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Effects of Minor Ginsenosides, Ginsenoside Metabolites, and Ginsenoside Epimers on the Growth of *Caenorhabditis elegans*

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In the previous report, we have demonstrated that ginsenoside Rc, one of major ginsenosides, is a major component for the restoration for normal growth of worms in cholesterol-deprived medium. In the present study, we further investigated the roles of minor ginsenosides, such as ginsenoside Rh_1 and Rh_2, ginsenoside metabolites such as compound K (CK), protopanaxadiol (PPD), and protopanaxatriol (PPT) and ginsenoside epimers such as 20(*R*)- and 20(*S*)-ginsenoside Rg_3 in cholesterol-deprived medium. We found that ginsenoside Rh_1 almost restored normal growth of worms in cholesterol-deprived medium in F1 generation. However, supplement of ginsenoside Rh_2 caused a suppression of worm growths in cholesterol-deprived medium. In addition, CK and PPD also slightly restored normal growth of worms in cholesterol-deprived medium but PPT not. In experiments using ginsenoside epimers, supplement of 20(*S*)- but not 20(*R*)-ginsenoside Rg_3 in cholesterol-deprived medium also almost restored worm growth. These results indicate that the absence or presence of carbohydrate component at backbone of ginsenoside, the number of carbohydrate attached at carbon-3, and the position of hydroxyl group at carbon-20 of ginsenoside might plays important roles in restoration of worm growth in cholesterol-deprived medium.

Keywords: *Panax ginseng*, *Caenorhabditis elegans*, Ginsenoside metabolites, Ginsenoside epimers, Growth

INTRODUCTION

Ginseng, the root of *Panax ginseng* Meyer, has been used as a representative tonic for two thousand years in the Far East countries like Korea, China, and Japan. Now, ginseng is one of the most famous and precious herbal medicines consumed around the world [1]. Although ginseng exhibits multiple pharmacological actions *in vitro* or *in vivo* studies, its mechanisms on various efficacies are still elusive. Recent accumulating evidences show that ginseng saponins (or ginsenosides) are the main active ingredients of ginseng (Fig. 1). Ginseng root contains 3% to 4% of ginseng saponins. Ginseng saponins are especially abundant in fine roots rather than main body of ginseng root. Ginseng saponins are one of glycoside saponins and one of the derivatives of triterpenoid dammarane consisting of thirty carbon atoms. Each ginsenoside has a common hydrophobic four ring cholesterol-like backbone structure with sugar moieties attached. About 30 different types of ginseng saponins have been isolated

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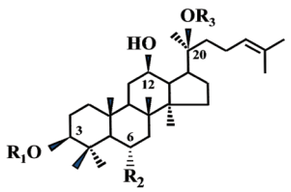

Ginsenosides	R1	R2	R3
Rb_1	-Glc_2-Glc	-H	-Glc_6-Glc
Rc	-Glc_2-Glc	-H	-Glc_6-Ara(fur)
Rh_1	-OH	-Glc	-H
Rh_2	-Glc	-H	-H
Rg_3	-Glc_2-Glc	-H	-H
PPD	-H	-H	-H
PPT	-H	-OH	-H
CK	-H	-H	-Glc

Fig. 1. Structures of the representative ginsenosides and ginsenoside metabolites. They differ at three side chains attached the common steroid ring. Subscripts indicate the carbon in the glucose ring that links the two carbohydrates. Glc, glucopyranoside; Ara(fur), arabinofuranose; PPD, protopanaxadiol; PPT, protopanaxatriol; CK, compound K.

and identified from the root of *P. ginseng* (Fig. 1).

Caenorhabditis elegans is one of nematode species and is one of free living animals under the earth rather than is parasitic in host animals or plants. *C. elegans* takes its food from dead animals or plants. Since *C. elegans* has short life cycle and large amounts of worms can be easily grown for biochemical assays or genetic studies in the laboratory, the organism is one of the well-established genetic models and its development processes have well characterized. Interestingly, *C. elegans* cannot synthesize sterols unlike other animals and requires dietary sterol, which is usually supplied as cholesterol in the laboratory [2,3]. Although the functions of sterols in *C. elegans* have not been fully characterized, *in vitro* sterol deprivation results in decreased fertility, delayed development, and short life span throughout the first (F1), second (F2), and third (F3) generations [3,4].

