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Research Article

# Korean Red Ginseng and Rb1 facilitate remyelination after cuprizone diet-induced demyelination

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ABSTRACT

*Background:* Demyelination has been observed in neurological disorders, motivating researchers to search for components for enhancing remyelination. Previously we found that Rb1, a major ginsenoside in Korean Red Ginseng (KRG), enhances myelin formation. However, it has not been studied whether Rb1 or KRG function in remyelination after demyelination *in vivo*.

*Methods:* Mice were fed 0.2% cuprizone-containing chow for 5 weeks and returned to normal chow with daily oral injection of vehicle, KRG, or Rb1 for 3 weeks. Brain sections were stained with luxol fast blue (LFB) staining or immunohistochemistry. Primary oligodendrocyte or astrocyte cultures were subject to normal or stress condition with KRG or Rb1 treatment to measure gene expressions of myelin, endoplasmic reticulum (ER) stress, antioxidants and leukemia inhibitory factor (LIF).

*Results:* Compared to the vehicle, KRG or Rb1 increased myelin levels at week 6.5 but not 8, when measured by the $LFB^{+}$ or GST-$pi^{+}$ area within the corpus callosum. The levels of oligodendrocyte precursor cells, astrocytes, and microglia were high at week 5, and reduced afterwards but not changed by KRG or Rb1. In primary oligodendrocyte cultures, KRG or Rb1 increased expression of myelin genes, ER stress markers, and antioxidants. Interestingly, under cuprizone treatment, elevated ER stress markers were counteracted by KRG or Rb1. Under rotenone treatment, reduced myelin gene expressions were recovered by Rb1. In primary astrocyte cultures, KRG or Rb1 decreased LIF expression.

*Conclusion:* KRG and Rb1 may improve myelin regeneration during the remyelination phase *in vivo*, potentially by directly promoting myelin gene expression.



## 1. Introduction

The myelin sheath enwraps neuronal axons, enabling saltatory conduction in the nervous system [1]. Myelin plasticity is essential for a wide range of physical and mental functions including learning and memory [2]. While dysregulation of myelin is observed in the normal aging process [3,4], it is aggravated under neuropsychiatric conditions such as Alzheimer's disease [5] as well as demyelinating diseases like multiple sclerosis [6].

*Panax ginseng* Meyer has been widely used as a health food, and has been examined extensively for its efficacy in improving health and combating disease [7,8]. Previous investigations have also indicated that ginseng and its components can enhance higher brain functions such as cognition, learning and memory [9,10], proposing ginseng as a possible complementary therapy for related diseases such as dementia [11]. Although myelin is critical for brain plasticity involved learning [2] and whose dysfunction is related with several neurological diseases [12,13], investigations on the effects of ginseng on myelin regulation or oligodendrocytes (OLs) which are myelin-forming cells are scarce.

Ginseng extracts include saponin [14], non-saponin fractions [15] and gintonin [16]. Interestingly, we previously found that the non-saponin fraction [17] or gintonin [18] can increase the proliferation of OL precursor cells (OPCs), while the saponin fraction, especially Rb1, improves myelin sheath formation *in vitro* and *in vivo* under physiological conditions [17]. Rb1 has been reported for its multiple functions such as neuroprotection [19] and anti-

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diabetic features [20]. Recent studies have suggested that Rb1 performs these functions possibly by modulating mitochondrial behaviors such as energy metabolism, oxidative stress, and reactive oxygen species release [21]. Another ginsenoside Rg1 has recently been reported to exert a positive effect on myelin sheath protection and increase the number of mature OLs in a chronic Parkinson's disease mouse model [22]. These suggest the potential health benefits of ginseng extract via stimulating the proliferation of OL-lineage cells and myelin formation.

However, there are still no studies which have investigated the effects of ginseng or its components on remyelination itself in an *in vivo* demyelination model. Based on our previous findings where we showed that Rb1 facilitates myelin formation *in vitro* and *in vivo* under physiological conditions [17], we investigated the effects of Rb1 and Korean Red Ginseng (KRG) extract, whose most abundant component corresponds to Rb1 [17], on remyelination in the cuprizone-induced demyelination model in this study. Cuprizone is a copper chelator and feeding 0.2 % cuprizone-containing chow for 5 weeks preferentially induces apoptosis of mature OLs with the minimal effect on other cell types. This model has been relatively well-studied, with its time-dependent effects, and the main location of demyelination (i.e corpus callosum) previously characterized [23]. In a previous research [24], reactive oxygen species (ROS) formation was increased and membrane potential was reduced in mitochondria isolated from brains of cuprizone-feeding mice for 5 weeks. Similarly, cuprizone-feeding has been reported to change mitochondrial state of OLs in mouse central nervous system [25–28]. Moreover, 500 μM cuprizone treatment for 48 hrs on primary OL cultures significantly reduced mitochondrial membrane potential and differentiation into mature OLs [29]. In the current study, rotenone, which inhibits complex I of mitochondrial respiratory chain, was utilized to mimic the feature of mitochondrial dysfunction observed in *in vivo* cuprizone model, and the rotenone treatment has been known to inhibit differentiation of OLs [30,31].

Myelin synthesis in OLs requires a sufficient amount of membrane protein and lipid, resulting in a high endoplasmic reticulum (ER) capacity requirement. Indeed, during myelination, ER stress pathway is activated [32]. In usual condition, three ER stress sensors [IRE1 (inositol-requiring enzyme 1), PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6α/β)] are bound to BIP (binding-immunoglobulin protein, also known as GRP-78) and inactivated, however once ER stress arises, BIP is dissociated from those sensors and unfolded protein response (UPR) is initiated [33]. Activation of PERK, one of the ER stress sensors, phosphorylates eIF2a (eukaryotic translation initiation factor 2a), resulting in inducement of DDIT3 (DNA Damage Inducible Transcript 3, also known as CHOP) expression, which can proceed into both directions of pro-apoptotic or pro-survival. When it heads towards pro-survival, GADD34 (Growth arrest and DNA damage-inducible protein, one of the small number of genes which are actively translated under phosphor-eIF2a conditions during ER stress), a downstream of DDIT3, dephosphorylates eIF2a and functions as a feedback loop for recovery of general protein synthesis and promotes cell recovery from stress [34,35]. Cuprizone can increase DDIT3 expression in OLs, suggesting its influence on ER stress pathway activation [36].

In this study, after the induction of demyelination by 5 weeks of cuprizone feeding, mice were supplemented daily with vehicle, KRG, or Rb1 for a further 1.5 or 3 weeks. Myelin content was measured by using luxol fast blue (LFB) staining and the levels of mature OLs, OPCs, astrocyts and microglial cells were examined by immunohistochemistry, in order to understand the mechanism of how KRG and Rb1 modulates myelin levels during the remyelination process. To further understand molecular mechanism, we performed *in vitro* primary OL cultures under physiological differentiation condition or pathological condition treated with cuprizone or rotenone to mimic the *in vivo* environments of the cuprizone model and examined the expression levels of myelin, ER stress, and antioxidant-related genes after KRG or Rb1 treatment. Furthermore, *in vitro* primary astrocyte cultures were also investigated to know the indirect effects of KRG or Rb1 on OL myelination.

