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Effects of immune-challenged domestic silkworm hemolymph on the regulation of *SIRT5* and *PRDx1* expression

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Abstract

SIRT5 and PRDx1 play crucial roles in cancer and are involved in the basic mechanisms of reactive oxygen species detoxification. In our previous studies, we showed that hemolymph extracts of immune-challenged *Bombyx mori* have antioxidant properties. Following H_2O_2 stimulation, immune-challenged *B. mori* hemolymph extracts elicited SIRT5 downregulation activity, reaching effective activity at the highest concentration of 100 ppm. Additionally, cells treated with immune-challenged *B. mori* hemolymph extracts demonstrated increased PRDx1 mRNA expression compared to that of PBS-treated cells. Therefore, immune-challenged *B. mori* hemolymph extracts offer a potential auxiliary means of treating drug-resistant tumors through downregulation of *SIRT5* and upregulation of *PRDx1* expression. Nevertheless, further studies on the effects of *B. mori* hemolymph on *SIRT5* and *PRDx1* regulation are pertinent for using it as a food or pharmaceutical material and understanding its therapeutic effect on tumors, including those that are drug-resistant.

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Introduction

Reactive oxygen species (ROS) induce intracellular oxidative stress, which can cause several diseases, such as aging, chronic diseases, and cancer, owing to the oxidation of proteins, RNA, and DNA and the peroxidation of membrane lipids (Balakrishnan *et al*., 2014; Baynes, 1991; Felton and Summers, 1995; Kim *et al*., 2020; Kim and Park, 2021; Oghenesuvwe and Paul, 2019; Pardini, 1995; Sosa *et al*., 2013; Zhang *et al*., 2016; Zielińska *et al*., 2017). The Sirtuin family is a group of proteins that are involved in metabolic regulation. It consists of seven types, namely SIRT1-7, and has been studied in mammals for the past two decades. They mainly function as nicotinamide adenine dinucleotide-dependent histone deacetylases (Singh *et al*., 2018). SIRT5 belongs to the Sirtuin family and is vital in cancer biology, including stress response, apoptosis, and autophagy (Li *et al*., 2015; Liang *et al*., 2017; Liu *et al*., 2013; Polletta *et al.,* 2015; Shi *et al.,* 2019; Sun *et al*., 2019; Wang *et al*., 2018a). For example, increased SIRT5 expression has been observed in non-small cell lung cancer, hepatocellular carcinoma, colorectal cancer, Waldenstrom's macroglobulinemia, and breast cancer compared to that in normal tissues (Chang *et al*., 2018; Lu *et al*., 2014; Lv *et al*., 2015; Shi *et al*., 2019; Sun *et al*., 2011; Sun *et al*., 2019; Wang *et al*., 2018b). Specifically, a trend toward

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SIRT5 downregulation was observed in liver cancer, highlighting its tumor-suppressive role (Nakagawa *et al*., 2009; Sun *et al*., 2019). SIRT5 is also involved in the basic mechanisms of ROS detoxification because it inhibits oxidative stress-induced apoptosis in cardiomyocytes and neuroblastoma cells (Sun *et al*., 2019; Liang *et al*., 2017; Liu *et al*., 2013).

Peroxiredoxin 1 (PRDx1) is an antioxidant that plays an important role in H_2O_2-mediated cell signaling, similar to SIRT5 (Ren *et al*., 2013). According to Ding *et al*., abnormal expression of PRDx1 has been observed in several types of human solid cancers (Ding *et al*., 2017). Similar to SIRT5, PRDx1-based anticancer studies are mainly based on antioxidant activities (Ding *et al*., 2017).

