Anti-oxidative effects of *Phellinus linteus* and red ginseng extracts on oxidative stress-induced DNA damage

Byung-Jae Park[1], Yeong-Seok Lim[1], Hee-Jung Lee[1], Won Sik Eum[2], Jinseu Park[2], Kyu Hyung Han[2], Soo Young Choi[2,*] & Kil Soo Lee[1,*]

[1]Department of Life Science, [2]Department of Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym University, Chunchon 202-702, Korea

Anti-oxidative effect of *Phellinus linteus* (*P. linteus*) and red ginseng extracts on DNA damage induced by reactive oxygen species (ROS) were investigated in this study. *P. linteus* (PLE) and red ginseng extracts (RGE) inhibited the breaking of *E. coli* ColE1 plasmid DNA strands as well as nuclear DNA of rat hepatocytes damaged by oxidative stress. In addition, a reaction mixture of PLE and RGE showed synergistic inhibitory effect against DNA damage. These results suggest that PLE and RGE have a cellular defensive effect against DNA damage induced by ROS. [BMB reports 2009; 42(8): 500-505]

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide ($\cdot O2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) are produced as by-products of the normal metabolism of aerobic organisms (1). ROS cause oxidative damage in biological macromolecules such as DNA, protein, and fatty acids with various effects including cancer, the aging process (1), cell death (2), and in functional changes to the proteosome and lysosomal systems of lipid metabolism (3).

Oxidative damage is considered a major type of endogenous damage leading to the aging process. Animals have numerous antioxidant defenses, but these defenses weaken and become inadequate with age. Superoxides are transformed into H_2O_2 by superoxide dismutase (SOD), and H_2O_2 is degraded into H_2O by catalase. However, some of the hydrogen peroxide is transformed into hydroxyl radicals by Fenton reactions and Haber-Weiss reactions which may cause serious cellular damage in response to oxidative damage in certain conditions (4). Therefore, it is suggested that a combination of antioxidants might be of greater benefit against oxidative dam-

age incurred both at the cellular and molecular levels (5). Consequently regular intake of antioxidants in the form of natural products may be beneficial for health, longevity, and vitality by means of removing, directly or indirectly, the generated ROS.

Phellinus linteus (*P. linteus*) is a mushroom which is classified as Basidiomycotina, Aphylloporales, Hymenochaetaceae, *Phellinus*. It is usually used in traditional oriental medicine, and has been reported to have many pharmaceutical attributes, including anti-mutagenicity and anti-cytotoxicity (6), anti-cancer as well as enhancement of immunity (7, 8), and anti-oxidant properties (9). Reports suggested that the anti-cancer effect of *P. linteus* might be an indirect action by activating cytotoxic cells and macrophages in order to increase the potential immune response potential (10).

Ginseng (*Panax ginseng* C.A. Meyer) is a perennial herb which belongs to *Panax* Araliaceae, and its roots are used as a general tonic in traditional oriental medicine to increase health, longevity, and vitality, especially in the elderly (11). Red ginseng is made by steaming and drying fresh ginseng, suggesting chemical transformations of active physiological properties occur via the production process (12). Ginseng is considered to have various effects such as the suppression of the growth and metastasis of cancer cells (13), activation of glutathione peroxidase (GPX) and SOD to protect against damage done by free radicals in rats (14), as well as decreasing malondialdehyde (MDA), and increasing the activities of SOD and catalase in humans (15). Non-Saponins in red ginseng could improve learning and memory (16), while acid-polysaccharides activate natural killer cells and the production of interferons (17).

Although a number of studies have reported various (antioxidant, antitumor, anti-mutagenicity, including immune responsive potentials) medical benefits of *P. linteus* and red ginseng, the effects of *P. linteus* and red ginseng on free radical DNA damage have not yet been reported. This study investigated the protective effects of *P. linteus* and red ginseng extracts (PLE, RGE) on bacterial plasmid DNA strand breaks caused by hydroxyl radicals produced by H_2O_2 and $FeCl_2$ in DNA fragmentation assays (18, 19). The effects of these extracts on the degradation of eukaryotic nuclear DNA in rat liv-

er cells caused by hydroxyl radicals produced by H_2O_2 were examined using single cell gel electrophoresis (SCGE, comet assay). In addition, the possibility of a synergistic inhibition of DNA damage by a reaction mix of PLE and RGE was tested.

