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Red ginseng polysaccharide alleviates cytotoxicity and promotes anti-inflammatory activity of ginsenosides

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Abstract

Although Ginsenosides are a major physiological component of red ginseng (RG), some isolated ginsenosides are known to have cytotoxic activity. This study was intended to reduce cytotoxicity and promote anti-inflammatory activity of ginsenosides by mixing the crude ginsenosides (RG-CG) and polysaccharides (RG-CP). RG-CG-CM, fractionated from RG-CG, not only had higher anti-inflammatory activity than that of RG-CG at 100 μg/mL, but also showed increased cytotoxicity, while RG-CP-4P and RG-CP-8P, fractionated from RG-CP, showed no anti-inflammatory activity and cytotoxicity. To reduce the cytotoxicity without affecting the high anti-inflammatory activity of RG-CG-CM, RG-CG-CM and RG-CP-4P were mixed in a ratio of 2:8. The anti-inflammatory activity was significantly increased at a concentration of 75 μg/mL, while no cytotoxicity was observed. A combination of RG-CP and RG-CG effectively reduced the cytotoxicity and enhanced the anti-inflammatory activity of RG-CG, specifically RG-CG-CM fraction, providing scope for its industrial application.

Keywords: anti-inflammatory activity; cytotoxicity; ginsenoside; Korean Red ginseng; polysaccharide.

Practical Application: Preparation of ginsenoside extract without cytotoxicity.

1 Introduction

The concept of medicinal plant-medicinal plant or medicinal plant-drug combination to increase its physiological activity has been used as a traditional medicine for thousands of years (Che et al., 2013). The use of herbal-drug combinations to enhance their therapeutic effect, especially in cancer chemotherapy, is of great interest. In general, since natural medicinal components exhibit superior safety characteristics as compared to chemical synthetic substances, the phytochemical activity of various medicinal plants has been investigated (Ashraf, 2020). Moreover, some medicinal plants, such as ginseng, possess various physiological activities, such as anticancer activities (Davis & Behm, 2019; Park, 2019), owing to the presence of ginsenosides (Majid, 2019; Smolinski & Pestka, 2003).

Various extraction methods and applied techniques, including pressurized liquid extraction, high-pressure microwave-assisted extraction, supercritical fluid extraction, pulsed electric field extraction, water-in-oil-in water nanoemulsion, spray-dried nanoencapsulation and head-space solid-phase microextraction, have been used to enhance the content of ginsenoside and volatile components in ginseng (Cui et al., 2020; Jegal et al., 2019; Min et al., 2018). Ginsenosides, contained in red ginseng (RG), are considered anti-cancer compounds as they impart toxicity in various cancer cells (Dong et al., 2011; Nag et al., 2012), which is a desirable phenomenon. However, it is also often toxic to normal cells at high concentrations (Yu et al., 2018a). Furthermore, increasing the dose enhances the physiological activity, such as anticancer activity, but the dosage used is further limited owing to the side effects (Yu et al., 2018b).

Several studies have been conducted to predict the survival improved by and therapeutic effects of RG-derived polysaccharides. For example, compared to Paclitaxel alone, a combination regimen of paclitaxel (5 or 15 mg/kg) and acidic polysaccharide (25 mg/kg) caused a 30-day lifetime increase by 28.6 or 42.8% in ICR mice (Shin et al., 2004). Moreover, the survival rate of BALB/c mice was 10% when only cyclophosamide was administered, while the maximum survival rate increased to 53% when cyclophosphosamide was combined with acidic polysaccharide (25 mg/kg) (Shim et al., 2007).

High-molecular weight substances, such as polysaccharides and proteins, isolated from RG are regarded as safer than low-molecular weight materials, including ginsenosides, because they have relatively low toxicity owing to high solubility in water (Cho et al., 2014). RG polysaccharides, in particular, have a wide spectrum of therapeutic properties, including anti-tumor, immune-stimulating, anti-diabetes, anti-coagulant, anti-viral, and antioxidant, and are therefore, thought to be utilized as medicinal and functional food components for human health (Yu et al., 2018b).

