

Research Article

Ginsenoside Rg3 inhibits grass carp reovirus replication in grass carp ovarian epithelial cells

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Abstract

Ginseng exhibits multiple medicinal properties, including the improvement of immune function and enhancing disease resistance. In this study, we investigated the inhibitory effects of ginsenoside Rg3 on Grass Carp Reovirus (GCRV) infection of grass Carp Ovarian (CO) epithelial cells, in order to provide a baseline framework for future high-efficacy antiviral drug screening investigations. Ginsenoside Rg3 was added to GCRV-infected CO cells, and cells were cultured at 27°C before cell proliferation was measured by MTT assays. Label-free Real-Time Cellular Analysis (RTCA) after 72 h of experimentation demonstrated that 100 µg/mL ginsenoside Rg3 treatment had the highest inhibitory effect on GCRV (among 1,10,100µg/mL treatments). We then measured the capacity for cellular antioxidant ability. Cells treated with 1,10,100µg/mL ginsenosideRg3 exhibited increases in Total Antioxidant Capacity activity relative to controls, respectively. Furthermore, Antioxidant assay and Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) showed that ginsenoside Rg3 were efficient to restrain the replication of GCRV in CO cells. Expression analysis of immune-related genes via RT-qPCR showed that treatment with ginsenosideRg3 promoted expression of IRF-3 and IRF-7 increases, respectively. Moreover, expression of IFN-1 was induced, which then inhibition the expression of Tumor Necrosis Factor-Alpha (TNF-α). In conclusion, we demonstrated that ginsenoside Rg3 promotes CO cell proliferation, inhibits GCRV activity, promotes CO cell immune activities, and thereby enhances the resistance of CO to GCRV infection.

Keywords: Ginsenoside Rg3; Grass carp ovarian epithelial cells; Grass carp reovirus; Antioxidant capability; Immune response

Introduction

Grass carp is an economically important freshwater fish, and is the most cultured fish in China. Grass Carp Reovirus (GCRV) was first identified in China in 1984 [1], and infection leads to hemorrhagic disease states that result in high morbidity and mortality. GCRV is one of the most severe grass carp diseases and results in significant problems for the grass carp culture industry [2,3]. The viral structure [4], infection mechanism [5], methods for rapid viral detection [6], and vaccine development [7] have all been investigated intensively for GCRV. These studies have indicated that GCRV is a Double-Stranded RNA (dsRNA) virus that consists of a double-layer capsid and 11 double-stranded RNAs that encode 7 structural proteins (VP1-VP7) and 5 non-structural proteins (NS16, 26, 31, 38 and 80) [8].

Ginseng is a slow-growing perennial herbaceous plant [9] and is an important herbal medicine ingredient in Asia [10]. Ginseng features multiple medicinally-important components and exhibits significant biological activities that act on different targets [11], thus making ginseng an ideal anti-viral medicinal herb. Ginseng has been shown to regulate the immune system [12] and alleviate pathological symptoms, in addition to reducing inflammation [13], reducing oxidation [14], and preventing mutagenesis [15] and cancer [16]. Ginsenoside (GS) is the major pharmacologically active ingredient extracted from ginseng [17]. Tao Yu et. al., [18] demonstrated that ginsenoside molecules significantly inhibit the expression of macrophage-derived cytokines, including TNF-α and IL-1. By stimulating p38 MAPK and ERK-dependent signal pathways, the ginsenoside Rh2-B stimulates T cell

proliferation, T cell cytotoxicity, and IFN-γ secretion [19]. However, there are few reports about the protective effect of ginsenosides on viral infection. Yang et al., [20] discovered that 20(S)-ginsenoside-Rg3 is able to protect against rotavirus infection. The aim of this study was to investigate the influence of ginsenosideRg3 to grass carp ovarian (CO) epithelial cells with GCRV on the antioxidant capability (SOD, CAT, GSH, T-AOC) and immune-related genes(IRF3, IRF7, Myd88, IFN-1, TNF-α) .