On the other hand, some species of parasitic nematodes are found in wild and cultivated ginseng root. For example, *Pratylenchus subpenetran* is one of main parasitic animals and causes damage to ginseng roots [5]. It penetrates into fine roots or outer spaces of ginseng root from soil and makes a lot of small humps in fine roots, because they die if they are exposed to air. Its parasitic actions in fine roots of ginseng interfere to absorb nutrients from soil. It is known that if it is not treated with anti-nematode agents, it causes to severe damages on normal development of ginseng during young period, resulting in delay of the growth and further make ginseng fragile to other microbial infections. Although these observations suggest a possibility that ginseng roots might provide suitable environment(s) for survival of the parasitic nematodes, little is known on effects of ginseng saponins or individual ginsenosides as main ingredients of ginseng on the growth, development, and life span of nematodes.

In the previous report, we have shown that supplements of ginseng total saponins (GTS) fraction and ginsenoside Rb_1 and Rc restored normal growth of *C. elegans* grown in cholesterol-deprived-medium [6]. These results show a possibility that the individual ginsenosides might have differential effects on growth of *C. elegans*. On the other hand, recent works have shown that ginsenosides administered via the oral route may pass into the large intestine without being decomposed by either gastric juices or digestive enzymes [7]. Ginsenoside Rh_1 is metabolized into protopanaxatriol (PPT) by intestinal microorganisms, whereas ginsenoside Rh_2 is metabolized into protopanaxadiol (PPD). They possess only the backbone structure of ginsenosides without any carbohydrate component [7] (Fig. 1). Some of these ginsenoside metabolites have shown to have anticancer activity [7]. We have also shown that ginsenoside epimers have differential effects on voltage-gated ion channel regulations [8]. For example, 20(*S*)-ginsenoside Rg_3 but not 20(*R*)-ginsenoside Rg_3 inhibited voltage-dependent Ca^+, K^+, and Na^+ channel currents [8].

In the present study, we examined whether supplements of minor ginsenosides, ginsenoside metabolites, or ginsenoside epimers show any effects on the growth of *C. elegans* grown in cholesterol-deprived-medium. We found that minor ginsenoside, ginsenoside metabolites, and ginsenoside epimers showed differential effects on worm growth in cholesterol-depleted medium, depending on the number of carbohydrate attached at carbon-3 and on the position of hydroxyl group at carbon-20 of ginsenoside Rg_3. Thus, it appears that the number of carbohydrate attached at carbon-3 and the position of hydroxyl group at carbon-20 of ginsenoside Rg_3 might contribute to the restoration of worm growth in cholesterol-deprived medium.

MATERIALS AND METHODS

Materials

Electrophoresis grade agarose was obtained from Becton, Dickinson and Company (Sparks, MD, USA) and peptone was obtained from Amresco (Solon, OH, USA). Cholesterol and all other analytical agents were obtained

from Sigma (St. Louis, MO, USA). Cholesterol stock solution for cholesterol treatment group was prepared at 5 mg/mL of ethanol. The final ethanol concentration was 0.01%. Minor ginsenosides, ginsenoside metabolites, and ginsenoside epimers, isolated according to the method of Tanaka *et al*. [9] and Shibata *et al*. [10], respectively, was kindly provided by the Korea Ginseng Corporation (Seoul, Korea). After chromatography, we confirmed no existence of phytosterols by HPLC. Fig. 1 shows the structures of ginsenoside metabolites and ginsenoside epimers.

Media and *Caenorhabditis elegans* growth

The nematodes were grown and maintained on NGM agarose plates (3 g/L NaCl, 2.5 g/L peptone, 5 mg/L cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4, pH 6.0, and 17 g/L agar) with the *Escherichia coli* OP50 strain in an incubator at 20°C [11]. To obtain cholesterol-free conditions, agar was replaced by agarose, which was extracted three times with chloroform [12]. We also extracted peptone with ether in a large beaker in a fume hood [3]. For this, the peptone powder was mixed with an excess volume of ether, allowed to settle, decanted, and the process was repeated twice more. The extracted peptone was allowed to dry overnight in the hood to remove the remaining ether [3]. *E. coli* strain OP50 was also grown directly in this sterol-free medium. Wild-type N2 *C. elegans* (Bristol type) was provided by the Caenorhabditis Genetics Center and was maintained according to the methods of Brenner [11]. For definition of various generations of worms, we first cultured worms (Po, n=10-20) in each treatment group such as cholesterol (5 μg/mL)-fed, cholesterol-deprived, minor ginsenosides+cholesterol-deprived, ginsenoside metabolites+cholesterol-deprived, or ginsenoside epimers+cholesterol-deprived group. When eggs from Po animals grown in each treatment were again placed on various treatment group plates as mentioned above, the resulting animals are referred to as F1 generation. When eggs from F1 animals were grown in various treatment group plates, the resulting animals are referred to as F2 generation.