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Recently, we isolated 2 polysaccharides, RG-CW-EZ-CP-4 and RG-CW-EZ-CP-8 from the starch-eliminated crude polysaccharide from RG (RG-CP), by the ethanol fractionation method without column chromatography (Kim et al., 2019). These polysaccharides had higher macrophage-stimulating and bone marrow cell proliferating activities than those of other polysaccharide fractions. Additionally, these polysaccharides were mainly composed of pectic-like acidic polysaccharide (141 kDa) and arabinose-rich polysaccharide (147 kDa; main peak), respectively. However, to the best of our knowledge, the synergistic effect of polysaccharides and ginsenosides on the inflammatory response has not been investigated to date. Ginsenosides are expected to increase anti-inflammatory activity and cytotoxicity at high concentration. In this study, increased anti-inflammatory activity without toxicity to LPS-stimulated RAW 264.7 macrophages was expected following the combination treatment with active polysaccharides with low cytotoxicity and ginsenosides at high concentrations.

2 Materials and methods

Sample was obtained from the Korean Ginseng Corporation (Seoul, Republic of Korea) at the beginning (January/February) and end (September/October) of the 2018. Korea red ginseng (*Panax ginseng*) variety was used. A voucher specimen (KNUT 16-01) was deposited at the Major in Food and Nutrition, Korea National University of Transportation (Chungbuk, Republic of Korea).

2.1 Preparation and fractionation of crude ginsenosides (RG-CG) from RG by silica-gel column chromatography

To obtain RG-CG, 8 volumes of 95% ethanol (w/v) was added to RG and incubated at 80 °C for 2 h for extraction. After the extraction, the supernatant was recovered by centrifugation (5,800 × *g*, 5 °C), concentrated using a rotary evaporator, and then lyophilized. The lyophilized extract was treated with Sep-pak C18 (Waters Co., Milford, MA, USA) to remove water-soluble components, and the fraction (RG-CG) was eluted with acetonitrile and freeze-dried. RG-CG was further fractionated by silica gel column (2 × 30 cm) (Liu et al., 2015) as follows: RG-CG-HE eluted by hexane mixed with ethyl acetate (1:1), RG-CG-CM eluted by chloroform mixed with methanol (5:1), and RG-CG-M eluted with methanol.

2.2 Preparation and fractionation of RG-CP by ethanol fractionation

RG-CP were prepared and fractionated according to the method reported by Kim et al. (2019). The RG slices were washed with cold water 20 times, stirred at room temperature for 12 h, and centrifuged to obtain a supernatant. After adding 5 volumes of ethanol to the supernatant, it was set aside for 6 h and centrifuged to recover the precipitate. The recovered precipitate was dissolved in a minimum volume of water and then treated with α-amylase and amyloglucosidase (Sigma-Aldrich, St Louis, MO, USA) to remove starch-like polysaccharides. RG-CP were obtained by ethanol precipitation, dialysis, and lyophilization. The ethanol fractionation method was performed as previously described (Lee et al., 2015). To fractionate RG-CP, the amount of ethanol added (water:ethanol = 1:0.5, 1:1, 1:4, and 1:8) varied, thus sequential fractions (RG-CP-0.5P, RG-CP-1P, RG-CP-4P, and RG-CP-8P) were obtained.

2.3 Quantification of ginsenoside contents in ginsenoside fractions

For ginsenoside analysis, pretreatment was performed using a C18 ODS cartridge. The content of major ginsenosides was analyzed using HPLC (Agilent 1260 infinity, Agilent Technologies Inc., Santa Clara, CA, USA) (Lee et al., 2009), with the detection wavelength 203 nm and the Imtakt Cadenza CD-C18 column (4.6 mm × 75 mm; Imtakt Co., Kyoto, Japan). Solvent A (10% acetonitrile) and solvent B (90% acetonitrile) were used, with the gradient conditions as follows: 0 → 11 min, 11% B (isocratic); 11 → 15 min, 11 → 16% B; 15 → 16 min, 16 → 20% B; 16 → 18 min, 20 → 21%; 18 → 24 min, 21% B (isocratic); 24 → 25 min, 21 → 22% B; 25 → 35 min, 22% B (isocratic); 35 → 36 min, 22 → 23% B; 36 → 40 min, 23% B (isocratic); 40 → 41 min, 23 → 24%; 41 → 45 min, 24% B (isocratic); 45 → 53 min, 24 → 37% B; 53 → 61 min, 37 → 45% B; 61 → 66 min, 45 → 46%; 66 → 73 min, 46 → 48% B; 73 → 75 min, 48% B (isocratic); 75 → 77 min, 48 → 11%; 77 → 85 min, 11% B (isocratic).