## 2. Materials and methods

### 2.1. Mouse

For the cuprizone diet, seven weeks-old C57BL/6N female mice were purchased from ORIENT BIO Inc (Seongnam, Korea) and utilized in the experiments from the age of eight weeks-old. Mice were housed in a specific pathogen free environment (23 ± 2 °C, 12-h light/dark cycle). Food and water were supplied ad libitum. Mouse Body weights were measured every week during the experimental period (Supplementary Fig. 1). For primary cultures, postnatal day 0-1 ICR pups were purchased from the same provider and sacrificed on the day of the arrival. All experiments were performed in accordance with the relevant laws and institutional guidelines, and were approved by the University of Brain Education's Animal Care and Use Committee.

### 2.2. Reagents and antibodies

KRG total extract were provided by Korean Society of Ginseng. Rb1 was purchased from Chem Faces (Wuhan, China). Both KRG and Rb1 were dissolved in distilled water with following concentrations: 25, 250, 100 mg/kg for KRG; 0.1, 1, 5 mg/kg Rb1. Treatment solutions were prepared freshly every day before the injection. Rabbit polyclonal antibodies were used as follows: GST-pi (Glutathione S-transferase P1-1, #312, MBL, CA, USA); NG2 (Neuron-glial antigen 2, #ab129051, Abcam, Cambridge, UK); Iba-1 (Ionized calcium-binding adapter molecule1, #1919741, Wako, Osaka, Japan); GFAP (Glial fibrillary acidic protein, #Z033429-2, Dako, Copenhagen, Denmark). Secondary antibodies were purchased from Jackson ImmunoResearch. Mouse chow containing 0.2% cuprizone (Bis-cyclohexanone oxaldihydrazone, #C9012, Sigma-Aldrich, St. Louis, MO, USA) was purchased by order-made from DooYeol Biotech (Seoul, Korea). Rotenone was purchased from Sigma-Aldrich.

### 2.3. Cuprizone-induced demyelination

The C57BL/6N female mice were assigned to the indicated groups of ten mice each (Fig. 1). Mice were fed with a chow containing 0.2% cuprizone during 5 weeks and returned to normal diet with daily oral injection of distilled water, KRG (25, 250, 1000 mg/kg), or Rb1 (0.1, 1, 5 mg/kg) over additional 3 weeks. Mice were sacrificed at 0, 5, 6.5, 8 week time points since the initiation of the experiment and perfused with 4% paraformaldehyde to isolate brain samples. The brain tissues were then dehydrated in ascending grades of alcohol and xylene and embedded in paraffin according to standard procedures.

### 2.4. Luxol fast blue and immunohistochemistry

Paraffin blocks were sectioned by 5 μm of thickness and the sections were processed for rehydration under xylene and descending grades of alcohol and subjected to LFB and immunohistochemistry staining. LFB staining was performed according to the manufacturer's instructions (VitroView Luxol Fast Blue Stain Kit, #VB-3006, Vitrovivo Biotech). For immunohistochemistry,

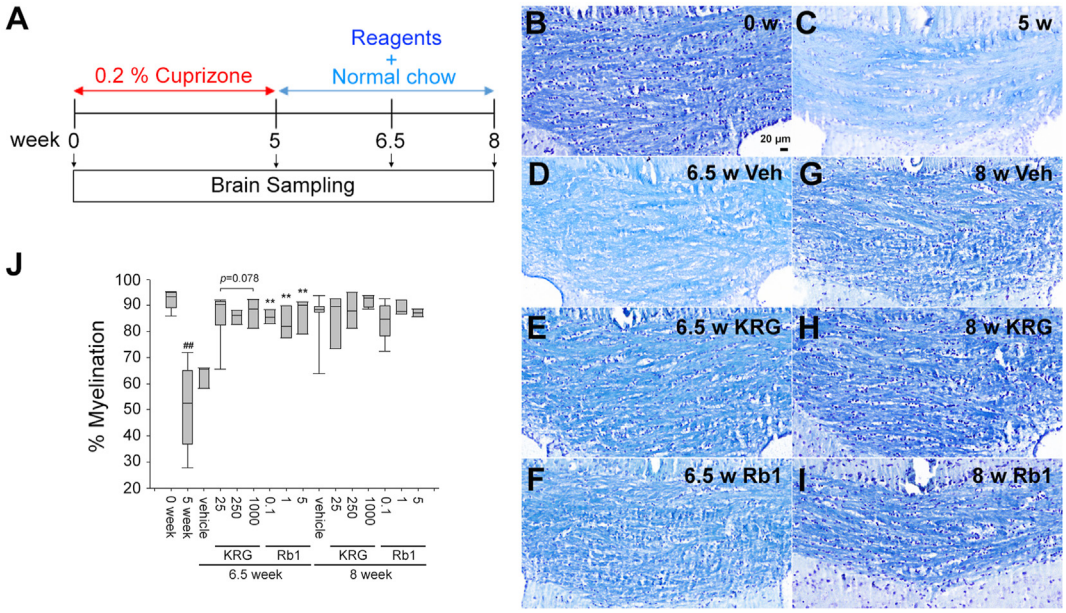


**Fig. 1.** Korean Red Ginseng and Rb1 facilitate remyelination after cuprizone diet-induced demyelination. (A) Experimental design. Mice were fed with 0.2% cuprizone-containing chow for 5 weeks and returned to a normal diet with daily oral injection of the vehicle, 25, 250, 1000 mg/kg Korean Red Ginseng (KRG), or 0.1, 1, 5 mg/kg Rb1 and sacrificed for brain sampling at the 0, 5, 6.5, and 8 week time points. (B–I) Luxol fast blue staining. The location of the picture is the center of the corpus callosum at bregma 0.94-0.62 mm. Brains injected with KRG (E, H) and Rb1 (F, I) indicate brains injected with the concentrations of 25 mg/kg and 0.1 mg/kg, respectively. Scale bar, 20 μm. (J) Percent myelination. Data were presented by box-and-whisker plot. Kruskal-Wallis one way ANOVA on Ranks within the vehicle controls at 0, 5, 6.5, and 8 weeks: $P = 0.001$; All pairwise multiple comparison procedures (Dunn's method): $^{\#\#}P = 0.001$ (0 vs 5 weeks), $P = 0.063$ (5 weeks vs 8 weeks). One way ANOVA on Ranks within 6.5 weeks according to KRG concentration: $P = 0.078$. One way ANOVA within 6.5 weeks according to Rb1 concentration: $^{**}P = 0.002$; All pairwise multiple comparison procedures (Holm-Sidak method): $^{**}P = 0.004$ (0.1 mg/kg Rb1 vs vehicle), $^{**}P = 0.007$ (1 mg/kg Rb1 vs vehicle), $^{**}P = 0.004$ (5 mg/kg Rb1 vs vehicle). N = 3-7 female mice per group.

sections were washed with phosphate-buffered saline (PBS) and blocked for 45 min at room temperature (RT) in PBS/5% normal goat serum/0.2% Triton X-100, then incubated overnight at 4 °C with the primary antibodies diluted in blocking solution. At the next day, sections were washed with PBS, incubated with secondary antibodies for 45 min at RT, washed again in PBS, and mounted in mounting medium.

### 2.5. Primary cultures

Methods for glia mixed cultures and OPC isolation are described in our previous paper [17]. OPCs were maintained in differentiation medium to induce differentiation into mature OLs. For astrocyte cultures, the astrocyte layer after the OPC isolation from the glia mixed cultures was washed with PBS, trypsinized, seeded and maintained in Glia medium. Both OL or astrocyte cultures were incubated with KRG (1, 10, 100 μg/ml) or Rb1 (1 μg/ml) under normal condition or stress condition (100 μM cuprizone or 1 μM rotenone) for 48 hrs.

