumor strategy is to deliver excess oxidative stress into tumor cells or to target the disruption of the antioxidative defense systems of tumor cells. This strategy has been termed "oxidation therapy" in cancer treatment [126]. Several studies have reported that certain dietary anticancer/cancer preventive agents cause generation of ROS specifically in tumor cells, not in normal cells [56, 127, 128]. Through adaptation, normal cells that are exposed to pro-oxidant chemopreventive agents which generate oxidative stress can acquire resistance to transformation via adjusting the normal redox tone of these cells. In contrast, transformed cells, which typically endure an oxidizing intracellular environment, would ultimately succumb due to an excess of ROS caused by the same agent. ROS and cellular redox tone are exploitable targets in cancer chemoprevention via the stimulation of cytoprotection in normal cells and/or the induction of apoptosis in malignant cells [129]. Dietary intake of such chemopreventive agents could be a prefect strategy to achieve this purpose.

(a) Sulfur-Containing Compounds. Diallyl disulfide (DADS) and diallyltrisulfide (DATS) which are found in abundance in garlic are among the dietary factors studied extensively for their anticancer action involving induction of oxidative stress in the human body, as reviewed elsewhere [130]. The pro-oxidant and thiol-adducting activities of these electrophilic organosulfur compounds are attributed to their reactive isothiocyanate (R–N=C=S) pharmacophore. Dietary isothiocyanates include sulforaphane, phenethyl isothiocyanate (PEITC), benzyl-isothiocyanate, and 6-methylsulfinylhexyl-isothiocyanate (Figure 6). Originally, copper-mediated oxidative DNA damage induced by these isothiocyanates was considered to be carcinogenic [131]; however, later studies demonstrated that these phytochemicals exhibit preferential cytostaticity in premalignant and tumor cells via their pleiotropic pro-oxidant activities as reviewed elsewhere [106].

(b) Curcumin. Curcumin (diferuloylmethane) from turmeric, like isothiocyanates, is a pleiotropic redox modulator that is involved in multiple cellular activities as a pro/antioxidant and metal chelator, as recently reviewed [59]. Curcumin, which contains an electrophilic Michael acceptor as an active moiety, can also mediate strand scission of DNA in the presence of Cu (II) [132]. The compelling anticancer activities of curcumin have been widely demonstrated across different cancer cell lines and animal systems, as a function of above-mentioned reactive pharmacophores targeting various cellular molecules. Currently, the cancer preventive/therapeutic potential of curcumin, as single or combinatorial agent, is under evaluation in various clinical trials, including multiple myeloma, rectal cancer, metastatic colon cancer, advanced osteosarcoma, and pancreatic cancer [59].

(c) Sesquiterpene Lactones. The sesquiterpene lactones (SLs) have also gained considerable attention for their effectiveness in treating inflammation, headaches, infections, and other human diseases. SLs contain Michael acceptors that act as electrophiles that can increase cellular ROS and modulate specific redox sensitive targets in cancer cells. Artemisinin and parthenolide (Figure 6) are SL-derived drugs now being evaluated in cancer clinical trials [133–138]. Artemisinin, isolated from *Artemisia annua* (qinhao, sweet wormwood), possesses an endoperoxide bridge in the reactive pharmacophore that can be activated and cleaved by endogenous ions, leading to the generation of radical species and ROS through the Fenton reaction, which was observed to be a common mechanism underlying both the antimalarial and anticancer activities of the compound [139]. Parthenolide, identified from feverfew (*Tanacetum parthenium*), contains an electrophilic α-methylene-γ-lactone as the active moiety underlying its anticancer activity related to the Michael acceptor electrophile [66, 67]. Phytochemicals with prooxidant properties such as the SLs with Michael acceptor electrophiles have the potential to sensitize tumors in cancer treatment. For example, concurrent delivery of the SL parthenolide and the clinical drug paclitaxel in mixed micelles greatly improved the therapeutic response of resistant lung cancer cell lines to paclitaxel treatment [140]. In a mouse peritoneal dissemination model, parthenolide also improved the chemosensitivity of paclitaxel against gastric cancer through deregulation of the NF-κB signalling pathway [141]. Nevertheless, parthenolide and dehydrocostus lactone can also suppress cancer cell activity through downregulating other molecular targets, such as mitogen-activated protein kinase (MAPK) and protein kinase C, and induction of c-Jun-N-termial kinase (JNK) [142].

In our laboratory, we identified a germacranolide SL deoxyelephantopin (DET) from a medicinal plant *Elephantopus scaber* (Asteraceas) which contains an α-methylene-γ-lactone, an α,β-unsaturated lactone and a methacrylate ester side chain [62]. DET could induce ROS in breast cancer cells which became the upstream stimulus for the formation of centrosomal ubiquitinated protein aggregates and the

induction of protein carbonylation that might subsequently restrict cancer cell motility [63]. DET was also observed to activate ER stress- and JNK pathway-mediated apoptosis in mammary carcinoma cells triggered by ROS [62]. However, it is not yet clear whether DET caused oxidative DNA damage through the involvement of transition metals. Illustration that the anticancer activity of DET, the same as artemisinin, is through its role as a pro-oxidant suggests that pro-oxidant intervention using SLs may constitute a promising anticancer strategy.

3.4. Cancer-Associated Transition Metals in Phytochemical-Mediated Redox Regulation. Several essential transition metals, such as zinc, iron, copper, cobalt, and manganese, are known to regulate various metabolic and signaling pathways. For example, iron is an essential element in oxygen transportation [143] while copper is an essential component of several antioxidant enzymes [144]. In cancer cells, high metal ion concentration is one factor that contributes to the observed high base level of oxidative stress, which raises the possibility of killing cancer cells by dosing with metal supplements [145]. However, the prooxidant effect of metal ions is also known to initiate carcinogenesis [30], which raises concerns about applying metal supplementation as a therapeutic strategy against cancer. However, some studies indicated that cancer cells are prone to proliferate in environments with high levels of copper and iron and therefore suggested that these ions maybe be functionally involved in carcinogenesis [146, 147]. In a national cohort of the United States adults, serum concentrations of iron and copper were shown to correlate with mortality rate in cancer patients [148]. Due to the significant role of these metal ions in cancer epidemiology, their levels in different cancers were reviewed by Gupte and Mumper [145]. In comparison to normal individuals, the Cu (Zn, Se, Fe) ratios are usually higher in patients suffering from breast [149], cervical [150], ovarian [150], lung [151], prostate [152], bladder [153], and stomach cancer [154], and leukemia [155]. Increased levels of copper have also recently been correlated with poor survival in breast cancer patients [156]. The major metal ion contained in chromatin, copper is closely associated with the DNA bases, especially guanine [157]. As one of the redox active metals, copper can directly catalyze the formation of ROS via the Fenton reaction and cause oxidative stress in the cells [158]. The intracellular level of transition metal ions can determine whether phytoantioxidants act as cytoprotective antioxidants or cytotoxic prooxidants. Figure 7 summarizes the current understanding of the interplay between phytoagents and transition metal ions and the antioxidant/pro-oxidant role switch of phytoagents in response to the level of metal ions. The level of transition metal ions determines whether a phytoagent ultimately functions as cytoprotective antioxidant or cytotoxic pro-oxidant. Under normal level of transition metal ions, phytoantioxidants serve as radical scavengers and Nrf2/ARE activators that confer a cytoprotective effect that can be applied in chemoprevention. When the level of intracellular transition metal ion is high, such as in cancer cells, phytoagents recycle the metal ions and thus facilitate ROS production through the Fenton or Fenton-like reactions. Otherwise, metal ions catalyze the cleavage of phytoagents and generate radical cleavage products that can cause ROS. Such a prooxidant effect further drives the redox-sensitive cancer cells to their antioxidant limit and leads to cytotoxicity that can be applied as a chemotherapeutic strategy. On the other hand, metal-chelating phytoagents reduce metal ion levels and thus block the ROS producing Fenton(-like) reaction and provide a cytoprotective effect.

3.4.1. Ion Chelation by Phytoagents. Increasing numbers of studies are evaluating the antioxidant properties of phytochemicals through assessment of their ability to chelate metal ions that lead to attenuated reactivity of free radicals. Water extracts of pine needles inhibited oxidative DNA damage probably due to their strong hydroxyl radical and intracellular ROS scavenging activity and the chelating action of the iron (Fe2+) ion [159]. Antioxidant activity was reported for lunasin, a novel preventive peptide purified from *Solanum nigrum* L, which is also found in soy, barley, and wheat. The peptide did not scavenge endogenous hydroxyl radicals but inhibited the Fenton reaction by chelating iron ions, thus protecting DNA from oxidative damage [160]. The antioxidant properties of phenolic compounds are clear; however, the contribution of metal ion chelation to the antioxidative effect of these compounds is not yet conclusive. One study showed that the orthodihydroxy polyphenols bearing catechol or galloyl groups exhibit strong metal chelating activity [161]. In the study by Andjelkovic and colleagues, the ability of the phenolic compounds which chelate iron was ranked based on iron binding constants in ascending order. Protocatechuic acid was the weakest chelator, followed by hydroxytyrosol, gallic acid, and caffeic acid, with chlorogenic acid as the strongest chelator [162]. Iron chelation by phenolic compounds, phytochemicals in pine needle extracts, or by the peptide lunasin, which subsequently inhibited DNA oxidation, may deserve further exploration for their potential in cancer prevention.

The reactivity of metal ions can be attenuated indirectly through inhibition of their transportation. Dihydroartemisinin was reported to decrease iron uptake and disturb iron homeostasis in cancer cells through down regulating cell-surface transferrin receptor-1, which may be a novel mechanism of dihydroartemisinin independent of oxidative damage that has been previously mentioned as anticancer property of artemisinin [163]. The disturbance of iron homeostasis in cancer cells via irondepletion by natural or synthetic iron chelators has recently been shown to inhibit tumor growth by therapeutically manipulating iron level [164]. The effect of phytocompounds on deregulation of reactive ion metabolism in tumor cells is worth further exploration.

It is interesting to note that a prokaryotic glutathione analog, namely, ergothioneine, can protect cells from oxidative damage as measured by 4-HNE and partially rescue cell death caused by irradiation [165]. Another report showed that ergothioneine forms a chelation complex with copper and therefore protects cells from copper-induced DNA damage [166].

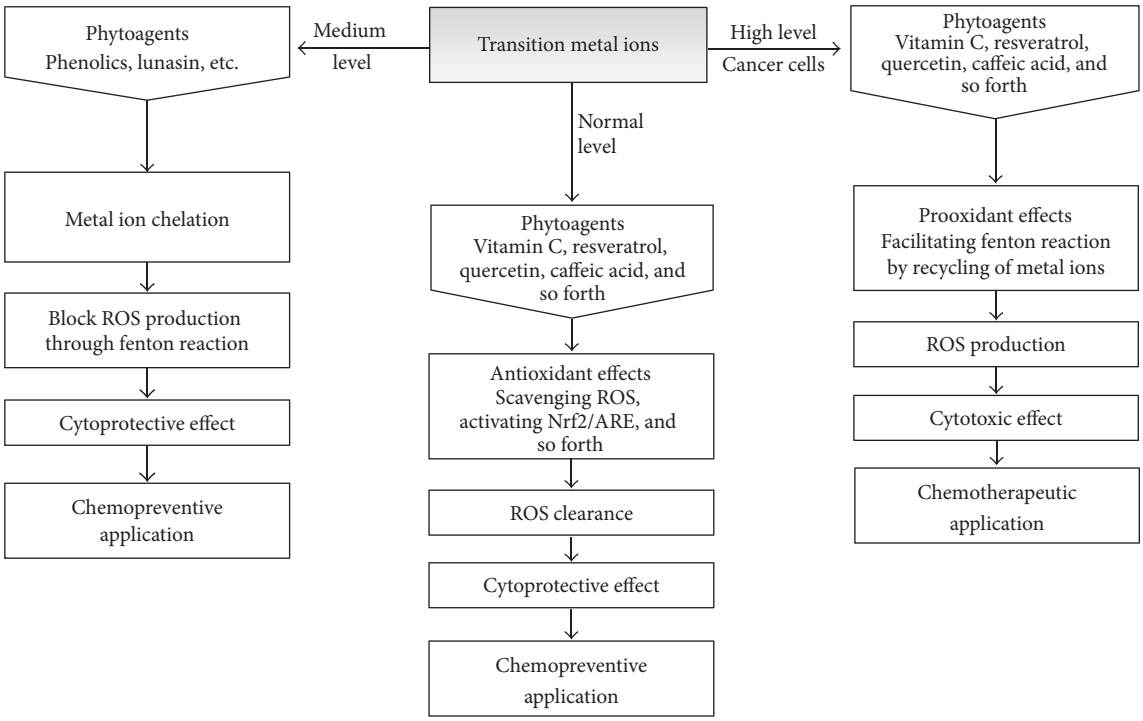

Figure 7: Role switches under different conditions—phytoagents function as both antioxidants and prooxidants in concert with transition metal ions. The level of transition metal ions determines whether a phyto-antioxidant ultimately functions as cytoprotective antioxidant or cytotoxic prooxidant. Under normal levels of transition metal ions, phytoantioxidants serve as radical scavengers and Nrf2/ARE activators that confer a cytoprotective effect that can be applied in chemoprevention. When the level of intracellular transition metal ion is high, such as in cancer cells, phytoantioxidants recycle the metal ions and thus facilitate ROS production through the Fenton or Fenton-like reactions. Otherwise, metal ions catalyze the cleavage of phytoagents and generate radical cleavage products that can cause ROS. Such a prooxidant effect further drives the redox-sensitive cancer cells to their antioxidant limit and leads to cytotoxicity that can be applied as a chemotherapeutic strategy. On the other hand, metal-chelating phytoagents reduce metal ion levels and thus block the ROS producing Fenton(-like) reaction and provide a cytoprotective effect.

3.4.2. Transition Metal-Mediated Prooxidant Properties of Phytochemicals in Anticancer Activity. Under certaincon-ditions, antioxidants can act as prooxidants [167]. Caffeic acid produces hydrogen peroxide which is activated by transition metals to cause oxidative DNA damage *in vitro* and in cultured human cells in the presence of Mn(II) or Cu(II) [168]. In another study using DNA fragments isolated from the human *p53* gene, quercetin increased 8-oxoG levelsignificantly in the presence of copper ions (Cu^{2+}), whereas 8-oxoG formation by kaempferol or luteolin was insignificant [169]. These early studies raised concern about whether ingestion of these phytochemicals may lead to increased risk of cancer. Lately, rats treated with 7,12-dimethylbenz[a]anthracene (DMBA) have become a widely used model for mammary carcinogenesis and in recent study, dietary supplementation with copper alone or together with the grape polyphenol resveratrol was found to promote carcinogenesis through increased frequency of microsatellite instability [170]. Later, a similar result was observed in the DMBA-model treated with combined supplementation with zinc ions and resveratrol [171]. However, a different mechanism was reported for resveratrol action in another cancer model with different stage of carcinogenesis. Resveratrol and its derivatives increase copper-mediated oxidative DNA damage by their pro-oxidant properties coupled with higher apoptosis induction in human leukemia cell lines [172].

The well-known antioxidant vitamin C, for example, was also found to act as a pro-oxidant *in vitro* when mixed with transition metal ions [173]. In healthy humans, Rehman and colleagues observed an increased level of oxidative DNA damage after 6-week supplementation of a mixture of ferrous sulphate andvitamin C, suggesting that this combination acts as a pro-oxidant; however, a longer period of sup-plementation by 12 weeks did not show significant effect [174]. Intriguingly, catalytic therapy that involves hydroxyl radical induction through a redox active mixture of vitamin C/medicinal herbal extracts and copper has been employed to improve the treatment of cancer patients [175, 176]. The Bhat group that established a model that involves human peripheral lymphocytes and comet assay carried out a series of studies on plant-derived polyphenolic antioxidants and proved that the mechanism is not restricted to vitamin C [177–179]. The most recent finding from the group is that the polyphenolic compound gossypol from the cotton plant and its derivative apogossypolone also cause oxidative damage to DNA by mobilizing endogenous copper in lymphocytes [180]. Although the reported mechanism was mainly the result obtained from lymphocytes; nevertheless, it could

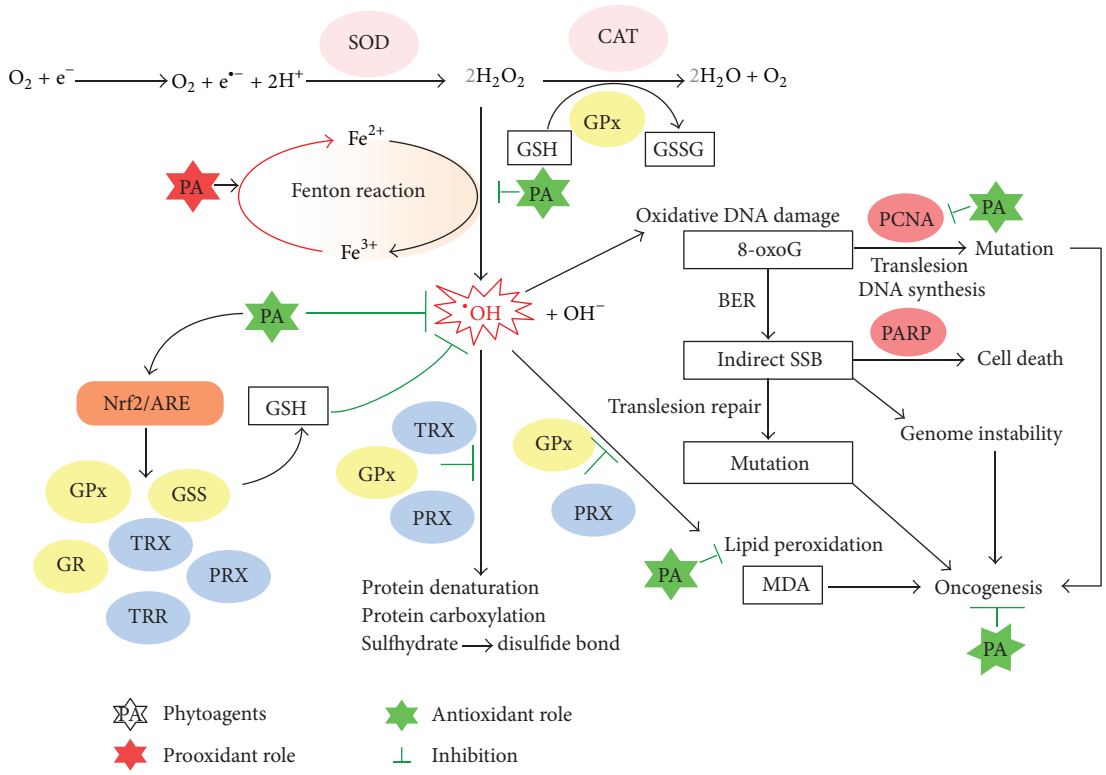

Figure 8: Summary of mechanisms of action of phytoagents in chemoprevention and chemotherapeutics through modulating oxidative stress. In the presence of ferrous ions (or other transition metal ions), phytoagents recycle the metal ion and thus promote the Fenton reaction that generates the highly reactive hydroxyl radical from hydrogen peroxide. Such prooxidant effects of phytoagents in the presence of metal ion can overwrite their cytoprotective roles because the production of ROS may be faster than the induction of antioxidant defense. Hydrogen peroxide imposes oxidative damage on biomolecules, such as proteins, lipids, and DNA, and leads to protein carbonylation, lipid peroxidation, and DNA base oxidation, which can be prevented by phytoantioxidants. Phytoantioxidants can activate Nrf2/ARE signaling and thus transcriptionally upregulate a panel of antioxidant genes that can provide further antioxidant capacity. Glutathione synthetase (GSS) can raise the level of glutathione (GSH) which can reduce oxidative damage by scavenging hydroxyl radicals, which otherwise cause oxidative DNA damage and increase the chance of point mutation and genome instability during the DNA repair process while glutathione reductase (GR) recycles the oxidized form of GSH and maintains the level of the reduced form of GSH. Glutathione peroxidase (GPx), thioredoxin (TRX), and peroxiredoxin (PRX) can prevent oxidative insults on proteins and lipids.

imply the anticancer property of polyphenols based on the abundant copper detected in different types of tumors [145, 153]. The enhanced electron transfer between transition metals and phytochemicals probably occurs in cancer cells with higher levels of copper ions, which may induce ROS generation subsequently leading to DNA damage [178, 180].