2.4 Evaluation of cytotoxic and anti-inflammatory effects

The murine macrophage RAW 264.7 cell line was obtained from Korean Cell Line Bank (Seoul, Korea). The cells were cultured overnight in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific) and 10% heat-inactivated fetal bovine serum (FBS; ThermoFisher Scientific) and seeded at a density of 2×10^5 cells/well in 96-well plates. The culture supernatant was replaced with DMEM supplemented with 10% FBS (160 μL), and 20 μL sample solution was added to the cells and incubated for 30 min. The cells were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, St. Louis, MO, USA) for 24 h, washed with phosphate buffered saline, and incubated with 100 μL of 10% Ez-Cytox (DaeilLab Service, Seoul, Republic of Korea) diluted in phosphate buffered saline (pH 7.4) for 1 h. Cytotoxic effect was evaluated as cell viability against the LPS control group. Furthermore, cell-free cultured supernatant was collected and the pro-inflammatory mediators interleukin (IL)-6 and nitric oxide (NO) in the supernatant were quantified by an ELISA kit (BD bioscience Inc., San Diego, CA, USA) and Griess assay kit (Promega, Madison, WI, USA), respectively.

2.5 Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences, version 12.0 (SPSS, Inc., Chicago, IL, USA). The differences between samples and saline control were assessed using the Student's *t*-test at 95% significance. Data are presented as the mean ± standard deviation (SD).

3 Results and discussion

Inflammatory reaction is one of the important defense systems of the human body, which plays pivotal role in eliminating

pathogens during an infection and repairing damaged tissue (Choi, 2008). However, unregulated and continuous inflammation can lead to chronic inflammatory diseases such as arteriosclerosis, rheumatoid arthritis, Crohn's disease, allergy, diabetes, and cancer (Landskron et al., 2014; Libby, 2006). To date, various inflammatory mediators, such as cytokines, chemokines, nitric oxide, prostaglandins, histamine, complement proteins, and adhesion molecules, and their mechanisms of action in inflammation have been investigated and identified; proper regulation of these mediators can prevent the development various diseases that are caused by chronic inflammation (Abdulkhaleq et al., 2018). Traditionally, drugs consisting of a single compound, such as non-steroidal anti-inflammatory drugs, have been widely used to regulate the production of inflammatory mediators. However, several adverse effects, such as kidney disease, gastrointestinal hemorrhage, and hypertension, can be triggered by the long-term use of these drugs, explaining increasing attention and efforts to discover anti-inflammatory agents from herbal extracts (Ghasemian et al., 2016; Wang et al., 2013).

RG-CG and RG-CP were prepared from Korean RG and the yield was 3.5% and 2.8%, respectively, against dried raw material. The anti-inflammatory activity of RG-CG and RG-CP on RAW 264.7 macrophages was compared at equivalent doses ranging from 1 to 1,000 μg/mL. When the cells were treated with RG-CG at concentration below 100 μg/mL, the cell proliferation showed a dose-dependent pattern and 103.1–151.4% increase was noted against the LPS control (Figure 1A). Contrarily, cell viability was dramatically decreased at a high dose (1,000 μg/mL), indicating high toxicity of RG-CG at high concentrations. At concentrations below 100 μg/mL, RG-CG did not significantly inhibit IL-6 production (Figure 1B), but the inhibition (99.4%) was noted at a concentration of 1,000 μg/mL due to the cytotoxic effect. None of the concentrations of both RG-CG and RG-CP inhibited IL-6 production without inducing cytotoxicity. However, RG-CG significantly inhibited NO production at 100 μg/mL, a non-toxic concentration, by 33.2% compared to that in the LPs-treated control group (Figure 1C).

