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Brief Communication

Upregulation of Kruppel-like Factor 4 Gene expression by *Allomyrina dichotoma* Hemolymph in the INS-1 Pancreatic β-cells

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The hemolymph of Korean rhinoceros *Allomyrina dichotoma* consists of blood and lymph in which various kinds of proteins function physiologically. We have previously demonstrated that *A. dichotoma* hemolymph has the potential to treatment and prevent diabetes through activating transcription factor 3-gene (ATF3) regulation. In this study, we investigate the expression of Kruppel-like factor 4 (KLF4) in *A. dichotoma* hemolymph-treated INS-1 pancreatic β-cells. The new findings show that *A. dichotoma* hemolymph, which upregulates KLF4 gene expression in a dose-dependent and time-dependent manner. In addition, hemolymph combine with mild endoplasmic reticulum (ER) stress, which also differentially regulates KLF4 gene expression. These results may provide insights to KLF4 gene-related disease therapies through KLF4 gene regulation.

Key Words: *Allomyrina dichotoma,* Hemolymph, Kruppel-like factor 4 (KLF4)

Recently, Food and Agriculture Organization of the United Nations (FAO) reported that edible insects such as grasshoppers (*Sphenarium purpurascens*), crickets (*Gryllus bimaculatus*), mealworms (*Tenebrio molitor*), and buffalo worms (*Alphitobius diaperinus*), should be recognized as one of the great potential future food sources in which high quality protein, vitamins and amino acids can be obtained (Nowak et al., 2016; Tao and Li, 2018; van Huis, 2013). Korean rhinoceros beetles (*Allomyrina dichotoma*) are regarded as a traditionally medicine for liver-related diseases in Korea (Choi et al., 2006; Kim et al., 2007). It was reported that *A. dichotoma* boiled-extract demonstrates effectiveness as anti-hepatofibrotic, anti-neoplastic, antibiotic, anti-diabetic and anti-obesity at the experimental level (Miyanoshita et al., 1996; Yoshikawa et al., 1999; Sagisaka et al., 2001; Chung et al., 2014; Kim et al., 2015; Kim et al., 2016). In addition, our previous study has shown that hemolymph of *A. dichotoma* activates transcription factor 3 (ATF3) gene, which indicates a potential for the possible treatment of diabetes (Kim et al., 2018). Hemolymph of insect is the circulating fluid functionally analogous to the blood and lymph of vertebrates, and consists of some kinds of hemocytes and various ions, carbohydrates, lipids, proteins and hormones (Blow and Douglas, 2019; Pendar et al., 2019). Up to date, in all experiments of insect bioactivity, two types of insect sample were used either crushed dried-insects into a powder or boiled extract of insect. However, in this study, we used raw insect hemolymph directly for the functional

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study for purposes of maintaining the protein activity. Kruppel-like factor 4 (KLF4) is known as a transcription factor which has zinc finger DNA-binding capacity that regulates gene expression associated with proliferation, anti-inflammation, and apoptosis of the cells (Tang et al., 2019). Some of the disorders that have reportedly resulted from KLF4 gene-related diseases include secretory meningioma, cerebral cavernous malformations, skin squamous cell carcinoma, germ cell, embryonal cancer, and familial adenomatous polyposis (Morales-Martinez et al., 2019).

Herein, we demonstrate the upregulation of KLF4 gene in *A. dichotoma* hemolymph-treated INS-1 pancreatic β-cells. The gene expression of KLF4 is significantly up regulated in transcriptional level from our previous cDNA microarray experiment (Kim et al., 2018). Hemolymph exposure in INS-1 cells for 2 h resulted in 7.06-folds increase in KLF4 levels as compared to the control genes. As such, KLF4 was used as the target gene for the current experiment.

