A High-Fat Diet Increases Oxidative Renal Injury and Protein Glycation in d-Galactose-Induced Aging Rats and Its Prevention by Korea Red Ginseng

Sok Park1,*, Chan-Sik Kim2,1,*, Jinah Min3, Soo Hwan Lee3 and Yi-Sook Jung5,6,**

1Division of Sports Industry & Science, Mokwon University, Daejeon 302–729, Republic of Korea
2Korean Medicine Based Herbal Drug Development Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon 305–811, Republic of Korea
3Department of Physiology, Ajou University School of Medicine, Suwon 443–749, Republic of Korea
4Department of Exercise Nutrition, Teachers College, Kyungpook National University, Daegu 702–701, Republic of Korea
5Department of Pathophysiology, College of Pharmacy, Ajou University, Suwon 443–749, Republic of Korea
6Research Institute of Pharmaceutical Sciences and Technology, Ajou University, Suwon 443–749, Republic of Korea

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Summary
Declining renal function is commonly observed with age. Obesity induced by a high-fat diet (HFD) may reduce renal function. Korean red ginseng (KRG) has been reported to ameliorate oxidative tissue injury and have an anti-aging effect. This study was designed to investigate whether HFD would accelerate the d-galactose-induced aging process in the rat kidney and to examine the preventive effect of KRG on HFD and d-galactose-induced aging-related renal injury. When rats with d-galactose-induced aging were fed an HFD for 9 wk, enhanced oxidative DNA damage, renal cell apoptosis, protein glycation, and extracellular high mobility group box 1 protein (HMGB1), a signal of tissue damage, were observed in renal glomerular cells and tubular epithelial cells. However, treatment of rats with HFD plus d-galactose-induced aging with KRG restored all of these renal changes. Our data suggested that a long-term HFD may enhance d-galactose-induced oxidative renal injury in rats and that this age-related renal injury could be suppressed by KRG through the repression of oxidative injury.

Key Words
age-related renal injury, d-galactose, high-fat diet, Korean red ginseng, oxidative stress

Aging is characterized by a decline in renal function and susceptibility to age-related renal insufficiency (1). Aging features a progressive accumulation of oxidative agents associated with decreased efficiency of antioxidant defense mechanisms (2, 3). The oxidative stress in renal tissue occurs due to the alterations in redox homeostasis with the effect of the elevation of reactive oxygen species (ROS) levels (4–6). Antioxidant enzyme levels decrease with age, leading to inadequate ROS elimination (3). The increased oxidative modification of the electron transport chain complex may lead to age-associated oxidative renal injury (7).

Advanced glycation end products (AGEs) are formed by non-enzymatic irreversible protein glycosylation/glycation (8). The accumulation of glycated proteins in the tissues is harmful in several diseases states (9, 10). Aging is associated with a more common appearance of AGEs in various tissues (11, 12). In humans and animals, the rate of AGE accumulation correlates inversely with species longevity (13). However, it is not clear whether AGE accumulation is due to aging or age-related renal injury.

d-Galactose is a reducing sugar that can be metabolized at normal concentrations. However, at high levels, it can be converted into aldose and hydroperoxide under the catalysis of galactose oxidase, resulting in the generation of a superoxide anion and oxygen-derived free radicals (14). d-Galactose also reacts readily with the free amines of amino acids in proteins and peptides, both in vivo and in vitro, to form AGEs. Evidence shows that AGEs could cause ROS accumulation (15). When rats are injected with d-galactose for 6 wk, free radical production is increased in renal and other tissues (16). d-Galactose has been used to induce oxidative stress in vivo to mimic the natural aging process in rats and mice (17–20). In addition, a high-fat diet (HFD) was found to be a significant risk factor for kidney disease (21–23). HFD-induced obesity may expedite the aging process (24). Animals fed a long-term HFD show increased oxidative stress and dysfunctional mitochondria in many organs (25, 26). Both HFD- and d-galactose-induced aging lead to oxidative stress in the inner ear of rats (27).
Panax ginseng, one of the best health foods for vitality and combating fatigue, increases energy and eliminates chronic fatigue while improving health. Ginseng has been used as a tonic for >2,000 years and is a well-known energy booster and dietary supplement in Asian countries. Among the several kinds of P. ginseng products, Korean red ginseng (KRG) has the most potent pharmacological effects against immune deficiency, metabolic syndrome, and cancer (28–30). KRG is produced by steaming and drying fresh ginseng. During this process, ginsenosides undergo chemical changes that have the potential to create special physiologic activities in vivo (31). To date, more than 30 different ginsenosides from KRG have been isolated and characterized as having different pharmacological effects (32). The KRG extract is reported to reduce AGE formation in hyperglycemic rats (33).