Materials and Methods

Materials

The grass Carp Ovarian cell line (CO) was obtained from the Aquatic Laboratories at Jilin Agricultural University. CO cells were cultured in M199 complete medium containing 10% FBS that was supplemented with 1×10^6 IU/mL of penicillin and streptomycin (purchased from Gibco, US). Cells were cultured in an incubator at 27°C, with a 5% CO₂ atmosphere. Media was exchanged every other day, and cells were passage at 80% confluence. After three to four passages, cells were used for experiments. Grass Carp Reovirus (GCRV) was also obtained from the Aquatic Laboratories at Jilin Agricultural University. The Ginsenoside Rg3 used in this study was kindly provided by the School of Chinese Medicinal Materials at Jilin Agricultural University. Ginsenoside Rg3 was dissolved in dimethyl sulfoxide, sterilized by filtration through a 0.45-µm pore filter (Millipore, Billerica, MA, USA), and stored at 4°C until use.

Effect of ginsenoside Rg3 on cell proliferation

CO cells in the logarithmic growth phase were trypsinized, and then plated in 96-well plates at a concentration of 2.5×10^4 cells/well. After 24 h of tissue culture, the medium was removed and the medium was removed and replaced with 100 µL of ginsenoside Rg3 and different content of M199 cell culture medium (without FBS). Final

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concentrations of ginsenoside Rg3 were respectively 1µg/mL, 10µg/mL and 100µg/mL. An equal volume of M199 cell culture medium (without FBS) was used as the blank control. After another 24 h of cell culture, 20 µL of MTT (5 mg/mL) was added to each well, and they were further incubated at 37°C for 4 h. The culture media in each well was carefully removed and replaced by 150 µL DMSO. After 10 min of incubation on a shaker, the Optical Density (OD) of each well was measured using a plate reader at 490 nm.

RTCA Real-time Label-free Cell Analysis

CO cells were plated for real-time label-free analysis at a concentration of 400 cells/well. Five different treatments were included with three replicates each: cell-only blank controls, GCRV treated + 1µg/mLginsenosideRg3, GCRV treated + 10µg/mLginsenosideRg3, GCRV-treated + 100µg/mL ginsenoside Rg3, and a GCRV-treated positive control. After 24 h of cell incubation, cells were rinsed three times with PBS and treated with 10–4 GCRV for 2 h. ginsenoside Rg3 was then added as described above, and cells were monitored for 72 h using the real-time cell detection system.

Measuring cellular antioxidant capacity

Single cell suspensions were prepared by trypsinizing log phase cells with 1 mg/ml trypsin. A density of 1.5×10^6 cells were plated in each well of a six-well plate. Cells were then cultured in an incubator at 25°C, with a 5% CO₂ atmosphere for 24 h. The treatment groups were designated as above, with three replicates in each group. After 24 h, antioxidant capacity was measured. Cells were collected, sonicated at 4°C, and centrifuged at 13,000 g for 10 min. The cell lysate was then stored at -80°C until further analysis. Antioxidant index were measured according to manufacturer instructions (Nanjing Jiancheng Bioengineering Institute). One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 mL reaction solution per mg tissue protein. The CAT activity was calculated at 240nm by measuring the initial rate of H₂O₂ (10 mM) decomposition. One unit of T-AOC was defined as the concentration of T-AOC that made the absorbance of 1 mL of serum increase 0.001 per minute at 37°C. The GSH was examined by the dithio-bis-nitrobenzoic acid (DTNB) method. The GSH concentration was monitored at 423nm on a Thermo UV-6300 spectrophotometer.

Detection of mRNA expression by RT-qPCR

The total RNA was extracted from the kidney using a Trizol kit (TaKaRa, Dalian, China).The purified of total RNA was analyzed by the A260/A280 ratio and 1% agarose gel electrophoresis was conducted to test the RNA integrity. According to the manufacturer's instructions, synthesis of cDNA was accomplished using Prime Script RT reagent Kit (Takara).

