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Korean red ginseng attenuates doxorubicin-induced testicular dysfunction in rats by modulating inflammatory, oxidative, and autophagy responses

Kyu-Min Cha[a,1], Spandana Rajendra Kopalli[a,1], Sang Yun Han[b,1], Sang-Ho Lee[a], Min-Sik Jeong[a], Jae Youl Cho[b], Chang-Gyun Han[c], Seung-Ho Lee[c], Soo-Nam Kim[d], Jin-Chul Kim[d], Si-Kwan Kim[a,*]

[a] Department of Biomedical Chemistry, College of Biomedical & Health Science, Konkuk University, Chungju 380-701, Republic of Korea
[b] Department of Genetic Engineering, Sungkyunkwan University, Suwon 16419, Republic of Korea
[c] Botanical Drug Laboratory, Korea Ginseng Research Institute, Korea Ginseng Corporation, Daejeon 300-716, Republic of Korea
[d] Department of Natural Constituents Research Center, Korea Institute of Science and Technology, Gangneung, Gangwon-do 210-340, Republic of Korea

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ABSTRACT

The effect of Korean red ginseng (KRG; *Panax ginseng* Meyer) against doxorubicin (DOX)-induced testicular inflammation was investigated. Male Sprague-Dawley rats were treated with DOX (1 mg mL^{-1} kg^{-1} $week^{-1}$; intra-peritoneally) for 8 weeks, and KRG water extract (EX1; 100 and EX2; 200 mg/kg/day; orally) was administered for 9 weeks starting one week before DOX exposure. The expression levels related to spermatogenesis, inflammatory and autophagy markers were evaluated using western blotting and reverse transcriptase polymerase chain reaction. DOX-induced a significant ($p < .05$) alteration in the expression of antioxidation spermatogenesis and sex hormone receptors genes when compared with control groups, which were attenuated with KRG-WE treatment significantly ($p < .05 \sim p < .01$). KRG-WE also ameliorated the DOX-induced alteration in pro-inflammatory cytokines via the mitogen-activated protein kinases/nuclear factor kappa-B pathway and autophagy in the testes of rats. In conclusion, KRG might be used as a functional food for prevention of chemotherapy-induced testicular inflammation.

1. Introduction

Korean red ginseng (*Panax ginseng* Meyer; Araliaceae), one of the most widely used functional foods, is known to improve immune function and physical performance and to enhance vitality, health, and longevity (Lee & Kim, 2014; Nocerino, Amato, & Izzo, 2000). In particular, the roots of six-year-old Korean red ginseng (KRG) have been extensively reported to exhibit various pharmacological activities, including anti-stress effects (Kitts & Hu, 2000; Yeh et al., 2007), and to provide multiple therapeutic benefits against diverse pathological conditions including cardiovascular issues, diabetes, allergies, insomnia, gastritis, hepatotoxicity, and sexual dysfunction (Hwang et al., 2010; Leung & Wong, 2013; Nah, Kim, & Rhim, 2007; Radad, Gille, Liu, & Rausch, 2006; Rausch, Liu, Gille, & Radad, 2006; Won et al., 2014; Xiong et al., 2010; Yayeh et al., 2012; Zheng et al., 2011).

The risk versus benefit ratio or toxicity versus effective dose is a critical parameter in the process of drug development, as well as in clinical practice. The wider the gap between the toxic dose and effective dose, the safer the compound is as a potential therapeutic. However, if there is no alternative therapy for patients with a life-threatening disease, they must put up with the toxicity of the agent. This applies to many drugs, including the anticancer agents used in chemotherapy, which produce toxic effects in multiple organ systems. The therapeutically effective doses of many anticancer drugs may produce irreversible damage to many organs, including male reproductive organs. Doxorubicin (DOX), an anthracycline antibiotic, is used to treat a wide spectrum of malignancies, but produces a toxic response in normal tissues, including the testes (Nambu & Kumamoto, 1995).