However, the mixture of a polyphenol and a transition metal was shown to promote tumor growth in mice with carcinogen induction that mimics the process of cancer initiation [170, 171]. These studies raise concerns about the potential carcinogenic activities of phytoagents. It is not clear whether the mixture of antioxidant phytochemical and transition metal resembles the oxidative stress that could possibly initiate tumorigenesis in normal cells, but that such a prooxidant effect drives the redox-sensitive cancer cells to their antioxidant limit and leads to cytotoxicity that has been applied in catalytic therapy. More studies are required to clarify the interaction of phytoagents and redox active metals as their oxidative potential may initiate tumors in a healthy individual.

4. Future Prospects

This review provides updated and integrative information about the regulation of nucleic acid oxidation by phytoagents in cancer. Animal models and human epidemiological studies have revealed that phytochemicals prevent carcinogenesis through direct ROS scavenging or induction of cellular antioxidant defense systems that consist of detoxifying enzymes, defense machinery mediated by Nrf2-antioxidative stress, and inhibiting inflammatory signaling pathways that together protect cells from DNA damage by ROS and reactive metabolites of carcinogens [42, 57, 58] (Figure 8). Investigation of oxidative modulation of proteins and lipids as well as DNA by phytochemicals provides a comprehensive picture of their functions as redox regulators in cancer. In general, antioxidant phytoagents are potentially useful in cancer prevention because they can protect healthy cells from oxidative DNA damage through radical scavenging, antioxidant defense system stimulation, and metal ion chelation; prooxidant phytoagents, on the other hand, are particularly effective in treating aggressive tumors with abnormally

radical-reactive cellular environments by exceeding the limit of oxidative stress that can be tolerated by tumor cells. Cancer cells, in general, have a higher basal redox level due to either defects in antioxidant defense or increased production of ROS during oncogenic transformation [122, 126]. Therefore, when challenged with similar quantities of ROS, cancer cells fail to buffer/clear excessive ROS and cell death ensues. In contrast, normal cells with lower redox levels are capable of buffering/clearing ROS by inducible antioxidant defense regulated by Nrf2/ARE signaling and are thus protected from cell death. Recently, dietary levels of phytochemicals have been suggested to trigger induction of low levels of oxidative stress that may "prime" cellular antioxidant defense systems to resist higher level of oxidative insults, thus offering greater protection against carcinogenic insult [60].

However, several studies have also hinted at a "dark" side of these cell-protective mechanisms. For example, the cytotoxicity of the anticancer drug platinum was attenuated by base excision repair of ROS-induced formation of 8-oxoG, indicating that repairing genotoxic damage could contribute to multidrug resistance of cancer cells [181]. Restoration of glutathione level by overexpression of γ-glutamylcysteine synthetase was found to prevent DNA damage and subsequent apoptosis caused by genotoxic agents in a resistant human ovarian carcinoma cell line [182]. Overexpression of catalase was found to cause drug resistance in breast cancer cell lines in chemotherapy [183]. These findings imply that alteration of the expression of antioxidant enzymes could be a mechanism through which cancer-cell resistance to redox-based chemotherapeutic agents is promoted. On the other hand, several phytochemicals have been indicated to upregulate the Nrf2 pathway which stimulates the defense system and leads to cancer prevention. However, overexpression of Nrf2 and its downstream genes was also observed in several cancer cell lines and human tumors, rendering cancer cells at an advantage for survival and unlimited proliferation. In addition, increased Nrf2 activity was found in some resistant cancer cells; in other words, to overcome chemoresistance in tumors, the Nrf2 pathway has to be deregulated [184]. Therefore, sophisticated design is necessary and caution has to be taken when administrating and handling Nrf2-dependent (as discussed above) phytochemicals in cancer patients, given that transformed cancer cells that are "overprotected" by antioxidants could possibly develop drug resistance.

Nrf2 is one of the key players in phytoagent-mediated antioxidant defense whose activation confers a chemopreventive effect. However, recent studies indicate that Nrf2 itself also plays a double-bladed-sword role in cancer management [185]. On one hand, Nrf2 orchestrates gene expression that protects cells from oxidative damage and detoxifies xenobiotics; on the other hand, the same effects confer chemoresistance to cancer cells. It is important to discern when and how to manipulate Nrf2 and so we can make use of its advantages while minimizing potential disadvantages. The major negative sides of Nrf2 activation include promoting bioactivation of xenobiotics whose glucuronide conjugation form is genotoxic and forms adducts with DNA [186–188], neutralizing the chemotherapeutic effects in which oxidative stress plays a significant role in mediating cytotoxicity to cancer cells, and facilitating drug excretion from cell through increasing the expression levels of multidrug resistant pumps. Thereby, to minimize potential disadvantages, the use of phytoagents as Nrf2 activators for chemoprevention should carefully avoid coadministration of drugs that are bioactivated by Nrf2-regulated phase II enzyme processing. On the other hand, for pro-oxidant cancer chemotherapy, Nrf2 activation is deemed as a side-effect and should be suppressed by coadministration of Nrf2 inhibitor [185]. Still, more future studies are required to confirm these points and thus provide a more accurate prediction, and therefore application, of phytoagents in cancer management.

For phytochemicals that function as both antioxidants and prooxidants, further characterization of the factors that determine the transition from antioxidative to prooxidative effects in the biosystem is essential. One contributing factor is the presence of transition metals. In addition, the doses of phytochemicals used in each treatment at different times may be crucial. In this regard, we propose some considerations on context-dependent, dual function of phytoagents that may help to understand and to predict the chemotherapeutic role of phytoagents. By comparing normal and cancer-bearing individuals, we know that the oxidative DNA marker 8-oxoG correlates well with basal redox level [8, 189]. Cancer cells with higher basal redox level demonstrated elevated levels of 8-oxoG, whereas normal cells had lower levels of basal redox level and 8-oxoG. The overexpression of transferrin receptor in cancer cells increased intracellular level of ferrous ions and, presumably, increased the rate of the Fenton reaction. It can be assumed that high levels of ferrous ions in cancer cells switch the functions of phyto-antioxidants to those of pro-oxidants resulting in further elevation of ROS level in cancer cells but not in normal cells, and the selective killing of cancer cells. More studies are required to determine the concentration threshold of metal ions that switche phytoagents to their prooxidant roles, so that potential chemotherapeutic applications can be better characterized. In summary, two main points form the base of the concept of the context-dependent dual role of phytoagents. One is the level of intracellular level of transition metal ions and the other is the basal redox level. The higher the two, the more likely the agent to produce a pro-oxidant effect, whereas the lower the two, the more likely the agent to produce an antioxidant effect.

Continued rigorous research to identify molecular targets and conduct human studies with bioactive phytochemicals are important to provide potential alternatives or novel approaches for plant-based cancer prevention or therapy. It is likely that the anticancer properties of phytochemicals result from modulation of a number of molecular mechanisms that regulate different stages of carcinogenesis. In this regard, increased antioxidant strength may be important prior to dysregulation of signaling pathways during tumorigenesis, whereas prooxidant cytotoxicity may be critical in eliminating transformed tumor cells that exhibit dysregulated redox balance and metal ion absorption. In conclusion, careful dose-response and stage-dependent studies that compare enhancement of antioxidant capacity and induction of oxidative stress by phytochemicals are essential to clarify when

and to what extent these phytoagents can be used in cancer prevention or therapy.

Authors' Contribution

Wai-Leng Lee and Jing-Ying Huang contributed equally to this paper as the co-first authors.

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Review Article

DNA Damage in Inflammation-Related Carcinogenesis and Cancer Stem Cells

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Infection and chronic inflammation have been recognized as important factors for carcinogenesis. Under inflammatory conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated from inflammatory and epithelial cells and result in oxidative and nitrative DNA damage, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-nitroguanine. The DNA damage can cause mutations and has been implicated in the initiation and/or promotion of inflammation-mediated carcinogenesis. It has been estimated that various infectious agents are carcinogenic to humans (IARC group 1), including parasites (*Schistosoma haematobium* (SH) and *Opisthorchis viverrini* (OV)), viruses (hepatitis C virus (HCV), human papillomavirus (HPV), and Epstein-Barr virus (EBV)), and bacterium *Helicobacter pylori* (HP). SH, OV, HCV, HPV, EBV, and HP are important risk factors for bladder cancer, cholangiocarcinoma, hepatocellular carcinoma, cervical cancer, nasopharyngeal carcinoma, and gastric cancer, respectively. We demonstrated that 8-nitroguanine was strongly formed via inducible nitric oxide synthase (iNOS) expression at these cancer sites of patients. Moreover, 8-nitroguanine was formed in Oct3/4-positive stem cells in SH-associated bladder cancer tissues and in Oct3/4- and CD133-positive stem cells in OV-associated cholangiocarcinoma tissues. Therefore, it is considered that oxidative and nitrative DNA damage in stem cells may play a key role in inflammation-related carcinogenesis.

1. DNA Damage in Inflammation-Related Carcinogenesis

Infection and chronic inflammation have been recognized as important risk factors for carcinogenesis and malignancies [1–3]. The International Agency for Research on Cancer (IARC) has estimated that approximately 18% of cancer cases worldwide are attributable to infectious diseases caused by bacteria, viruses, and parasites [4]. The burden of cancer caused by infectious agents is shown in Table 1. Inflammation can be induced not only by chronic infection, but also by many other physical, chemical, and immunological factors [5, 6]. It has been estimated that chronic inflammation accounts for approximately 25% of human cancers.

Under inflammatory conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated from inflammatory and epithelial cells [7]. ROS and RNS are capable of causing damage to various cellular constituents, such as nucleic acids, proteins, and lipids. ROS are generated from multiple sources, including inflammatory cells, carcinogenic chemicals and their metabolites, and the electron transport chain in mitochondria [2, 3]. ROS can induce the formation of oxidative DNA lesion products, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is considered to be mutagenic [7, 8]. During DNA synthesis,

Table 1: Possible markers for cancer stem cells in inflammation-related cancer.

Etiologic agent/ pathologic condition		Associated cancer	Detection of 8-nitroguanine	Possible markers for cancer stem cells related to each cause [references]
Parasites	SH	Bladder cancer	Patients [10, 11]	Oct3/4 (patients with SH) [10]
				CD44v6 (patients without SH) [11]
	OV	Cholangiocarcinoma	Hamsters [12–15]	—
			Patients [16, 17]	CD133, Oct3/4 [17]
Viruses	HCV,	Hepatocellular carcinoma	Patients with HCV [18]	CK19 [19]
	HBV		Mice with HBV [DN]	Nanog, CD133 [20]
	HPV	Cervical carcinoma	Patients [21]	CK17 [22, 23]
				CD44 (HPV16) [24]
				Oct3/4 (HPV16) [25]
	EBV	Nasopharyngeal carcinoma	Patients [26]	LMP2A ([27] and a lot)
				LMP1, Bmi-1 [28]
Bacteria	HP	Gastric cancer	Patients [29, 30]	SALL4, KLF5 [31]
				KLF5 [32]
				LgR5 [33]
Inflammatory diseases	IBD	Colorectal cancer	Mice [34]	—
	LP	Oral squamous cell carcinoma	Patients [35]	Bmi-1 [36]
				KRT15 [37]
	BE	Barrett's esophageal adenocarcinoma	Patients [38]	Oct3/4 [39]
				CD133 [40]
				Musashi-1 [41]
Others	Asbestos	Mesothelioma, lung carcinoma	Mice [42]	—

SH: *Schistosoma haematobium*, OV: *Opisthorchis viverrini*, HCV: hepatitis C virus, HBV: hepatitis B virus, HPV: human papillomavirus, EBV: Epstein-Barr virus, HP: *Helicobacter pylori*, IBD: inflammatory bowel diseases, LP: lichen planus, BE: Barrett's esophagus, DN: data not shown.

adenine is misincorporated opposite 8-oxodG, leading to G:C to T:A transversions [9].

Nitric oxide (NO) is synthesized by NO synthases. There are three isoforms: neuronal NO synthase (nNOS, also known as NOS1), inducible NO synthase (iNOS or NOS2), and endothelial NO synthase (eNOS or NOS3) [43, 44]. iNOS is activated to drastically generate NO in inflammatory and epithelial cells under inflammatory conditions, while eNOS and nNOS are constitutively expressed and produce relatively small amounts of NO. iNOS can be also upregulated by transcription factors such as NF-κB, HIF1-α, STAT, and TNF-α. NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes, including iNOS, and functions as a tumor promoter in inflammation-associated cancer [45].

Excess NO production plays a crucial role in an enormous variety of pathological processes, including cancer [43]. NO reacts with superoxide ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), a highly reactive species causing 8-oxodG and 8-nitroguanine [46, 47]. The reaction of guanine with $ONOO^-$ forms 8-nitroguanine as the major compound [46], while adenine nitration is minor compared to its C8-oxidation [48]. Akaike et al. have demonstrated that 8-nitroguanine is formed via NO production associated with inflammation in mice with viral pneumonia [49]. 8-Nitroguanine is considered to be not only a marker of inflammation, but also a potential mutagenic DNA lesion, leading to carcinogenesis [50]. 8-Nitroguanine formed in DNA is chemically unstable and thus can be spontaneously released, resulting in the formation of an apurinic site [51]. The apurinic site can form a pair with adenine during DNA synthesis, leading to G:C to T:A transversions [52]. However, the discovery of translesion DNA polymerases and their role in the mutagenesis in living cells made this paradigm rather obsolete [53, 54]. AP sites are indeed mutagenic, but the A-rule does not really describe its mutagenic potential. Cells deficient in Rev1 and Rev3, subunits of DNA polymerase ζ, were hypersensitive to nitrative stress, and translesion DNA synthesis past apurinic sites mediated by this polymerase might contribute to extensive point mutations [55]. It has been reported that adenine is preferentially incorporated opposite 8-nitroguanine during DNA synthesis catalyzed by polymerase η and $\kappa\Delta$C in

a cell-free system, suggesting that G:C to T:A transversions can occur [56]. In the $ONOO^-$-treated *supF* shuttle vector, which was replicated in host *Escherichia coli* cells, the majority of mutations occurred at G:C base pairs, predominantly involving G:C to T:A transversions [57]. Thus, 8-nitroguanine is a potentially mutagenic DNA lesion that can participate in initiation and promotion in infection-related carcinogenesis.

We have investigated the formation of 8-nitroguanine and 8-oxodG in various clinical specimens and animal models in relation to inflammation-related carcinogenesis, as summarized in Table 1. It is noteworthy that DNA damage was specifically induced at sites of carcinogenesis under chronic infection and various inflammatory conditions, as reviewed previously [2, 3]. It has been estimated that 11 infectious agents are carcinogenic to humans (Group 1) by IARC: parasites (*Schistosoma haematobium* (SH), *Opisthorchis viverrini* (OV), and *Clonorchis sinensis* (CS)), viruses (hepatitis B and C virus (HBV, and HCV), human papillomavirus (HPV), Epstein-Barr virus (EBV), human T-cell lymphotropic virus (HTLV-1), Kaposi's sarcoma herpesvirus (KSHV), and human immunodeficiency virus-1 (HIV-1)), and bacterium *Helicobacter pylori* (HP) [4, 58]. We demonstrated that 8-nitroguanine was strongly formed via iNOS expression at related cancer sites of SH, OV, HBV, HCV, HPV, EBV, and HP [2, 3, 10, 11]. The IARC classification of CS has been recently updated from 2A to 1, so we have not yet collected enough data for 8-nitroguanine. The mechanism of carcinogenesis by HTLV-1, KSHV, or HIV-1 seems not to be associative to inflammation. We could not observe 8-nitroguanine in leukaemia samples from patients infected with HTLV-1 (data not shown). 8-Nitroguanine was also formed in tissues from patients with inflammatory diseases, such as inflammatory bowel diseases (IBD), Lichen planus (LP), and Barrett's esophagus (BE) [3, 38]. Recently, we have reported that the formation of 8-nitroguanine and 8-oxodG increased significantly in the order of Barrett's esophageal adenocarcinoma > Barrett's esophagus > normal tissues. Treatment of BE patients with proton pump inhibitors (PPIs), which is expected to reduce the risk of Barrett's esophageal adenocarcinoma, suppressed these DNA lesions probably via activation of an antioxidant enzyme Mn-SOD [38]. Regarding inflammation-related carcinogenesis without infection, we describe the formation of 8-nitroguanine in lung tissues of mice intratracheally administered asbestos [42], although the precise mechanism of nitrative DNA damage remains to be clarified. Nitrative stress is involved in the asbestos-derived inflammatory response via myeloperoxidase [59–62] that plays a significant role in asbestos-induced carcinogenesis [63]. Interestingly, immunoreactivities of 8-nitroguanine, iNOS, and NF-κB significantly increased in the order of carcinogenic potential: crocidolite (blue asbestos) > chrysotile (white asbestos) > control [42].