RESULTS

P. linteus extracts (PLE) and red ginseng extracts (RGE) could inhibit bacterial plasmid DNA breaks caused by H_2O_2 and $FeCl_2$

To investigate the effects of *P. linteus* and red ginseng extracts on bacterial plasmid DNA breaks caused by oxidative stress, DNA fragmentation assays were performed under various concentrations of PLE (Fig. 1A), RGE (Fig. 1B) or both (Fig. 1C). It was observed that hydrogen peroxide (H_2O_2) and $FeCl_2$ could induce oxidative stress by generating hydroxyl radicals. Hydroxyl radicals broke the supercoiled form of plasmid DNA and markedly enhanced the nicked circular DNA form (lane 4
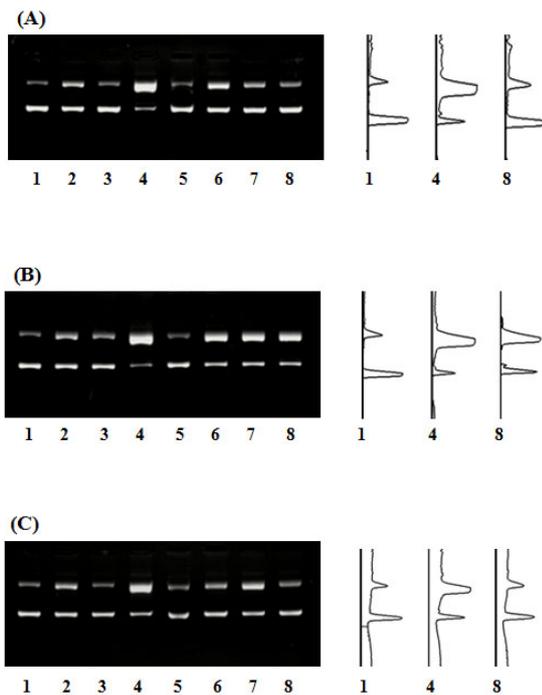

Fig. 1. Effects of PLE (A), RGE (B), and combined PLE and RGE (C) on ColE1 plasmid DNA damage. Total reaction volume (10 μl) was incubated for 2 h. Gel electrophoresis was performed in 0.8% agarose gel. Lanes in A and C are as follows: lane 1, Control (ColE1 plasmid DNA 0.21 μg); lane 2, 1 mM H_2O_2; lane 3, 0.05 mM $FeCl_2$; lane 4, 1 mM H_2O_2 + 0.05 mM $FeCl_2$; lane 5, 1 mM H2O2 + 0.05 mM $FeCl_2$ + catalase 13.4 μg; lane 6, 1 mM H_2O_2 + 0.05 mM $FeCl_2$ + PLE, RGE, or PLE and RGE 5 μg; lane 7, 1 mM H_2O_2 + 0.05 mM $FeCl_2$ + PLE, RGE, or PLE and RGE 10 μg; lane 8, 1 mM H_2O_2 + 0.05 mM $FeCl_2$ + PLE, RGE, or PLE and RGE 20 μg.

in Fig. 1). However, the DNA breaks were prevented when the hydroxyl radical scavenger (catalase) was incubated with re-action mixture. As shown in Fig. 1 (lane 5), the catalase sig-nificantly protected the DNA from oxidative damage. On the other hand, PLE inhibited the DNA break in a dose-dependent manner as shown in Fig. 1A, lanes 6-8. On the basis of the DNA band intensities of the supercoiled and nicked circular forms, the inhibitory effects on DNA strand breaks was also observed by the RGE. The inhibitory effects of RGE were much lower compared to those of PLE under the same conditions (Fig. 1B, lane 6-8).