Hemolymph used in this experiment was extracted from a healthy third-instar larva of *A. dichotoma*. The collected hemolymph was incubated for 5 min using 5 mL of thrombin to 500 mL of hemolymph at room temperature. In order to remove the insoluble matters including several kind of blood cells, the hemolymph was centrifuged for 5 min at 11,000 x g at temperatures of 4℃. After dialysis and subsequent filtering through 0.22 mm syringe filter, the supernatant was divided and stored at -70℃ until following experiment. The concentration of the final protein was 2.5 mg/mL as measured by Bio-Rad protein assay kit. INS-1 pancreatic β-cells were cultured in RPMI-1640, and supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM/mL L-glutamine, 10 mM/mL HEPES, 1 mM/mL sodium pyruvate, and 50 mM/mL 2-mercaptoethanol, in a humidified environment (5% CO_2, 37℃). Analysis to identify genes that are differentially expressed was conducted using Agilent's Gene Expression Hybridization. Scan and image analysis conducted using Agilent's DNA microarray scanner and DNA analysis was by Agilent's GeneSpring Software Kit (Agilent, Santa Clara, CA, USA). Total RNA was extracted using the SV Total RNA Isolation system (Promega, Madison, WI, USA). The mRNA in the samples was reverse-transcribed using a SuperscriptII™ First Strand Kit (Invitrogen Carlsbad, CA, USA). The resulting cDNA was amplified by PCR using the primer pair's mouse actin F (5'-GAAATCCACCAAA-GCTCAC-3') and R (5'-TCTCGGTCAAGTTCAACATC-

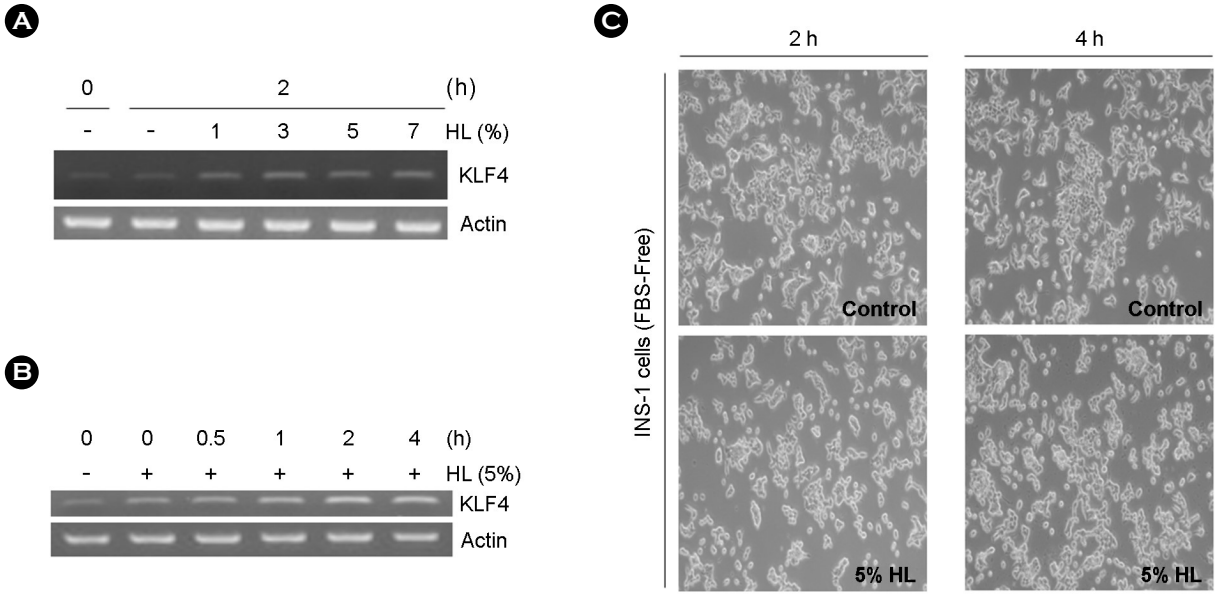

Fig. 1. *A. dichotoma* hemolymph enhances KLF4 gene expression in the INS-1 pancreatic β-cells. (A) Dose-dependent effect of *A. dichotoma* hemolymph for KLF4 gene expression. (B) Time-dependent effect of *A. dichotoma* hemolymph for KLF4 gene expression. (C) Photomicrograph of INS-1 pancreatic β-cells morphology because of *A. dichotoma* hemolymph treatment.