### 2.6. Quantitative real time PCR

Cells were washed and thoroughly scraped and total RNA was extracted using TRI reagent (Sigma-Aldrich) and reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Thermo Fisher). Real time PCR was performed using PowerUP SYBR Green Master Mix (Life Technologies) and the primer sequences are like followings:

bActin (forward, 5′-CTTCTACAATGAGCTGCGTG-3’; reverse, 5′-GGGTGTTGAAGGTCTCAAAC- 3′), MYRF (forward, 5′-TGGCAACTTCACCTACCACA-3’; reverse, 5′-GTGGAACCTCTGCAAAAAGC- 3′), ZFP191 (forward, 5′-GCTCAGGGATTACCGAGTTC-3’; reverse, 5′-CTCTCCAGCTGAAGCCATCT- 3′), MBP (5′-CGAGAACTACCCATTATGGCTCCC-3’; reverse, 5′-TGGAGGTGGTGTTCGAGGTGTC- 3′), MAG (forward, 5′-GTTTGCCCCCATAATCCTTCTG-3’; reverse, 5′-TCCCTCTCCGTCTCATTCACAGTC- 3′), CNP (forward, 5′-GTTCTGAGACCCTCCGAAAA-3’; reverse, 5′-CCTTGGGTTCATCTCCAGAA- 3′), BIP (forward, 5′-ACTCCGGCGTGAGGTAGAAA-3’; reverse, 5′-AGAGCGGAACAGGTCCATGT- 3′), DDIT3 (forward, 5′-GGAGGTCCTGTCCTCAGATGAA-3’; reverse, 5′-GCTCCTCTGTCAGCCAAGCTAG- 3′), GADD34 (forward, 5′-CCCTCCAACTCTCCTTCTTCAG-3’; reverse, 5′-CAGCCTCAGCATTCCGACAA- 3′), NQO1 (forward, 5′-CAGCCAATCAGCGTTCGGTA-3’; reverse, 5′-CTTCATGGCGTAGTTGAATGATGTC-3′), HO1 (forward, 5′-TGCAGGTGATGCTGACAGAGG-3’; reverse, 5′-GGGATGAGCTAGTGCTGATCTGG- 3′), LIF (forward, 5′-TCAACTGGCACAGCTCAATGGC-3’; reverse, 5′-GGAAGTCTGTCATGTTAGGCGC-3′).

### 2.7. Image analysis

LFB staining pictures were recorded using a digital slide scanner (PANNORAMIC SCAN II, 3D HISTECH, Budapest, Hungary). Fluorescent staining pictures were taken by LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) and ECLIPSE Ts2 (Nikon, Tokyo, Japan). Image analysis was performed by using Image J. Percent myelination (Fig. 1) was calculated by [(LFB$^{+}$ area within the total corpus callosum)/(the total corpus callosum area)x100]. Marker positive area in corpus callosum (%) (Figs. 2–5) was calculated by [(marker$^{+}$ area within the medial corpus callosum)/(the medial corpus callosum area)x100].

### 2.8. Statistical analyses

The data were analyzed using Sigmaplot 14.0 software. Statistical comparisons between different treatments were performed

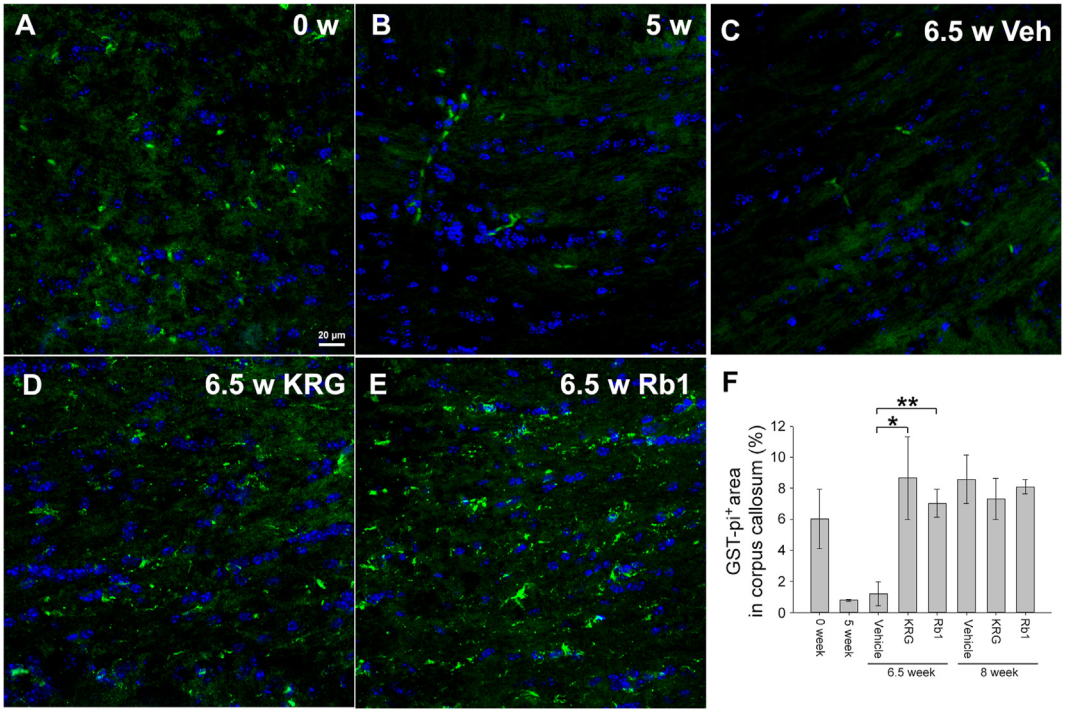


**Fig. 2.** Korean Red Ginseng and Rb1 upregulate mature oligodendrocyte marker expression after 1.5 weeks of oral injection during the remyelination phase in the cuprizone-induced demyelination model. (A-E) Representative pictures of GST-pi immunostaining (green) on the medial corpus callosum of mice oral-injected with vehicle, Korean Red Ginseng (KRG, 25 mg/kg), and Rb1 (0.1 mg/kg) at 0 (A), 5 (B), and 6.5 week (C-E). Blue indicates dapi-positive nucleus. Scale bar, 20 μm. (F) Graph of GST-pi$^+$ area in the medial corpus callosum. Student's *t*-test: $*P = 0.044$ (6.5w veh vs 6.5w KRG), $**P = 0.008$ (6.5w veh vs 6.5w Rb1). N = 3-6 female mice per group. Bars represent mean ± standard error of the mean.