Cellular RNA was reverse-transcribed to cDNA using the Aii-in-One First-Strand Synthesis Master Mix and then stored at -20°C. qRT-PCR was then performed using the Light Cycler 480 Software following the manufacturer instructions for SYBR Premix Ex Taq TM (Perfect Real Time; TaKaRa Bio Inc). The PCR conditions were as follows: pre-denaturation at 95°C for 5 min, and then 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. Primers that were used are listed in Table 1.

Statistical analysis

Real-time PCR data were analyzed using the 2-ΔΔCt method. The results from three replicates are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 20.0 and the difference between each treatment group was tested using one way ANOVA and Tukey HSD for post hoc comparisons.

Results

Effect of ginsenoside Rg3 on CO cell viability

To investigate the effects of ginsenoside Rg3 on CO cell viability, MTT assay was performed. As shown in Figure 1, the viability of CO cell was obviously enhanced by ginsenoside Rg3 at 24h. Thus, ginsenoside Rg3 (1, 10, 100 µg/mL) exerted no significant cytotoxicity on CO cell.

RTCA Real-time Label-free Cell Analysis

The data is showed in Figure 2. After the addition of ginsenoside Rg3 at 8 h, the activities of cells treated with 1µg/mL, 10µg/mL or 100µg/mL ginsenoside Rg3 were higher than that of the cell-only blank controls. These results indicated that ginsenoside Rg3 exhibited a significant inhibitory effect on viral activity in the early phase of infection.

Cell antioxidation capacity

To better understand the mechanism underlying the effect of ginsenoside Rg3, we investigated the antioxidant responses of T-AOC, SOD, CAT and GSH. The results showed that GCRV significantly decreased the expression of T-AOC, SOD, CAT and GSH. Ginsenoside Rg3 dose dependently increased T-AOC, SOD, CAT and GSH activity in GCRV-infection CO cell (Figure 3).

Table 1: Primers used for the analysis of mRNA expression by RT-qPCR.

Primer name	Sequence (5'/3')	Accession no.
β-actin	Fwd:ACTGCTGCTTCCTCCTCCTC Rev:CCAAGAAGGATGGCTGGAAC	M25013
Myd88	Fwd: CCTTTGCCAGGAAGTCACT Rev: ACCTGTGTGGAGGGTCTGGT	XM_019067529.1
IRF3	Fwd:GGTCCGACTGCCTATTCCAG Rev:CCACACCATCCAATCTCCTG	XM_019101982.1
IRF7	Fwd:GGTCTGCAATCCGACACTAA Rev: TGAACGAGTGGCGAAGACAG	KF844251.1
IFN-1	Fwd: TCAATGCTCTGCTTGCGAAT Rev: TTCACCTGGTCCCTCCACCTT	DQ357216.1
TNF-α	Fwd:TGAACGAGTGGCGAAGACA Rev: GATGGCAGCCTTGAAGTG	JQ670915.1