The domestic silkworm *Bombyx mori* (*B. mori*) was bred from the wild silkworm (*Bombyx mandarina*) approximately 5,000–10,000 years ago (Park, 2022). In our previous studies, we compared the antioxidant activities of hemolymph extracts derived from immune-challenged and unchallenged *B. mori* larvae (Kim *et al*.,2020; Kim and Park, 2021; Park, 2022). Notably, immune-challenged *B. mori* hemolymph extracts exhibited antioxidant properties (Kim *et al*.,2020; Kim and Park, 2021; Park, 2022). In HepG2 cells, immune-challenged *B. mori* hemolymph decreased the expression of SIRT5, which was increased by hydrogen peroxide, and conversely increased PRDx1. This indirectly indicates that immune-challenged *B. mori* hemolymph is effective against human hepatocellular carcinoma.

Materials and Methods

Silkworm Collection and Immune-challenged Hemolymph

The immune-challenged silkworm hemolymph used in this study was prepared according to the methods and procedures in a study conducted by Kim *et al*. (2020). Briefly, the freeze-dried *Lactobacillus plantarum* (*Lb. plantarum*) cell walls were dissolved in 0.1M sodium citrate buffer (pH 4.7) and subjected with ultrasonication. The same amount of n-butanol was added and extracted by stirring for 30 min at room temperature. After extraction, centrifugation was performed at 10,000 rpm for 30 min, and the supernatant was recovered. It was then evaporated, dialyzed with sterilized distilled water, and freeze-dried. The freeze-dried extract was weighed, dissolved in sterilized saline solution, and used for silkworm immunity induction tests. Day 5 fifth instar silkworm larvae were used for immune challenge with *Lb. plantarum* extracts. Fifty microliters of *Lb. plantarum* cell wall extracts dissolved in sterile saline solution was injected dorsolaterally into the hemocoel using 1-mL disposable syringes. For antibacterial and anti-inflammatory activity assays, circulating hemolymph in the body fluid was directly collected 24 h after injection into sterile tubes.

Cell Culture and Cell Viability Assay

The human liver cancer cell line HepG2 was supplied by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (Welgene, Republic of Korea) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and incubated with phosphate buffered saline (PBS) (control), 50, and 100 ppm at 37°C for 24 h. Cell numbers were measured using the Cell Titer 96 Aqueous One solution, which contained phenazine ethosulfate (PES) and 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfophenyl)-2H-tetra zolium, inner salt (MTS; Promega Corporation, Madison, WI, USA). Absorbance was determined at 490 nm with background subtraction at 650 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

ROS Induction and Treatment with Immune-challenged Hemolymph

To induce oxidative stress, hydrogen peroxide (H_2O_2) was prepared from a 30% stock solution. At 24 h after seeding, HepG2 cells were exposed to oxidative stress for 2 h. H_2O_2 was diluted in a complete culture medium. After treatment with H_2O_2, the cells were rinsed twice with PBS and incubated in DMEM. Oxidative stress-induced HepG2 cells were incubated with immune-challenged hemolymph. Two different concentrations (50 and 100 ppm) of immune-challenged *B. mori* hemolymph extracts were added to determine the dose dependence of its activity. Total RNA was isolated from the cultured HepG2 cells.

cDNA Synthesis and Reverse Transcription–Quantitative Polymerase Chain Reaction

Total RNA was isolated from HepG2 cells treated with immune-challenged hemolymph using the TRIzol reagent solution (Life Technologies, Frederick, Maryland, USA)

Table 1. Primer pairs used in qPCR

Name		Sequences (5` to 3`)	Product Length (bp)
SIRT5	Forward	GTCCACACGAAACCAGATTTGCC	148
	Reverse	TCCTCTGAAGGTCGGAACAACA	
PRDx1	Forward	CACTGACAAACATGGGGAAGT	82
	Reverse	TTTGCTCTTTTGGACATCAGG	
GAPDH	Forward	GAGTCAAACGGATTTGGTGGT	238
	Reverse	TTGATTTTGGAGGGATCTCG	