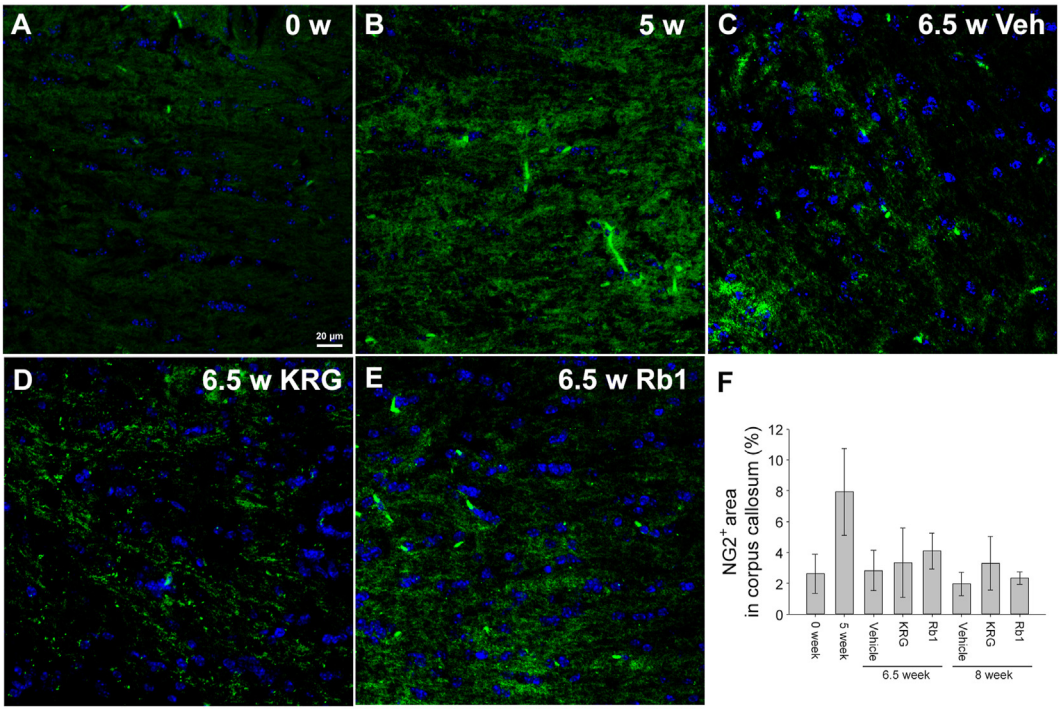


**Fig. 3.** Korean Red Ginseng and Rb1 do not induce significant changes in the protein expression level of oligodendrocyte precursor cell marker NG2 during the remyelination phase in the cuprizone model. (A-E) Representative pictures of NG2 immunostaining (green) on the medial corpus callosum of mice oral-injected with vehicle, Korean Red Ginseng (KRG, 25 mg/kg), and Rb1 (0.1 mg/kg) at 0 (A), 5 (B), and 6.5 week (C-E). Blue indicates Dapi-positive nucleus. Scale bar, 20 μm. (F) Graph of NG2$^+$ area within the medial corpus callosum. N = 3 female mice per group. Bars represent mean ± standard error of the mean.

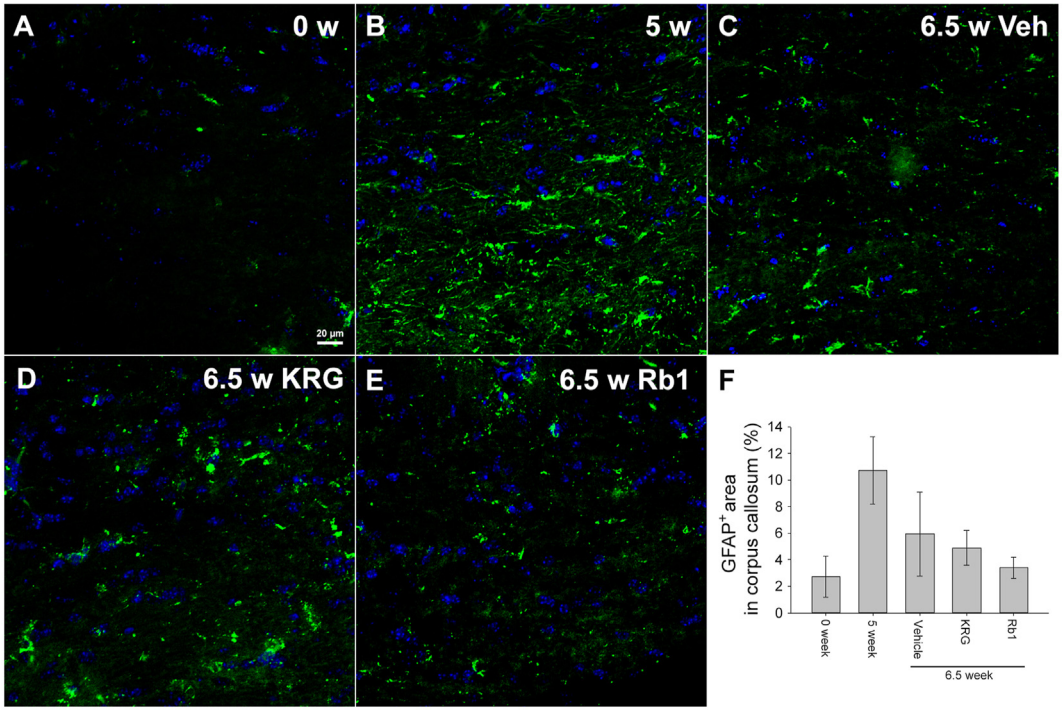


**Fig. 4.** Korean Red Ginseng and Rb1 do not induce significant changes in astrocyte cell marker expression during the remyelination phase in the cuprizone model. (A-E) Representative pictures of GFAP immunostaining (green) on the medial corpus callosum of mice oral-injected with vehicle, Korean Red Ginseng (KRG, 25 mg/kg), and Rb1 (0.1 mg/kg) at 0 (A), 5 (B), and 6.5 weeks (C-E). Blue indicates Dapi-positive nucleus. Scale bar, 20 μm. (F) Graph of $GFAP^+$ area within the medial corpus callosum. N = 3 female mice per group. Bars represent mean ± standard error of the mean.

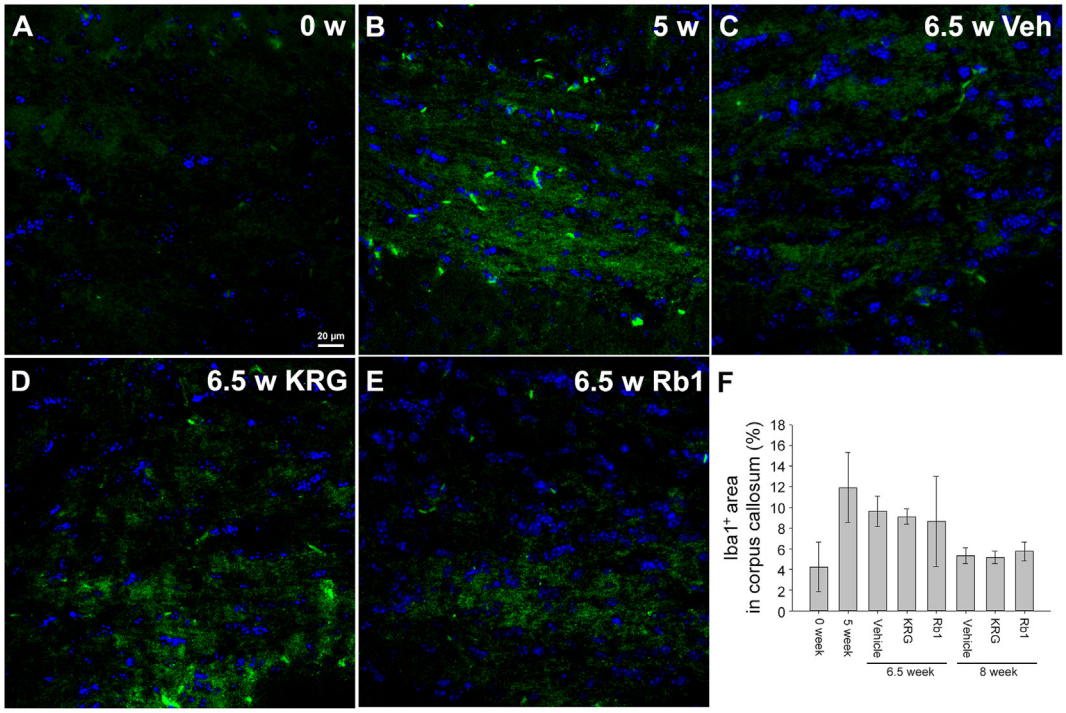


**Fig. 5.** Korean Red Ginseng and Rb1 do not induce significant changes in microglia cell marker expression during remyelination phase in the cuprizone model. (A-E) Representative pictures of Iba1 immunostaining (green) on the medial corpus callosum of mice oral-injected with vehicle, Korean Red Ginseng (KRG, 25 mg/kg), and Rb1 (0.1 mg/kg) at 0 (A), 5 (B), and 6.5 weeks (C-E). Blue indicates Dapi-positive nucleus. Scale bar, 20 μm. (F) Graph of $Iba1^+$ area within the medial corpus callosum. N = 3 female mice per group. Bars represent mean ± standard error of the mean.

using following calculations: Kruskal-Wallis one way ANOVA on Ranks with Dunn's *post hoc* test; one-way ANOVA with Holm-Sidak *post hoc* test; Student's *t*-test. *P*-values <0.05 were considered to be statistically significant.