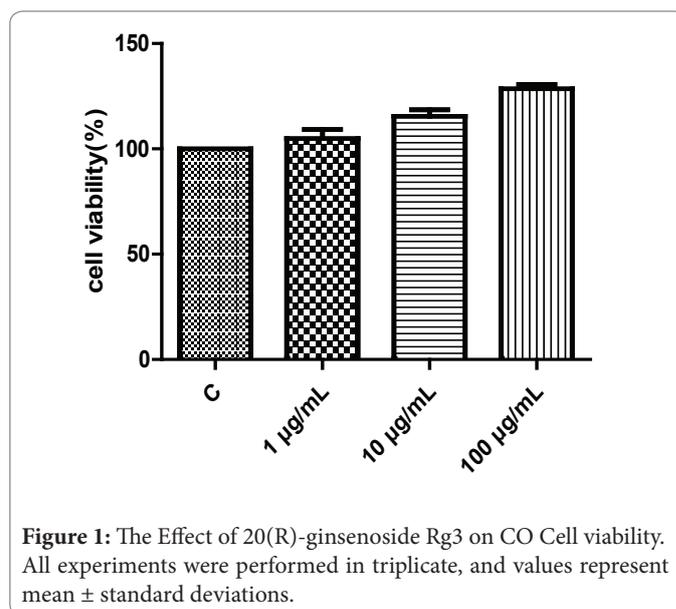


Figure 1: The Effect of 20(R)-ginsenoside Rg3 on CO Cell viability. All experiments were performed in triplicate, and values represent mean ± standard deviations.

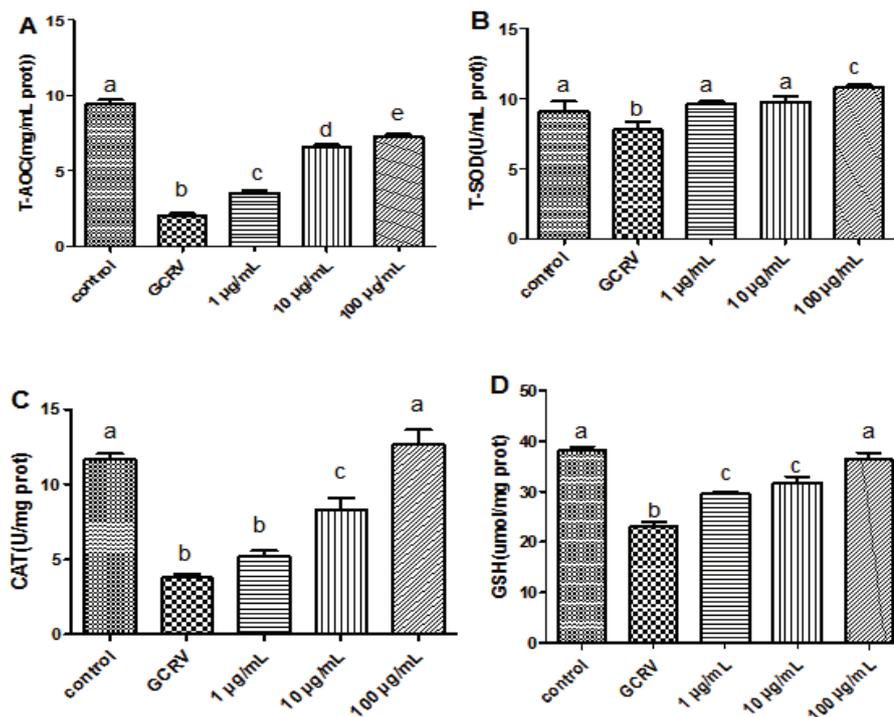


Figure 2: T-AOC content in GCRV infected CO cells after treatment with different concentrations of ginsenoside Rg3. **B:**T-SOD content in GCRV infected CO cells after treatment with different concentrations of ginsenoside Rg3. **C:**CATcontent in GCRV infected CO cells after treatment with different concentrations of ginsenoside Rg3. **D:**GSH content in GCRV infected CO cells after treatment with different concentrations of ginsenoside Rg3. All experiments were performed in triplicate and are shown as the mean ± standard deviation. Statistically significant values that differed between groups are labeled with different letters ($p < 0.05$).