Table 1. Effects of minor ginsenosides, ginsenoside metabolites, and ginsenoside epimers on growth rate of *Caenorhabditis elegans* grown in cholesterol-deprived medium

	Growth rate (h) (egg to adult)		
	L4	Young adult	Adult
First generation			
+Chol	65±0.2	76±0.2	96±0.2
-Chol	74±0.1**	92±0.1***	115±0.1***
-Chol + Rh_1	65±0.1	89±0.1***	96±0.1
-Chol + Rh_2	72±0.2**	96±0.2**	145±0.2***
-Chol + CK	73±0.2**	114±0.1***	139±0.1***
-Chol + PPD	80±0.2**	112±0.2***	136±0.2***
-Chol + PPT	94±0.2***	118±0.2***	142±0.2***
-Chol + 20(*R*)Rg_3	73±0.1**	95±0.1***	119±0.1***
-Chol + 20(*S*)Rg_3	69±0.1**	80±0.1	96±0.2
Second generation			
+Chol	65±0.1	76±0.2	96±0.2
-Chol	76±0.1**	100±0.1***	123±0.1***
-Chol + Rh_1	72±0.1**	95±0.1**	120±0.1***
-Chol + Rh_2	72±0.2**	95±0.2**	144±0.2***
-Chol + CK	90±0.2**	119±0.1***	144±0.1***
-Chol + PPD	95±0.2***	120±0.2***	144±0.2***
-Chol + PPT	95±0.2***	120±0.1***	144±0.1***
-Chol + 20(*R*)Rg_3	73±0.1**	95±0.2***	120±0.1***
-Chol + 20(*S*)Rg_3	69±0.2	80±0.1	99±0.2

Data are mean±SE (n=20 for each stage).
L4, larvae 4; Chol, cholesterol; CK, compound K; PPD, protopanaxadiol; PPT, protopanaxatriol.
$^{**}p$<0.01, $^{***}p$<0.001, significantly different from cholesterol-fed group.

Measurement of growth rate and worm length

Larvae 4 (L4) hermaphrodites (n=20-30) grown in cholesterol-fed, cholesterol-deprived, minor ginsenosides+cholesterol-deprived, ginsenoside metabolites+cholesterol-deprived, or ginsenoside epimers+cholesterol-deprived group were individually cloned onto agarose plates at 20°C. Growth rate of worms on each agarose plate was determined by observation of characteristic stage-specific morphology every 24 h until the end of the growth. We put individual worm at a specific stage on an agarose pad and measured the length of it. We also analyzed growth rate of F1 and F2 progenies with the same procedure as described above.

RESULTS

Effects of minor ginsenosides on worm growth in cholesterol-deprived medium

Worms grown in NGM agarose plates at 20°C were divided into three groups: cholesterol (5 μg/mL)-fed, cholesterol-deprived, or cholesterol-deprived but supplemented with minor ginsenosides such as ginsenoside Rh_1 or Rh_2 (300 μM, each). We first examined the growth rate in cholesterol-deprived or fed-medium. As shown in Table 1 and Fig. 2A, in F1 generation the growth rate (i.e., the time to reach at adult stage from egg) of worms grown in cholesterol-deprived medium were significantly retarded by 9, 16, and 19 h at L4, young adult, and adult