Some clinical and experimental studies have reported that P. ginseng has a preventive effect on age-related organ dysfunction and anti-glycation activity (33–36). In earlier studies, KRG inhibited advanced glycation end product-mediated renal injury (37) and cyclosporine-induced renal injury (38). KRG also has an anti-obesity effect (39) and has been shown to extend the lifespan of Drosophila melanogaster (40). However, the effects of KRG on age-related renal injury and cross-link formation of glycated proteins remain unknown. To elucidate this issue, we examined whether HFD would interact with D-galactose to accelerate age-related renal injury and evaluate the preventive effects of KRG on age-related renal injury in rats with HFD- and D-galactose-induced aging.

**MATERIALS AND METHODS**

**Animals and experimental design.** Thirty-two male 8-week-old Sprague-Dawley rats were used in this study. Each rat was housed individually in a temperature-controlled room with a 12-h light/dark cycle and had free access to drinking water. After a 2-wk acclimation period, the rats were divided into 4 groups: young control rats (CON, n = 8), rats with D-galactose-induced aging (GAL, n = 8), rats with HFD-+D-galactose-induced aging (HFD+GAL, n = 8), and rats with HFD-+D-galactose-induced aging treated with KRG (200 mg/kg/d, KRG, n = 8). The rats in the CON and GAL groups were fed standard laboratory chow (3.34 kcal/g; PMI Nutrition International, St. Louis, MO). The rats in the HFD+GAL group were fed a high-fat diet containing 60% kcal fat (5.24 kcal/g; D12492; Research Diets, New Brunswick, NJ) for 9 wk. The rats in the GAL, HFD+GAL, and KRG groups were injected with D-galactose (100 mg/kg/d) intraperitoneally, while the rats in the CON group were injected with an equal volume of vehicle (0.9% saline). The KRG treatment was begun 1 wk after the onset of HFD feeding, and KRG was administered orally for 8 wk. Each rat’s body weight was regularly monitored. All of the experimental procedures were performed under the supervision of our Institutional Animal Care and Use Committee.

**KRG preparation.** The KRG extracts were provided by the Korean Tobacco and Ginseng Corp. (Daejon, Korea) and contained the following 7 glycosides known as ginsenosides (mg/g): Rg1 (0.71), Rb1 (4.62), Rg3 (s) (2.14), Re (0.93), Rc (2.41), Rb2 (1.83), and Rd (0.89).

**In vitro assay of the cross-linking of glycated proteins.** For the AGE cross-linking inhibition assay, AGE-BSA (TransGenic Inc., Kobe, Japan) was incubated in the presence or absence of KRG or aminoguanidine, a well-known AGE inhibitor, in collagen-coated 96-well plates. Collagen-AGE-BSA cross-linking was detected using a mouse anti-AGE antibody (6D12; Wako Pure Chemical Industries, Ltd., Osaka, Japan), a horseradish peroxydase-linked goat anti-mouse IgG antibody, and a H2 O2 substrate containing the ABTS chromogen. The inhibition of collagen-AGE-BSA cross-linking was expressed as a percent decrease in optical density (OD=410 nm). We calculated the IC50 concentration (μg/mL) as the concentration at which collagen-AGE-BSA cross-linking was inhibited by 50%.

**Immunohistochemical staining.** Immunohistochemistry was performed as previously described (41). Antibodies included a rabbit anti-high mobility group box 1 protein (HMGB1, 1:200; Epitomics, Burlingame, CA), a mouse anti-AGE (6D12; Wako), and a mouse anti-8-hydroxygluanine (8-OHdG) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For the detection of HMGB1, AGEs, and 8-OHdG, the sections were incubated using an Envision kit (Dako, Carpinteria, CA) and visualized using 3,3′-diaminobenzidine tetrahydrochloride. Negative controls for immunohistochemistry were run by incubating the sections with non-immune serum instead of the primary antibody. The immunohistochemical intensity was analyzed in 5 randomly selected areas (mm2) using ImageJ software (NIH, Bethesda, MD).

**Apoptosis assay.** To evaluate apoptosis in renal tissues, a TUNEL assay was performed (DeadEnd Apoptosis Detection System; Promega, Fitchburg, WI) according to the manufacturer’s instructions. Apoptotic cells were detected using peroxidase-conjugated streptavidin. The number of TUNEL-positive cells per unit area (mm2) was then determined in a total of 5 fields.

**Statistical analysis.** Comparisons between groups
were performed using one-way analysis of variance followed by Tukey’s multiple comparison test using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). A p-value < 0.01 was considered statistically significant.

RESULTS

Inhibitory effect of KRG on glycated protein cross-linking in vitro

To investigate whether KRG could inhibit AGE cross-linking, AGE-BSA was incubated with KRG in collagen-coated plates. KRG (IC$_{50}$ = 55.65 µg/mL) exhibited much stronger inhibitory activity on AGE-BSA binding with collagen than aminoguanidine (IC$_{50}$ = 563.54 µg/mL), a well-known glycation inhibitor (Fig. 1).

