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Research article

Protective effect of Korean Red Ginseng against chemotherapeutic drug-induced premature catagen development assessed with human hair follicle organ culture model

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ABSTRACT

Background: Chemotherapy-induced alopecia (CIA) is one of the most distressing side effects for patients undergoing chemotherapy. This study evaluated the protective effect of Korean Red Ginseng (KRG) on CIA in a well-established *in vitro* human hair follicle organ culture model as it occurs *in vivo*.
Methods: We examined whether KRG can prevent premature hair follicle dystrophy in a human hair follicle organ culture model during treatment with a key cyclophosphamide metabolite, 4-hydroperoxycyclophosphamide (4-HC).
Results: 4-HC inhibited human hair growth, induced premature catagen development, and inhibited proliferation and stimulated apoptosis of hair matrix keratinocytes. In addition, 4-HC increased p53 and Bax protein expression and decreased Bcl2 protein expression. Pretreatment with KRG protected against 4-HC-induced hair growth inhibition and premature catagen development. KRG also suppressed 4-HC-induced inhibition of matrix keratinocyte proliferation and stimulation of matrix keratinocyte apoptosis. Moreover, KRG restored 4-HC-induced p53 and Bax/Bcl2 expression.
Conclusion: Overall, our results indicate that KRG may protect against 4-HC-induced premature catagen development through modulation of p53 and Bax/Bcl2 expression.
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1. Introduction

Hair provides protective, sensory, and sexual attractiveness attributes and is also often used to indicate personal beliefs or social position. During postnatal life, hair cyclically undergoes the following three alternating phases of rapid growth: anagen (2–6 yr), apoptosis-mediated regression (catagen, 2–3 wk), and relative quiescence (telogen, 2–3 mo) [1]. Hair matrix keratinocytes at the anagen phase are some of the fastest dividing cells in the body, with 60% of them remaining in the S phase [1]. Because chemotherapeutic drugs target rapidly proliferating cell populations, they attack not only neoplastic cancer cells but also rapidly growing hair matrix keratinocytes in anagen, which leads to hair loss (alopecia) [2].

Chemotherapy-induced alopecia (CIA) is one of the most distressing side effects for patients undergoing chemotherapy [3–5]. Although the CIA is almost always reversible, CIA can lead to negative psychological perceptions for patients, even leading to refusal of treatment [2,6]. The incidence of CIA is ~65% among patients receiving chemotherapy [5,7]. As much as 47–58% of female patients consider hair loss to be the most traumatic aspect of chemotherapy and 8% would decline chemotherapy due to fears of hair loss [8,9]. Therefore, the pursuit of more efficient management strategies for CIA remains a major research challenge in clinical oncology [10].

Korean Red Ginseng (KRG; the steamed root of *Panax ginseng* Meyer) has been an established traditional herbal medicine for > 2,000 y [11]. KRG has been cultivated and aged for ≥ 4–6 yr,

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and goes through extensive cleaning, steaming, and drying processes to enhance its pharmacological activity and stability [12]. Recent studies have reported antitumor, antiviral, antidiabetic, antioxidative, and immune-modulatory activities of KRG [13–16]. Furthermore, a number of studies have also illustrated the role of KRG as a potent regulator of hair growth. KRG prevents apoptosis of hair follicles in irradiated mice, promotes hair growth in C57BL/6 mice, promotes human and murine vibrissae hair growth in organ culture, and improves hair regrowth in androgenetic alopecia and alopecia areata patients [17–21].

In a recently developed *in vitro* human hair follicle organ culture model for CIA, the cyclophosphamide (chemotherapeutic drug) metabolite 4-hydroperoxycyclophosphamide (4-HC) induces apoptosis followed by dystrophy in isolated human anagen hair follicles, like CIA *in vivo* [22]. This study assessed the ability of KRG to protect against CIA in a well-established *in vitro* human hair follicle organ culture model [22].

2. Materials and methods

2.1. Materials

The KRG extract was provided by the Korea Ginseng Corporation (Daejeon, Korea) through a standardized and reproducible process. The extract was manufactured by the Korea Ginseng Corporation (Seoul, Korea) from the roots of a 6-y-old red ginseng (*P. ginseng* Meyer), which was harvested in the Korea. KRG was prepared by steaming fresh ginseng at 90–100°C for 3 h and then drying it at 50–80°C. The KRG extract was prepared from red ginseng water extract, which was extracted three times at 85–90°C for 8 h in circulating hot water. The water content of the pooled extract was 36% of the total weight. KRG was analyzed by HPLC and contained the following major ginsenosides (Rb1, 7.44 mg/g; Rb2, 2.59 mg/g; Rc, 3.04 mg/g; Rd, 0.91 mg/g; Re, 1.86 mg/g; Rf, 1.24 mg/g; Rg1, 1.79 mg/g; Rg2, 1.24 mg/g; and Rg3, 1.39 mg/g) and other minor ginsenosides.