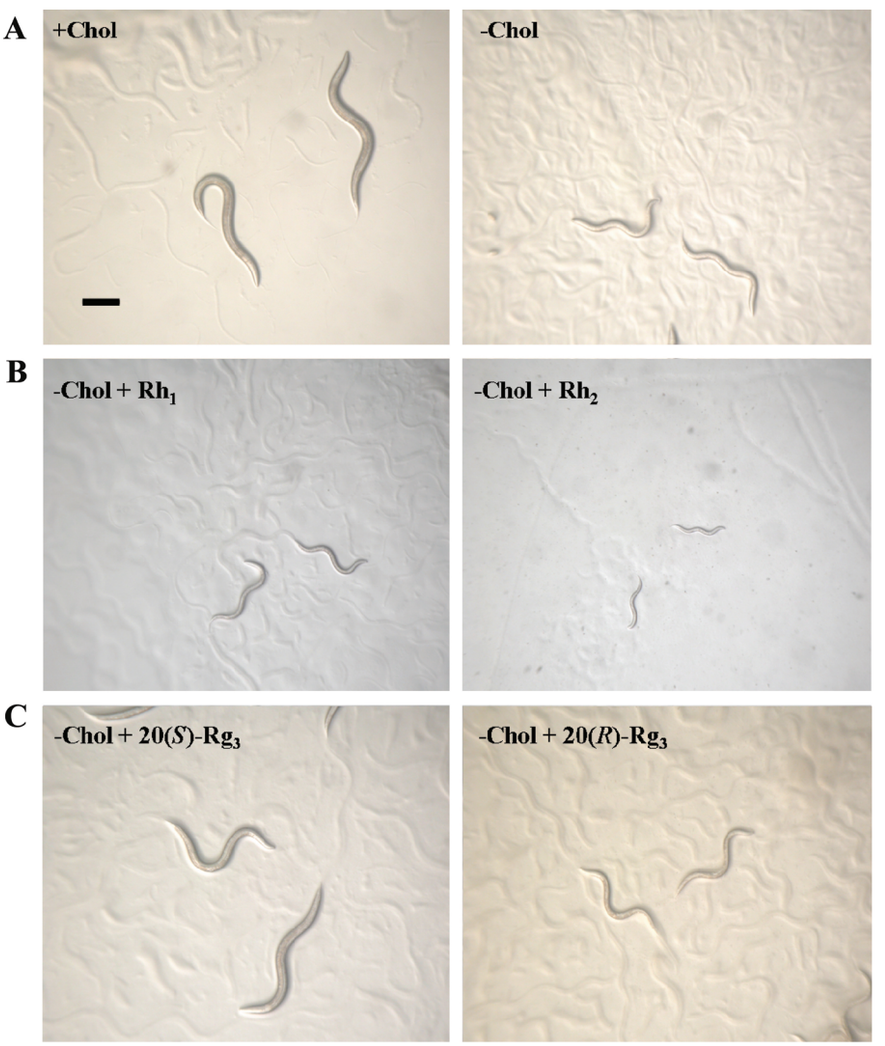

Fig. 2. Eggs laid by first generation animals were placed on plates containing the following concentrations: (A) cholesterol 5 mg/mL (+Chol), cholesterol-deprived (-Chol); (B) -Chol+300 μM Rh_1, -Chol+300 μM Rh_2; (C) -Chol+300 μM 20(*S*)-Rg_3, -Chol+300 μM 20(*R*)-Rg_3. Photographs were taken after 72 h growth of second generations at 20°C. All photographs were exposed for equal times under identical conditions. Scale bar=200 μm.