## 3. Results

### 3.1. KRG and Rb1 supplementation promotes remyelination after cuprizone-induced demyelination

In our previous study, we reported that Rb1, which is a major ginsenoside of KRG, improves myelin sheath formation *in vitro* as well as the expression of the major myelin protein marker, myelin basic protein (MBP) *in vivo* under physiological conditions [17]. However, we do not know if it can also enhance remyelination after demyelination. To answer this question, we utilized the cuprizone model, which induces demyelination in the brain, especially in the corpus callosum, by feeding the mice with 0.2% cuprizone-containing chow for 5 weeks. Subsequent resumption of a normal diet allows mice to recover spontaneous remyelination [23]. After 5 weeks of the cuprizone diet, mice were returned to a normal diet combined with daily oral injections of the vehicle control, KRG (25, 250, 1000 mg/kg), or Rb1 (0.1, 1, 5 mg/kg) (Fig. 1A). Brain samples were collected at 0, 5, 6.5, and 8 weeks for LFB staining (Fig. 1B–I) and immunohistochemistry (Figs. 2–5, Supplementary Fig. 2-3). At 5 weeks after the initiation of the cuprizone diet, demyelination was evident at the corpus callosum ($^{\#\#}P = 0.001$, 0 vs 5 week, Fig. 1B, C, J). After returning to a normal diet, the remyelination was subsequently followed over a 3-week period ($P = 0.063$, 5 vs 8 week, Fig. 1B–D, J). We observed that the lowest concentration of both KRG and Rb1 (i.e. 25 mg/kg for KRG and 0.1 mg/kg for Rb1) were effective at restoring myelin levels after 1.5 weeks of the administration (at 6.5 weeks), and we maintained these concentrations for subsequent experiment analyses. At 6.5 weeks, we observed that 25 mg/kg KRG exhibited restored myelin levels to 86% compared to the vehicle control (63%) ($P = 0.078$), while 0.1 mg/kg Rb1 exhibited 86% myelin levels compared to the vehicle control ($^{**}P < 0.01$). At 8 weeks, both KRG and Rb1 showed no significant differences in % myelination compared to the control.

In the cuprizone diet-induced demyelination model, cuprizone induces demyelination through the selective cell death of mature OLs [23]. Therefore, in order to assess directly the changes of mature OL cells, we used GST-pi which is a marker for mature OLs. When we compared the GST-pi$^+$ area at 6.5 weeks, both KRG- or Rb1-injected mice exhibited a significant increase compared to the vehicle control ($^{*}P = 0.044$ for KRG, $^{**}P = 0.008$ for Rb1, Fig. 2, Supplementary Fig. 2), although there were no significant differences at the 8 week time point. This is consistent with previous *in vitro* and *in vivo* data studying the effects of KRG and Rb1 under physiological conditions [17]. Our current results indicate that both KRG and Rb1 can enhance myelin formation, during the remyelination phase after demyelination.

### 3.2. KRG and Rb1 supplementation does not affect the protein expression of oligodendrocyte precursor cell marker after cuprizone-induced demyelination

Next, we wondered whether the upregulated remyelination in KRG or Rb1-treated mice at 6.5 weeks was due to increased level of OPCs. From previous work, we had observed that Rb1 facilitates the increased myelin formation but not OPC cell number *in vitro* [17], thus it was assumed that the OPC number may not be changed by the treatment here. To examine this, we used NG2 as an OPC marker for immunostaining and measured OPC levels under vehicle, KRG or Rb1 treatment (Fig. 3). Consistent with previous *in vitro* results, both KRG and Rb1 did not induce significant changes in the NG2$^+$ area within the medial corpus callosum at the 6.5 and 8 week time points (Fig. 3F).

### 3.3. KRG and Rb1 do not significantly impact on the protein expression levels of astrocyte or microglial cell markers after the cuprizone-induced demyelination

Previous findings suggest that KRG or Rb1 can affect the expression or function of other glial cell types [37,38]. To examine the expression level of other glial cell types by KRG or Rb1 injection, we performed immunostaining with GFAP for astrocyte cell marker (Fig. 4) and Iba1 for microglial cell marker, respectively (Fig. 5). The percentage of marker$^+$ area was measured within the medial corpus callosum. The expression of each marker was upregulated after the cuprizone diet, however, both the GFAP$^+$ area (%) and Iba1$^+$ area (%) did not exhibit significant differences in KRG or Rb1 compared to the vehicle control (Figs. 4F and 5F).

### 3.4. KRG accelerates the expression of myelin genes, ER stress genes, and antioxidant genes in primary oligodendrocyte cultures

In order to reveal the detailed molecular biological mechanism, we used primary cultures of OLs isolated from glia mixed cultures (*days in vitro* 10) derived from postnatal day 0-1 mouse cortex. First of all, to investigate whether KRG or Rb1 directly affect the myelin dynamics by modulating myelin gene expression, primary OL cultures under differentiation condition were treated with KRG (1, 10, 100 μg/ml) or Rb1 (1 μg/ml) for 48 hrs and examined the expression levels of MYRF (myelin gene transcription factor), ZFP191 (required for myelin formation in the late OL developmental stage), and major myelin genes (MBP, MAG, CNP). Intriguingly, KRG significantly increased the expression of transcription factor of myelin genes (MYRF) at 100 μg/ml ($p = 0.035$) and exhibited a tendency of increment in other concentrations (1, 10 μg/ml) as well (Fig. 6A). Moreover, KRG exhibited a facilitatory tendency on expression of ZFP191, MBP, and MAG genes (Fig. 6B–D). Rb1 also exhibited a slight increase but did not reach a significant change on expression of MYRF, ZFP191, MBP, and MAG genes (Fig. 6E–H). While 100 μM cuprizone induced no or slight reduction in MAG, CNP level (Fig. 6I–K), 1 μM rotenone treatment significantly reduced myelin genes such as MBP, MAG, MYRF, and ZFP191 (Fig. 6L–S). Rb1 exhibited a tendency or a significant difference in the recovery for the expression of MAG and ZFP191 under rotenone treatment ($p = 0.07$ for MAG, Fig. 6Q; $p = 0.021$ for ZFP191, Fig. 6S).