On the basis of our studies, various pathogenic factors induce inflammatory responses and the production of ROS and RNS from inflammatory and epithelial cells via iNOS expression, which is regulated by transcriptional factors including NF-κB, STAT, and HIF-1α [2, 3]. Oxidative and nitrative stresses cause DNA damage, contributing to the accumulation of genetic alterations in tissues throughout the carcinogenic process. Particularly, 8-nitroguanine formation may participate in inflammation-related carcinogenesis as a common mechanism. Therefore, 8-nitroguanine could be used as a potential biomarker of inflammation-related carcinogenesis. Importantly, experimental evidence has suggested that 8-nitroguanine can lead to mutations, preferentially G:C to T:A transversions [46, 64], in addition to 8-oxodG [9, 65]. Indeed, G:C to T:A transversions have been observed in vivo in the ras gene [66] and the *p53* tumor suppressor gene in lung and liver cancer [67, 68]. We also revealed that 8-nitroguanine and 8-oxodG were apparently formed in adenocarcinoma caused by mutated K-ras, by using conditional transgenic mice with K-ras^{val12} [69]. 8-Nitroguanine was colocalized with iNOS, NF-κB, IKK, MAPK, MEK, and mutated K-ras, suggesting that oncogenic K-ras causes additional DNA damage via signaling pathways involving these molecules. It is noteworthy that K-ras mutation mediates not only cell overproliferation but also the accumulation of mutagenic DNA lesions, leading to carcinogenesis. These findings imply that DNA damage mediated by ROS and RNS may participate in carcinogenesis via activation of protooncogenes and inactivation of tumor suppressor genes.

2. Cancer Stem Cell Markers in Inflammation-Related Carcinogenesis

The cancer stem cell concept is widely accepted as important for overcoming cancer. Several studies have revealed that cancer cells show accumulation of mutations, genetic instability, and epigenetic change suggesting that cancer is also a disease of genes [70]. The most important question is how to generate cancer stem cells. Recently, many studies have reported on the expressions of stemness cell markers in various kinds of cancer. Table 1 summarizes possible markers of cancer stem cells, especially related to each inflammatory causative agent. We reported that 8-nitroguanine was strongly formed at all of these cancer sites from animals and patients with infectious agents, inflammatory diseases, and exposure to asbestos. Importantly, we also detected colocalization of 8-nitroguanine and stemness marker in infection-related carcinogenesis, as mentioned in the next section. On the basis of our recent studies, it is considered that chronic inflammation can increase mutagenic DNA lesions through ROS/RNS generation and can promote proliferation via stem cells activation for tissue regeneration. This idea is also supported by other papers about the association of cancer stem cells with infection and inflammation [71–74].

3. DNA Damage and Mutant Stem Cells Induced by *Schistosoma haematobium* Infection

Chronic infection with SH is associated with urinary bladder cancer [76]. Contact with contaminated freshwater is the major risk factor for infection. SH-associated bladder cancer is a common malignancy, especially in the Middle East and Africa. It is believed that the parasite's eggs in the host

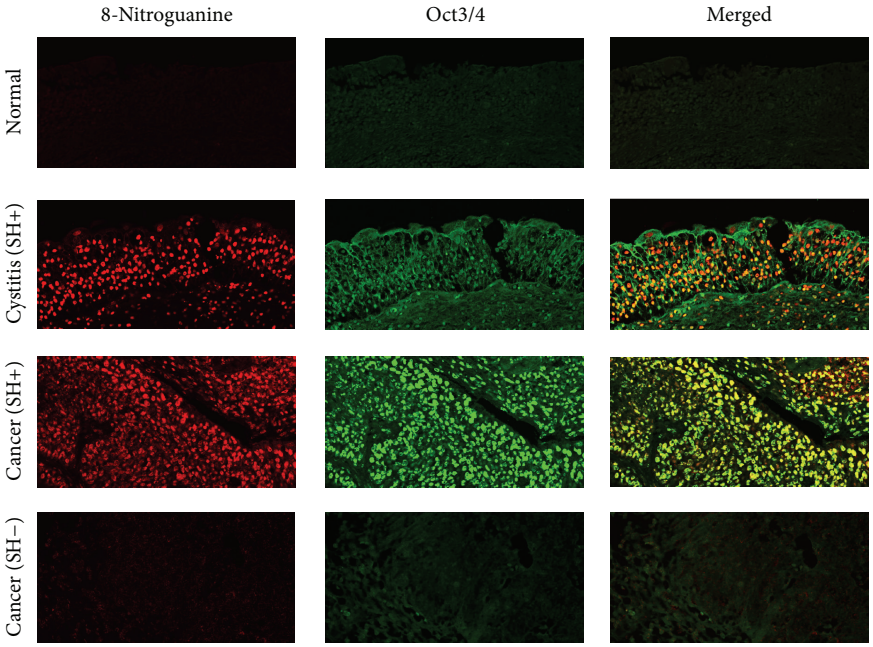

Figure 1: The formation of 8-nitroguanine (red) and the expression of Oct3/4 (green) were assessed by double immunofluorescence staining [10]. In the merged image, co-localization of 8-nitroguanine and Oct3/4 is indicated in yellow. Original magnification in all pictures is 200x (SH: *Schistosoma haematobium*). Formalin-fixed and paraffin-embedded biopsy and surgical specimens were obtained from normal subjects and patients with SH-induced cystitis and bladder cancer. Normal tissues and urinary bladder cancer tissues without SH infection were obtained from a commercial urinary bladder tissue array (Biomax.us, USA). Normal tissues with cystitis were excluded. SH-egg antigens in sera were detected by Sandwich ELISA assay [75]. This study was performed in accordance with the Ethical Guidelines for Epidemiological Research enacted by the Japanese government. Deparaffinized and antigen-retrieved sections were incubated first with 5% skim milk, and then with a rabbit polyclonal anti-8-nitroguanine antibody (2 μg/mL, prepared as described previously [11]) and mouse monoclonal anti-Oct3/4 antibody (2 μg/mL, Santa Cruz Biotechnology, CA, USA) overnight at room temperature. The sections were then incubated for 3 h with Alexa 594-labeled goat antibody against rabbit IgG and Alexa 488-labeled goat antibody against mouse IgG (each 1 : 400, Molecular Probes, Eugene, OR, USA).

bladder result in irritation, eventual fibrosis, and chronic cystitis, leading to carcinogenesis. To investigate whether oxidative and nitrative DNA damage participate in inflammation-related carcinogenesis, we performed immunohistochemical analysis using bladder tissues obtained from cystitis and bladder cancer patients infected with SH. We demonstrated for the first time that 8-nitroguanine is formed in the tumors of bladder cancer patients with SH infection [10]. The formation of 8-nitroguanine and 8-oxodG was significantly higher in bladder cancer and cystitis tissues than in normal tissues. Oxidative DNA damage and SH infection were strongly correlated [10, 77]. iNOS expression was co-localized with NF-κB in 8-nitroguanine-positive tumor cells from bladder cancer patients. NF-κB can be activated by TNF-α, a major mediator of inflammation, which has been reported to increase in peripheral blood mononuclear cells stimulated by SH egg antigen [78]. It is reasonable to conclude that both 8-nitroguanine and 8-oxodG are formed by iNOS-mediated NO overproduction via NF-κB activation, under SH-caused chronic inflammation.

A stemness marker Oct3/4 is generally expressed in pluripotent embryonic stem and germ cells [79]. Expression of Oct3/4 is reportedly necessary for maintaining the self-renewing, cancer stem-like, and chemoradioresistant properties of tumorigenic stem-like cell populations [80, 81] and is thus considered to play roles in the carcinogenesis process. Another stemness marker, CD44, has been identified as a cell surface marker associated with cancer stem cells in several types of tumors [82, 83], including urinary bladder cancer [84]. Expression of CD44v6, a splicing variant of CD44, is correlated with proliferation of poorly differentiated urothelial cells and the characteristic phenotype of tumor-initiating bladder cancer stem cells [85–87]. Our previous reports show that different risk factors induce different levels of expression of stemness markers in urinary bladder carcinoma. SH-induced urinary bladder cancer correlates with the expression of Oct3/4 [10], while urinary bladder cancer without the infection correlates with the expression of CD44v6 [11]. Moreover, 8-nitroguanine was formed in Oct3/4-positive stem cells in SH-associated cystitis and cancer tissues [10] as shown in Figure 1. Inflammation by SH infection may increase the number of mutant stem cells, in which iNOS-dependent DNA damage occurs via NF-κB activation, leading to tumor development.

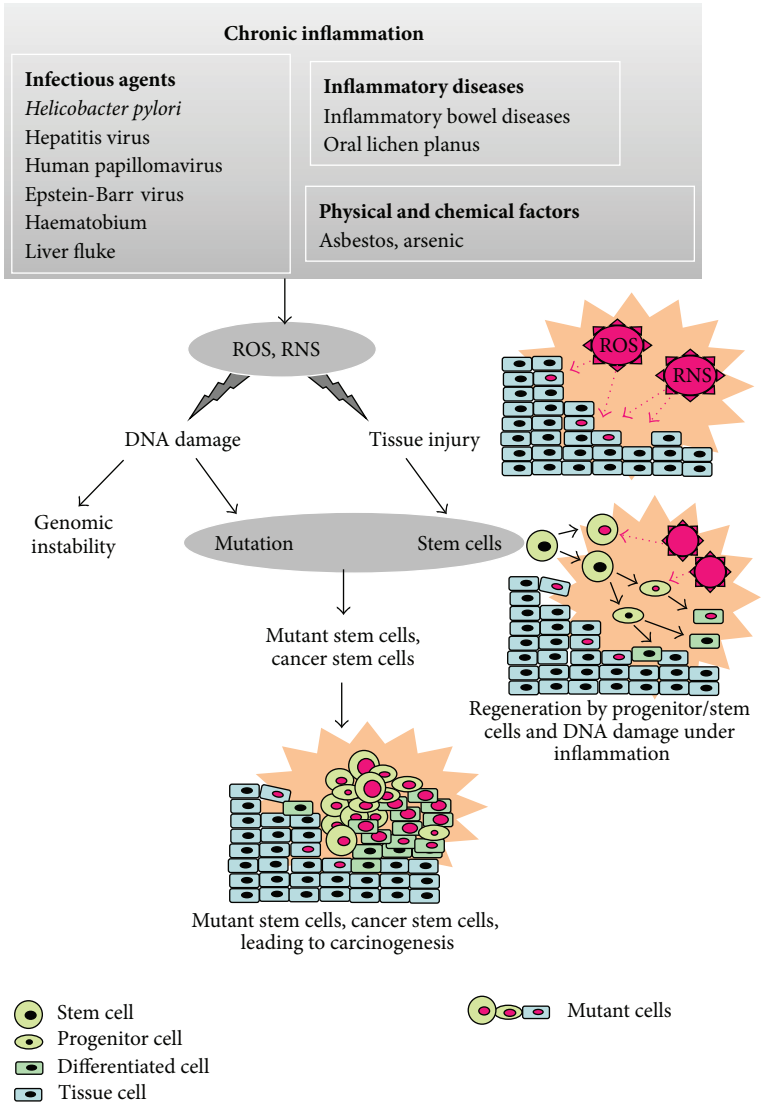

Figure 2: Postulated mechanism for generating cancer stem cells by inflammation.

4. DNA Damage and Mutant Stem Cells Induced by *Opisthorchis viverrini* Infection

Chronic infection with the liver fluke OV is associated with cholangiocarcinomas [58]. Infection with this parasite is repeatedly caused by eating raw fish containing the infective stage of the fluke. We have demonstrated that 8-nitroguanine is formed in relation to inflammation-related carcinogenesis using an animal model [12–15]. 8-OxodG and 8-nitroguanine were formed in inflammatory cells and epithelium of bile ducts, and their formation increased in a manner dependent on infection times. The anthelmintic drug praziquantel dramatically diminished these DNA lesions and iNOS expression in OV-infected hamsters. Thus, repeated OV-infection can induce the iNOS expression in bile ducts and subsequently cause nitrative and oxidative damage to nucleic acids, which may participate in cholangiocarcinoma.

In our study with patients, the formation of 8-oxodG and 8-nitroguanine occurred to a much greater extent in cancerous tissue than in noncancerous tissue in intrahepatic cholangiocarcinoma patients, indicating that these DNA lesions contribute to tumor progression [16]. Our proteomic study showed that carbonylation of serotransferrin and heat shock protein 70 kDa protein 1 (HSP70.1) is significantly associated with poor prognoses [88]. Carbonylation of protein is an irreversible modification induced by oxidative stress. We have proposed that carbonylations of serotransferrin and HSP70.1 may induce oxidative stress by iron-accumulation and dysfunction of antioxidative property, leading to increased oxidative DNA damage and progression of cholangiocarcinoma.

Recently, we observed high expression and co-localization of hepatocyte marker and cholangiocyte marker in OV-associated cholangiocarcinoma patients, suggesting the

involvement of stem cells in cholangiocarcinoma development [17]. Cholangiocarcinoma tissues with positive stemness markers (CD133 or Oct3/4) showed significantly lower expression of antioxidant enzyme Mn-SOD and significantly higher levels of 8-oxodG, 8-nitroguanine, and DNA damage response protein γ-H2AX. Moreover, CD133- and Oct3/4-positive cholangiocarcinoma patients had significant associations with tumor histology types, tumor stage, and poor prognoses. These findings suggest that CD133 and Oct3/4 in cholangiocarcinoma are highly associated with formation of DNA lesions, which may be involved in genetic instability and lead to tumor development with aggressive clinical features. In our study, proliferating cell nuclear antigen (PCNA) accumulated in the epithelium of bile ducts of hamsters after repeated OV infection, supporting the hypothesis that cell proliferation is promoted by inflammation-mediated DNA damage [14]. Inflammation by OV infection may increase the number of mutant stem cell, in which oxidative stresses, such as carbonylation of proteins and oxidative DNA damage, and cell proliferation are promoted, leading to progression of cholangiocarcinoma.

5. Conclusions

Nitrative and oxidative DNA lesions with mutagenic properties are formed in various types of inflammation-related cancer tissues. We have proposed a mechanism for the generation of cancer stem cells by inflammation in Figure 2. Chronic inflammation by infectious agents, inflammatory diseases, and other factors causes various types of damage to nucleic acids, proteins, tissue, and so on, via ROS/RNS generation. Tissue injury under chronic inflammation may activate progenitor/stem cells for regeneration. In these cells, ROS/RNS from inflammation can cause multiple mutations, which may generate mutant stem cells and cancer stem cells, leading to carcinogenesis. Indeed, 8-nitroguanine was formed in stemness marker-positive cells in parasite-associated cancer tissues. The mechanism for generation of cancer stem cells will be explained by our ongoing studies on the formation of 8-nitroguanine in stem-like cells of target tissues associated with other inflammation-related cancers.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Is the Oxidative DNA Damage Level of Human Lymphocyte Correlated with the Antioxidant Capacity of Serum or the Base Excision Repair Activity of Lymphocyte?

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A random screening of human blood samples from 24 individuals of nonsmoker was conducted to examine the correlation between the oxidative DNA damage level of lymphocytes and the antioxidant capacity of serum or the base excision repair (BER) activity of lymphocytes. The oxidative DNA damage level was measured with comet assay containing Fpg/Endo III cleavage, and the BER activity was estimated with a modified comet assay including nuclear extract of lymphocytes for enzymatic cleavage. Antioxidant capacity was determined with trolox equivalent antioxidant capacity assay. We found that though the endogenous DNA oxidation levels varied among the individuals, each individual level appeared to be steady for at least 1 month. Our results indicate that the oxidative DNA damage level is insignificantly or weakly correlated with antioxidant capacity or BER activity, respectively. However, lymphocytes from carriers of *Helicobacter pylori* (HP) or *Hepatitis B virus* (HBV) tend to give higher levels of oxidative DNA damage ($P < 0.05$). Though sera of this group of individuals show no particular tendency with reduced antioxidant capacity, the respective BER activities of lymphocytes are lower in average ($P < 0.05$). Thus, reduction of repair activity may be associated with the genotoxic effect of HP or HBV infection.

1. Introduction

The endogenous level of DNA damage due to oxidative stress in human peripheral blood lymphocytes (PBL) has been extensively used as biomarkers in studying the genotoxic effects associated with diseases, microbial infection, ageing, or the exogenous agents [1–7]. However, the DNA damage levels presented in the previous studies were often collected at a single point time; it is unclear whether the damage level of concern is steady for a certain period of time, for example, a week or a month or longer. In this report, we showed that the endogenous level of DNA oxidation in lymphocytes from each individual was constant at least for 1 month (see the following). We measured the level of DNA oxidation in lymphocytes with a modified comet assay, which includes a step of enzymatic cleavage by bacterial Fpg/Endo III, recognizing oxidized purines and pyrimidines, respectively [8, 9]. We considered that such damage levels of PBL may be modulated by the antioxidant capacity of serum or the repair activity of lymphocytes. Like the measurement of DNA damage in PBL, serum or plasma antioxidant capacity has long been used as biomarker for various studies [10]. In contrast, the repair activity of PBL as biomarker just began to receive attention [11]. The repair activity can be assessed with plasmids damaged with specific agents or the synthetic oligonucleotides specific for particular lesions [12, 13]. Repair of the DNA lesions (including excision and gap-filling activities) is indicated by the incorporation of P^{32}-labled nucleotide. On the other hand, the activity to excise oxidative DNA lesions can be evaluated with a modified version of comet assay, in which the cell or nuclear extract is used to replace the Endo III/Fpg. Cells (not for extracts) treated with a constant amount of H_2O_2 or other oxidative agents are embedded in gels and used as substrate for the purpose [14, 15].

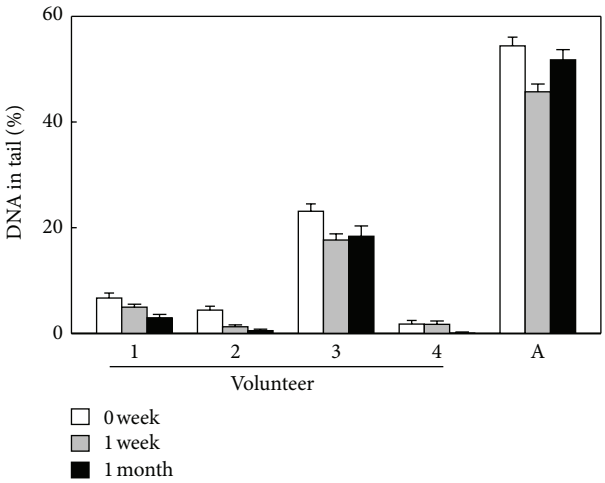

Figure 1: Oxidative DNA damage level of lymphocyte is steady. Lymphocyte samples of four volunteers collected at the indicated period of time were analyzed for the oxidative DNA damage with comet-Fpg/Endo III assay. 1–4: individual labels of the four volunteer; A: human AGS cells treated with 5 mM amoxicillin for 1 h, a positive control for enzyme (Fpg/Endo III) activity.

To test if the steady state levels of oxidative DNA damage of PBL are modulated by the antioxidant capacity of serum or the repair activity of PBL, 24 peripheral blood samples obtained from the individuals for routine health check-up were examined. Our results indicate that the level of oxidative DNA damage in PBL is not correlated with the serum antioxidant capacity but is weakly correlated with the BER activity in lymphocyte NE. Also, we found that PBL from carriers of *Helicobacter pylori* or *Hepatitis B virus* had higher levels of oxidative DNA damage, to which the BER activity but not the serum antioxidant capacity may attribute.