Body weight

Body weight gain over 9 wk is summarized in Table 1. The body weight in the galactose group was slightly increased compared to that in the CON group. In rats that received both D-galactose and HFD, body weight was significantly increased compared to that in rats in the CON group and was reduced by KRG treatment.

Oxidative DNA damage in renal tissues

To determine whether HFD would interact with D-galactose to accelerate the age-related oxidative renal injury, we examined the oxidative DNA damage in renal tissues using 8-OHdG immunostaining. The oxidation of guanine to form 8-OHdG acts as a marker of oxidative DNA damage (42). As shown in Fig. 2, the 8-OHdG marker exhibited nuclear and/or perinuclear localization in the renal tubular epithelial cells. The immunoreactivities of 8-OHdG in the GAL and HFD+GAL groups were significantly higher than that in the CON group. Moreover, HFD+GAL group showed significantly higher staining intensity than the GAL group, which indicated that the HFD accelerated the age-related oxidative renal injury induced by D-galactose. KRG treatment suppressed the expression of 8-OHdG compared to that was observed in the HFD+GAL group (Fig. 2B).

Apoptosis assay in renal tissues

To characterize renal cell injury, we used TUNEL staining. TUNEL analysis detects cells in which the DNA is fragmenting; it is widely used as a marker for apoptosis (43). In the CON group, a TUNEL-positive nucleus was barely detected. In the GAL and HFD+GAL groups, many TUNEL-positive cells were observed in the renal glomeruli and tubular epithelial cells. There were significantly more TUNEL-positive cells in the HFD+GAL group than in the GAL group. However, KRG treatment prevented increased numbers of positive cells compared to the CON group renal tissues (Fig. 3).

Protein glycation in renal tissues

To determine whether HFD would interact with
D-galactose to accelerate the cross-link formation of glycated proteins in vivo, we performed immunohistochemical staining for AGEs in renal tissues. As shown in Fig. 4, the immunoreactivities of AGE in the GAL and HFD+GAL groups were significantly higher than that in the CON group. AGE expressions were mainly distributed in the glomerulus and renal tubules. In particular, high AGE expression was observed in the proximal tubular epithelial cells. Moreover, the staining intensity in the HFD+GAL group was significantly higher than that in the GAL group, which indicated that the HFD had an accelerating effect on the renal AGE cross-linking induced by D-galactose. KRG treatment suppressed the AGE expression compared to the level seen in the HFD+GAL group (Fig. 4B).

**HMGB1 cytoplasmic translocalization in renal tissues**

HMGB1, a nuclear DNA-binding protein, can be released extracellularly and acts as a pro-inflammatory cytokine or alarm signal for tissue damage (44). In the renal tissues of rats with diabetic nephropathy, HMGB1 is extensively released from the renal glomerular cells and renal tubular epithelial cells (45). To investigate the pathogenic status of HMGB1 in age-related renal injury, we used immunohistochemical staining in renal tissues.
HMGB1 was expressed only in the nuclei in the CON group, whereas it was expressed in the nuclei and diffusely in the cytoplasm in the renal tissues of the GAL and HFD+GAL groups. This cytoplasmic translocation of HMGB1 was markedly inhibited by KRG administration (Fig. 5).

**DISCUSSION**

The present study provides novel evidence of HFD-accelerated age-related renal injury in animal models. We showed that rats fed an HFD for 9 wk become obese and demonstrated enhanced oxidative stress and cross-link formation of glycated proteins in the renal tissues of rats with D-galactose-induced aging. Our results described the link between an HFD and aging in renal tissues. In addition, we showed that KRG has a renoprotective effect in this animal model.