As myelin production requires an ER capacity to provide more proteins and lipids, enhanced myelin gene expressions by KRG (Fig. 6A) were accompanied by enhanced ER stress markers such as BIP and DDIT3 (Fig. 7A and B). As a downstream of ER stress pathway as well as a pro-survival indicator, GADD34 was also increased by KRG (Fig. 7C). Adaptation response induced by UPR increases antioxidant expression and modulates ROS production in ER [39]. As myelin gene expression increases by KRG (Fig. 6A–D), ER stress also increases (Fig. 7A–C), simultaneously, antioxidant expression such as NQO1 and HO-1 is also increased by KRG (Fig. 7D–E). Rb1 which is the highest content among ginsenosides of KRG slightly increased the expression of BIP, DDIT3 and GADD34 without significant changes (Fig. 7F–H) and significantly increased the expression of antioxidant (NQO1) (Fig. 7I). Rb1 also slightly increased HO1 expression without a significant difference (Fig. 7J).

Cuprizone increases DDIT3 expression in OLs, suggesting its influence on ER stress pathway activation [36]. Indeed, in our study, cuprizone treatment significantly increased expression of ER stress-related factors such as BIP and DDIT3 (Fig. 7K and L). In cuprizone-induced toxic environments, KRG treatment

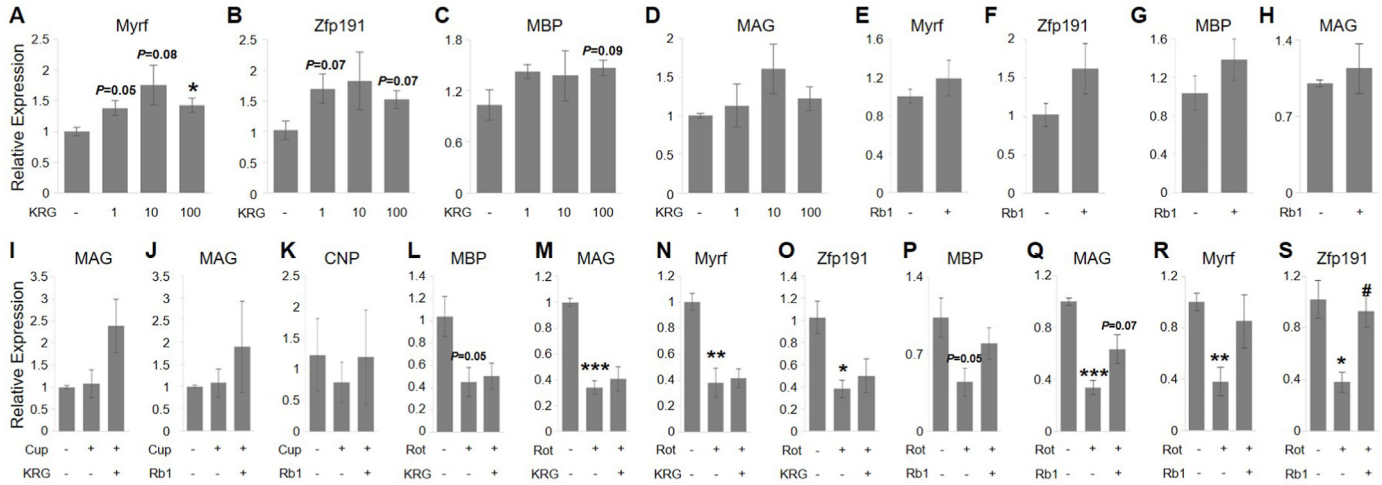


**Fig. 6.** Korean Red Ginseng facilitates expression of myelin genes in primary oligodendrocytes. Under physiological differentiation condition (A-H) or pathological condition induced by cuprizone or rotenone (I–S), primary oligodendrocytes were treated with Korean Red Ginseng (KRG) (A-D, I–O) or Rb1 (E-H, P–S) for 48 hrs and myelin-related genes (MYRF, ZFP191, MBP, MAG, CNP) were examined by quantitative real time PCR, normalized by bActin. (A-D) Relative expression of myelin gene transcription factor MYRF (A: *, $P = 0.035$, Control vs KRG 100 μg/ml, Student's *t*-test), oligodendrocyte late stage developmental marker ZFP191 (B), MBP (C), MAG (D) depending on KRG concentration in differentiating primary oligodendrocytes. (E-H) Relative expression of MYRF (E), ZFP191 (F), MBP (G), MAG (H) by 1 μg/ml Rb1 in differentiating primary oligodendrocytes. (I–K) Relative expression of MAG and CNP by 1μg/ml KRG or 1 μg/ml Rb1 under 100 μM cuprizone treatment. (L-S) Relative expression of MBP, MAG, MYRF, and ZFP191 by 1 μg/ml KRG or 1 μg/ml Rb1 under 1 μM Rotenone treatment. Rotenone treatment significantly reduced the expression of MAG (M, Q: ***, $P = 0.0004$, Control vs Rot, Student's *t*-test), MYRF (N, R: **, $P = 0.0089$, Control vs Rot, Student's *t*-test), and ZFP191 (O, S: *, $P = 0.018$, Control vs Rot, Student's *t*-test). Rb1 significantly reversed the expression pattern in ZFP191 (S: #, $P = 0.021$, Rot vs Rot + 1 μg/ml Rb1,). N = 3 different primary cultures. *, $P < 0.05$, compared with control; #, $P < 0.05$, compared with cuprizone or rotenone; Student's *t*-test. MYRF, myelin regulatory factor; ZFP191, zinc finger protein191; MBP, myelin basic protein; MAG, myelin associated glycoprotein; CNP, 2′, 3′-cyclic nucleotide 3′ phosphodiesterase.

significantly reduced the expression of these factors (Fig. 7K-M). Therefore, KRG seems to reduce the activation of ER stress pathway induced by cuprizone. Rb1 also exhibited a tendency of reduction in the expression of BIP, but not DDIT3 and GADD34, induced by cuprizone (Fig. 7P–R). Rotenone treatment did not affect ER stress marker expression and KRG or Rb1 also did not change these marker expression under rotenone-induced toxic environments (Fig. 7U–W, Z-AB).

Toxic conditions induced by either cuprizone or rotenone increased expressions of antioxidant, i.e., NQO1 and HO1, while they were reduced by KRG treatment. However, although KRG reduced the enhanced expression of antioxidants induced by cuprizone or rotenone, antioxidant expression seemed to be increased along with KRG concentrations even under toxic environments (Fig. 7N, O, X, Y). Rb1 also exhibited contrasting results: reduced antioxidant under cuprizone treatment (Fig. 7S and T) or increased antioxidant under rotenone treatment by Rb1 (Fig. 7AC, AD).

LIF released by astrocytes was reported to promote myelination [40]. In order to check the indirect effects of KRG or Rb1 on myelination by astrocytes, we examined LIF expression in primary astrocyte cultures. Unlike expected, KRG decreased LIF expression in astrocytes in dose-dependent manner (Fig. 8A). Rb1 also exhibited similar results (Fig. 8B)Cuprizone treatment did not induce significant changes in LIF expression of astrocytes (Fig. 8C–D). Under cuprizone treatment, KRG exhibited U shape changes in LIF expression (Fig. 8C) and Rb1 slightly reduced the expression without a significant change (Fig. 8D). Rotenone dramatically increased the expression of LIF in astrocytes and neither KRG or Rb1 affect it (Fig. 8E–F).