2. Materials and Methods

2.1. Patients and Samples. The 24 blood samples were randomly collected from individuals (males and females) who were 20–40 years old, nonsmoking, nondrinking, and lived/worked in similar environment. The four volunteers (males and females) for the experiment described in Figure 1 were 25–35 years old, nonsmoking, nondrinking, apparently healthy, and lived/worked in similar environment. The study was approved by Institutional Review Board of Taiwan (13MMH IS 189).

2.2. Isolation of the Lymphocytes. Lymphocyte were isolated from blood samples with the modified procedures of those described previously [16]. The blood samples were centrifuged at 3000 rpm (1400 ×g) for 15 min at 22°C for separation of serum from blood cells, and the serum on the top of the centrifuge tube was collected for antioxidant capacity measurement. The cells at the bottom of the centrifuge tube were resuspended in PBS at 1 : 1 ratio and the suspension was layered at 4 : 3 ratio to the ficoll-paque PLUS (GE Healthcare 17-1440-02) in another centrifuge tube. The whole assembly of blood cells was centrifuged at the same experimental condition described previously and the lymphocytes were collected from the layer between plasma and ficoll-paque layers. The cells were washed twice with PBS before analyses. One part of the cells was used for oxidative DNA damage experiment and the other part of the cells was used for repair activity experiment.

2.3. Detection of Oxidative DNA Damage with the Comet-EndoIII/Fpg Assay. The method described previously [17] was followed. In brief, conventional alkaline comet assay procedures were performed to obtain nucleoids in agarose gels on slides. Each slide was incubated with 2 units of both EndoIII and Fpg (Trevigen, Inc., Gaithersburg, MD, USA) in buffer of 10 mM Tris-HCl pH 7.4 for 1 h at 37°C in a sealed, humid chamber. EndoIII and Fpg are bacterial glycosylases that specifically recognize oxidized pyrimidines and purines, respectively. After enzyme digestion, DNA in nucleoids was alkaline denatured and then separated with electrophoresis. Nucleoids after staining with propidium iodide were examined with a fluorescence microscope; images of at least 50 nucleoids per slide were recorded with a closed-circuit display CCD camera (Zeiss/Axioskop 2 Mot plus). The migration of DNA from the nucleus of each cell was measured with a computer program (http://tritekcorp.com/) and is expressed as % DNA in the tail.

2.4. Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The standard TEAC assay described in [18] and in [19] has been used with minor modifications for determination of the TEAC value. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS (Sigma Aldrich, NO. A9941-5TAB) radical ($ABTS^{\bullet}$) in 6 min. The blue-green $ABTS^{\bullet}$ was produced through the reaction between 7 mM ABTS and 2.45 mM potassium persulfate in water. This solution was stored in the dark for 12–16 h at 4°C before use. The extinction coefficient of $ABTS^{\bullet}$ at 734 nm is 1.5×10^4 mol^{-1} l cm^{-1}. The concentrated $ABTS^{\bullet}$ solution was diluted with phosphate buffered saline (PBS), pH 7.4 to a final absorbance of 0.8 ± 0.02 at 734 nm at 37°C (i.e., an $ABTS^{\bullet}$ concentration of approximately 47 μM). Stock solutions of trolox (Sigma Aldrich, NO. 23881-3) were prepared in ethanol and serve as the standard curve. For the samples, 100 μL serum were added to 900 μL $ABTS^{\bullet}$ solution and the absorbance at 734 nm was measured in time. This was compared to a blank where 100 μL of the PBS was added to 900 μL of the $ABTS^{\bullet}$ solution. The initial reduction of absorbance was determined 3 min after addition of the antioxidant with constant mixing time (about 10 sec). The TEAC of serum was calculated by relating this decrease in absorbance to that of variation trolox concentrations served as a standard curve and normalized with protein amount determined with BCA kit (Pierce Chemical Co., Chester, UK).

2.5. Preparation of Nuclear Extract (NE) from Lymphocytes and Comet-NE Assay to Determine the Base Excision Activity in NE of Lymphocytes from 24 Blood Samples. Preparation of nuclear extract for comet-NE assay (see below) is done as

described in our previous study [17]. In brief, the isolated lymphocytes were first treated with 2.5 mM hydroxyurea and 25 μM cytosine-β-D-arabinofuranoside for 16 h. Hydroxyurea, an inhibitor of ribonucleotide reductase, and cytosine-β-D-arabinofuranoside, an inhibitor of DNA polymerase, are used here to prevent DNA synthesis in comet-NE assay. The cells were washed with hypotonic buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 M sucrose) and were allowed to swell in the hypotonic buffer without sucrose for 10 min on ice. The swollen cells were then ruptured with 10 strokes of a Dounce homogenizer and homogenates were forced through a 22 G needle 10 times. Each mixture was centrifuged at 2000 ×g for 5 min and nuclear pellets were resuspended in buffer (20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 10% sucrose) and stored at −70°C. The nuclei were thawed on ice and allowed to swell in 100 mM NaCl on ice for 1 h. The ruptured nuclei were centrifuged at 15,000 ×g for 20 min at 4°C, and the supernatants were filtered through a YM-10 Microcon filter (Millipore, Bedford, MA, USA). Protein concentrations were determined with a BCA Protein Assay Kit; bovine serum albumin was used as a standard.

The procedures of Comet-NE assay are similar to comet-EndoIII/Fpg assay unless in the enzyme incubation NE of lymphocytes but no Endo III/Fpg was performed. Constant amount of NE (about 0.6 μg) in NE buffer contained 50 mM Hepes-KOH (pH 7.9), 70 mM KCl, 5 mM $MgCl_2$, 0.4 mM EDTA, 2 mM ATP, and 40 mM phosphocreatine, and 2.5 mM creatine phosphokinase was added on each slide. Also, to determine base excision activity, the amoxicillin-treated (5 mM for 1 h) human AGS cells were used as the cell substrate in the comet-NE assay. Previously, we have reported that amoxicillin caused oxidative DNA damage [8, 20].

2.6. Cell Cultures. The AGS human gastric adenocarcinoma cell used in this study were originally obtained from the American Type Culture Collection (Manassas, VA, USA). AGS cells were grown in 1X RPMI medium (Sigma Aldrich, NO. R6594). All cell culture media were supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, NO. 19003C), and 0.03% glutamine and cells were grown at 37°C in a water-saturated atmosphere containing 5% CO_2.

2.7. Immunological Test. Infection of *Helicobacter pylori* (HP) or carriers of *hepatitis B virus* (HBV) were identified with DPC IMMULITE 2000 (Global Siemens, Germany) tested for presence of IgG against *H. pylori* or surface antigen of HBV.

2.8. Data Analysis. All experiments were performed independently at least three times. Data are expressed as means ± SE. Student's t-test was used for statistical analyses. A P value of <0.01 is considered to be statistically significant.

3. Results

3.1. The Oxidative Damage Level of Lymphocyte Is Stable. Although the oxidative DNA damage levels of human lymphocytes have been used as biomarker for investigating the intervention of antioxidants, often the measurement was done as the single time point. Whether the oxidative DNA damage level of lymphocyte from an individual is steady over a period of time, for example, months, remains unclear. Thus, we conducted an experiment involving four apparently healthy volunteers, and use the comet-*EndoIII/Fpg* assay to monitor the DNA damage level of lymphocyte for a month. The results indicate that the oxidative damage levels varied among the individuals; however, levels of each individual were relatively steady over a month unless a slightly decreasing trend of the damage levels of individuals was noted over the period of the time particularly in those with low damage levels (Figure 1). Human AGS cells treated with amoxicillin, an oxidative stress inducer, were used in the experiment as positive control (lane A of Figure 1).

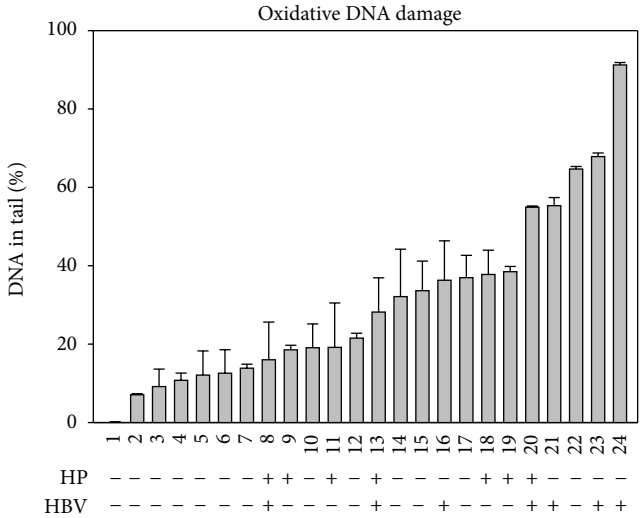

Figure 2: Variation of oxidative DNA damage levels of lymphocytes from 24 blood samples. Oxidative DNA damage levels of the lymphocytes isolated from 24 blood samples obtained from individuals who participated in health checkup. The oxidative DNA damage levels, represented as % DNA in tail, were aligned in the order from low to high, and each sample was coded (1–24) based on the respective DNA damage level. Carriers and noncarriers of HP or HBV are indicated with + or −, respectively.

3.2. Individual Variation of Oxidative DNA Damage of Lymphocytes of Peripheral Blood. The oxidative DNA damage levels of twenty-four blood samples randomly collected from individuals for ordinary health checkup were measured. The tested samples were given labels according to the respective oxidative DNA damage levels in the order from low to high (see Figure 2), showing that the variation can be up to 10 folds.

The levels of comet assay without EndoIII/Fpg, determined for 10 of the 24 blood samples, were, as expected, very small (1.58 ± 1.3% DNA in tail). Such levels are negligible if compared with the levels determined by comet assay with EndoIII/Fpg.

Among the tested samples, those from the individuals with infection of *Helicobacter pylori* (HP) or carriers of *hepatitis B virus* (HBV) were identified after the measurement

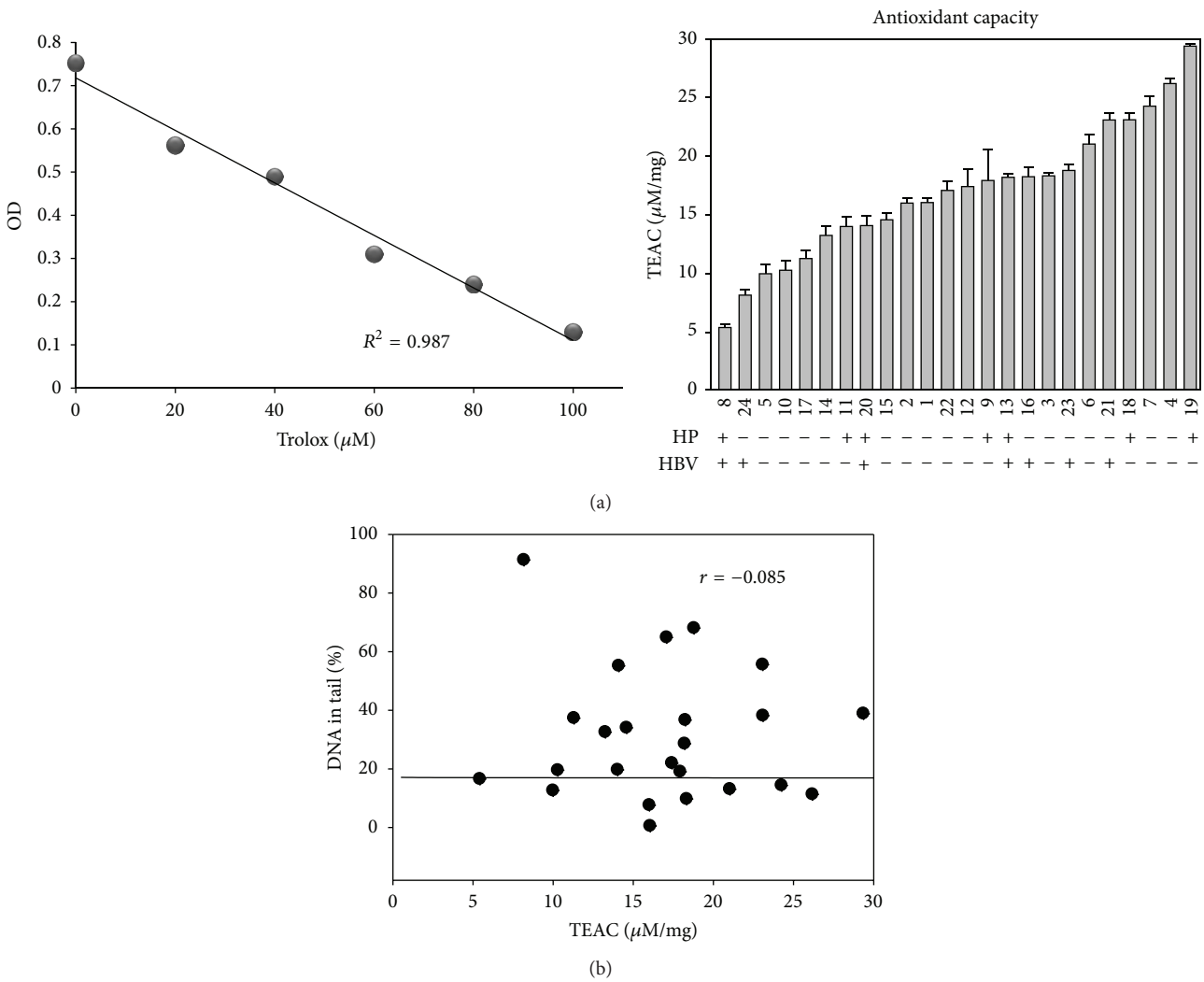

Figure 3: Variation of antioxidant capacities of sera from 24 blood samples. (a) Antioxidant capacities of sera isolated from 24 blood samples as described in Figure 2. Top part: trolox standard curve. Bottom part: The sample code was given according to those described in Figure 2, and the carriers and noncarriers of HP or HBV are indicated with + or −, respectively. (b) Correlation between the oxidative DNA damage levels described in Figure 2 and the antioxidant capacities of Figure 3(a). The r value (= −0.085), the correlation coefficient, was obtained from statistical analysis.

of oxidative DNA damage levels. The samples from HP or HBV carriers apparently tend to be at the right-handed side, that is, the side with high oxidative DNA damage levels. This is supported by comparing the means of the oxidative DNA damage levels: 31.0 ± 5.0% for HP carriers; 51.1 ± 9.2% for HBV and 21.5 ± 4.6% for noncarriers ($P < 0.05$). Thus, lymphocytes from HP or HBV carriers have significantly higher oxidative DNA damage than those from noncarriers.

3.3. Individual Variation of Oxidative DNA Damage of Lymphocytes of Peripheral Blood Is Not Correlated with the Antioxidant Capacity of Serum. To explore the factors that influence the oxidative DNA damage level of lymphocyte, the antioxidant capacity of serum was measured and the respective measurements were also shown in the order from low to high, while the sample labels remain the same as those given previously (Figure 3(a)). Since the order of antioxidant capacity is no longer 1 to 24, it is readily to conclude that there is no correlation (either positive or negative) between oxidative DNA damage level and antioxidant capacity of serum. In addition, the variation of antioxidant capacities among serum samples is at most 6 folds, not as dramatic as that with oxidative DNA damage. This conclusion is also true if the oxidative DNA damage level is plotted against the antioxidant capacity shown in Figure 3(b).

Moreover, the antioxidant capacities of serum samples from HP or HBV carriers do not show any preference to low or high side (Figure 3(a)), suggesting that antioxidant capacities of serum is not greatly affected due to the infection with either HP or HBV.

3.4. Helicobacter pylori or Hepatitis B Carriers Have Lower NE Activity to Repair Oxidative DNA Damage. To explore whether the repair capacity plays any role in the oxidative DNA damage level of lymphocyte, the activity to repair amoxicillin-induced DNA damage was assayed for

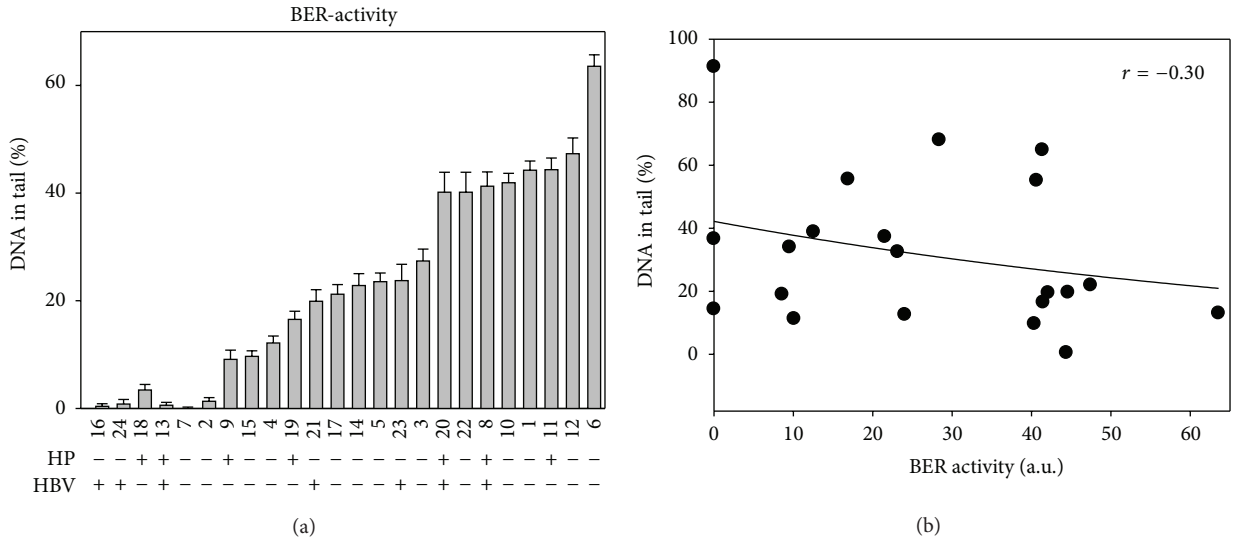

Figure 4: Variation of excision activities in nuclear extracts of lymphocytes from 24 blood samples. (a) To test the lymphocyte's nuclear etract's (NE's) activity, the AGS cells were served as substrate. AGS cells were treated with 5 mM amoxicillin to induce oxidative DNA damage and examined by comet-NE assay with lymphocyte's NE. The sample code was given according to those described in Figure 2, and the carriers and noncarriers of HP or HBV are indicated with + or −, respectively. (b) Correlation between the oxidative DNA damage levels of Figure 2 and the excision activities of Figure 4(a) (*note*: samples coded 2, 13 and 18 were excluded from the correlation because the concern of artifact due to sample preparation; their respective NER activities shown in Figure 5(a) are also low). The r value (= −0.30), the correlation coefficient, was obtained from statistical analysis.

the nuclear extracts of lymphocyte of tested blood samples. Amoxicillin has been shown by us to cause oxidative stress [8, 20]. The results of the repair activity assay shown in Figure 4(a) indicate that the variation among the tested samples can be more than 10 folds, as dramatic as the variation of oxidative DNA damage levels. The repair activity and the oxidative DNA damage level are weakly correlated in negative way ($r = -0.30$; see Figure 4(b)).