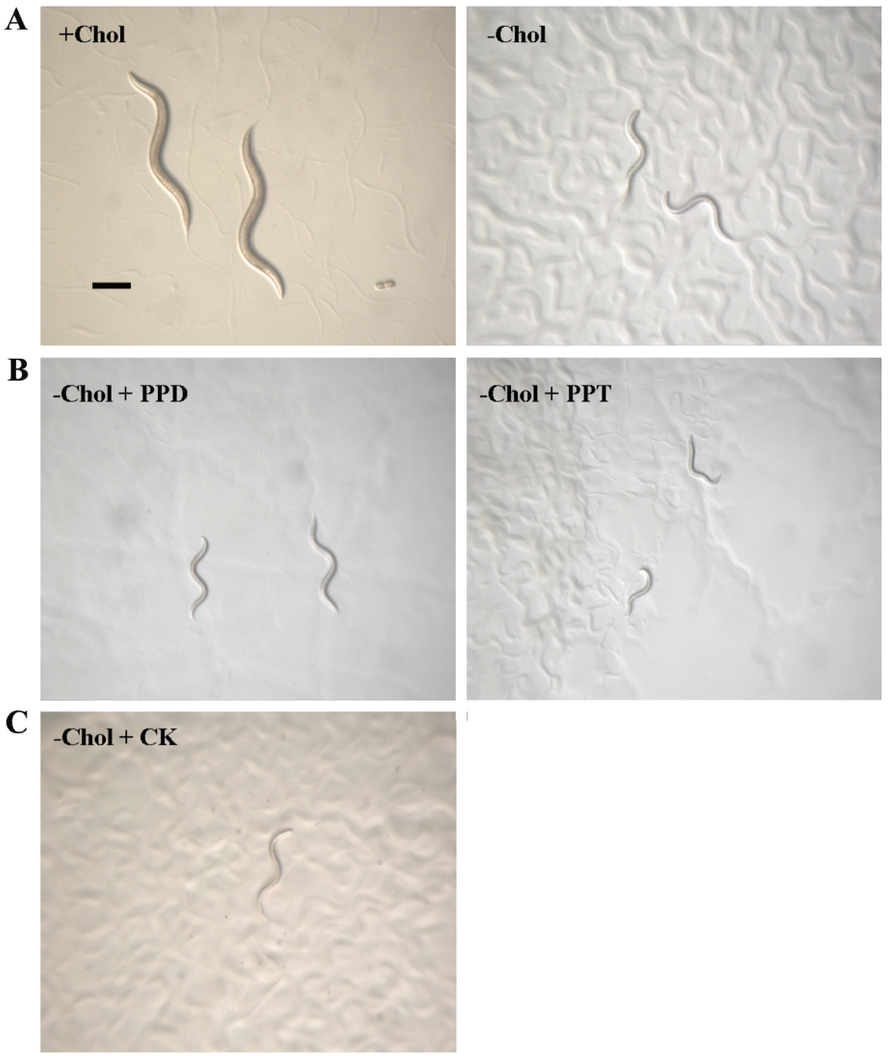

Fig. 3. Eggs laid by first generation animals were placed on plates containing the following concentrations: (A) cholesterol 5 mg/mL (+Chol), cholesterol-deprived (-Chol); (B) -Chol+300 μM protopanaxadiol (PPD), -Chol+300 μM protopanaxatriol (PPT); (C) -Chol+300 μM compound K (CK). Photographs were taken after 72 h growth of second generations at 20°C. All photographs were exposed for equal times under identical conditions. Scale bar=200 μm.

stage, respectively, and by 11, 24, and 27 h at L4, young adult, and adult stage in F2 generation, respectively, compared with worms grown in cholesterol-fed medium. In addition, we could observe the significant differences in the worm length between worms grown in cholesterol-deprived medium and worms grown in cholesterol-fed medium at L4, young adult, and adult stage (Figs. 2-4).

Supplement of Rh_1 to cholesterol-deprived medium almost restored the growth rate and worm length in all stages of F1 generation (Table 1 and Fig. 4A). However, in F2 generation, supplement of Rh_1 to cholesterol-deprived medium retarded the growth by 7, 19, and 24 at L4, young adult, and adult stage, respectively, compared with worms grown in cholesterol-fed medium (Table 1). In F2 generation worm length was slightly restored (Fig. 4B). Interestingly, supplement of Rh_2 to cholesterol-deprived medium strongly suppressed worm growth, decreased worm size, and further retarded the worm growth by 7, 20, and 49 h at L4, young adult, and adult stage in F1 generation, respectively and by 7, 19, and 48 h at L4, young adult and adult in F2 generation, respectively, compared with worms grown in cholesterol-fed medium (Table 1), indicating that ginsenoside Rh_1 or Rh_2, which differs in the position of one carbohydrate, shows a differential effect on worm growth in cholesterol-deprived medium.