Oxidative stress is an important feature of aging (46). ROS are associated with the inflammatory response and frequently contribute to the tissue-damaging effects of inflammatory reactions (15, 47). D-Galactose can induce renal injury, including oxidative stress and inflammation, in mice, which can lead to renal dysfunction (18, 48). Moreover, obesity is reported to expedite the aging process (24). A long-term HFD showed increased oxidative stress and dysfunctional mitochondria in many organs (25, 26). Although a large body of evidence suggests that both enhanced intracellular lipid accumulation and oxidative stress were significantly associated with cellular dysfunction, the relationship between HFD and oxidative stress in age-related renal injury still remain unknown. In this study, we did not include an only HFD group. The reason for the omission is that obesity induced by HFD cannot directly decrease renal function in a several animal models. Liu et al. showed that the treatment of D-galactose increase levels of urea, uric acid and creatinine in serum and the renal histological injury in rats (48). However, Aguila et al. showed that HFD for 18 mo did not induce the increases of serum creatinine and proteinuria in rats (49). In another previous study, 14-wk-old Zucker rats exhibited pronounced hyperlipidemia and obesity, and the level of proteinuria of these rats was 12±4 mg/d. In age-matched lean control rats, the level of proteinuria was 11±5 mg/d. No significant difference was also found among the groups for serum creatinine at 14 wk (50). There is an intrinsic limitation in the use of rat models in elucidating the aging process in humans. However, these results suggest that an HFD cannot directly decrease renal function in rats. Although we did not check urinary markers for renal function, it is well known that renal histological alterations are highly correlated with renal function (51, 52). In the present study, we demonstrated the HFD plus the long-term injection of D-galactose in the rat kidney induces more severe oxidative stress and leads to apoptotic renal cell death. In contrast, KRG by oral gavage administration markedly decreased the expression of 8-OHdG in the HFD- and D-galactose-treated rats, which might be attributed to its ability to scavenge and prevent free radical generation (53–56).
ROS has been reported to injure DNA by breaking single DNA strands and DNA-protein cross-links as well as modifying base residues such as by introducing a hydroxyl group into the C-8 position of guanosine and guanine residues to form 8-OHdG and 8-hydroxyguanine: it is widely used as a sensitive biomarker of DNA oxidation (57–59). In this study, the 8-OHdG level was significantly increased in the kidneys of d-galactose-treated rats and highly increased in the HFD+GAL group compared to that seen in the GAL group, suggesting that obesity may aggravate the age-related renal injury induced by d-galactose. KRG treatment produced a significant decrease in the renal 8-OHdG level. Our findings indicate that KRG may effectively protect kidney function against oxidative DNA damage by decreasing the 8-OHdG level in the kidneys of the HFD- and d-galactose-treated rats.

Renal cell apoptosis or necrosis will inevitably affect glomerular filtration rate and endothelial function, resulting in renal failure (60–62). Moreover, high ROS concentrations contribute to apoptotic cell death whenever they are generated in the context of the apoptotic process (63, 64). The present study showed that the HFD plus d-galactose increased the number of TUNEL-positive cells in the rat kidney. TUNEL staining followed the 8-OHdG pattern. This result also indicates that an HFD can amplify galactose-induced apoptotic cell death in renal tissues. However, we found that KRG markedly decreased the number of TUNEL-positive cells in the HFD plus d-galactose-treated rats. Thus, our findings suggest that the antioxidant activity of KRG has a potential anti-apoptotic effect.

We also demonstrated here that d-galactose-injected rats fed an HFD showed accelerated renal injury associated with more pronounced renal AGE content. Our data indicate that HFD plus d-galactose induced extensive AGE cross-linking formation and renal deposition. Accelerated renal injury was observed in rats in the HFD+GAL group compared with the GAL group, indicating that an HFD was associated with enhanced AGE deposition. Oxidative stress or pro-apoptotic cytokine through AGE interaction and its receptor were involved in renal glomerular cell apoptosis (65). We also examined the effect of KRG intervention on protein glycation increases. It was previously reported that KRG has an anti-AGE activity under hyperglycemic conditions (33). Similarly, we showed that the KRG intervention inhibited AGE cross-linking and renal deposition in HFD- and d-galactose-treated rats. Our results indicated that the reduction of AGE cross-linking by KRG might be a major mechanism of renoprotection in rats with HFD- and d-galactose-induced aging.

A ubiquitous nuclear protein, HMGB1, can be released by various immune cells in response to infection or injury (66) and is a warning signal that alerts the immune system (67). Endotoxin, pro-inflammatory cytokines, and oxidative stress are also capable of inducing active or passive HMGB1 release (68). To our knowledge, our result is the first evidence that HMGB1 is extensively expressed in the renal tissues of the HFD- and d-galactose-treated rats and that HMGB1 was released from the renal glomerular cells and renal tubular epithelial cells. The distribution of HMGB1 in these cells was clearly translocated from the nucleus to the cytoplasm. This observation indicated that renal glomerular cells and renal tubular epithelial cells might secrete HMGB1 and represent the major source of HMGB1 in age-related renal injury. KRG treatment also inhibited the cytoplasmic translocation of HMGB1 in the HFD- and d-galactose-treated rats. These results also showed that the antioxidant activity of KRG may exert a renoprotective effect against age-related renal injury.

In conclusion, our data indicate that long-term HFD may enhance d-galactose-induced oxidative renal injury and glycated protein cross-linking in rats. However, the underlying mechanisms of renal injury in the long-term HFD-fed rats and those with d-galactose-induced aging require further investigation. We also demonstrated that KRG protects against aged-related renal injury by inhibiting oxidative stress and AGE cross-linking. Hence, our findings suggest a potential clinical use of KRG in the prevention of age-related oxidative renal injury.

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REFERENCES