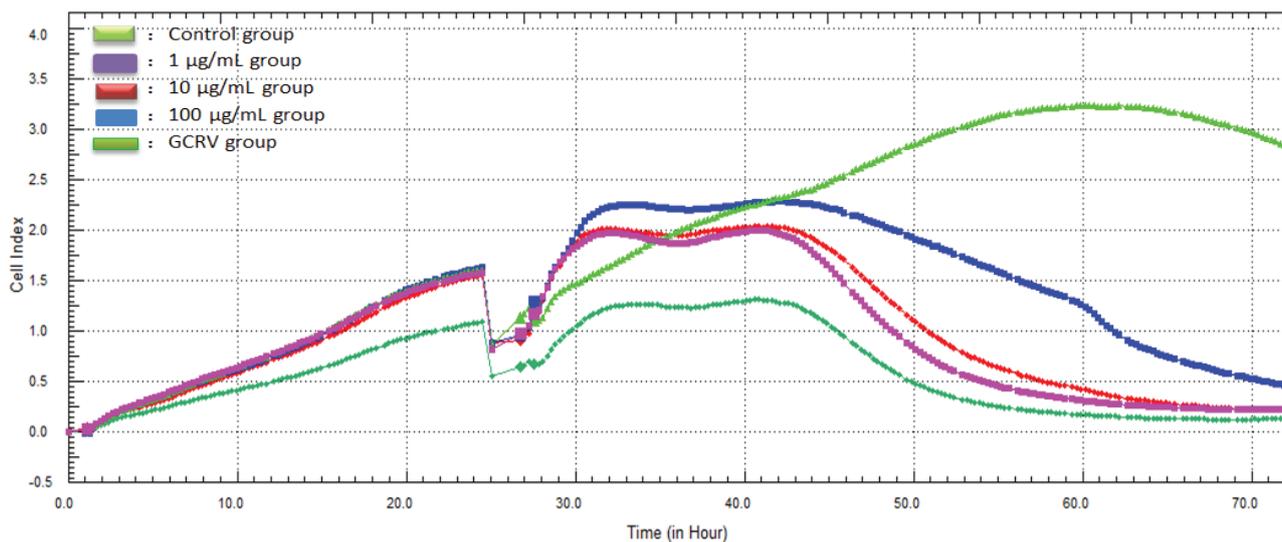


Figure 3: Changes in cell activity.

The Effect of ginsenoside Rg3 on the expression of immune-related genes in CO Cells

As shown in Figure 4, we investigated the gene expression of IRF7, IRF3, TNF- α , Myd 88 and IFN-I by qRT-PCR. Compared with GCRV group the level of IRF7, IRF3, Myd88 and IFN-I in CO cells were enhanced significantly by ginsenoside Rg3, as well as ginsenoside Rg3 inhibits the expression of pro-inflammatory cytokines TNF- α and showed concentration-dependent trend.

Discussion

The antioxidant defense system clears Reactive Oxygen Species (ROS) in cells and plays a significant role in animal immune systems. The capacity for cellular oxidation resistance is tightly associated with disease-resistance capabilities. The antioxidation system of fish comprises both non-enzymatic and enzymatic antioxidant system components. The non-enzymatic system mainly comprises glutathione (GSH), vitamin C, β -carotene, vitamin A, vitamin E, iron metal sulfur