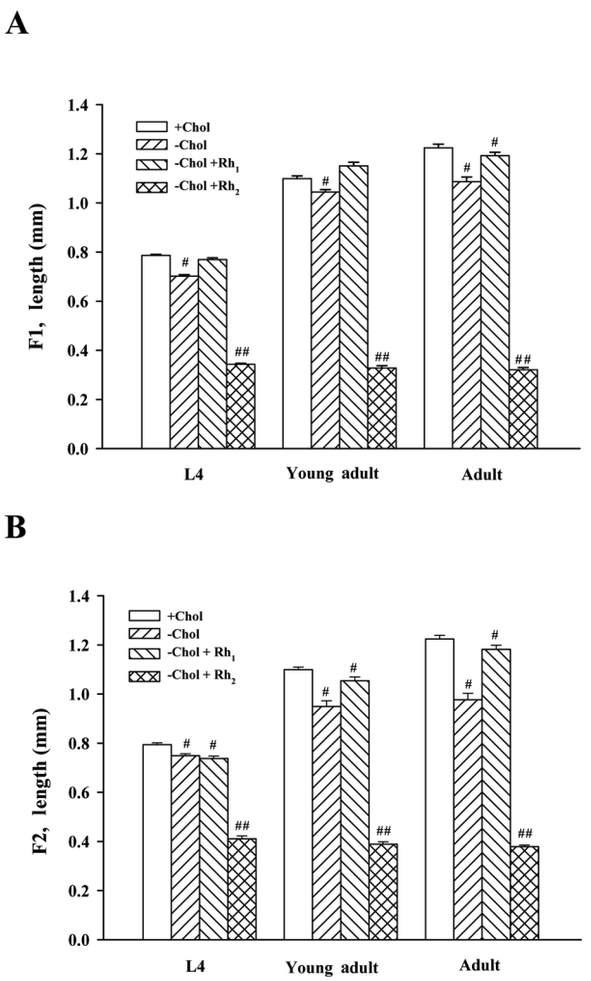

Fig. 4. Comparison of body size of worms treated with 300 μM ginsenoside Rh_1 or 300 μM ginsenoside Rh_2. (A) In first generation (F1), specific stages (larvae 4 [L4], young adult, or adult) are indicated on the bottom of each bar with supplemented ginsenoside Rh_1 or Rh_2. The average value of twenty worms is presented. (B) In second generation (F2), specific stages (L4, young adult, or adult) are indicated on the bottom of each bar with supplemented ginsenoside Rh_1 or Rh_2. The average value of twenty worms is presented. Data are mean±SE. $^{\#}p<0.01$, different from cholesterol (Chol)-fed animals; $^{\#\#}p<0.005$, different from Chol-fed animals.

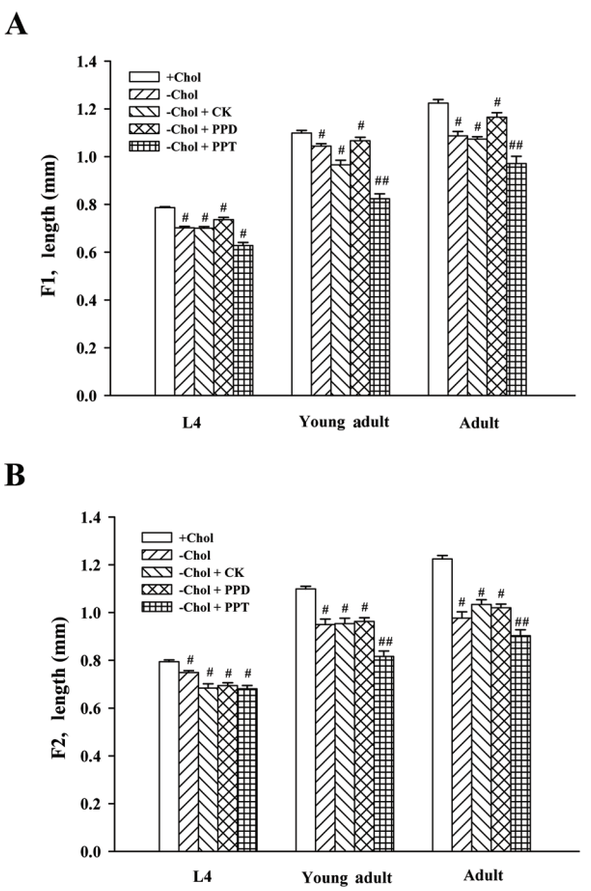

Fig. 5. Comparison of body size of worms treated with compound K (CK), 20(*S*)-protopanaxadiol (PPD), or 20(*S*)-protopanaxatriol (PPT) (300 μM each) in cholesterol-deprived medium. (A) The average value of twenty worms is presented. (B) In second generation (F2), specific stages are indicated on the bottom of each bar. The average value of twenty worms is presented. Data are mean±SE. F1, first generation; L4, larvae 4. $^{\#}p<0.01$, different from cholesterol (Chol)-fed animals; $^{\#\#}p<0.005$, different from Chol-fed animals.

Effects of ginsenoside metabolites on worm growth in cholesterol-deprived medium

We first examined the effects of compound K (CK) on the worm growth in cholesterol-deprived medium. Supplement of CK to cholesterol-deprived medium did not restore the growth rate rather further retarded the growth of worm retarded by 8, 38, and 43 h at L4, young adult and adult stage in F1 generation, respectively and by 25, 43, and 48 h at L4, young adult and adult in F2 generation, respectively, compared with worms grown in cholesterol-fed medium (Fig. 3C) and Table 1. Thus, CK, which contains one carbohydrate at carbon-20, did not help much to restore worm growth in cholesterol-deprived condition. In addition, we could observe that supplement of CK to cholesterol-deprived medium did not restore the worm length throughout all stages of F1 and F2 and generations (Fig. 5).