In addition, PLE and RGE extracts were applied together to determine if the combination could enhance or suppress the defensive effects against DNA damage compared to either alone. When both extracts were added in combination to the DNA under oxidative stress induced by H_2O_2 and $FeCl_2$, the intensities of the DNA bands of the supercoiled forms in-creased to the control levels (Fig. 1C, lane 8), in comparison to those treated singly with PLE or RGE (Fig. 1C lanes, 6 and 7). These results suggest that PLE and RGE could synergistically protect against DNA strand breaks caused by H_2O_2 - and $FeCl_2$ - induced oxidative stress.
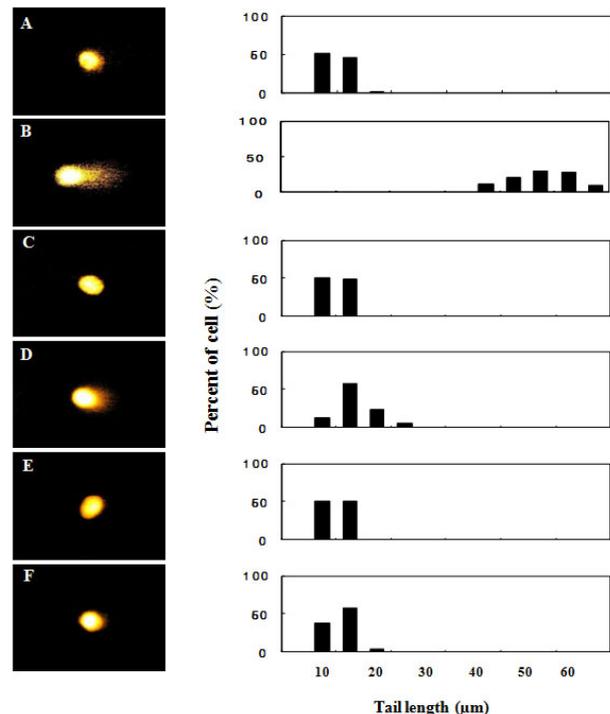

Fig. 2. The effect of PLE on DNA damage caused by hydrogen peroxide in rat hepatocytes. Cell number: 60 cells/plate, Magnifi-cation: ×400. (A) Control (Rat hepatocyte); (B) 0.5 mM H_2O_2; (C) 0.5 mM H_2O_2 + catalase 23.6 μg; (D) 0.5 mM H_2O_2 + PLE 50 μg/ml; (E) 0.5 mM H_2O_2 + PLE 100 μg/ml; (F) PLE 100 μg/ml.

PLE and RGE showed protective effects on eukaryotic nuclear DNA damage induced by oxidative stress

A single cell gel electrophoresis (comet assay) was conducted to investigate the effects of PLE and RGE on eukaryotic nuclear DNA damage using freshly prepared rat hepatocytes. Typically in comet assays, the tail lengths of nuclear DNA are observed as a mark of DNA damage. Here, the tail lengths were estimated at 0-10 μm in control treatments (Fig. 2A, 3A, and 4A) which increased to 40-50 μm in 0.5 mM H_2O_2 treated reactions indicating serious nuclear DNA damage (Fig. 2B, 3B, and 4B). When catalase was added to the damaged DNA, the tail lengths of nuclear DNA protected to the control levels (Fig. 2C, 3C, and 4C), indicating that catalase completely protects against nuclear DNA damage induced by H_2O_2. These results served as positive controls for the current experiment to assess the antioxidant activities of PLE and RGE. In the case of PLE treatment following oxidative stress, the tail lengths of nuclear DNA were reduced to the same sizes as shown in the control experiments in a PLE dose-dependent manner (Fig. 2D and 2E). In addition, PLE alone did not cause any damage to hepatocyte nuclear DNA (Fig. 2F). These results suggest that PLE may have antioxidant properties and therefore able to inhibit nuclear DNA damage induced by H_2O_2 in eukaryotic cells.