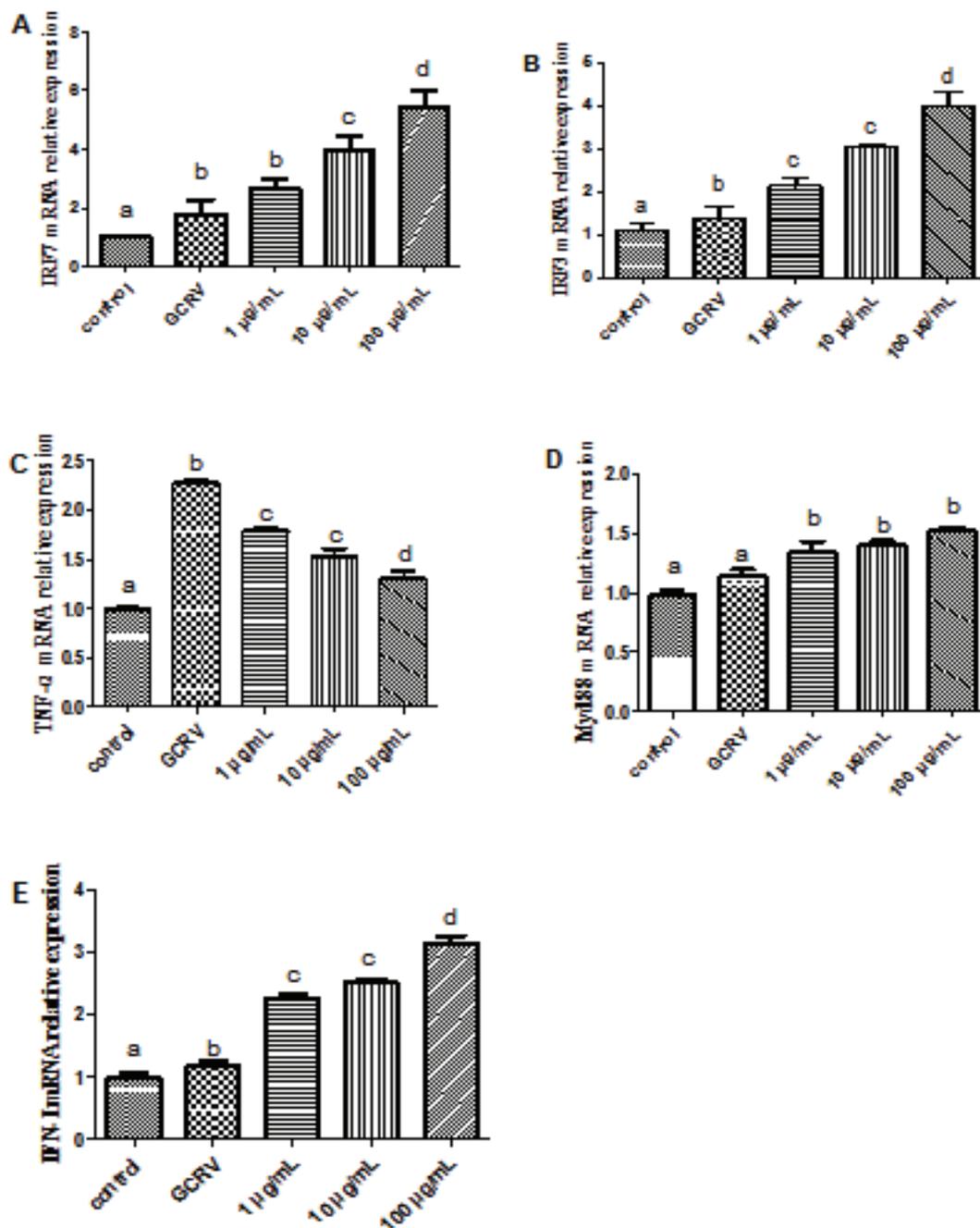


Figure 4: Relative gene expression of IRF7, IRF3, TNF- α , Myd88 and IFN-1 in GCRV-infected CO cells after treatment with different concentrations (0,1,10,100 μ g/mL) of ginsenoside Rg3. Data are expressed as the mean \pm SD. The markers (a,b,c,etc.) indicated among the groups differences significant by the Tukey's multiple range test ($P < 0.05$).

proteins, and iron proteins, among others. In contrast, the antioxidant enzyme system components mainly comprise Superoxide Dismutase (SOD), CAT, and Glutathione Peroxidase (GPX), among others. The activity of SOD mainly reflects the ability of an organism to clear oxygen free radicals [21], while CAT is the primary enzyme that degrades hydrogen peroxide (H_2O_2) to H_2O . The general antioxidant capacity of an organism is also closely related to its overall condition of health. GSH is a low-molecular-weight scavenger that serves as the substrate for GSH-PX and GST, and is required for the decomposition of hydrogen peroxide. Thus, GSH is an important indicator of the capacity for oxidation resistance.