To test the possibility that the low activity levels (Figure 4(a)) may be the artifact of nuclear extract preparation, the respective activities to repair UV-induced DNA damage were assayed. The results indicate that some of these NE samples (e.g., samples coded 16 and 7) exhibited high NER activities (Figure 5(a)), suggesting that their low BER activities cannot be attributed to the artifact during NE preparation. Also, complementation of the repair activity could be seen when the NE samples with undetectable activities were mixed (Figure 5(b)).

Moreover, it is noted that repair activities in NE samples from HP or HBV carriers are lower than those of noncarrier: 18.9 ± 4.6% versus 29.38 ± 5.4% (mean ± SE; $P < 0.05$).

4. Discussion

4.1. The Oxidative Damage Level of Human Lymphocyte Is Not Correlated with the Serum Antioxidant of Capacity or the Base Repair Excision Activity in Nuclear Extracts of Lymphocyte. To know if the oxidative damage level of human lymphocyte is correlated with the serum antioxidant of capacity, we first examined if the individual oxidative DNA damage level of lymphocyte is steady over a period of time, for example, weeks or months. Previous studies about the oxidative damage level of human lymphocyte mostly showed the values of single time point. Our study based on the results of four individuals indicates that the oxidative DNA damage levels of lymphocytes are steady at least for one month. The results suggest that the daily meals, particularly food taken occasionally or medicine administered probably, have little influence on the oxidative DNA damage level of lymphocyte. Since the damage level varied among the individuals, the genetic or pathological background of chronic illness appears to be decisive. On the other hand, the serum antioxidant capacity has been shown to be not affected by the sampling time [21]. Our results show that the level of oxidative DNA damage in PBL is not correlated with the serum antioxidant capacity but is weakly correlated with the BER activity in lymphocyte NE; in other words, DNA damage repair activity of lymphocyte but not serum antioxidant capacity may play some role in determining the steady state of oxidative DNA damage level of lymphocyte.

The oxidative DNA damage levels in lymphocytes may be measured as the levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) with HPLC or the EndoIII/Fpg sensitive sites with comet assay. Similar to our observation, marked variations in oxidative DNA damage levels among individuals have been noted by the previous investigators [22–25]. The levels of 8-oxodG among the healthy individuals can vary about 10 folds [24]. Previous study has indicated that the mean levels of oxidative DNA damage of PBL varied with different population [24], supporting that genetic background plays role in this regard. Other studies have indicated that diseases such as cancer, diabetics, and cardiopathologic

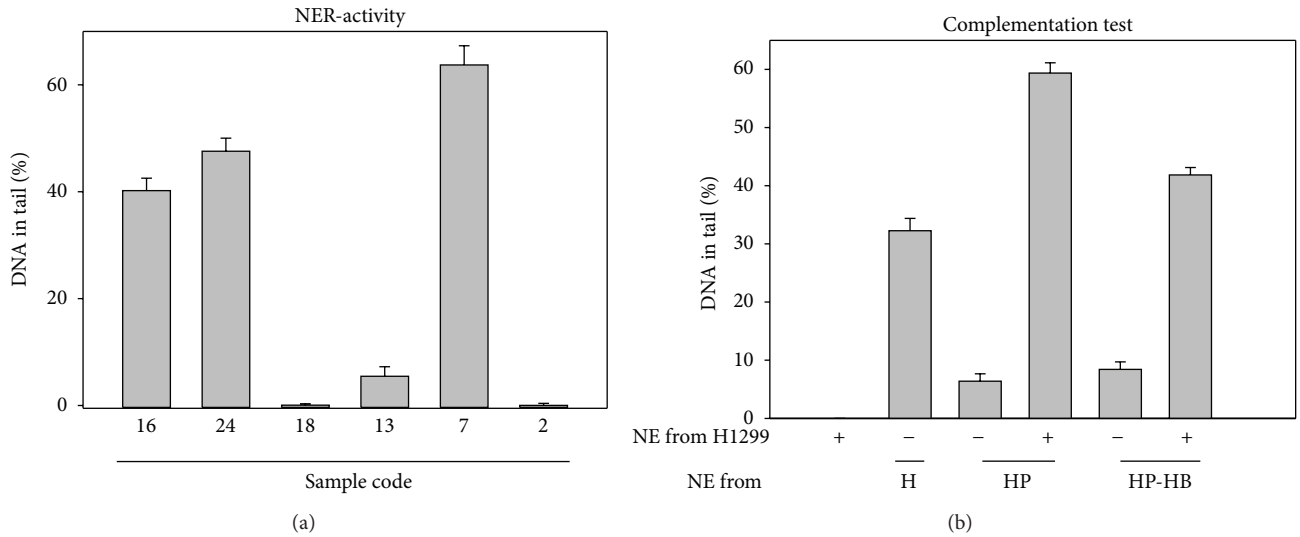

Figure 5: Control experiments for excision activities measured by the experiment described in Figure 4(a). (a) Nucleotide excision activities in some nuclear extracts of lymphocytes from the 24 blood sample. Nuclear extracts which failed to excise amoxicillin-induced DNA lesions were selected and used for examining their activities to excise UV-induced lesions. To determine the nucleotide excision activity, AGS cells treated with 5 J/m^2 UV were used as substrates in comet-NE assay. (b) Complementation test. Nuclear extracts samples from *Helicobacter pylori* carrier (HP) and HP-HBV (HP-HB) double carrier were mixed with the NE from p53 deficient cell line H1299 and used as in comet-NE assay. The NE from H1299 cell and healthy (H) people were severed as negative and positive control.

condition increase the mean of levels oxidative DNA damage of PBL [26, 27]. Collins et al. [28] have shown that oxidative DNA damage levels are positively correlated with colorectal cancer in men and negatively correlated with stomach cancer in women.

The antioxidant capacity in our study is total antioxidant capacity (expressed in TEAC), which is contributed by various components of serum including proteins, lipids, and small molecules. Previous study has indicated that proteins and urate contribute about 52% and 38% of total antioxidant capacity, respectively [29]. Since serum concentrations of proteins and urate are usually not affected by short-term dietary treatment, the total antioxidant capacity would be little influenced by food or other nutrient supplements. Although it is more precise to know the antioxidant capacity of individual component in serum, it is technically more challenging to do so as separation and identification of the individual component are necessary. Actually, the correlation between plasma levels of antioxidants such as α-tocopherol or β-carotene or ascorbate and endogenous oxidative DNA damage has been found insignificant [30]. On the other hand, the overall or total antioxidant capacity (TEAC used in our study) is wildly used in previous studies. Previous study has shown that TEAC is comparable with other similar methods, indicating little variation (<10%) among the total antioxidant capacities of healthy individuals [29]. Thus, TEAC assay is a relevant assay for our purpose.

The conclusion in the present study *should not* be interpreted that dietary supplement of antioxidant has no influence on the level of oxidative DNA damage in PBL. In fact, Duthie et al. [30] have reported that the oxidative DNA damage levels are not correlated with the plasma levels of α-tocopherol or β-carotene or ascorbate of individuals either smokers or nonsmokers of normal healthy males. This is consistent with our conclusion: no correlation between oxidative DNA damage levels and TEAC levels. However, in their study, supplement of diet for 20 weeks with vitamin C, vitamin E, and β-carotene reduce the endogenous oxidative DNA damage in PBL (from 60.5 ± 7.0 to 38.0 ± 5.9). It should be noted that the reduction is less than 2 folds, suggesting that the dietary effect is not enough to explain the wild variation of endogenous oxidative DNA damage in PBL. Thus, our study does not go against the findings by Brevik et al. [31], who have reported that dietary supplement with some plant products increases BER by 55% and decreases NER by 39%, with kiwifruit NER (−38%) and BER (no effect). The two studies are of different context.

4.2. The Oxidative Damage Levels of Lymphocytes and the Base Excision Repair Capacity from Helicobacter pylori (HP) or Hepatitis B Virus (HBV) Carriers. Our results suggest that individuals infected with HP or HBV tend to have higher oxidative DNA damage levels in their lymphocytes. Such observation is consistent with the conclusion of the previous reports that infection of HP increases the DNA damage of lymphocyte [32] and is in agreement with the clinic observation that carriers of HP or HBV have higher risk of cancer affliction [33, 34]. Our study suggests that the reduced DNA damage repair activities but not antioxidant capacities are more relevant to their high oxidative DNA damage levels. Why the infection of HP or HBV impairs the repair capacity of lymphocyte is unclear. The effect of HP infection on BER has been studied. APE1, the human endonuclease to process abasic sites formed during BER,

may be downregulated or upregulated depending on the experimental conditions [35, 36]. As reactive oxygen species have been well known to be associated with HP infection, the role of BER to protect against tumorigenesis due to HP infection has been recognized. On the other hand, the recent study indicates that X protein of HBV, which structurally resembles human thymine DNA glycosylase (TDG), a key enzyme of BER, inhibits TDG-dependent BER [37]. Our preliminary study on repair activity complementation shows promising for the kind of research to explore the molecular mechanism leading to low BER activity.

5. Conclusion

In this research, we have demonstrated that the lymphocyte's DNA oxidative level is steady more than one month. The level of oxidative DNA damage in PBL is not correlated with the serum antioxidant capacity but is weakly correlated with the BER activity in lymphocyte NE. Our study shows that lymphocytes of blood samples from carriers of *Helicobacter pylori or hepatitis B virus*, as compared to those of noncarriers, have higher oxidative DNA damage levels and lower repair activities.

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Research Article

Oxidative Stress Is Involved in the Pathogenesis of Keshan Disease (an Endemic Dilated Cardiomyopathy) in China

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Oxidative stress and selenoprotein deficiency are thought to be associated with the pathogenesis of Keshan disease (KD). However, to our knowledge, the level of oxidative stress and expression of selenoproteins have not been investigated in the myocardium of patients with KD. In this study, 8-hydroxy-2-deoxy guanosine (8-OH-dG), a marker of oxidative stress, was used to assess the level of oxidative stress, and thioredoxin reductase 1 (TrxR1) and glutathione peroxidase 1 (GPx1) were assessed to reflect the level of selenoproteins. Myocardial samples from 8 patients with KD and 9 non-KD patients (controls) were immunohistochemically stained for 8-OH-dG, TrxR1, and GPx1. The staining intensities were subsequently quantified using Olympus Image-Pro Plus 6.0 software. The data showed that the positive rate of 8-OH-dG expression in myocardial nuclei was higher in the KD group (68.6%) than that in the control group (2.4%). In addition, a positive correlation between the positive rate of 8-OH-dG and the degree of myocardial damage was observed in the KD group. The distribution of TrxR1 and GPx-1 was not associated with the distribution of myocardial damage. The expression of these two selenoproteins was higher in the control group than that in the KD group. Our study represents the first report on the expression profiles of oxidative stress and selenoproteins in the myocardium of patients with KD. The level of oxidative stress significantly increased and was positively correlated with the degree of myocardial damage in patients with KD. The selenoproteins, TrxR1 and GPx1, may have a role in the pathogenesis of KD.

1. Introduction

Keshan disease (KD) is an endemic cardiomyopathy of unknown cause and is named after Keshan County in Heilongjiang Province where the disease was first identified in 1935. It is clinically categorized into four groups: acute, subacute, chronic, and latent. The epidemiological characteristics of KD show a regional distribution. It is distributed in a narrow low-selenium belt from Northeast to Southwest China [1].

There is growing evidence showing that selenium deficiency is closely related to KD. The selenium levels in soil and food in the external environment of the endemic area are significantly lower than those in the nonendemic area. Blood and hair selenium levels in patients with KD are also significantly lower than those in healthy individuals [2]. In particular, the incidence of the acute and subacute types of KD significantly decreased after selenium supplementation [2]. Researchers have proposed that oxidative stress induced by selenium deficiency plays a pivotal role in the pathogenesis of KD [3]. As a vital antioxidant element, selenium is incorporated into selenoproteins to fulfill its biological functions. Glutathione peroxidase1 (GPx1) and thioredoxin reductase 1 (TrxR1), two selenoproteins, are important members of the body's antioxidant system. They catalyze the reduction of hydrogen peroxide to eliminate harmful reactive oxygen species from the tissues and protect biological membranes and large molecular structures from oxidative damage [4]. 8-OH-dG is a modified product of oxidative DNA damage and is usually used as a biomarker for assessing oxidative stress [5].

Previous research showed that the blood GPx activity in patients with KD was lower than that in subjects in the nonendemic area [6]. However, there has been no report on the expression of selenoproteins and the level of oxidative stress in the myocardium of patients with KD. In this study, the level of oxidative stress was evaluated by determining 8-OH-dG and the level of selenoproteins was assessed by the detection of TrxR1 and GPx1 in the myocardium of subjects with or without KD. We found that oxidative stress was detected in the myocardium of subjects with KD. A positive correlation between the positive rate of 8-OH-dG expression and the degree of myocardial damage was observed. The expression of TrxR1 and GPx1 decreased significantly in the KD group compared with the controls. Oxidative stress may be induced by a decrease in the expression of TrxR1 and GPx1 in patients with KD.

2. Methods

2.1. Ethic Statement. The experimental protocols and procedures used in this study were approved by the Medical Ethics Committee of Harbin Medical University.

2.2. Tissue Samples. Tissue samples from 17 autopsies were obtained from Harbin Medical University. These tissue samples were divided into the KD group ($n = 8$) and the non-KD group (the control group) ($n = 9$) according to the pathological diagnostic criteria of KD (WS210-2001). The KD group included 4 acute KD and 4 chronic KD patients (4 males and 4 females, mean age 28 ± 6). The specimens in the control group were obtained from a non-KD area (4 males and 5 females, mean age 30 ± 5). The subjects in the control group all died in the traffic accident. All specimens were embedded in paraffin and 5 μm sections were cut.

2.3. Antibodies. Mouse anti-human 8-OH-dG antibody (sc-66036), mouse anti-human GPx1 antibody (sc-74498), and mouse anti-human TrxR1 antibody (sc-28321) were bought from Santa Cruz Biotechnology, Inc., USA. Mouse anti-enterovirus VP1 (E3310-01) was bought from USBio Company, USA. The horseradish peroxidase-conjugated goat anti-mouse IgG and diaminobenzidine dye kit were bought from Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China.

2.4. Immunohistochemical Staining. Immunohistochemical staining for 8-OH-dG, TrxR1, and GPx1 was performed using standard techniques as previously described [7]. Formalin-fixed, paraffin-embedded tissue sections of 5 μm thickness were deparaffinized and hydrated. Endogenous peroxidase was inactivated by covering the tissue with 3% hydrogen peroxide for 10 min. The slides were washed three times with phosphate buffer (pH 7.2; 3 min each). Heat-induced epitope retrieval was performed twice using citrate buffer (pH 6.0) and a microwave at 100°C (10 min each). After microwave irradiation, the sections were allowed to cool down to room temperature. Subsequently, the slides were washed with phosphate buffer (pH 7.2) 3 times (3 min each). The slides were blocked with 5% bull serum albumin solution at room temperature for 20 min. Tissue sections were then incubated overnight with anti-8-OH-dG antibody (1 : 100), anti-TrxR1 antibody (1 : 100), or anti-GPx1 antibody (1 : 100) in a humidified chamber at 4°C. After three washes with phosphate buffer, tissue sections were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Zhong Shan-Golden Bridge) at 37°C for 20 min. The slides were then washed five times with phosphate buffer (3 min each). Freshly prepared diaminobenzidine substrate was added to the sections at room temperature for 3 min. The sections were then rinsed with water and counterstained with hematoxylin. Samples were dehydrated using a general protocol and sealed with neutral balsam. Immunohistochemical staining was examined by two pathologists blinded to the array composition.

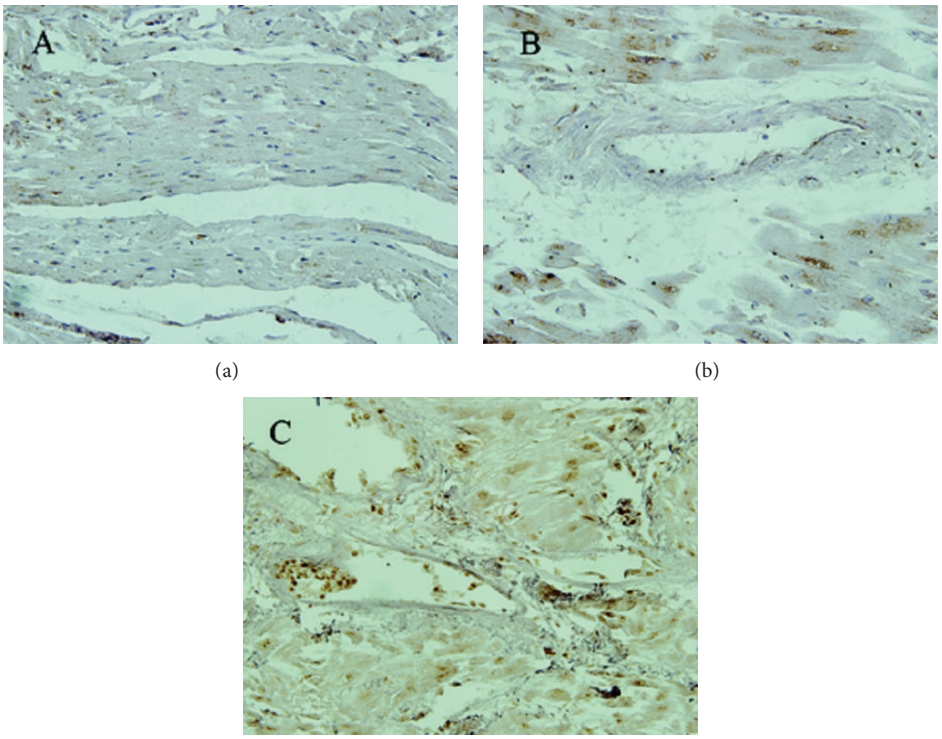
2.5. Quantitative Analysis of the Immunohistochemistry. Observations were performed using a light microscope (BX51, Olympus, Japan) with a 40x objective (UplanSApo, Olympus). The images were captured using a digital camera (DP72, Olympus). Five photographs of each specimen were randomly taken under the same conditions including light source, color saturation, brightness, gain, and contrast. The photographs were then quantified using Image-Pro Plus 6.0 (IPP 6.0) software. Quantitative analysis of the immunohistochemistry findings was performed after color segmentation based on the fixed threshold value of hue, saturation, and intensity (HSI). The positive rate of 8-OH-dG was obtained through the number of cardiac myocytes in the view divided by the number of 8-OH-dG positive cardiac myocytes which reflected the level of oxidative stress. The quantity of TrxR1 and GPx1 was expressed as integrated optical density (IOD, area × average optical density).