## 4. Discussion

In this study, in order to analyze the effects of KRG or Rb1 on the remyelination process, we orally injected KRG or Rb1 after the cessation of a 5-week cuprizone diet over an additional period of 3 weeks (Fig. 1A). In the medial corpus callosum of mice injected with KRG or Rb1, we observed a significant increase in myelin levels during the remyelination process (Fig. 1) as well as increased mature OL marker (GST-pi) immunostaining over the vehicle (Fig. 2, Supplementary Fig. 2). The myelin-enhancing features of KRG or Rb1 were also observed in primary OL cultures (Fig. 6A–H). This is consistent with the *in vitro* effect of Rb1 on the increased myelin sheath formation as well as the *in vivo* effect of Rb1 in enhancing myelin basic protein expression in our previous report [17]. Another ginsenoside Rg1 maintained the iron-regulated protein homeostasis in mature OLs and was able to protect the myelin sheath and maintain the number of mature OLs in a chronic Parkinson's Disease mouse model [22]. In addition, Rg1 protects myelin from cuprizone-induced demyelination via CXCL10-mediated glial response [41]. Regarding the modulation on OLs, Rg1 mainly functions in OL or myelin protection [22,41], while Rb1 directly facilitates OL maturation or myelin production in addition to OL protection.

After the demyelination, myelin repair involves active participation of the endogenous OPC population [42–45]. However, we did not see any significant differences in immunostaining with OPC markers between vehicle- and KRG- or Rb1-treated mouse brains (Fig. 3). In our previous studies, saponin fraction containing major ginsenosides such as Rb1 does not affect OPC proliferation in the *in vitro* pure OPC culture [17,18], consistent with our current result *in vivo*. Therefore, the increased myelin levels during the remyelination phase in this study seems not to be due to increased OPC levels but probably by accelerated maturation or myelin production of OLs. Indeed, in primary OL cultures, KRG significantly increased the expression of MYRF (Fig. 6A). MYRF is autoproteolytically cleaved and functions as a transcription factor to directly activate myelin genes [46]. As myelin production requires a large amount of proteins and lipids, ER stress pathway is activated during myelination [32]. Consistently our results also showed a higher MYRF expression by KRG (Fig. 6A) was consistent with a higher ER stress marker expression such as BIP and DDIT3 (Fig. 7A and B).

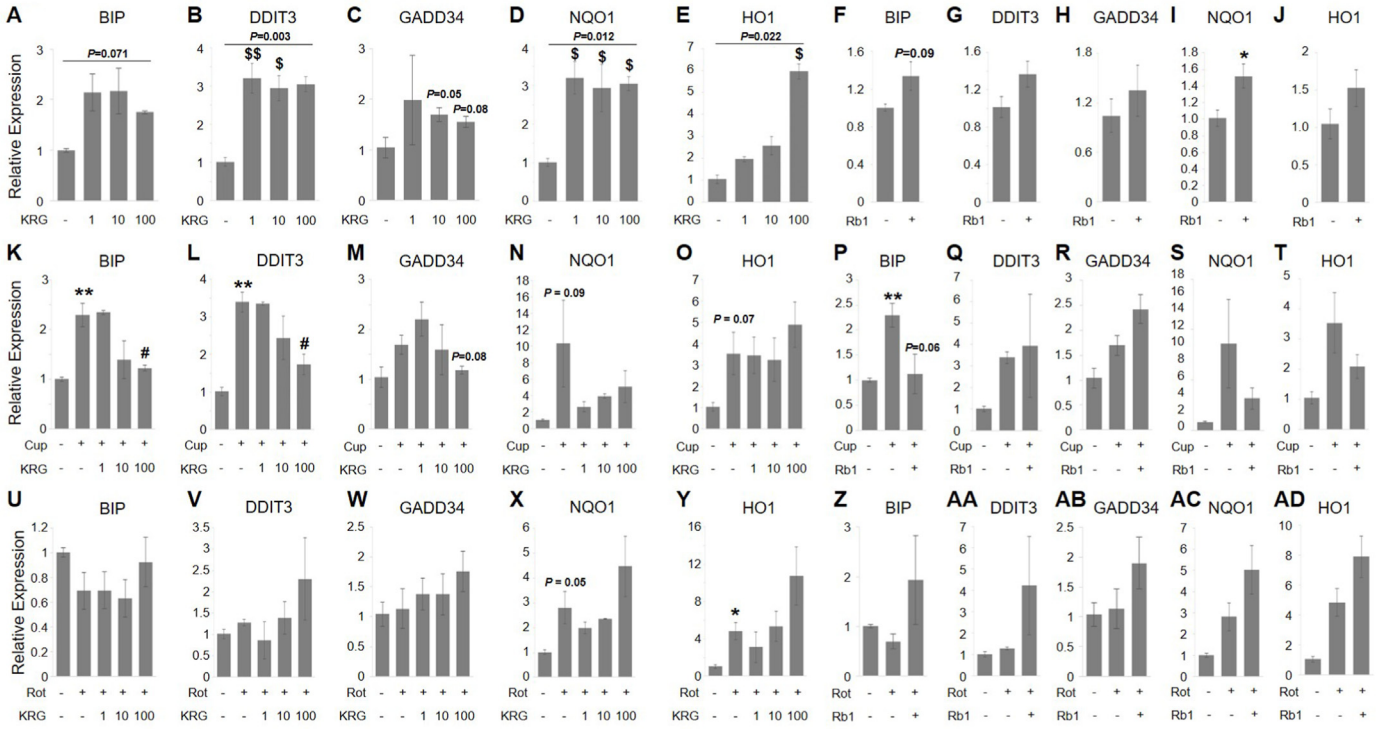


**Fig. 7.** Dynamic expression changes in the ER stress-related markers and antioxidant genes by Korean Red Ginseng under physiological and pathological conditions in primary oligodendrocytes. Under physiological differentiation condition (A-J) or pathological condition induced by cuprizone (K-T) or rotenone (U-AD), primary oligodendrocytes were treated with Korean Red Ginseng (KRG) (A-E, K–O, U–Y) or Rb1 (F-J, P-T, Z-AD) for 48 hrs and ER stress-related genes (BIP, Ddit3, GADD34), antioxidant genes (NQO1, HO1) were examined by quantitative real time PCR, normalized by bActin. (A-C) Relative expression of ER stress marker BIP (A: $P = 0.071$, one-way ANOVA), DDIT3 (B: $P = 0.003$, one-way ANOVA; $$, $P = 0.003$, Control vs KRG 1; $, $P = 0.031$, Control vs KRG 10, Holm-Sidak method), GADD34 (C: $P = 0.05$, Control vs KRG 10; $P = 0.08$, Control vs KRG 100, Student's *t*-test), depending on KRG concentration in differentiating primary oligodendrocytes. (D-E) Relative expression of antioxidant NQO1 (D: $P = 0.012$, one-way ANOVA; $P = 0.023$, Control vs KRG 1; $P = 0.031$, Control vs KRG 10; $P = 0.029$, Control vs KRG 100, Holm-Sidak method) and HO1 (E: $P = 0.022$, Kruskal-Wallis one way ANOVA on Ranks; $, $P = 0.012$, Control vs KRG 100, Turkey test), depending on KRG concentration in differentiating primary oligodendrocytes. (F-J) Relative expression of BIP (F), DDIT3 (G), GADD34 (H), NQO1 (I: $P = 0.043$, Student's *t*-test), HO1 (J) by 1 μg/ml Rb1 in differentiating primary oligodendrocytes. (K-T) Relative expression of ER stress and antioxidant genes by KRG or Rb1 under 100 μM cuprizone treatment in primary oligodendrocyte cultures. (K–O) Relative expression of BIP (K: **, $P = 0.006$, Control vs Cup); #, $P = 0.012$, Cup vs Cup + KRG100, Student's *t*-test), DDIT3 (L: **, $P = 0.0023$, Control vs Cup; #, $P = 0.026$, Cup vs Cup + KRG100, Student's *t*-test), GADD34 (M), NQO1 (N), HO1 (O) by KRG under cuprizone treatment. (P-T) Relative expression of BIP (P: **, $P = 0.0059$, Control vs Cup, Student's *t*-test), DDIT3 (Q), GADD34 (R), NQO1 (S), HO1 (T) by Rb1 under cuprizone treatment. (U–Y) Relative expression of ER stress and antioxidant genes by KRG or Rb1 under 1 μM rotenone treatment in primary oligodendrocyte cultures. (U–Y) Relative expression of BIP (U), DDIT3 (V), GADD34 (W), NQO1 (X), HO1 (Y: *, $P = 0.015$, Control vs Rotenone, Student's *t*-test) by KRG under rotenone treatment. (Z-AD) Relative expression of BIP (Z), DDIT3 (AA), GADD34 (AB), NQO1 (AC), HO1 (AD) by Rb1 under rotenone treatment. N = 3 different primary cultures. $, $P < 0.05$, $$, $P < 0.01$, post hoc test of one way ANOVA; *, $P < 0.05$, Student's *t*-test compared with control; #, $P < 0.05$, Student's *t*-test compared with cuprizone or rotenone; μg/ml, KRG unit. BIP, binding-immunoglobulin protein; DDIT3, DNA damage inducible transcript 3; GADD34, growth arrest and DNA damage-inducible protein; NQO1, NAD(P)H: quinone oxidoreductase 1; HO1, heme oxygenase1.