The key cyclophosphamide metabolite 4-HC was purchased from Niomec (Bielefeld, Germany).

2.2. Isolation and culture of follicular keratinocytes

Human occipital scalp skin specimens were obtained from patients undergoing hair transplantation surgery after obtaining informed consent. The Institutional Ethics Committee of the Yonsei University, Wonju College of Medicine, Wonju, Korea, approved all described studies. The study was conducted according to the principles of the Declaration of Helsinki.

For culture of follicular keratinocytes (FKCs), anagen hair follicles were cut off from the hair bulb region and then dermal sheathes were removed from the upper part of the hair follicles. Hair shafts, including part of the outer root sheath, were treated with 0.05% trypsin–EDTA (Invitrogen, Waltham, Massachusetts, USA). The dissociated cells were rinsed in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and centrifuged for 5 min at 1,500 rpm. Cells were then resuspended in EpiLife medium (Cascade Biologics, Portland, OR, USA) with EpiLife defined growth supplement (Cascade Biologics) and antibiotics and seeded onto a culture dish. Second-passage FKCs were used in this study.

2.3. Cell viability assay

The cytotoxic effects of KRG on FKCs were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [23]. In brief, 1×10^4 cells were seeded in each well containing 100 μL of the growth medium in a 96-well plate. Cells were permitted to adhere for 24 h, and then were treated with serial doses of KRG extract (from 0 μg/mL to 1,000 μg/mL) for 1–2 d. After treatment, the medium in each well was removed and replaced with a phosphate-buffered saline solution containing 5 mg/mL MTT. Then the plate was incubated at 37°C for 4 h. The remaining supernatant was then completely removed and 100 μL of dimethyl sulfoxide was added to each well and mixed thoroughly to dissolve the crystallized formazan. After 10 min of incubation to ensure that all formazan crystals were dissolved, the optical density at 570 nm was determined using an enzyme-linked immunosorbent assay reader. The mean absorbance of the treated group was expressed as the cell viability percentage of the control group's absorbance. Three repeated experiments were performed.

2.4. Human hair follicle organ culture

Human anagen hair follicles were isolated as previously described [24]. Isolated human anagen hair follicles were maintained in Williams E medium (Invitrogen) supplemented with 10 μg/mL insulin (Sigma, St. Louis, MO, USA), 10 ng/mL hydrocortisone (Sigma), 2mM L-glutamine (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) for 1 d. Isolated anagen hair follicles were cultured in each type of medium for 6 d. In brief, control hair follicles were cultured with vehicle for 6 d. Test groups were pretreated with or without KRG (100 μg/mL or 500 μg/mL) on Day 0. Furthermore, a key cyclophosphamide (chemotherapeutic drug) metabolite, 4-HC (20μM), was added on Day 1 [22]. Western blot analysis and immunofluorescence staining were performed after 2 d of culture. The same experiment was repeated three times.

2.5. Measurement of hair follicle length and morphology

The hair follicle length was defined as the entire length from the base of the hair bulb to the tip of the hair shaft. Measurements were made every 2 d using the measuring scales attached to the objective lens of the microscope until the 6th d of cultivation. The measured values were then statistically analyzed. At the same time, the hair follicle morphology (anagen, early catagen, mid catagen, and late catagen) was observed and the hair cycle score was measured according to the following system: anagen VI, 100; early catagen, 200; mid catagen, 300; and late catagen 400. Experiments were repeated three times.

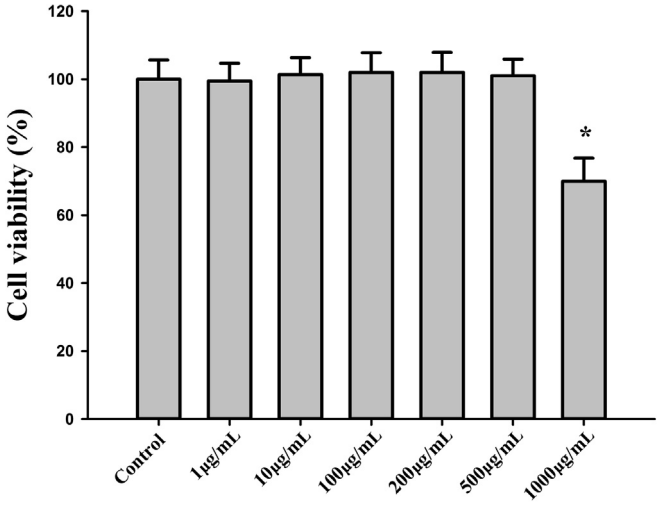

Fig. 1. Follicular keratinocytes viability assay. Cell viability (%) = (mean absorbency in test wells)/(mean absorbency in control wells) × 100. All the values are shown as mean ± standard deviation. * $p < 0.05$ versus control cells incubated with media alone.

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