2.6. Statistical Analysis. The data were expressed as mean ± SD. SPSS 11.0 software was used for all statistical analyses. Comparisons between two groups were evaluated using the t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The Expression of 8-OH-dG. In order to detect the level of oxidative stress in the myocardium of KD patients, the expression of 8-OH-dG was determined by immunohistochemistry. Figure 1 shows that a positive signal for 8-OH-dG was claybank or brown and was predominantly expressed in cell nuclei. In the KD group, positive nuclei were diffusely distributed in the myocardium and were strongly positive. A few nuclei were weakly positive or positive in focal myocardial necrosis. Fibrocytes were negative for 8-OH-dG. However, in the control group, myocardial nuclei were negative or weakly positive.

The data in Table 1 show that the positive rate of 8-OH-dG expression in myocardial nuclei was higher in the KD group than in the control group. In addition, the positive rate of 8-OH-dG expression in the acute KD (91.7%) was significantly higher than that in the chronic KD (53.2%). These results



(a) (b) (c)

Figure 1: The expression of 8-OH-dG in myocardium. The positive signal of 8-OH-dG was claybank or brown and predominantly expressed in cell nuclei. The negative nuclei of 8-OH-dG were blue dyed by hematoxylin. (a) Most of myocardial nuclei are negative in the control group. (b) More than half of myocardial nuclei were positive in chronic KD. (c) Myocardial nuclei almost were positive in acute KD. 400x.

Table 1: The expression of 8-OH-dG in subjects with or without KD.

Group	$\overline{X} \pm$ SD (%)
KD group ($n = 8$)	$68.6 \pm 20.4^*$
Control group ($n = 9$)	2.4 ± 1.5

$^*P < 0.05$ compared with the control group.

suggest that the level of myocardial oxidative damage was higher in the KD group than that in the control group.

3.2. The Expression of TrxR1 and GPx-1. Immunohistochemistry was used to investigate the expression of TrxR1 and GPx1 in the myocardium. Figure 2 shows that a positive signal for TrxR1 and GPx1 was claybank or brown and predominantly expressed in the cytoplasm. The distribution of TrxR1 and GPx1 was not associated with the distribution of myocardial damage. Table 2 shows that the IOD of these two antioxidant enzymes was higher in the control group than that in the KD group. These results indicate that a deficiency of selenoproteins existed in the myocardium of patients with KD.

Table 2: The expression of TrxR1 and GPx1 in subjects with or without KD.

Protein	Group (KD = 8, control = 9)	Mean ± SD (IOD)	t	P
TrxR1	KD group	401340 ± 59865	−28.493	$P < 0.01$
	Control group	2790300 ± 379298		
GPx 1	KD group	497590 ± 197082	−6.016	$P < 0.01$
	Control group	1348400 ± 615840		

4. Discussion

The role of oxidative stress in the pathogenesis of cardiomyopathy has been a topic of research. Studies have shown that oxidative stress may result in cardiomyopathy through activation of the apoptosis program in myocardial cells [8]. Many researchers have suggested that a deficiency in selenium is one of the principal factors responsible for KD [1]. The level of oxidative stress induced by selenium deficiency is higher in patients with KD than that in healthy subjects in non-KD areas [9]. This was corrected after selenium supplementation, and the incidence of acute KD significantly decreased [2, 10]. These results suggested that oxidative stress has a role in the pathogenesis of KD. Currently, little is known about the level of oxidative stress in the myocardium of patients with KD. 8-OH-dG is an oxidative stress biomarker and has been widely used to assess the level of oxidative injury [11]. In this study, we assessed the level of oxidative stress in the myocardium

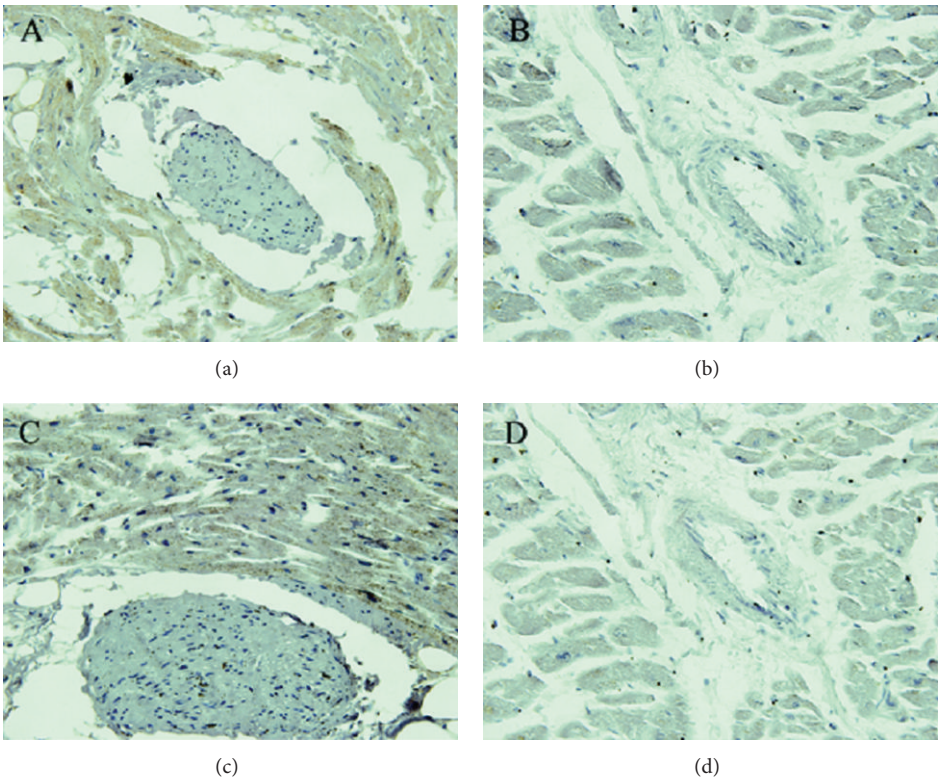


Figure 2: The expression of TrxR1 and GPx1 in myocardium. The positive signal of TrxR1 and GPx1 was claybank or brown and predominantly expressed in the cytoplasm. (a) Control group (TrxR1). (b) KD group (TrxR1). (c) Control group (GPx-1). (d) KD group (GPx-1). 400x.

of patients with KD by detecting the expression of 8-OH-dG. It was found that the positive rate of 8-OH-dG expression in myocardial nuclei was higher in the KD group than that in the control group. In addition, a positive correlation between the positive rate of 8-OH-dG and the degree of myocardial damage was observed in the KD group. Our results provide evidence that oxidative stress is involved in the pathogenesis of KD.

TrxR1 is an isozyme of thioredoxin reductase and is expressed in the cytoplasm. It participates in the oxidative stress reaction due to its catalytic reduction activity. Research has shown that TrxR1 knockout mice showed diminished TrxR1 activity, and oxidative stress increased leading to the emergence of heart diseases [12]. To date, there are few studies on the relationship between TrxR1 and KD. The expression of TrxR1 in the myocardium of KD patients was first observed by us. It was found that the expression of TrxR1 was significantly lower in the KD group than that in the control group. This indicated that there was not only a deficiency in selenium but also a decrease in TrxR1 in the myocardium of KD patients.

GPx1, an important antioxidant enzyme, is a member of the glutathione peroxidase family and is found in the cytoplasm [13]. It was found that selenium supplementation could protect the myocardium against oxidative stress damage by increasing the activity of GPx1; therefore, the risk of cardiovascular disease decreased [14]. This research showed that GPx1 activity in peripheral blood was lower in KD patients than in healthy individuals [15]; however, the activity of GPx1 in the myocardium of KD patients is still unknown. Our results showed that GPx1 was similar to TrxR1, and the expression of GPx1 was lower in the KD group than that in the control group. The above results indicate that selenium deficiency in the myocardium of patients with KD leads to the combined deficiency of selenoenzymes such as GPx1 and TrxR1. This may decrease the capacity of the antioxidative system in the myocardium and lead to oxidative damage. This would subsequently result in the emergence of cardiomyopathy.

In summary, although the sample size was small in this study, our results strongly demonstrated that damage due to oxidative stress was present in the myocardium of patients with KD. Furthermore, a positive correlation was found between the expression of 8-OH-dG and the degree of KD myocardial damage. The selenoproteins, TrxR1 and GPx1, may be involved in the pathogenesis of KD. These results provide evidence that selenium supplementation may prevent cardiomyopathy through the oxidative stress pathway.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Junrui Pei and Wenqi Fu contributed equally to this work.

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Review Article

Oxidative Stress and Nucleic Acid Oxidation in Patients with Chronic Kidney Disease

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Patients with chronic kidney disease (CKD) have high cardiovascular mortality and morbidity and a high risk for developing malignancy. Excessive oxidative stress is thought to play a major role in elevating these risks by increasing oxidative nucleic acid damage. Oxidative stress results from an imbalance between reactive oxygen/nitrogen species (RONS) production and antioxidant defense mechanisms and can cause vascular and tissue injuries as well as nucleic acid damage in CKD patients. The increased production of RONS, impaired nonenzymatic or enzymatic antioxidant defense mechanisms, and other risk factors including gene polymorphisms, uremic toxins (indoxyl sulfate), deficiency of arylesterase/paraoxonase, hyperhomocysteinemia, dialysis-associated membrane bioincompatibility, and endotoxin in patients with CKD can inhibit normal cell function by damaging cell lipids, arachidonic acid derivatives, carbohydrates, proteins, amino acids, and nucleic acids. Several clinical biomarkers and techniques have been used to detect the antioxidant status and oxidative stress/oxidative nucleic acid damage associated with long-term complications such as inflammation, atherosclerosis, amyloidosis, and malignancy in CKD patients. Antioxidant therapies have been studied to reduce the oxidative stress and nucleic acid oxidation in patients with CKD, including alpha-tocopherol, N-acetylcysteine, ascorbic acid, glutathione, folic acid, bardoxolone methyl, angiotensin-converting enzyme inhibitor, and providing better dialysis strategies. This paper provides an overview of radical production, antioxidant defence, pathogenesis and biomarkers of oxidative stress in patients with CKD, and possible antioxidant therapies.

1. Introduction

Chronic kidney disease (CKD) and/or end-stage renal disease (ESRD) have a high incidence of cardiovascular disease and malignancy [1, 2]. Several factors contribute to both types of health consequences including immune system dysfunction, chronic inflammation and infection, reduced antioxidant levels, and accumulation of uremic toxins. The mortality rate is substantially higher in patients with CKD than in the general population, and increased oxidative stress has been observed in patients with CKD [3, 4].

Oxidative stress results from an imbalance between free radical production and insufficient endogenous antioxidant defense mechanisms and has been documented in uremic patients [5, 6]. Most free radicals in biological systems are aerobic metabolism-generated reactive oxygen species (ROS), but there are also derivatives of nitrogen (reactive nitrogen species, RNS) [7]. Increased concentration of

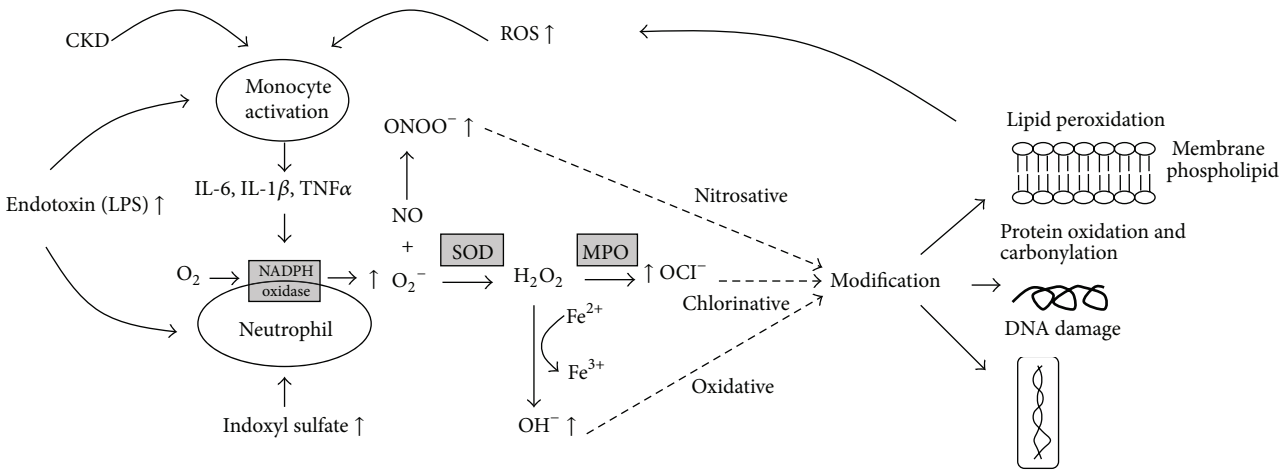

Figure 1: Synthesis of reactive oxygen species (ROS) in patients with chronic kidney disease (CKD). Excessive reactive ROS including $ONOO^-$, OH^-, and OCl^- are generated from oxygen through several main enzymes (NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase (MPO)). Several factors can also increase ROS generation, including cytokines (IL-8, IL-1β, and TNF-α) released from activated monocytes, uremic toxin (indoxyl sulfate), and endotoxin (LPS) from the HC procedure. The resulting excessive ROS can lead to nitrosative ($ONOO^-$), chlorinative (OCl^-), and oxidative (OH^-) modifications to lipids, proteins, and DNA.

malondialdehyde generated by lipid peroxidase [8] and impaired function of antioxidant systems because of low levels of superoxide dismutase and glutathione (GSH) peroxidase have been reported in hemodialysis (HD) patients [9]. These products can also induce chemical changes in many substances such as proteins, lipids, and nucleic acids.

Oxidative nucleic acid damage is defined as the imbalance between the excess formation and insufficient removal of highly reactive molecules (ROS and RNS) in response to environmental or behavioral stress [10]. Oxidative stress can induce DNA or nucleic acid damage, such as base and sugar modifications [11], covalent crosslinks, and single- and double-stranded breaks [12]. The DNA bases, especially guanine (G), are particularly susceptible to oxidation, leading to oxidized guanine products. Nucleobase modifications most frequently involve 8-hydroxy-2′-deoxyguanosine (8-OH-dG), one of the most abundant oxidative products of nucleic acids [13]. In CKD patients, impaired function of the antioxidant systems and imbalance between free radicals and endogenous antioxidant forces may contribute to the accelerated development of oxidative nucleic acid damage, which may increase the risk of later cancer development [14]. The purpose of this review is to provide an overview of pathogenesis, biomarkers, and consequences of oxidative stress in patients with CKD and the possible antioxidant therapies to reduce oxidative stress and nucleic acid oxidation.

2. Pathogenesis of Oxidative Stress and Nucleic Acid Oxidation in CKD

2.1. Increased Production of RONS in Patients with CKD (Figure 1)

2.1.1. Generation of RONS by the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Complex. The mitochondrial respiratory chain represents the most powerful cellular source of oxidants in the body. During respiration, in the mitochondrial electron transport chain, the electrons are passed individually to oxygen. Each oxygen molecule needs four electrons to be reduced completely; intermediate stages of reduction are formed during electron transport, thereby producing free radicals, which are atoms or molecules with one or more unpaired electrons that are capable of independent existence. Free radicals are particularly reactive molecules. Most free radicals in biological systems are derivatives of oxygen, but there are also derivatives of nitrogen (reactive nitrogen species, RNS) [7]. ROS and RNS are terms used collectively to describe highly reactive oxygen and nitrogen radicals, as well as nonradical derivatives.

Superoxide anion (O_2^-) is the major free radical generated *in vivo* by the reduction of molecular oxygen through the action of the NADPH oxidase enzyme complex. As soon as O_2^- is formed, it is converted into hydrogen peroxide (H_2O_2). Excessive production of ROS by NADPH oxidase is commonly thought to be responsible for tissue injury associated with a range of chronic inflammatory diseases and has long been considered a unique property of phagocytic cells [15]. Both O_2^- and H_2O_2 are precursors for the production of more powerful oxidants. O_2^- has a high affinity for reacting with the free radical nitric oxide (NO), which rapidly produces the RNS peroxynitrite ($ONOO^-$) [16], whereas H_2O_2 reacts with intracellular iron to form the hydroxyl radical (OH^-) via the Haber-Weiss cycle. The resulting $ONOO^-$ and OH^- can lead to extensive nitrosative and oxidative modifications to proteins [17], lipids [18], and nucleic acids [19].

2.1.2. Generation of Chlorinated Oxidants by the Myeloperoxidase System. Myeloperoxidase, an abundant enzyme in macrophages and neutrophils, catalyzes the generation of the oxidant hypochlorous acid (OCl^-) from H_2O_2 in the presence of Cl^-. $HOCl^-$ is a powerful compound capable

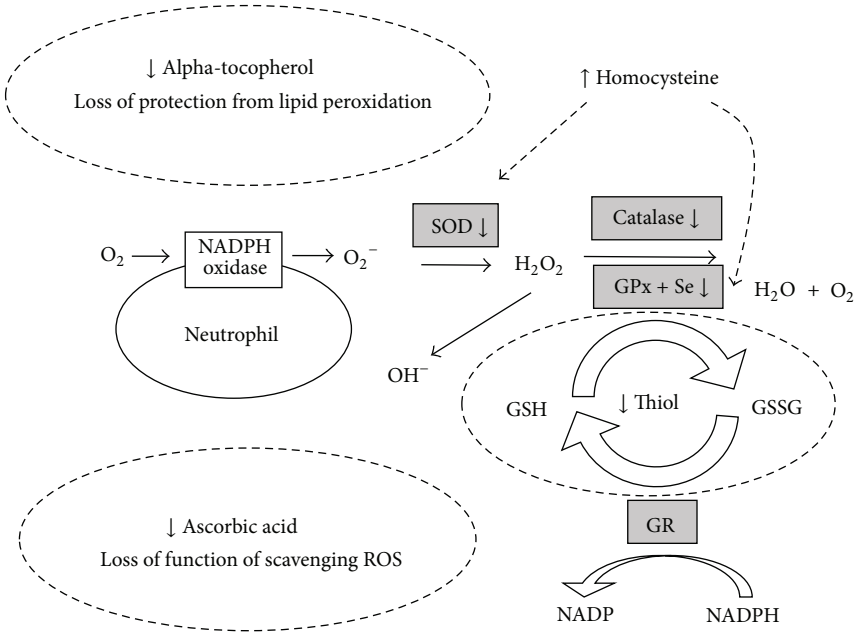

Figure 2: Impairment of antioxidant system in patients with CKD. Antioxidant systems, including nonenzymatic systems (thiol, alpha-tocopherol, and ascorbic acid) and enzymatic systems (superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)), are impaired or deficient in patients with CKD. Hyperhomocysteinemia can lead to inhibition of the activity of the antioxidant enzymes SOD and GPx. GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulfide; Se: selenium.

of oxidizing chlorination of many molecules such as lipids, proteoglycans, amino acids, and other membranous or intracellular constituents [20]. Some studies have provided biochemical, experimental, and human clinical data supporting the important role of myeloperoxidase-catalyzed oxidation in atherosclerotic disease in individuals with uremia [21, 22].