Functional foods from natural herbs are being increasingly used in combination with conventional anticancer therapeutics to decrease their toxic side effects. In our previous study, we found that KRG protects against DOX-induced testicular damage in rats by modulating the antioxidant system and hormonal imbalance (Kopalli, Cha, et al., 2016; Kopalli, Won, et al., 2016). KRG significantly ameliorated the DOX-induced damage in the rat testes, and improved the sex hormone levels, spermatogenesis, seminiferous tubular diameter, and testes weight. Further, the altered protein expression levels of peroxiredoxin, glutathione-*S*-transferase, nectin-2, inhibin-α, and cAMP response element

* Corresponding author.
E-mail address: skkim@kku.ac.kr (S.-K. Kim).
[1] These authors contributed equally to this work.

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binding protein were attenuated by KRG in DOX-treated rats. Apart from the oxidative mechanisms that are involved in DOX toxicity (Wei et al., 2015), mechanisms related to inflammation might also play a significant role in damaging testicular tissue. An earlier report indicated that DOX treatment not only induced oxidative stress, but also increased the inflammatory response, including the release of cytokines such as tumour necrosis factor (TNF)-α (Bien et al., 2007). Reports also indicated that DOX-induced cytotoxicity could occur via damage-associated molecular patterns (DAMPs), leading to inflammation and TNF production. This process would amplify the inflammatory response and might trigger several other signalling pathways, including autophagy (Balkwill, 2009; Krysko et al., 2011). These findings suggest that counteracting the inflammatory damage to the testes associated with DOX treatment might prevent testicular dysfunction. With these facts in mind, we further investigated the effects of KRG on the mRNA expression levels of antioxidant enzymes, key molecules involved in spermatogenesis, and sex hormone receptors, including the effects on inflammatory responses and autophagy signalling in the DOX-exposed rat testis.

2. Materials and methods

2.1. Preparation of KRG-WE

The Korea Ginseng Corporation in Daejeon, South Korea kindly supplied 6-year-old roots of *P. ginseng*. The extraction procedure was followed as described previously (Hwang, Kim, Wee, Choi, & Kim, 2004). Briefly, the roots were washed thoroughly with tap water and steamed at 98 °C for 60 min. The steamed roots were dried at 70 °C for 72 h and extracted with 10 volumes of water at 90 °C for 48 h, followed by filtration. The filtrate obtained was concentrated under reduced pressure to obtain dark-brown coloured syrup named KRG-WE, which was used throughout the study.

2.2. Experimental animals

The experimental protocol was described previously (Kopalli, Cha, et al., 2016; Kopalli, Won, et al., 2016). Briefly, twenty-eight male Sprague-Dawley rats (8 weeks old; weighing 240 ± 20 g) purchased from the Daehan Biolink Co., Ltd. (Eumseong-gun, Chungbuk, Korea) were allowed to acclimatize to the animal facility for 2 weeks before the experiment. The animals were provided with standard AIN-76A diet pellets and water *ad libitum*, and were kept at a constant temperature (23 ± 2 °C) and relative humidity (55 ± 10%) in a 12 h light/dark cycle. The animals were randomly divided into four groups (n = 7). The control group (CON) was administered 0.9% normal saline solution orally. The other three groups were the DOX group (DXR), KRG-WE 100 mg/kg plus DOX group (EX1), and the KRG-WE 200 mg/kg plus DOX group (EX2). The DXR group was injected with DOX (1 mg mL^{-1} kg^{-1} $week^{-1}$) intraperitoneally (*i.p.*) for 8 weeks (Prahalathan, Selvakumar, & Varalakshmi, 2005) and KRG-WE (100 or 200 mg/kg/day) was administered orally for 9 weeks starting one week before DOX exposure in the KRG-WE plus DOX groups (EX1 and EX2). The doses of KRG-WE were selected based on our previous reports (Hwang et al., 2004; Kopalli, Cha, et al., 2016; Kopalli, Won, et al., 2016). KRG-WE was mixed evenly with sterilized standard powder-type diet, pelletized and provided *ad libitum*. The concentration of KRG in the pellets was adjusted every week after considering the changes in body weight and the daily dietary intake of the rats. All animal experiments were approved and conducted in accordance with the guidelines of the Institution of Experimental Animal Ethics, Konkuk University, South Korea (Permission No. KU12051).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