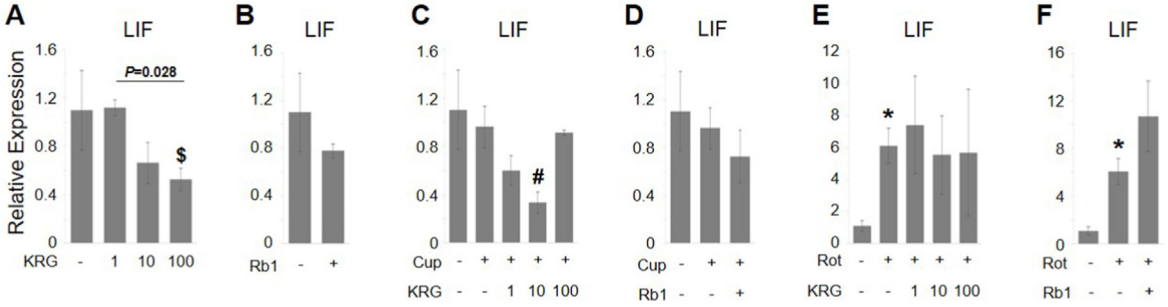


**Fig. 8.** Expression changes of leukemia inhibitory factor by Korean Red Ginseng in primary astrocytes. Under physiological condition (A-B) or pathological condition induced by cuprizone (C-D) or rotenone (E-F), primary astrocytes were treated with Korean Red Ginseng (KRG) (A, C, E) or Rb1 (B, D, F) for 48 hrs and leukemia inhibitory factor (LIF) was examined by quantitative real time PCR, normalized by bActin. (A-B) Relative expression of LIF depending on KRG (A: $P = 0.028$, one-way ANOVA; $, $P = 0.036$, KRG 1 vs 100, Holm-Sidak method) or Rb1 (B) under physiological condition. (C-D) Relative expression of LIF depending on KRG (C: #, $P = 0.03$, cuprizone vs cuprizone + KRG10, Student's *t*-test) or Rb1 (D) under cuprizone treatment. (E-F) Relative expression of LIF depending on KRG (E) or Rb1 (F) under rotenone treatment (*, $P = 0.012$, control vs rotenone, Student's *t*-test). N = 3 different primary cultures. $, $P < 0.05$, post hoc test of one way ANOVA; *, $P < 0.05$, Student's *t*-test compared with control; #, $P < 0.05$, Student's *t*-test compared with cuprizone or rotenone.

Furthermore, enhanced antioxidant expression in OLs by KRG or Rb1 (Fig. 7D, E, I) suggest that KRG or Rb1 can supply a better environment for myelin formation with less oxidative stress by providing antioxidant within OLs. The enhanced expression of myelin genes or antioxidant genes in the *in vitro* results support the promotive function of KRG or Rb1 on remyelination in *in vivo* model.

The effects of KRG on antioxidant expression under cuprizone or rotenone-induced toxic environments may involve two contrasting pathways (Fig. 7N, O, X, Y). ① KRG itself has an promoting effect on antioxidant expression (Fig. 7D and E). ② Because antioxidant expression is also increased as downstream of ER stress [47,48], signal reduction for antioxidant expression by ER stress-reducing effects of KRG under stress condition (Fig. 7K,L) may be transmitted simultaneously. The contrasting effects on antioxidant as described in ①, ②, might result in a tendency of reduction on antioxidant expression by KRG under toxic environments as well as gradual increase of antioxidant expression as KRG concentration rises (Fig. 7N, O, X, Y).

In the central nervous system, other glial cell types such as astrocytes and microglia also participate to modulate myelination [49,50]. Astrocytes promote myelination of mature OLs via their release of leukemia inhibitory factor in concordance with axonal activity [40]. However, in our *in vitro* primary astrocyte cultures, there was no stimulation for astrocytes by neuronal axon activation. Our results suggest that KRG, under absence of neuronal activation, promotes myelin gene expression in OLs (Fig. 6A–D), and decreases LIF production in astrocytes (Fig. 8A). LIF is an IL6 class cytokine. KRG induced antioxidant expression (Fig. 7D and E). These antioxidants reduces the expression of inflammatory cytokines such as IL-6 and inhibits inflammation [51]. Therefore, in astrocytes, reduced expression of LIF by KRG under physiological condition as well as cuprizone-induced pathological conditions (Fig. 8A, C) is consistent with cell protective effects such as increased antioxidants expression by KRG.

During cuprizone-induced demyelination, astrocytes also regulate myelin clearance through the recruitment of microglia [52]. Another major glial type, microglia, function in facilitating remyelination through the clearance of myelin debris, secretion of growth factors, as well as the extracellular matrix remodeling [53]. The expressions of astrocyte or microglia markers were increased at 5 weeks under cuprizone-induced demyelination (Figs. 4 and 5), as previously reported [23]. However, administration of KRG or Rb1 after the cessation of cuprizone diet did not affect the expression levels of the astrocyte or microglia markers at 6.5 or 8 weeks (Figs. 4 and 5), suggesting that neither KRG nor Rb1 significantly change astrocyte or microglia behaviors during the remyelination phase. If KRG or Rb1 were administered at an early time point before or during demyelination, they might significantly impact astrocyte or microglia phenotypes. Previously, KRG has been reported to protect myelin via modulating immune cells when administered daily from 10 days prior to immunization with myelin basic protein peptide in experimental autoimmune encephalomyelitis model [38,54].

In conclusion, we found that KRG and Rb1 can help to increase myelin levels when supplemented during the remyelination phase after the demyelination pathology, potentially by accelerating myelin production, but not by increasing the OPC proliferation. As the purpose of this study was to reveal the impact of KRG or Rb1 during the remyelination phase, we chose time points after the demyelination period for measurement (i.e., after the cessation of the cuprizone diet). Future studies on the myelin protective function of KRG or Rb1 as a pretreatment before or during the cuprizone diet would provide more insight into this process.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.09.005.

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