according to the manufacturer's instructions. The amount of RNA was determined spectrophotometrically by measuring the absorbance at 260 nm. Total RNA was treated with DNase I (Life Technologies) for 15 min at 37°C to remove genomic DNA. After purification, oligo dT-primed cDNA was prepared from the total RNAs using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription–quantitative polymerase chain reaction (qRT-PCR) was performed using a StepOnePlus Real-Time PCR system with Power SYBR Green PCR Master Mix (Takara, Japan). PCR was conducted using the following protocol: 40 cycles of denaturation at 95°C for 5 s and annealing and elongation at 60°C for 35 s. Fluorescence was detected at the end of every 60°C extension phase. Quantification of gene expression data was performed using the $2^{-\Delta\Delta Ct}$ method, and the crossing point of the target genes with β-actin was calculated using the formula $2^{-(\text{target gene-}\beta\text{-actin})}$ for quantification of relative expression. Sequences of the gene-specific primers used in this study (Bioneer Corporation, Daejeon, Korea) are listed in Table 1.

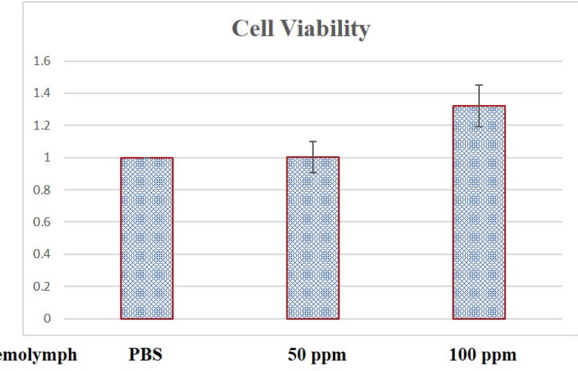

Fig. 1. Effects of immune-challenged *B. mori* hemolymph on the proliferation of human liver cancer cells (HepG2). HepG2 cells were treated with immune-challenged hemolymph for 24 h. Cell viability was then determined with a PES/MTS-based assay. The data are representative of three independent experiments performed in triplicate.

Results and Discussion

The cytotoxic effect on HepG2 cells was examined by exposing the cells to effective concentrations of immune-challenged hemolymph for 24 h, and cell proliferation was examined using a PES/MTS-based assay. As shown in Fig. 1, treating HepG2 cells with immune-challenged hemolymph for 24 h did not affect cell viability, indicating that the concentrations selected for this study did not damage cell integrity during the incubation period.

To assess the SIRT5 regulatory activities of the immune-challenged hemolymph in the ROS-induced HepG2 cells with H_2O_2, we measured *SIRT5* expression using RT-qPCR. First, the mRNA expression of each antioxidant marker gene isolated from target cells treated with H_2O_2 was compared with that of control cells treated with PBS. The C_T values for the mRNA expression of SOD-1, SOD-2, and GPx1 indicated their expression of the antioxidant-specific markers examined. Consistent with the previous study, cells treated with different concentrations (50 and 100 ppm) of immune-challenged *B. mori* hemolymph extracts showed antioxidant activity (Data not shown). As shown in Fig. 2, immune-challenged *B. mori* hemolymph extracts showed SIRT5 downregulation activity, reaching effective activity at the highest concentration of 100 ppm.

PRDx1 expression rate analysis of the antioxidant activity of immune-challenged *B. mori* hemolymph was performed based on ROS induction with H_2O_2, and all experiments were performed at least three times. Following H_2O_2 stimulation, cells treated with immune-challenged *B. mori* hemolymph extracts exhibited increased PRDx1 mRNA expression compared to that

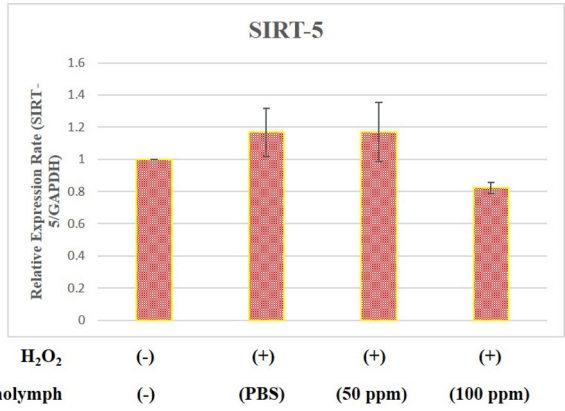

Fig. 2. Effects of the immune-challenged *B. mori* hemolymph on the expression of *SIRT5* in HepG2 cells. Immune-challenged hemolymph extract showed downregulation activity of SIRT5 at 100 ppm concentration compared to that of the PBS control group.