Limited studies have investigated the high-molecular weight fractions on the effect of inflammatory reaction, except for Kang et al. (2019) who reported the effect of non-ginsenoside fractions isolated from RG on the inhibition of macrophage differentiation and inflammatory responses. Here, no cytotoxicity was observed at all concentrations of RG-CP, (106.2-118.1% increase; Figure 2A), including 1000 μg/mL. Further, there was no significant difference in IL-6 and NO production in LPS-induced RAW 264.7 cells between the RG-CP- and LPS-treated groups The results indicated that the anti-inflammatory effect of RG seems to be attributed to ginsenoside, a low-molecular weight component of RG. RG-CG was loaded into open column chromatography packed with Silica gel 60 and fractionated into RG-CG-HE, RG-CG-CM, and RG-CG-M, and the yield against RG-CG was 0.7%, 1.0%, and 82.6%, respectively. RG-CG-HE was excluded from further evaluation following anti-inflammatory activity because of the lowest yield and ginsenoside contents. RG-CG-M did not exhibit cytotoxicity at 10 and 100 μg/mL concentrations, but exhibited inhibitory activity on NO production (Figure 2C). Contrarily, RG-CG-CM at a concentration of 100 μg/mL exhibited significant toxic effects (77.2% decrease in RAW 264.7 cell viability against LPS control) (Figure 2A) and inhibition of IL-6 (Figure 2B) and NO (Figure 2C) production. These results indicated that RG-CG-CM-induced cytotoxicity and influenced IL-6 and NO production. Moreover, RG-CP was fractionated into RG-CP-8P, RG-CP-8P, RG-CP-4P, RG-CP-1P, and RG-CP-0.5P, and the yield against RG-CP was 3.6%, 3.6%, 17.9%, 10.7%, and 46.4%, respectively. RG-CP and its five fractions did not significantly decrease the cell viability against LPS-treated control (Figure 2A). Additionally, the polysaccharide fractions did not influence IL-6 (Figure 2B) and NO (Figure 2C) production.

RG-CG and RG-CP fractions were analyzed for ginsenoside and sugar compositions, respectively. Total ginsenoside contents were 11.1, 124.3, and 40.4 mg/g in RG-CG-HE, RG-CG-CM, and RG-CG-M, respectively (Table 1). RG-CG-HE contained the lowest ginsenoside content, comprising of four ginsenosides: Rb2 (7.8 mg/g), compound K (2.3 mg/g), Rg3(r) (0.9 mg/g), and Rg3(s) (0.1 mg/g). As mentioned earlier, RG-CG-HE was excluded in follow-up experiments because of the lowest ginsenoside content. In contrast, RG-CG-CM had the highest content of total

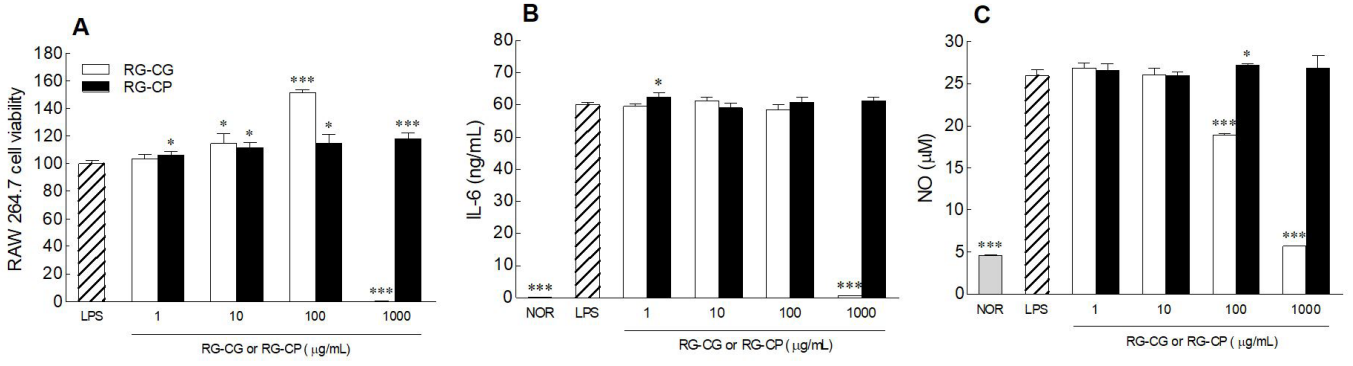

Figure 1. Cytotoxicity and anti-inflammatory activity of RG-CG and RG-CP on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with vehicle (DMSO) or samples at various doses for 30 min and stimulated with vehicle or LPS for 24 h. The cell viability was determined by MTT assay (A). Inhibitory effect on IL-6 (B) and NO production (C) was examined by Griess reaction and ELISA, respectively. Data are presented as the mean of triplicate experiments and standard deviation. Asterisk indicates significant difference (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$) against the LPS-stimulated control. Normal: only vehicle treatment, LPS: only LPS treatment.