A comet assay was also conducted using extracts of red ginseng on eukaryotic nuclear DNA damage. Our results showed that treatment with RGE resulted in decreased nuclear DNA tail lengths and furthermore displayed increased protective effects (Fig. 3D and 3E). However, the inhibitory effects of RGE were lower than that of the PLE (Figs. 2E and 3E).

To verify whether a combination of both extracts could enhance the protective effects of each extract, we added both extracts (50 and 100 μg/ml) in the same reaction mixture and examined nuclear DNA tail lengths. The mixture containing both extracts did not significantly increase or decrease the tail lengths, indicating that no additional damage was done to nuclear DNA (Fig. 4F) by the extracts. However, when both the extracts were added following oxidative stress, the tail lengths were completely reduced to the control levels and both extracts were more effective than either individually (Fig. 4D and 4E). These results indicate that PLE and RGE have antioxidant properties and protect synergistically from oxidative stress.
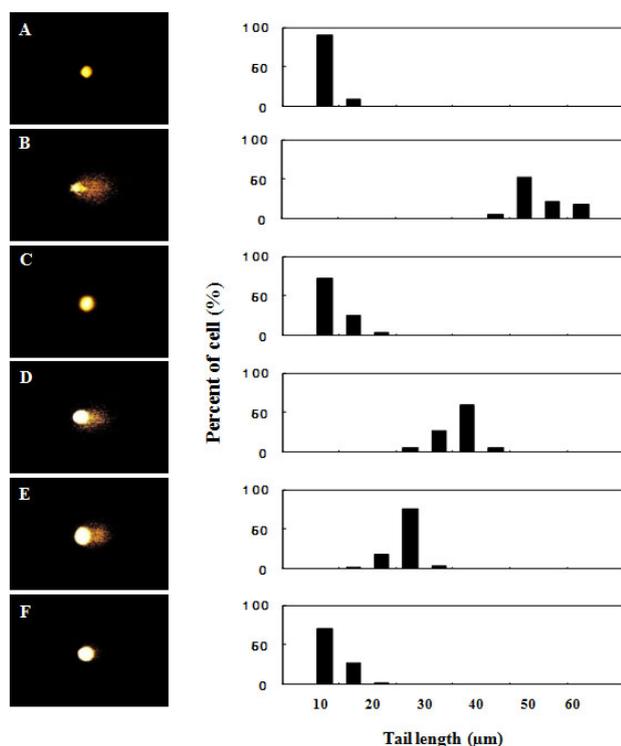

Fig. 3. The effect of RGE on DNA damage caused by hydrogen peroxide in rat hepatocytes. Cell number: 60 cells/plate, Magnification: ×400. (A) Control (Rat hepatocyte); (B) 0.5 mM H_2O_2; (C) 0.5 mM H_2O_2 + catalase 23.6 μg; (D) 0.5 mM H_2O_2 + GRE 100 μg/ml; (E) 0.5 mM H_2O_2 + GRE 200 μg/ml; (F) GRE 200 μg/ml.
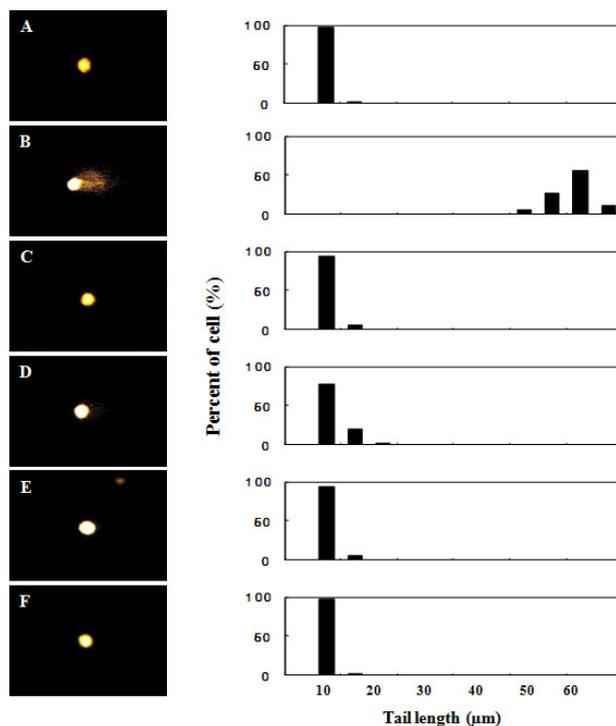