In the progression of CKD, the redox balance is not in equilibrium and is tipped toward oxidation, resulting in the dysregulation of cellular processes and subsequent tissue injury. Several studies have reported on the overproduction of ROS in uremic patients treated by HD [23, 24], which occurs via priming of leukocytes to produce ROS and HD-induced activation of polymorphonuclear leukocytes.

2.2. Impairment of the Antioxidant System in Patients with CKD (Figure 2). To prevent oxidative stress, detoxification of specific free radicals and other oxidants requires several intracellular and extracellular antioxidant systems, including both enzymatic and nonenzymatic systems.

2.2.1. Antioxidant Nonenzymatic Systems. A thiol is a compound containing the functional group –SH (reduced) and can be oxidized via disulfide bond formation (oxidized). By reacting with almost all physiological oxidants, thiols function as key antioxidant buffers in the maintenance of the homeostatic intracellular and tissue reduction/oxidation (redox) state. Thiol-containing cysteine residues in proteins are sensitive to oxidation, and changes in enzymatic activity or binding characteristics caused by oxidation provide a mechanism for signal transduction [25, 26]. The major biological thiol/disulfide couples are GSH, thioredoxin, and other cysteine-containing proteins. GSH is a scavenger of H_2O_2, OH^-, and chlorinated oxidants. Extracellular thiols also constitute an important component of antioxidant defense, particularly in plasma and interstitial fluids. Antioxidant defense is impaired when protective thiols are depleted in acute and chronic kidney injury, and oxidized thiols are toxic to the endothelium [27, 28].

Vitamin E (alpha-tocopherol) can protect cell membranes from lipid peroxidation and can interrupt the radical cascade by forming a low-reactivity vitamin that does not attack lipid substrates [14]. Vitamin E appears to be important in the protection against free-radical-induced oxidative damage by low-density lipoprotein (LDL) in biological membranes.

Vitamin C (ascorbic acid) is distributed widely in both intra- and extracellular fluids. Vitamin C acts as a potent water-soluble antioxidant in biological fluids by scavenging ROS (O_2^- and OH^-) and RNS species by forming semidehydroascorbic acid and may thereby prevent oxidative damage to important biological macromolecules [14]. Vitamin C deficiency in CKD patients on HD may be secondary to dietary restriction of fresh fruits and vegetables to avoid hyperkalemia and to loss of the vitamin when receiving dialysis.

Other inflammation proteins such as ferritin, transferrin, ceruloplasmin, and even albumin may also act as antioxidants by sequestrating transition metal ions involved in the formation of the most reactive oxyradicals.

2.2.2. Antioxidant Enzymatic Systems. Superoxide dismutases (SODs) represent a major defense system against oxidative damage by enzymatically converting O_2^- to H_2O_2. There

are three types of SODs in mammalian tissues: copper-zinc-containing SOD (SOD1) localized in the cytosol, manganese-containing SOD (SOD2) localized in the mitochondrial matrix, and extracellular SOD (SOD3). SOD1, SOD2, and SOD3 are each highly expressed in the normal kidney, predominantly in the renal tubules, compared with other organs [25]. A recent study suggests that SOD1 is a major antioxidant enzyme in the regulation of oxidative stress during progressive renal injury [29].

Catalase, which is responsible for the reduction of H_2O_2 to water, is expressed in most cells, organs, and tissues and at high concentrations in the liver and erythrocytes. It may also be a key enzyme in antioxidant defense in the kidney during injury. A recent study reported that the loss of catalase-buffering capacity leads to an increase in oxidative products and more severe renal fibrosis, resulting in progressive kidney disease in catalase-deficient mice [30].

Other antioxidants, including peroxiredoxin, thioredoxin reductase, and GSH peroxidase (GPx), also represent an important group of predominantly intracellular enzymes that reduce and inactivate H_2O_2 and other organic peroxides to water and oxygen. Five isoforms of GPx have been identified; two are present in human blood, GPx 1 in red blood cells [31], and GPx 3, which is produced by the kidney, in plasma [32].

The thioredoxin system comprises thioredoxin, thioredoxin reductase, and peroxiredoxin, which represent highly abundant proteins that are distributed through the cytoplasm, mitochondria, and other cell compartments [33]. Selenium-containing GPx reduces all organic lipid peroxides and requires GSH as a hydrogen donor [34]. Profound deficiencies in the activity of the GSH system and in selenium have been reported in HD patients. GPx activity is altered significantly in the early stages of CKD, decreases with the progression of uremia, and decreases markedly in HD patients [35]. The potential deleterious effect of HD-induced ROS overproduction is augmented by the impairment of antioxidant defense mechanisms associated with uremia; the main disturbance concerns the GSH/GPx/selenium complex because selenium concentration decreases significantly in uremic patients [36].

2.3. hOGG1 Gene and Other Gene Polymorphisms. Genetic background is known to be involved in the control of damaged DNA repair. Genetic polymorphisms in DNA repair genes may affect DNA repair capacity, resulting in DNA damage accumulation. The base excision repair (BER) pathway, containing *hOGG1*, *MTH1,* and *MUTYH*, is a major protector from oxidative DNA damage in humans. A C $\rightarrow$ G polymorphism at position 1245 in exon 7 of the *hOGG1* gene is associated with the substitution of cysteine for serine at codon 326 (Ser326Cys) and is a determinant of genomic damage in leukocytes [37, 38]. A population with decreased enzyme activity of the *hOGG1* gene would be at risk of accumulating 8-OH-dG in nuclear DNA because of incomplete repair of oxidatively damaged DNA. In one study of patients undergoing chronic HD, leukocyte 8-OH-dG level was increased further among patients with the 1245 GG genotype compared with patients with the 1245 CG or CC genotype [37]. Another recent report in Turkey showed that *XRCC1* Arg399Gln polymorphism may increase the risk for the development of ESRD [39]. In a Chinese population study, the polymorphisms in BER system, including *MUTYH* c.972GG (rs3219489) and *AluYb8MUTYH* (rs10527342), increased the risk for ESRD development, especially their combined effect with *OGG1*c.977GG. Therefore, those homozygous or heterozygous BER polymorphisms might be candidate genetic factors for ESRD development [40]. Oxidative DNA damage among chronic HD patients is influenced by a combination of the overproduction of ROS, impaired antioxidant defense mechanisms, and genetic influence.

Single-nucleotide polymorphisms (SNPs) of antioxidant enzymes including SOD2, GPx, and catalase may contribute to diseases associated with oxidative stress [41]. These diseases include cancer, diabetes, Alzheimer's disease and other neurodegenerative diseases, cardiovascular disease, and CKD [42]. The antioxidant enzyme SNPs associated most frequently with disease in humans are SOD2 SNP Ala16Val, GPx1 SNP Pro197Leu, and catalase SNP C-262T. Most evidence supports associations between the SOD2 SNP Ala16Val genotype and diseases such as breast, prostate, and lung cancers, diabetes, and cardiovascular disease, whereas the GPx1 SNP Pro197Leu and catalase SNP C-262T SNP genotypes are associated with breast cancer [43]. It has been reported that CKD patients with the SOD Ala/Val and Val/Val genotypes have a significantly greater decline in estimated glomerular filtration rate (eGFR) compared with patients with the Ala/Ala genotype. The amino acid change from Ala to Val affects the structure of SOD, changing the alpha helix structure to a beta sheet. Therefore, the SOD genotype may be useful for identifying CKD patients at risk of more rapid CKD progression [41].

GSH S-transferase M1 (GST M1) is a member of the GST family of proteins, which protects cellular DNA against oxidative damage. Patients undergoing maintenance HD who lack GST M1 activity (a particular GST M1 polymorphism) are more vulnerable to oxidative stress and are at greater risk of death compared with those who possess GST M1 activity [44].

2.4. Other Factors That Can Induce Oxidative Stress in CKD

2.4.1. Uremic Toxins. In CKD patients, progressive deterioration of renal function can lead to accumulation of uremic toxins, which can induce oxidative stress [52]. Indoxyl sulfate (IS), a uremic toxin, is an organic anion that is normally excreted into urine and exists at high concentrations in the serum of patients with progressive CKD [53]. A high concentration of IS is considered a risk factor for cardiovascular disease in CKD patients and accelerates the progression of CKD. It has been reported that IS upregulates the expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) by ROS-induced activation of NADPH oxidase and nuclear factor-κB (NF-κB) in vascular endothelial cells. Thus, IS may play an important role in the development of cardiovascular disease in CKD patients by increasing the endothelial expression of ICAM-1 and MCP-1 [54].

Table 1: Biomarkers of oxidative stress and antioxidant status.

Lipid peroxidation	Oxidized low-density lipoprotein (LDL), HOCl-modified LDL, malondialdehyde (MDA), 4-hydroxynonenal (HNE), hydroxyoctadecadienoic acid (HODE), thiobarbituric-acid-reactive substances (TBARS), advanced lipoxidation end products (ALE), cholesteryl esters
Arachidonic-acid-derived oxidation	Isofurans, F_2-isoprostane, isolevuglandins
Protein oxidation	Advanced oxidation protein products (AOPPs), protein thiols oxidation
Protein carbonylation	Advanced glycation end products (AGEs)
Amino acid oxidation	3-Nitrotyrosine, 3-chlorotyrosine, dityrosine, carboxymethyl lysine, cysteine/cystine, homocysteine/homocystine
Nucleic acid oxidation	8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 8-hydroxy-2′-deoxyguanosine (8-OH-dG)
Antioxidant status	Oxidative stress index (OSI: ratio of total antioxidant capacity/total oxidant status), glutathione activity, superoxide dismutase, catalase, thioredoxin, arylesterase/paraoxonase

2.4.2. Homocysteine (Hcy). The sulfur-containing amino acid Hcy is a prominent uremic toxin and is a normal product in the metabolism of the essential amino acid methionine. Hcy is an intermediary amino acid formed endogenously by the conversion of methionine to cysteine. In healthy individuals, Hcy is remethylated to methionine (predominantly in the kidney) or can be metabolized by the transsulfuration pathway to cysteine. The vast majority of patients with end-stage renal disease (ESRD) have high plasma Hcy levels, but the reasons involve different mechanisms, which may include reduced renal and nonrenal organic clearance [55]. Vitamin deficiency, mainly of folic acid and vitamin B_{12} (cobalamin), is considered a major contributor to the hyperhomocysteinemia found in patients with CKD. High blood Hcy levels increase oxidative stress because Hcy is susceptible to autooxidation, with secondary generation of ROS, and Hcy can inhibit the activity of the antioxidant enzymes GPx and SOD [56]. Hyperhomocysteinemia in patients with CKD is considered a risk factor for malignancy because the DNA of these patients is hypomethylated [57, 58].

2.4.3. Arylesterase/Paraoxonase. Human arylesterase (PON1), member of the paraoxonase family of enzymes, hydrolyses organophosphate compounds and has a protective effect against lipoprotein oxidation in CKD. Most importantly, PON1 displays Hcy-thiolactonase activity and poses antiatherogenic properties. PON1 is diminished in CKD patients when compared to healthy controls and might be a sensitive marker of antioxidant status [59, 60].

2.4.4. HD-Induced Oxidative Stress. Uremic toxins, dialyzer interactions, and dialysate contaminants have been suggested as the three major causes of oxidative stress in HD patients. The dialysis membranes seem to play a central role in the increased production of ROS in these patients [61, 62]. Our previous study [62] to evaluate the influence of two different dialysis membranes, polysulfone compared with regenerated cellulose (RC), on oxidative stress during HD found that HD with RC membranes resulted in a significantly increased production of oxidants during a single HD session, whereas dialysis with a polysulfone dialyzer had a milder effect. HD may induce repetitive bouts of oxidative stress, which may trigger the generation of ROS, primarily through membrane bioincompatibility and endotoxin (LPS) challenge [63]. Hemoincompatibility of the dialysis system plays a critical role in the production of ROS. LPS in the dialysate may indirectly trigger ROS production by activating polymorphonuclear leukocytes. Moreover, because HD can also reduce the levels of oxidized protein thiols, HD modalities using highly permeable membranes can cause solute loss, including loss of hydrophilic nonenzymatic antioxidants [64]. However, HD treatment can also improve oxidative status and reverse the increased levels of oxygen radical production by neutrophils in the blood of patients with ESRD [64].

3. Biomarkers of Oxidative Stress and Nucleic Acid Oxidation in CKD

In 1994, Maggi et al. first reported that oxidative stress may contribute to atherosclerosis in uremic patients by measuring changes in lipid peroxidation [65]. Uremic oxidative stress can be characterized biochemically as a state of accumulation of reactive aldehyde and oxidized thiol groups, with a concomitant depletion of reduced thiol antioxidant groups. Numerous methods to estimate the degree of oxidative stress have been used, ranging from techniques with low dynamic ranges (such as immunostaining) to powerful analytical assays such as liquid chromatography or gas chromatography coupled with mass spectrometry. The most commonly used biomarkers in human and experimental models are listed in Table 1. Excess prooxidants or free radicals can oxidize macromolecules such as lipids, proteins, carbohydrates, and nucleic acids, causing DNA, cellular, and tissue injury.A promising, more complete approach may involve the simultaneous use of multiple biomarkers from both the oxidant-generating and antioxidant pathways to assess oxidative stress [66]. One recent systemic review reveals that several biomarkers emerged as well-suited indicators of oxidative stress and antioxidant status in patients with CKD including malondialdehyde, F2-isoprostanes, lipid hydroperoxides, asymmetric dimethylarginine (ADMA), protein carbonyls, advanced oxidation protein products (AOPPs), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), and glutathione-related activity [60].

3.1. Biomarkers of Lipid Peroxidation, Protein Oxidation, and Protein Carbonylation

3.1.1. Lipid Peroxidation. Peroxynitrite is generated from the reaction of NO with ROS and has several unfavorable vascular actions. Although the availability of NO is reduced, the increased production of advanced glycation end products (AGEs) adds to the atherogenic potential of renal insufficiency. Measurements of lipid hydroperoxides (oxidized low-density lipoprotein (LDL) and HOCl-modified LDL) and nitrated amino acids serve as excellent markers of cell and tissue oxidative stress because of their relative stability compared with the direct measurement of more transient free radicals. ROS can react with double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. Malondialdehyde is the secondary oxidation product of peroxidized PUFAs and has been shown to have mutagenic and cytotoxic effects and possibly to be involved in the pathogenesis of several human diseases, including atherosclerosis, neurodegenerative diseases, and cancer [67]. Lipid peroxidation products, namely, malondialdehyde [68], 4-hydroxynonenal (HNE) [69], advanced lipoxidation end products (ALE) [70], hydroxyoctadecadienoic acid (HODE) [71], F_2-isoprostanes [72] (enzymatically produced by free-radical-catalyzed peroxidation of arachidonoyl lipids), and isolevuglandins, have been reported to be elevated in HD patients [73]. The thiobarbituric-acid-reactive substances (TBARS) and lipoperoxides have been reported to be increased in patients after one year of HD [74, 75]. Cholesteryl esters (CE) in the hydrophobic core of LDL particles are oxidized to hydroperoxides. CE are sensitive markers of lipid damage and have been used to examine oxidatively damaged tissues [60].

3.1.2. Biomarkers of Protein Oxidation Damage. In contrast to lipids, reaction products of protein with various oxidants can accumulate, and the subsequent reactants may have toxic activities. Evaluation of oxidatively modified proteins may be useful in assessing oxidative stress status. Protein oxidation markers had not been documented thoroughly in HD patients until AOPPs were identified in the plasma of uremic patients [76]. AOPPs can be detected in HD patients and in CKD patients not yet on dialysis. Oxidation of plasma thiol groups is quantitatively the major manifestation of protein oxidation.

3.1.3. Biomarkers of Protein Carbonylation and Amino Acid Damage. Oxidative stress may contribute to the progression of renal disease through the generation of AGEs. AGEs are formed nonenzymatically by the reaction of carbonyl compounds with a free amino group from proteins, lipids, or amino acids and have been identified as markers of oxidative stress in uremic patients. The components of oxidative stress that increase production of AGEs by increasing the formation of carbonyl groups are termed carbonyl stress compounds [77, 78]. AGEs accumulate during aging and in the course of many degenerative diseases and can be removed by the kidney. AGEs have genotoxic effects and can react with DNA in a similar way to their reaction with proteins, resulting in the formation of DNA-bound AGEs [79].

The aromatic amino acids are very susceptible to oxidation by various ROS. For example, OH^--radical-mediated oxidation of tyrosine residues gives rise to dityrosine; reaction with RNS leads to the formation of 3-nitrotyrosine; reaction with HOCl leads to the generation of 3-chlorotyrosine; lysine residues are oxidized to carboxymethyl lysine; and cysteine residues are also oxidized to cysteine/cystine and Hcy/homocystine. 3-Nitrotyrosine is an oxidation byproduct that accumulates in fibrotic kidneys and in the serum of patients with CKD, suggesting that oxidative stress increases during the progression of kidney disease.

ADMA, an analogue of L-arginine, is a naturally occurring product of metabolism found in human circulation. ADMA can uncouple endothelial NO synthase, leading to the loss of NO and an increase in superoxide production in the vascular endothelium [60]. In progression of CKD, elevated levels of ADMA can inhibit NO synthesis and therefore impair endothelial function and thus promote atherosclerosis.

3.2. Biomarkers of Oxidative Nucleic Acid Products in Patients with CKD. The OH^- attacks DNA strands when it is produced adjacent to cellular and mitochondrial DNA (mtDNA), causing the addition of DNA bases containing new radicals, which lead to the generation of a variety of oxidation products [80]. The interaction of OH^- with the nucleobases of the DNA strand, such as guanine, leads to the formation of C8-hydroxyguanine or its nucleoside form 8-OH-dG. Initially, the reaction of OH^- addition leads to the generation of radical adducts, after which one electron abstraction leads to the formation of 8-OH-dG. 8-OH-dG undergoes keto-enol tautomerism, which favors the oxidized product 8-oxo-dG. In nuclear DNA and mtDNA, 8-OH-dG and 8-oxo-dG are the predominant forms of free-radical-induced oxidative lesions and are therefore used widely as biomarkers for oxidative stress and carcinogenesis. The biomarkers 8-OH-dG and 8-oxo-dG are pivotal markers for measuring the effects of endogenous oxidative damage on DNA and are factors involved in the initiation and promotion of carcinogenesis [81]. Usually, 8-OH-dG is measured as an index of oxidative DNA damage. Although the other nucleobases of DNA react with OH^- in a similar manner, lesions associated with 8-oxo-dG are the most abundant DNA lesions because they form easily; 8-oxo-dG is promutagenic and is therefore a potential biomarker of carcinogenesis [82]. 8-OH-dG level can be measured in animal organs and in human samples such as in the urine, organs, and leukocyte DNA and serves as a biomarker of oxidative stress, aging, and carcinogenesis [83, 84].

8-OH-dG is one of the most abundant oxidative DNA products among the base modifications elicited by ROS and may provide a new marker for the assessment of oxidative DNA damage in ROS-mediated diseases [13, 85–87]. The 8-OH-dG level in leukocytes is significantly higher in HD patients compared with nondialyzed patients with advanced renal failure and with healthy subjects [88]. Markedly elevated 8-OH-dG levels have been reported in patients on peritoneal dialysis therapy [89].