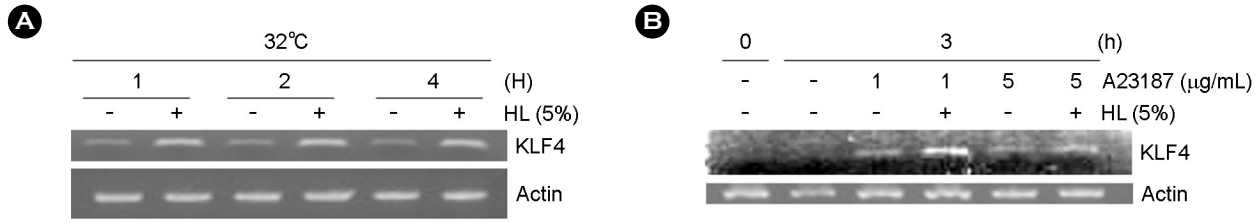

Fig. 2. (A) Low temperature regulates KLF4 gene expression. (B) ER stress inducible drug (A23187) regulates KLF4 gene expression.

3') and KLF4 F (5'-CTGAACAGCTCAGGGACTGTCA -3') and KLF4 R (5'-GTGTGGGTGGCTGTTCTTT-3'). RT-PCR conditions for 30 cycles were 94℃ for 30 s; 58℃ for 30 s; and 72℃ for 1 min (10 min in the final cycle), using both primers mentioned above with Taq DNA polymerase.

In this study, we investigate the effect of *A. dichotoma* hemolymph on the gene expression of KLF4 in the INS-1 pancreatic β-cells. As shown in Fig. 1A, KLF4 gene expression is upregulated dose-dependently on the *A. dichotoma* hemolymph after 2 h exposure. After the addition of 1% hemolymph, KLF4 gene expression was higher than its con trol. Fig. 1B presents the result of time-dependent expression of KLF4 gene under 5% *A. dichotoma* hemolymph. Gene expression of KLF4 is upregulated to 0.5 h exposure. It was confirmed that KLF4 gene expression was upregulated in both instances of dose-dependent and time-dependent aspects of hemolymph experiments. In addition, as a result of microscopic observation of the cells, the morphology of the cells is almost unchanged because the hemolymph is not cytotoxicity (Fig. 1C).

INS-1 pancreatic β-cells were incubated at 32℃ for 1, 2, and 4 h in 5% *A. dichotoma* hemolymph, in order to understand the possibility of hypothermia treatment through the regulation of KLF4 gene expression (Kim et al., 2018). As shown in Fig. 2A, there were no significant changes observed in KLF4 gene expression due to hypothermia treatment. ER comprises an advanced signal transducing system that functions in perpetuating cellular homeostasis through post-translational modification of secretory proteins (Baiceanu et al., 2016). Stresses in the ER are induced by unfolded protein response (UPR) to adaption and survival of cells and/or tissues by expression of ER chaperones such as binding immunoglobulin protein (BiP), calreticulin and protein ERp29 (Kwon et al., 2000; McCaffrey and Braakman, 2016). Next, testing of regulation of KLF4 gene expression by ER stress was observed using ER stress inducible drug (A23187). It was observed that KLF4 gene expression is upregulated by relatively weak (1 mg/mL) instead of strong (5 mg/mL) ER stress induction (Fig. 2B).

Many recent results provide evidences that effects of edible insect extracts against liver diseases (Ahn et al., 2014; Im et al., 2017; Kim and Chae, 2018; Lee et al., 2017a; Lee et al., 2017b). Our results also provide an insight to the development of KLF4-related disease therapies through gene expression regulation of KLF4 using *A. dichotoma* hemolymph combined with mild ER stress (Yoshida & Hayashi, 2014; Lu et al., 2015).

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CONFLICT OF INTEREST

The authors declare that no conflict of interest.

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