Supplement of PPD to cholesterol-deprived medium did not restore worm growth and further retarded the worm growth by 15, 36, and 40 h at L4, young adult and adult stage in F1 generation, respectively and by 30, 44, and 48 h at L4, young adult and adult in F2 generation, respectively, compared with worms grown in cholesterol-fed medium (Table 1 and Fig. 3B). Supplement of PPT to cholesterol-deprived medium further retarded the worm growth by 29, 42, and 46 h at L4, young adult, and adult

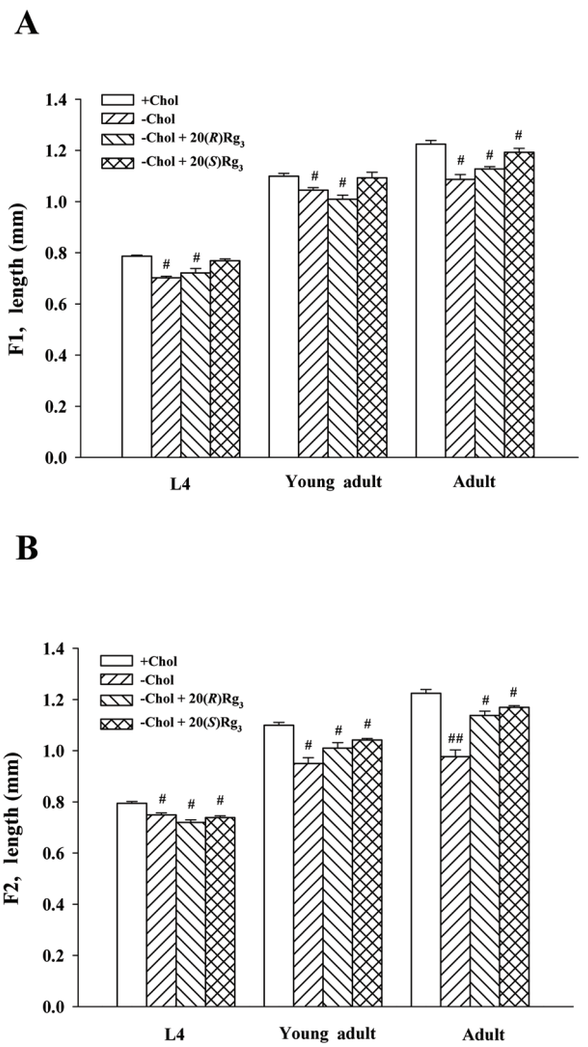

Fig. 6. Comparison of body size of worms treated with 300 μM 20(*R*)-Rg_3 or 300 μM 20(*S*)-Rg_3 stereoisomers. (A) In first generation (F1), specific stages are indicated on the bottom of each bar. The average value of twenty worms is presented. (B) In second generation (F2), specific stages are indicated on the bottom of each bar. The average value of twenty worms is presented. Data are mean±SE. L4, larvae 4. $^{\#}p<0.01$, different from cholesterol (Chol)-fed animals; $^{\#\#}p<0.005$, different from Chol-fed animals.

stage in F1 generation, respectively and by 30, 44, and 48 h at L4, young adult, and adult in F2 generation, respectively, compared with worms grown in cholesterol-fed medium (Table 1). Thus, supplement of PPD slightly restored worm length in cholesterol-deprived medium, whereas supplement of PPT to cholesterol-deprived medium rather retarded worm length in all stages of F1 and F2 generations (Fig. 5). These results indicate that the position of hydroxyl group of backbone of ginsenoside might also affect the worm growth in the cholesterol-deprived medium.

Effects of ginsenoside epimers on worm growth in cholesterol-deprived medium

Next, we examined the effects of ginsenoside epimers on worm growth in cholesterol-deprived medium. As shown in Table 1 and Fig. 2C, supplement of 20(*R*)-Rg_3 cholesterol-deprived medium retarded the worm growth by 8, 19, and 23 h at L4, young adult, and adult stage in F1 generation, respectively and by 8, 19, and 24 h at L4, young adult and adult in F2 generation, respectively, compared with worms grown in cholesterol-fed medium. In contrast, supplement of 20(*S*)-Rg_3 cholesterol-deprived medium almost restored worm growth and worm length was also restored at L4, young adult, and adult stage in F1 generation, respectively (Table 1, Figs. 2C and 6A) but in F2 generation supplement of 20(*S*)-Rg_3 cholesterol-deprived medium did not restore both worm growth and worm length as much as F1 generation compared with worms grown in cholesterol-fed medium (Table 1 and Fig. 6B). These results indicate that worms might have an ability to distinguish 20(*S*)-Rg_3 from 20(*R*)-Rg_3 for their normal growth in F1 and F2 generations.