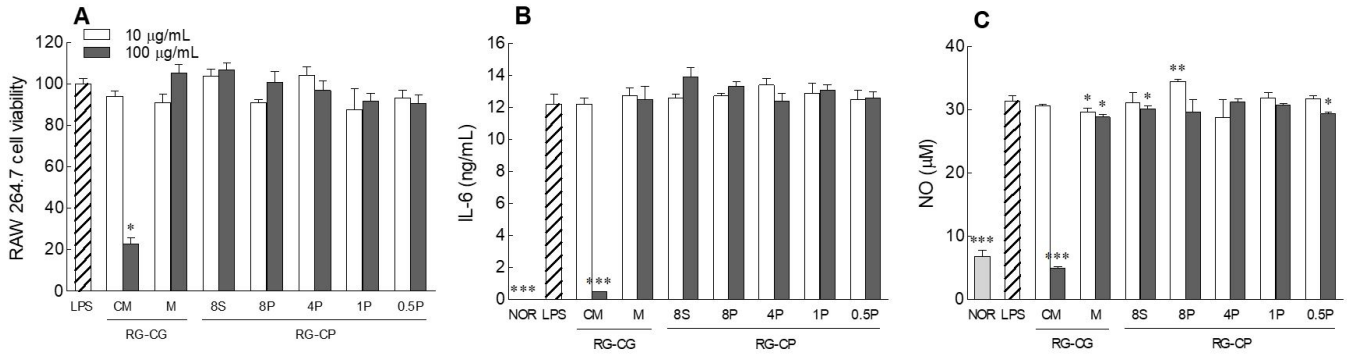

Figure 2. Cytotoxicity and anti-inflammatory activity of RG-CG and RG-CP fractions on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with vehicle (DMSO) or samples at various doses for 30 min and stimulated with vehicle or LPS for 24 h. The cell viability was determined by MTT assay (A). Inhibitory effect on IL-6 (B) and NO production (C) was examined by Griess reaction and ELISA, respectively. Data are presented as the mean of triplicate experiments and standard deviation. All experiments were performed in triplicate and data were presented as the mean and standard deviation. Asterisk indicates significant difference (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$) against the LPS-stimulated control. Normal: only vehicle treatment, LPS: only LPS treatment.

Table 1. Yields and ginsenoside contents of subfractions from RG-CG by Silica gel-packed column chromatography.

Ginsenoside	Fractions[1]		
	RG-CG-HE (mg g^{-1})	RG-CG-CM (mg g^{-1})	RG-CG-M (mg g^{-1})
Rg1	-	-	1.5 ± 0.1
Re	-	-	2.7 ± 0.2
Rf	-	-	1.7 ± 0.1
Rg1(s)+Rg2(s)	-	7.1 ± 0.8	1.1 ± 0.1
Rg2(r)	-	38.0 ± 5.4	3.2 ± 0.3
Rb1	-	-	6.5 ± 0.6
Rc	-	-	4.4 ± 0.5
Rb2	7.8 ± 0.4	-	2.6 ± 0.1
Rd	-	0.8 ± 0.0	1.2 ± 0.2
F2	-	47.5 ± 6.6	1.7 ± 0.1
Rg3(s)	0.1 ± 0.0	-	3.3 ± 0.4
Rg3(r)	1.2 ± 0.4	1.6 ± 0.1	1.8 ± 0.1
CK	2.4 ± 0.2	-	-
Rg5	-	26.0 ± 3.4	4.2 ± 0.4
Rk1	-	1.0 ± 0.1	5.1 ± 0.5
Rh2(s)+Rh(2(r)	-	-	-
Total	11.4 ± 0.3	122.0 ± 5.5	40.9 ± 0.6
Yield (%)[2]	0.7	1.0	82.6