The protocol for the RT-PCR analysis was followed as described previously (Kopalli et al., 2017). Briefly, total RNA was extracted from testicular tissue using the RNA-Bee reagent (AMS Bio, Abingdon, U.K.) according to the manufacturer's instructions, and the RNA (1 μg) was reverse-transcribed as described earlier (Won et al., 2014). The obtained PCR products were separated by electrophoresis on a 2.0% agarose gel containing ethidium bromide, and the bands were visualized by fluorescence imaging. The band intensity normalized to that of the GAPDH band was analysed using the ImageJ software package (version 1.41o; National Institutes of Health, NIH, Bethesda, MD, USA). The nucleotide sequences of the primers used for analysing the mRNA expression levels of inflammatory mediators and molecules related to autophagy and apoptosis are shown in Table 1.

Table 1
Sequence of the primers for inflammation, apoptosis and autophagy used in the study.

Parameter	Gene	Primer	Oligonucleotide sequence (5′-3′)
Inflammation	IL-1β	Forward	5′-AGG CTG ACA GAC CCC AAA AG-3′
		Reverse	5′-CTC CAC GGG CAA GAC ATA GG-3′
	IL-6	Forward	5′-GAA ATG ATG GAT GCT TCC AAA CTG G-3′
		Reverse	5′-GGA TAT ATT TTC TGA CAC AGT GAG G-3′
	TNF-α	Forward	5′-ACT CCC AGA AAA GCA AGC AA-3′
		Reverse	5′-CGA GCA GGA ATG AGA AGA GG-3′
	COX-2	Forward	5′-GTG GAA AAG CCT CGT CCA GA-3′
		Reverse	5′-TCC TCC GAA GGT GCT AGG TT-3′
Autophagy	mTORC1	Forward	5′-GAC AAC AGC CAG GGC CGC AT-3′
		Reverse	5′-ACG CTG CCT TTC TCG ACG GC-3′
GAPDH		Forward	5′-GTT ACC AGG GCT GCC TTC TC-3′
		Reverse	5′-GAT GGT GAT GGG TTT CCC GT-3′

Abbreviations: IL, interleukin; TNF-α, Tissue necrosis factor-alpha; COX-2, cyclooxegenase-2; mTORC-1, Mammalian Target of Rapamycin Complex 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

2.4. Western blot analysis

The protocol for western blotting was followed as described previously (Kopalli, Cha, et al., 2016; Kopalli, Won, et al., 2016). Briefly, equal amounts of testicular protein from each sample were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Each membrane was incubated for 1 h in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% skim milk to block non-specific binding. The membranes were then incubated with specific primary antibodies (1:1000, Santa Cruz Biotech, Santa Cruz, CA, USA) against inflammatory mediators such as interleukin (IL)-1B, IL-6, TNF-α, cyclooxygenase (COX-2), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), p-IκBα, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p-NF-κB, p65, p-(extracellular signal–regulated kinases) ERK, p38, p-(c-Jun N-terminal kinase) JNK, p-c-Jun, as well as autophagy marker (p-mTOR). The proteins were detected using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (GE Healthcare Life Sciences, Little Chalfont, UK). The band intensity was normalised to that of β-actin and analysed using the ImageJ software package (version 1.41o; National Institutes of Health, NIH, Bethesda, MD, USA).

2.5. High-performance liquid chromatography (HPLC) fingerprint analysis of KRG-WE

The profiles of the ginsenosides present in KRG-WE were analysed using HPLC (Agilent, Santa Clara, CA, USA) as reported in our previous study (Kopalli, Cha, et al., 2016; Kopalli, Won, et al., 2016). Briefly, HPLC was performed in room temperature containing a diode array detector and HS C18 column (4.6 × 250 mm, 5 m, Supelco, St. Louis,