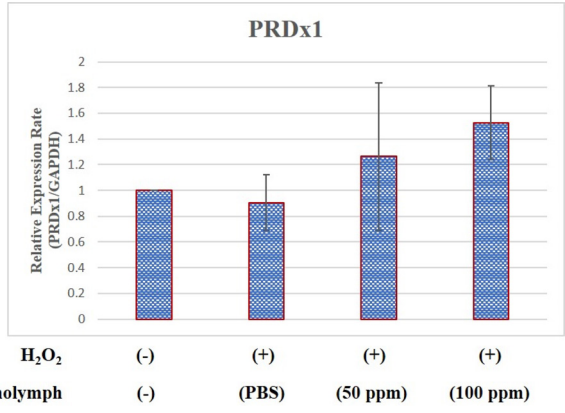

Fig. 3. Effects of immune-challenged *B. mori* hemolymph on the expression of *PRDx1* in HepG2 cells. Immune-challenged hemolymph extract showed upregulation activity of PRDx1 at both concentrations compared to that of the PBS control group.

of PBS-treated cells (Fig. 3).

Cisplatin, one of the most widely used chemotherapeutic agents for multiple solid tumors, including ovarian cancer, suggests that a novel cytotoxic effect of most genotoxic drugs is the promotion of ROS-dependent apoptosis or DNA damage (Chen *et al*., 2019; Liu *et al*., 2016; Luo *et al*., 2013; Marullo *et al*., 2013; Srinivas *et al*., 2019; Sun *et al*., 2019). SIRT5 positively regulates Nrf2, resulting in its nuclear translocation of Nrf2. Nuclear Nrf2 binds to antioxidant-responsive elements (ARE) and activates HO-1 transcription, thus eliminating cisplatin-induced ROS and leading to the inhibition of DNA damage and cisplatin resistance (Sun *et al*., 2019). Therefore, the tumor-suppressive effect in ROS-induced cancer cells is possible through downregulating SIRT5. In this study, immune-challenged hemolymph successfully decreased the expression rate of SIRT5 in ROS-induced HepG2 cells by H_2O_2. This indirectly indicated that it effectively treats solid tumors by promoting ROS-dependent apoptosis or DNA damage.

PRDx1 was first reported as an antioxidant enzyme, but its physiological role in oxidization–reduction balance remains unclear because it is highly susceptible to oxidative stress (Ding *et al*., 2017). It has been viewed as a tumor suppressor because PRDx1-knockout mice exhibit a shortened lifespan owing to the development of hemolytic anemia and cancer (Ren *et al*., 2013). Repression of PRDx1 expression promotes H_2O_2-induced senescence in breast cancer cells (Ding *et al*., 2017; Goncalves *et al*., 2012; McDonald *et al*., 2014). Cisplatin resistance is accompanied by a significant increase in the expression of the *PRDx1* in breast cancer cell strains (Ding *et al*., 2017; Kalinina *et al*., 2012; Turner-Ivey et al., 2013).

In this study, immune-challenged hemolymph increased the expression of the PRDx1 gene in H_2O_2-induced HepG2 cells compared with that in the control group. These results indicate that it can be an auxiliary means of treating drug-resistant tumors by downregulation of SIRT5 and upregulation of PRDx1 gene expression. Humans have used insects as food and traditional medicines for many years (Kim *et al*.,2020). Hemolymph is the circulating fluid in insects and a key component of the immune system (Kim *et al*.,2020). Immune-challenged *B. mori* hemolymph as a food and pharmaceutical material requires further research to increase its therapeutic effect on tumors, including drug-resistant tumors.

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