[1]RG-CG-HE; fraction from crude ginsenosides (RG-CG) by hexane and ethyl acetate, RG-CG-CM; fraction from RG-CG by chloroform and methanol, RG-CG-M; fraction from RG-CG by only methanol; [2]Yield (%) was calculated against RG-CG. All experiments were performed in triplicate and data were presented as the mean and standard deviation.

ginsenoside (124.3 mg/g), comprising mainly of F2 (47.9 mg/g), Rg2(r) (38.3 mg/g), and Rg5 (27.7 mg/g) as well as traces of Rh1(s)+Rg2(s) (7.1 mg/g), Rg3(r) (1.5 mg/g), Rk1 (0.9 mg/g), and Rd (0.8 mg/g). RG-CG-M contained 14 types of ginsenosides (total 40.4 mg/g), comprising mainly of Rb1 (6.5 mg/g), Rk1 (5.1 mg/g), Rg5 (4.2 mg/g), and Rc (4.1 mg/g). Therefore, RG-CG-CM was combined with polysaccharide fraction owing to high ginsenoside contents and anti-inflammatory activity with low cytotoxicity. When high concentration of ethanol is added to the water extract of medicinal plants, high molecular weight materials, such as polysaccharides and proteoglycans, form a precipitate due to differences in the solubility (Lee et al., 2015). Here, polysaccharides were fractionated by four different concentrations of ethanol (DIW:ethanol= 1:0.5, 1:1, 1:4, and 1:8). In our previous report (Kim et al., 2019), the yields of the fractions RG-CP-0.5P, RG-CP-1P, RG-CP-4P, and RG-CP-8P obtained by ethanol precipitation were 1.3%, 0.3%, 0.5%, and 0.1%, respectively. These fractions mainly contained polysaccharides containing neutral sugars (14.6-87.4%), uronic acids (12.7-85.4%), and proteins (0.22-0.44%) (data not shown). In addition, RG-CP-0.5P and RG-CP-1P contained 82.8% and 82.6% of galacturonic acid, respectively, and traces of galactose, arabinose, and glucose. However, the main sugar composition of RG-CP-4P and RG-CP-8P was arabinose/rhamnose/galactose/galacturonic acid at a ratio of 0.51/0.11/0.77/1.00 and 1.00/0.15/0.16/0.19, respectively. The results indicated that RG-CP-4P and RG-CP-8P contained different types of polysaccharides, predominantly pectic-like acidic and arabinose-rich polysaccharides, respectively (Kim et al., 2019).

The RG-CG-CM fraction exhibited anti-inflammatory activity at concentrations exhibiting cytotoxicity. Therefore, RG-CP-4P or RG-CP-8P were mixed with RG-CG-CM to maintain the anti-inflammatory activity and reduce the cytotoxicity of RG-CG-CM. At 25 μg/mL, none of the samples exhibited cytotoxicity (Figure 3A), while at 75 μg/mL, only RG-CG-CM fraction was cytotoxic. When polysaccharide fractions RG-CP-4P or RG-CP-8P were mixed with RG-CG-CM, no cytotoxicity was noted at any given concentration (Figure 3A), indicating that these combination treatments mitigated the cytotoxicity of the ginsenoside fraction. At 10 μg/mL, all samples significantly inhibited the IL-6 production compared with that of the LPS control (Figure 3B). Interestingly, the above mixtures, particularly RG-CP-4P and RG-CG-CM, at a ratio of 2:8 had significantly higher IL-6 inhibitory activity than that of the LPS control ($P < 0.01$), without cytotoxicity up to 75 μg/mL. The results were similar to those of RG-CG-CM at an equivalent dose, which showed cytotoxicity. Furthermore, RG-CG-CM combined with RG-CP-4P or RG-CP-8P inhibited NO production without cytotoxicity