Ginseng, which has been referred to as the “King of Herbs”, exhibits multiple activities related to immune system function. Ginseng has been shown to enhance antioxidation effects by increasing the activity of antioxidant enzymes like SOD and GSH-PX, and decreasing the oxidation of lipids such as malondialdehyde (MDA) [22-25]. Ginseng contains many pharmacologically active substances such as ginsenoside, polysaccharides, acetylenic compounds, sterols, and essential oils. Ginsenoside Rg3 is the main pharmacologically active component in ginseng and has been reported to ginsenoside Rg3 in reduction of the intracellular ROS level [26]. Rg3 can clearly alleviate the oxidative stress induced by TMT exposure [27]. In this study, ginsenoside Rg3 cells exhibited higher T-AOC, CAT and GSH contents compared to GCRV-infection, these results indicate that antioxidase activity increases in cells after ginsenoside Rg3.

Many antiviral medicines currently exist which includes nucleosides, interferons, and enzyme preparations [28,29]. However, recently developed antiviral therapeutic strategies that stimulate/operate host-endogenous antiviral mechanisms or target cytokines may be more effective than traditional approaches using viral proteins [30]. Importantly, ginseng is also an antiviral medicine that is used for many viral infections [31]. Oral administration of Korean Red Ginseng (KRG) for 28 days as an adjuvant in pigs enhanced the immune capability of pig spleen cells [32]. Ginseng Rg3 directly inhibits viral infections by inhibiting virus attachment, membrane permeation, and viral replication. More importantly, ginseng enhances host immunity. For example, ginsenoside Rg3 treatment significantly mitigated acute lung injury pathological damages and reduced production of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 [33].

Regarding fish genetic mechanisms that are important for immune function, IRF-3 and IRF-7 are transcription factors that are necessary for the expression of IFN-I that is induced by viral infection, and are key components in the IRFS-mediated antiviral immune response. IRF7 can be induced by both IFN-I and TNF [34], and exhibits low constitutive expression in cells that increases after viral induction [35]. Following stimulation with poly (I:C), murine dendritic Cells with IRF3 depletion exhibit low, but detectable interferon I expression, while murine DCs with IRF3 and IRF7 depletion exhibit no interferon I expression [36]. Following these observations, it is clear that IRF7 plays an important role in the TRIF-mediated IFN induction pathway. Further, viral infection of murine spleen pDCs with IRF7 depletion resulted in significantly affected IFN-I expression [37].

Myd88 is a protein that associates with multiple effector molecules and plays an important role in Toll-like receptor signaling pathways. Studies have shown that IRF7 and IRF3 can be activated via Myd88-mediated TLR pathways [38]. Further, in mouse fibroblasts with Myd88 gene depletion, IFN expression was not affected, suggesting that IFN expression that is induced by IRF7 is independent of Myd88 pathways [37]. TNF- α is a cell factor that regulates multiple biological activities, including immune response, and apoptosis, among others. The inhibition of NF- κ B reduces the expression of TNF- α mRNA,

indicating that TNF- α is involved in the NF- κ B pathway [39]. In addition, TNF- α and IL-1 decreased the infection efficiency of hepatitis B virus in HepaRG cells, which is mediated by the NF- κ B pathway [40]. In this study, we observed that ginsenoside Rg3 inhibited the expression of IRF3 and IRF7, induced the expression of IFN-I, as well as prevented the activation of TNF- α and thereby stimulated the host immune response.

In summary, treatment with ginsenoside Rg3 increased the expression of immune-related genes in the IFN-I pathway, and enhanced the antiviral capacity of CO cells, indicating that ginsenoside Rg3 has a significant inhibitory effect on GCRV. Moreover, our results provide the basis for further mechanistic studies of the antiviral effect of ginsenoside Rg3 and facilitate the development of antiviral medicines and our overall understanding of antiviral signaling pathways.

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