3.3. Techniques to Detect DNA Damage. Both before starting HD and during HD, peripheral blood leukocytes of CKD patients exhibit elevated genomic or nucleic acid damage compared with healthy controls. These changes have been demonstrated by analyzing the 8-OH-dG content in leukocyte DNA, micronuclei frequency, single-cell gel electrophoresis (comet assay), sister chromatid exchange or mitochondrial DNA deletions to detect the oxidatively damaged DNA, and nucleic acid damage. DNA and nucleic acid damage are measured in peripheral blood lymphocytes (PBL) as biomarkers of the early effects of genotoxic carcinogens in occupational and environmental settings.

3.3.1. 8-OH-dG. It is generally accepted that oxidatively damaged DNA can be repaired and that the repair products are released into the bloodstream and consequently appear in the urine without further metabolism [90]. The first report of an analysis of 8-OH-dG as a major oxidation product of DNA in the urine from experimental animals and humans was published in 1989 [91]. Studies have shown that urinary 8-OH-dG level is a good biomarker for risk assessment of various cancers and degenerative diseases. Various analytical techniques have been developed to measure oxidatively damaged DNA in the urine, including high-performance liquid chromatography with electrochemical detection, gas-chromatography-mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and liquid-chromatography-tandem mass spectrometry (LC-MS/MS) [92]. The first two methods have been established for >10 years, although they are labor intensive and have inadequate specificity or require chemical derivatization. A commercial ELISA kit is the most convenient method and is used frequently, although it often overestimates urinary 8-OH-dG concentration compared with chromatographic procedures. LC-MS/MS is a relatively new and powerful technology that can overcome the sensitivity and selectivity issues in the analysis of DNA adducts [93].

3.3.2. Micronuclei Frequency. Micronuclei are chromatin-containing structures surrounded by a membrane that are formed during mitosis [94]. The formation of micronuclei in dividing cells is the result of chromosome breakage caused by unrepaired or misrepaired DNA lesions, or chromosome malsegregation caused by mitotic malfunction. Micronuclei are sensitive *in vivo* and *in vitro* indicators of exogenous and endogenous genetic damage. Micronuclei and other nuclear anomalies such as nucleoplasmic bridges and nuclear buds are biomarkers of genotoxic events and manifestations of chromosomal instability that are often seen in cancer. The frequency of micronuclei in PBL is used extensively in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents [95]. Elevated rates of sister chromatid exchange and abnormal chromosomes have also been observed [96].

3.3.3. Single-Cell Gel Electrophoresis (Also Known as a Comet Assay) in PBL. The comet assay or single-cell gel electrophoresis is a simple and sensitive technique for detecting DNA damage at the level of the individual eukaryotic cell; this assay was first developed by Östling and Johansson in 1984 and later modified by Singh et al. in 1988 [97]. The comet assay measures DNA strand breaks, alkali labile sites, and relaxed chromatin in individual cells [97, 98]. In this assay, the cells are embedded in agar and exposed to an electrical field. Most of the genetic material from cells with damaged DNA migrates faster than does the material from cells with intact nuclear DNA. DNA migration is measured using computer assistance [99].

3.3.4. Mitochondrial DNA (mtDNA) Deletions. Mitochondria are a major intracellular source of ROS and free radicals. Because it lacks protective histones and has a low efficacy of DNA repair, mtDNA seems to be particularly sensitive to ROS [100]. Compared with nuclear DNA, human mtDNA is much more susceptible to damage induced by mutagens or carcinogens, such as free radicals, caused by the lack of proofreading and poor DNA repair during mtDNA replication. mtDNA deletions have been analyzed in hair follicles of patients with end-stage renal failure [101].

3.4. Biomarkers of Antioxidant Status in Patients with CKD. Antioxidant systems can stabilize free radicals, consequently reducing the oxidative stress. Enzymatic antioxidants are the most important defense against radical-induced damage. Therefore, recent researchers use different techniques to evaluate the activity of antioxidant status including oxidative stress index (OSI: ratio of total antioxidant capacity/total oxidant status), glutathione activity (GSH), superoxide dismutase (SOD), catalase, thioredoxin, and PON1/paraoxonase [60]. They also use different terms to express antioxidant status including total antioxidant efficiency, effectiveness, action, power, parameter, potential, potency, and activity. GSH is diminished in patients with CKD, even in the absence of dialysis. The ratios of GSH/GSSH and GSH-related makers including GSH-peroxidase, GSH-reductase, and GSH-S-transferase are also used to assess antioxidant status. Although SOD is a major antioxidant enzyme in the regulation of oxidative stress in CKD, the relationship between superoxide and SOD activity in CKD remains uncertain. Studies examining catalase activity in CKD are contradictory, but catalase might reflect antioxidant status in diabetes, rather than CKD. Besides, thioredoxin and PON1/paraoxonase are considered as the novel makers of antioxidant status in kidney disease [60].

4. Consequences of Oxidative Stress and Oxidative Nucleic Acid Damage in Patients with CKD

Oxidative stress, which can act synergistically with inflammation, is thought to be involved in the development of long-term complications such as inflammation, atherosclerosis, amyloidosis, and malignancy in CKD patients. Several lines of evidence indicate that nucleic acid damage associated with oxidative stress increases in CKD and HD patients. Stoyanova et al. [102] reported recently that oxidative nucleic

acid damage was higher in HD ($n = 77$) than in CKD patients ($n = 176$). Another study reported that patients with CKD exhibit upregulation of a number of genes involved in the oxidative phosphorylation system, suggesting that an impaired mitochondrial respiratory system contributes to increased oxidative stress [103]. Using comet studies, Stopper et al. showed significant increases in the percentage of DNA in the comet "tail" (indicating DNA damage) in 23 CKD patients compared with 21 healthy subjects; nucleic acid damage was higher in patients with high creatinine levels [104]. Reduced nucleic acid repair capacity in predialysis patients [105] also increases oxidative nucleic damage.

4.1. Inflammatory Response and Apoptosis. Oxidative stress may play multiple roles in the inflammatory response by cytokine-related ROS release and by regulation of transcription factors. First, oxidant generation is amplified by proinflammatory cytokines, especially interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α), produced by monocytes and the acute-phase reactant C-reactive protein released in response to ROS [14]. Second, O_2^- may activate NF-κB and activator protein-1 (AP-1), leading to the expression of cytokines, which may in turn stimulate overproduction of ROS by the NADPH oxidase complex [106]. Third, activated NADPH oxidase (NOX) is now recognized as an important modulator of a specific intracellular signal-transduction pathway by activating redox-sensitive kinases. NOX4-mediated generation of O_2^- leads to specific ERK1/2 and JNK activation. Angiotensin II and TNF-α can also lead to NOX2-mediated O_2^- generation via JNK activation [107]. In addition, receptor binding of TGF increases production of ROS via the NOX2 and activated mitogen-activated protein kinase pathways, and subsequent binding of Smad proteins to the plasminogen-activator inhibitor-1 (PAI-1) promoter activates gene transcription. The addition of the thiol-containing antioxidant GSH blocks the TGF-β-stimulated transcription of PAI-1 [108, 109], and attenuation of the NOX-mediated redox signal leads to dysregulation of the acute inflammatory process.

Apoptosis is initiated by a variety of stimuli, including DNA damage, toxins, oxidant stress, and cytokines (especially TNF-α) [110]. Galli et al. have shown that the apoptotic rate is increased in chronic HD patients and that this increase is associated with oxidative stress [111]. C-reactive protein levels and Fas and TNF-R2 expression are higher in lymphocytes and monocytes from chronic HD patients compared with normal controls [112]. Apoptosis can be initiated by death-signal-inducing receptors, of which Fas (CD95) is the best known. In our previous study, we reported increased immunofluorescence of the apoptosis marker for Fas, CD95, in monocytes from uremic patients [110].

4.2. Atherosclerosis. Atherosclerosis, a recognized inflammatory disease, is strongly related to oxidative stress. Several qualitative changes in LDL oxidizability have been shown in dialysis patients, including increased carbamylation, AGE transformation, and oxidation, which all favor the development of atherosclerosis [113]. LDL-mediated oxidation may occur through generation of O_2^- by macrophage 15-lipoxygenase and NADPH oxidase [114] or by endothelial NO synthase (eNOS) [115]. In the initial stages of atherosclerosis, eNOS activity may increase, supporting the concept of a role of oxidative stress in the early atherosclerosis of ESRD patients. The normal equilibrium between NO formation and ROS appears to be disturbed in the process of atherosclerotic plaque formation [116].

4.3. Amyloidosis. β_2-Microglobulin is a major constituent of amyloid fibrils in HD-associated amyloidosis. β_2-Microglobulin appears to be a good candidate for oxidative attack after the appearance of AGEs in amyloid deposits in long-term HD patients [117]. It has been reported that β_2-microglobulin can be fragmented and polymerized following exposure to O_2^- and OH^- generated by radiolysis [118].

4.4. DNA-Damage-Associated Malignancy. ESRD is associated with excessive morbidity and mortality because of cardiovascular disease resulting from inflammation and atherosclerosis and with increased occurrence of various types of cancer. The frequency of cancer is higher in patients with ESRD than in the general population [1, 2]. Oxidative stress and inflammation have been demonstrated to be of high pathogenetic relevance to cancer, especially because of the contribution of genomic damage. Oxidative damage to DNA may cause mutations of oncogenes and tumor-suppressor genes and may represent one mechanism underlying carcinogenesis [119–121]. AGEs are thought to be genotoxic. The most abundant of these lesions, 8-OH-dG, is also the most mutagenic and results in G $\rightarrow$ T transversion, which is found frequently in tumor-relevant genes. Other oxidative modifications of base and sugar residues in DNA occur frequently, but they have been studied less and their biological significance is unclear [120]. Stoyanova et al. [102] reported that 25% of patients with CKD have cancers, independent of whether they are undergoing HD treatment. Thus, DNA oxidative damage has also been identified as a useful index of oxidative stress and a possible indicator of cancer risk.

5. Antioxidant Therapies to Reduce Oxidative Stress and Nucleic Acid Oxidation in CKD and HD Patients (Table 2)

Antioxidant therapy may be beneficial for uremic patients with increased oxidative stress because an increase in oxidative stress contributes to uremic cardiovascular toxicity. Several products of oxidative metabolism have been reported to accumulate in the damaged kidney.

5.1. Vitamin E (Alpha-Tocopherol). Some small studies of uremic patients have shown that the chain-breaking antioxidant alpha-tocopherol (vitamin E) has a biochemical efficacy in beneficially altering the biomarkers of oxidative stress and in increasing erythropoiesis or reducing the required dose

Table 2: Antioxidant therapies to reduce oxidative stress in CKD and HD patients.

Study	Intervention	Subjects	Effect
Vitamin E			
Boaz et al. (2000) [45] SPACE study	High-dose alpha-tocopherol (800 IU once daily) or placebo	196 HD patients with preexisting cardiovascular disease followed for a median of 519 days	(1) Significant reduction in myocardial infarctions and other cardiovascular events (2) No significant difference in overall survival
Mann et al. (2004) [46] HOPE study	Vitamin E, 400 IU once daily	993 patients with mild-to-moderate renal insufficiency at high risk for cardiovascular events	No apparent effect on cardiovascular outcomes
Acetylcysteine			
Tepel et al. (2003) [47]	Acetylcysteine, 600 mg twice daily	134 HD patients followed for 2 years	(1) Cardiac events reduced by 30% (2) Ischemic stroke reduced by 36%
Vitamin C			
Tarng et al. (2004) [48]	Vitamin C, 300 mg three times weekly for 8 weeks	60 HD patients	Mean 8-OH-dG levels decreased significantly in all subjects
Losartan			
Kayabasi et al. (2013) [49]	Losartan 50–100 mg once daily	52 HD patients followed for 3 months	Decreasing oxidative stress index and increasing plasma thiol groups
Folic acid			
Delfino et al. (2007) [50]	Folic acid, 10 mg three times weekly for 6 months	46 HD patients	Effectively lowered plasma Hcy levels
Bardoxolone methyl			
Pergola et al. (2011) [51] BEAM study	Bardoxolone methyl at a target dose of 25, 75, or 150 mg once daily	Adults with CKD	Improved estimated glomerular filtration rate at 24 weeks

of erythropoietin [122]. High-dose vitamin E supplementation has been associated with inhibition of proatherogenic events such as monocyte O_2^- release, release of IL-1β from activated monocytes, lipid oxidation, platelet aggregation, *in vivo* smooth muscle cell proliferation, and monocyte adhesion to the endothelium. Vitamin E may help stabilize atherosclerotic plaque [123, 124]. The Secondary Prevention with Antioxidants of Cardiovascular Disease in End-Stage Renal Disease (SPACE) study reported a clinically and statistically significant reduction in the number of myocardial infarctions and other cardiovascular events in an alpha-tocopherol-treated group of HD patients compared with a group given placebo [45]. However, vitamin E failed to beneficially affect cardiovascular outcomes in the Heart Outcomes Prevention Evaluation (HOPE) study of 993 people with mild-to-moderate renal insufficiency treated with natural-source vitamin E (400 IU/day RRR-alpha-tocopherol acetate) or placebo [46]. Several reasons can explain the different cardiovascular outcomes between the SPACE and HOPE studies. First, the population enrolled in the SPACE trial was at higher cardiovascular risk. Second, participants in the SPACE study were treated with a higher dose of vitamin E than were those in the HOPE study. Third, most participants in the SPACE trial (43.3% of the vitamin E group) consumed vitamin C. Finally, the SPACE study included a smaller sample (196 HD patients) compared with other trials, which resulted in large confidence intervals based on a broad composite end point.

5.2. N-Acetylcysteine. Acetylcysteine, a thiol-containing antioxidant, has been used successfully to ameliorate the toxic effects of ischemia-reperfusion syndromes of the heart, kidney, lung, and liver [125, 126]. The activity of acetylcysteine is related to its action as a free radical scavenger or as a reactive sulfhydryl compound that increases the reducing capacity of the cell and may improve coronary and peripheral vascular function [127]. In patients undergoing HD (134 patients), treatment with oral acetylcysteine (600 mg two times/day) reduced the number of composite cardiovascular end points: cardiac events were reduced by 30%, and ischemic stroke was reduced by 36% [47]. Hsu et al. demonstrated that treatment of HD patients with acetylcysteine significantly improved anemia and decreased the plasma levels of 8-isoprostane and oxidized LDL [128]. Other studies have also shown that the use of N-acetylcysteine can reduce plasma Hcy concentration and improve endothelial function in HD patients, suggesting a direct mechanism for the improvement in endothelial function with antioxidant therapy [28, 129].

5.3. Ascorbic Acid (Vitamin C). Vitamin C is depleted in fluids under *in vivo* conditions of oxidative stress such as smoking and inflammation associated with rheumatoid arthritis. Vitamin C also decreases lymphocyte 8-OH-dG content, markedly reduces intracellular ROS production by lymphocytes, and upregulates *hOGG1* mRNA expression in lymphocytes [48].

5.4. Angiotensin-Converting Enzyme Inhibitors. There is some evidence that other medications, including angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists, might also exert antioxidant effects [95, 130–132]. Both AGEs and angiotensin II are known to upregulate NADPH oxidase, causing ROS formation and subsequent activation of NF-κB. The newly formed angiotensin II might amplify the AGE-induced DNA damage by increasing oxidative stress [133, 134]. The beneficial action of the angiotensin 1 receptor blockade in preventing DNA damage *in vivo* was confirmed in peripheral lymphocytes of rats with chronic renal failure (4/6 nephrectomy) [135]. Losartan has been found to decrease OSI and increase plasma levels of thiol groups in ESRD patients undergoing hemodialysis [49].

5.5. GSH and a Cysteine Prodrug. Oral administration of L-2-oxothiazolidine-4-carboxylate, a cysteine prodrug, has been shown to significantly elevate the whole-blood GSH level in chronic peritoneal dialysis patients [136].

5.6. Folic Acid and Vitamin B_{12}. Folic acid, which is required to metabolize Hcy to methionine, lowers plasma Hcy levels in patients with CKD. Adding vitamin B_{12} reduces plasma Hcy level further. Routine supplementations with folic acid and other antioxidant vitamins should be considered in HD patients to lower Hcy levels; although these supplements may not normalize the Hcy level, lowering the Hcy level may be beneficial by reducing the cardiovascular risk in this group [50, 137, 138]. Folic acid can lower the frequency of micronuclei in PBL *in vitro* [139]; micronuclei frequency was lowered by 6-month folic acid supplementation [140]. Vitamin B_{12} and folic acid are also important modulators of the micronuclei frequency in PBL of healthy individuals [141, 142].

5.7. Bardoxolone Methyl. Nuclear-factor-erythroid-2-related factor 2 (Nrf2) has a central role in the basal activity and coordinated induction of over 250 genes encoding antioxidant enzymes and related proteins, such as SOD, catalase, NADPH:quinine oxidoreductase-1, glutathione S-transferase, glutathione peroxidase, and thioredoxin. Nrf2 is held in the cytoplasm as an inactive complex bound to Keap 1 (Kelch-like ECH-associated protein 1), a repressor molecule that facilitates Nrf2 ubiquitination [143]. Bardoxolone methyl, an antioxidant modulator of inflammation, activates the Keap1-Nrf2 pathway, which plays an important role in maintaining kidney function and structure. In a recent study, treatment with bardoxolone methyl at a target dose of 25, 75, or 150 mg once daily for 52 weeks led to sustained, significant improvements in the eGFR rate in patients receiving standard medical care for CKD and type 2 diabetes [51].

Bardoxolone methyl interacts with cysteine residues on Keap1, allowing Nrf2 translocation to the nucleus and subsequent upregulation of a multitude of cytoprotective genes. The structure and activity profile of bardoxolone methyl resemble those of the cyclopentenone prostaglandins, endogenous Nrf2 activators that promote the resolution of inflammation. Bardoxolone methyl has anti-inflammatory effects by inhibiting the proinflammatory NF-κB pathway [51, 144–148]. Bardoxolone methyl appears to be an attractive therapeutic candidate for further study in patients with CKD.

5.8. New Dialytic Techniques. It has been reported that a daily HD (6 times/week) regimen can effectively lower the mean levels of glycation-related substances compared with standard HD (3 times/week). Therefore, daily HD can provide better control of AGEs produced in ESRD [149].

6. Conclusion

Patients with CKD have high morbidity and mortality compared with healthy people. Excessive oxidative stress resulting from an imbalance between RONS and antioxidative mechanisms has been documented in these patients and may reflect genetic influences. Different strategies to reduce oxidative nucleic acid damage in these patients and the development of biomarkers have provided evidence of the efficacy of antioxidants and new dialytic techniques in preventing oxidative stress in CKD patients, but further work is needed to evaluate these antioxidant therapies fully.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Chih-Chien Sung and Yu-Chuan Hsu contributed equally to this work.

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