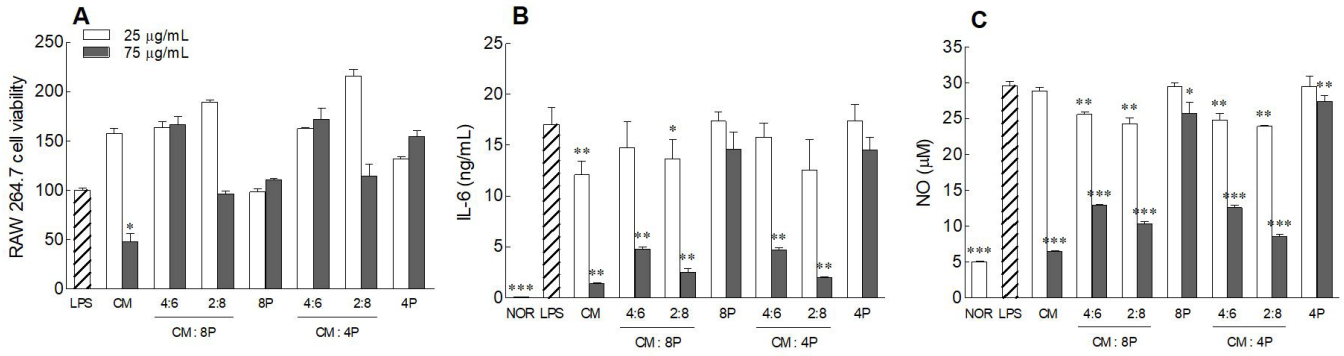

Figure 3. Cytotoxicity and anti-inflammatory activity of subfraction mixtures on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with vehicle (DMSO) or samples at various doses for 30 min and stimulated with vehicle or LPS for 24 h. The cell viability was determined by MTT assay (A). Inhibitory effect on IL-6 (B) and NO production (C) was examined by Griess reaction and ELISA, respectively. Data are presented as the mean of triplicate experiments and standard deviation. All experiments were performed in triplicate and data were presented as the mean and standard deviation. Asterisk indicates significant difference (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$) against the LPS-stimulated control. Normal: only vehicle treatment, LPS, only LPS treatment. CM, RG-CG-CM; 8P, RG-CP-8P; 4P, RG-CP-4P; CM:8P, mixture comprising RG-CG-CM and RG-CP-8P; CM:4P, mixture comprising RG-CG-CM and RG-CP-4.

(Figure 3C). In particular, mixture of RG-CP-4P or RG-CP-8P and RG-CG-CM at 2:8 ratio inhibited NO production (71.0% and 66.2% inhibition, respectively) more effectively than the mixture at 4:6 ratio (57.4% and 56.5% inhibition, respectively). The combination of RG-CG-CM and RG-CP-4P or RG-CP-8P at a ratio of 2:8 showed synergistic anti-inflammatory effects along with the decreased cytotoxicity than that when RG-CG-CM was treated alone at an equivalent dose. Therefore, we speculated that this synergistic effect could reflect decreased cytotoxicity.

The combination of berberine, coptisine, palmatine, and epiberberine, which are the active substances of *Rhizoma coptidis* (RC) extract, significantly improved the anti-diabetic activity in diabetic mice and toxicity in HepG2 cells than when berberine was used alone (Zhu et al., 2010). The antihyperglycemic ability of RC extract was higher than that of the alkaloid fraction (He et al., 2011). In addition, the LD50 values of berberine, coptisine, palmatine, and epiberberine in mice were 713.57, 852.12, 1533.68, and 1360 mg/kg, respectively, and were lower than that of the RC extract (2.95 g/kg). Cytotoxicity was alleviated when mixed with active substances alone (Ma et al., 2010; Yi et al., 2013). Different components can affect a variety of cellular messengers, which can play a role in the same reaction in the cells, resulting in synergistic effects.

4 Conclusion

In this study, combination of RG-CG-CM and RG-CP-4P or RG-CP-8P at a ratio of 2:8 exhibited synergistic anti-inflammatory effects along with the decreased cytotoxicity than those exhibited by RG-CG-CM alone at equivalent doses. The ginsenosides, the active compound of RG, were not expected to have a pharmacological effect due to their cytotoxicity at high concentration. However, its combination with polysaccharides, another active compound of RG, enabled pharmacological activity without cytotoxicity even at high concentration. Future studies investigating the effects of RG-CP-4P, RG-CP-8P, and RG-CG-CM, individually and in combinations are warranted to elucidate the mechanism underlying this synergistic effect.

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