DISCUSSION

Nematodes, including free-living *C. elegans*, require sterol for its normal development, growths, and life span as a nutritional source, since *C. elegans* is unable to biosynthesize sterol *de novo* [13]. However, *C. elegans* is usually able to obtain cholesterol or cholesterol-like sterols for their growth by metabolizing natural sterols such as phytosterols present in many plants or sterols from the animal body in the soil [14]. Although some species of nematodes are known to be parasitic in wild and cultivated ginseng roots [5], little is known on the physiological roles of ginseng saponins or ginsenosides to the reproduction, development, growth, and life span of *C. elegans*.

In the previous report, we could demonstrate that ginsenoside Rb_1 and Rc but not Rg_1 restored the average brood size, growth rate, percent development, and life span of *C. elegans* in cholesterol-deprived medium [6]. These results show a possibility that ginsenosides differentially affect development and growth of worm in cholesterol-deprived medium. In the present study, we examined the effects of minor ginsenosides, ginsenoside metabolites, or ginsenoside epimers on worm growth rate and worm length. We could observe that ginsenoside Rh_1 and ginsenoside Rh_2 exhibited a differential effect on worm growth in cholesterol-deprived medium. Supplement of ginsenoside Rh_1 almost restored growth of worm

compared with cholesterol-fed medium in F1 generation, whereas ginsenoside Rh_2 exhibited deleterious effects by causing a death or arrest at embryos or larvae stage. We could not maintain worms until F3 generation (data not shown). Worms that even did hatch were also very sluggish, nearly paralyzed without movement. Interestingly, these two ginsenosides have only one carbohydrate on backbone structure but the position of carbohydrate is different from each other (Fig. 1). Ginsenoside Rh_1 has a glucose at carbon 6 and ginsenoside Rh_2 has a glucose at carbon-3. Thus, ginsenoside Rh_2 could be utilized as anti-nematode agent (Fig. 4A, B). Taken together, these results indicate that the beneficial or harmful effects on *C. elegans* is depending on the type of ginsenosides, which is are different from the number and position of carbohydrates at carbon-3 or carbon-6 and position of hydroxyl group at carbon 20.

In experiments using PPD and PPT, we could observe that although the time to reach at adult stage from egg was almost same between PPD and PPT in cholesterol-deprived medium, PPT produced smaller worms than those of PPD size but did not restore growth rate and worm size as much as cholesterol-fed medium in F1 and F2 generations (Fig. 4A). CK also delayed time to reach adult stage and did not restore worm size as much as cholesterol-fed medium (Fig. 5A, B).

In regard to structure-activity of ginsenoside Rg_3 epimers, we have previously shown that 20(*S*)- but not 20(*R*)-Rg_3 inhibits voltage-dependent ion channel activities [7]. We also showed that in ex vivo experiments using swine coronary artery 20(*S*)- but not 20(*R*)-Rg_3 inhibited agonist-induced contraction of vessel [15]. These results indicate that 20(*S*)- rather than 20(*R*)-Rg_3 is a major active component for physiological or pharmacological actions. Furthermore, in the present *in vivo* study, we could demonstrate that supplement of 20(*S*)- but not 20(*R*)-Rg_3 to cholesterol-deprived medium almost restored worm growth rate and worm length as much as in cholesterol-fed medium. These results indicate that worms could distinguish 20(*S*)-Rg_3 from 20(*R*)-Rg_3, in which the position of hydroxyl group at carbon-20 differ, and utilize only 20(*S*)-Rg_3 for their growth under cholesterol-deprived condition. Again, we showed that 20(*S*)-Rg_3 but not 20(*R*)-Rg_3 could be biologically active.

In summary, using *C. elegans* as model system we herein used minor ginsenosides, ginsenoside metabolites, and ginsenoside epimers to know their roles in *C. elegans* growth in cholesterol-deprived-medium. We have obtained evidences that minor ginsenosides, ginsenoside metabolites, and ginsenoside epimers exhibit differential effects on *C. elegans* growth. Thus, ginsenoside 20(*S*)-Rg_3 and ginsenoside Rh_1 could be used as a sterol substitute in cholesterol-deprived medium for *C. elegans* growth, whereas ginsenoside Rh_2 could be used as an anti-nematode agent. Finally, these novel findings provide new insights that *C. elegans* could utilize subtypes of ginsenosides as sterol source.

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