Fig. 4. The effect of PLE and RGE on DNA damage caused by hydrogen peroxide in rat hepatocytes. Cell number: 60 cells/plate, Magnification: ×400. (A) Control (Rat hepatocyte); (B) 0.5 mM H_2O_2; (C) 0.5 mM H_2O_2 + catalase 23.6 μg; D, 0.5 mM H_2O_2 + PLE 50 μg/ml + RGE 50 μg/ml; (E) 0.5 mM H_2O_2 + PLE 100 μg/ml + RGE 100 μg/ml; (F), PLE 100 μg/ml + GRE 100 μg/ml.

DISCUSSION

All living organisms produce reactive oxygen species (ROS), such as $\cdot O_2^-$ (superoxide), H_2O_2 (hydrogen peroxide), and $\cdot OH$ (hydroxyl radical), and have various effective defense mechanisms to degrade ROS during normal metabolic processes in oxygen exposed environments (1). However, ROS can accumulate in organisms, incurring severe damage to the macromolecules including DNA, protein, and fatty acids (4). Natural products used in oriental medicine have been reported to have many physiological and pharmaceutical attributes, especially as antioxidants, anti-tumorogenic agents, in anti-aging, and in promoting longevity and general health (6-10). There is a continuous demand for health-aids and natural drugs in the increasing aging population. It is well known that the pharmaceutical beneficial effects of red ginseng and *P. linteus*. A variety of oligosaccharides, including β-glucan from *P. linteus*, were reported to be medically beneficial, including enhancing immunogenicity and activating macrophages, which in turn secrete cytokines to activate the antitumorogenic responses (20). However, their effects on DNA damage induced by ROS are still unclear.

In a DNA fragmentation assay, PLE effectively inhibited ColE1 plasmid DNA strand breaks induced by hydroxyl radicals produced by exposure to H_2O_2 and $FeCl_2$. The protective effects of PLE on DNA damage were significantly increased concentration-dependent manner.

Next, we performed a comet assay to investigate the effects of PLE on DNA damage to eukaryotic nuclei using primary cultured rat hepatocytes. Treatment with H_2O_2 caused damage, indicated by increased nuclear DNA tail lenghts (Fig. 2B). Catalase repaired the injury, evidenced by decreased tail lengths to control levels (Fig. 2C) and PLE showed inhibitory effects on nuclear DNA damage in a concentration-dependent manner (50 and 100 μg/ml) as shown in the DNA fragmentation assay (Fig. 2D and 2E). These results are consistent with the effects of ethanol extracts of PL on radical 1,1-diphenyl-2-picrylhyrazyl (DPPH), suggesting that both water extracts and ethanol extracts may inhibit the DNA damage by directly removing the hydroxyl radical (21). On the other hand, RGE displayed protective effects against DNA strand breaks, but protected weakly at high concentrations (20 μg/ml) in DNA fragmentation assays (Fig. 1B, lanes 6-8). Similar observations of RGE were reported earlier where RGE was shown to exhibit diminished ability to quench free radicals (22, 23). However, in the present work, RGE (200 μg/ml) was shown to markedly inhibit nuclear DNA damage by comet assays (Fig. 3D and 3E) when compared to DNA strand breaks in DNA fragmentation assays. This disparity was apparently due to RGE concentrations differences. Our results indicate that the PLE and RGE have antioxidant properties in oxidative stress by radical scavenging. Further studies remain to be elucidated why the PLE appeared to be more effective than RGE in DNA

fragmentation and comet assays in the present study.

To examine whether combinations of PLE and RGE could be more effective against DNA damage, we conducted DNA fragmentation and comet assays were performed. When both extracts were combined into the mixture of H_2O_2 and $FeCl_2$, the protection against DNA strand breaks was more effective at lower concentrations than in single applications of the extracts singularly (Fig. 1C, lane 6-8). Similar results were also obtained from comet assays (Fig. 4) where protective effects were displayed in a concentration-dependent manner. Although it is still unknown which components or mechanisms in both extracts operate to protect from oxidative injuries, it is likely that the two extracts are able to work synergistically to improve their inhibitory functions against DNA damage induced by oxidative stress.

Taken together, our results suggest that PLE and RGE extracts were potent anti-oxidant by protective effect against oxidative stress-induced bacterial DNA strand breaks. Furthermore, these extracts were more effective synergistically on the DNA damage incurred on eukaryotic nuclear DNA exposed to oxidative stress. Therefore, those data presented in this study may provide the possible therapeutic values of PLE and RGE extracts for the prevention of DNA damage progression and oxidative stress.

MATERIALS AND METHODS

Materials
ColE1 plasmid DNA (D-9683) from *E. coli* C600, agarose (Type II: Medium EEO, A-6877), ferrous chloride ($FeCl_2$), catalase (from *Aspergillus niger*, C-3515), and hydrogen peroxide (30%) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All of the other reagents were of the highest quality generally available.

Water extracts of *P. linteus* (PLE) and water fractions of red ginseng (RGE)
P. linteus was purchased locally and extracts were made using heat extraction fractionation. 10 volumes of distilled water were added and extracted at 100°C for 10 hours following filtration using 3 M filter paper (0.45 μM) to remove debris. The filtered extracts were freeze-dried and stored at 4°C prior to use. Red ginseng extracts were kindly provided by Hanil Insam Inc, Chunchon, Korea.

DNA fragmentation assay
ColE1 plasmid DNA (0.21 μg) was prepared to a final volume of 10 μl in sterile H_2O. The DNA was mixed with 1 mM of H_2O_2 and 0.05 mM of $FeCl_2$. The PLE (5, 10, 20 μg/ml) and/or RGE (5, 10, 20 μg/ml) were added in the reaction mixture and further incubated at 37°C for 2 h. Electrophoresis was immediately performed in 0.8% agarose gel and then the gels were stained with ethidium bromide solution for 30 minutes, and washed for 2 h. The bands in the gels were observed on a UV

transilluminator, and analyzed using a computer image analysis system (camera: Sony XC-75CE, program: BioCaptMW) (24, 25).

Single cell gel electrophoresis (comet assay)
The comet assay was performed as described by Ding *et al.* (1999) and Singh *et al* (1998) with slight modifications (26, 27). Rat hepatocytes were prepared from 4-5 week old male Spraque-Dawley Albino rats. Animals were purchased from the Experimental Animal Center, Hallym University, Chuncheon, Korea. Primary rat hepatocytes were isolated from animals using a two-step collagenase perfusion method as previously described (28). A suspension of hepatocytes (1.0×10^6 cells/ml, 100 µl) was treated with 0.5 mM H_2O_2, PLE (50 and 100 µg/ml) or RGE (100 and 200 µg/ml) and further incubated for 2 h. The cell suspensions (5 µl) were mixed with 0.7% low melting point (LMP) agarose, spread on a 0.7% normal agarose pre-coated microscope slide and placed at 4°C for 5 min to allow for solidification. The cells on the slides were lysed at 4°C for 1 h in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Triton X-100, with freshly added 1% DMSO, pH 10.0). The slides were then placed for 20 minutes in electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) to allow DNA unwinding and expression of alkali-labile sites. After electrophoresis, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 10 minutes and stained with EB solution (propidium iodide) for 1 h. All slides were analyzed under a fluorescence microscope system (Kinetic Imaging Ltd, UK) and Macro 2000 (Kinetic Imaging Ltd, UK).

Acknowledgements

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