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Original Article

Honey Bee Dry Venom Reduces Hepatitis B Virus Surface Antigen Secretion in PLC/PRF/5 Cell Line

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Background and Aims: Currently, many efforts are directed toward functional Hepatitis B virus (HBV) treatment. This is achievable by suppression of HBV surface antigen (HBsAg) secretion. In this regard, use of natural products has been the areas of interest by scientific communities.

Materials and Methods: Dried Honey Bee venom was extracted for assessing its anti-HBsAg secretion potential. Hepatoma cell-derived PLC/PRF/5 was propagated in complete medium. The cell line was treated by a serial dilution of Bee venom. Cell cytotoxicity (IC50) was measured by MTT colorimetric assay at three post-treatment times. HBsAg secretion was evaluated from PLC/PRF/5 supernatant treated by under-cytotoxic concentrations of Bee venom by using enzyme-linked immunosorbent assay.

Results: The results indicated that dried Bee venom extract is able to reduce secretion of HBsAg from the cell line with Selectivity Index (SI) of eight. Reduced levels of HBsAg were in dose-dependent manner and it was in its lower concentrations at 8 ppm after 12 hr post treatment. The IC50 was observed to be 63.78 ppm.

Conclusions: The Bee venom has anti-HBV activity. The way we used under-cytotoxic concentration of Bee venom, the HBsAg secretion was restored after 24 hr post treatment. Furthermore, mechanism of action of Bee venom in reducing HBsAg level needs to be further investigated.

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Introduction

The human hepatitis B virus (HBV) is a major etiological factor for causing chronic hepatitis (CHB) and consequently cirrhosis and hepatocellular carcinoma (HCC) [1–3]. There are over 250 million chronically infected people worldwide, and annually 600,000 death cases occur each year from HBV-related organ failure [4]. In order to reduce the risk of HCC and mortality, some Food and Drug Administration approved nucleos(t)ide analogues are recommended including lamivudine, adefovir, entecavir, telbivudine, and tenofovir that suppress or reduce viral replication [5]. The inhibitory effects of anti-HBV drugs are only transient because of the viral scape mutants. Furthermore, the drug only retains their antiviral abilities when they are used for a long treatment course. A functional cure for CHB is known by suppressing HBV surface antigen (HBsAg) secretion inhibition [4]. There are several significant approaches regarding HBsAg secretion inhibition. Recent advances in anti-HBV research has been around nucleic acid polymers [6]. Nucleic acid polymers block the release of HBsAg from infected hepatocytes by the selective targeting of the assembly and/or secretion of subviral particles [7]. There has been an approach for gene silencing human trial by using small interfering RNAs (siRNAs), and not completed due to integrated form of HBV in residual hepatocytes [8]. Further research in these approaches is still ongoing. Nature is a vast source of substances for discovering active products acting against human pathogens [9,10]. In this regard, Honey Bee (*Apis mellifera*) venom is known to have therapeutics potential for treatment of viral infections and cancer [11–15]. The major components of bee venom dry matter is peptides (>50%) and proteins (10-14%) [16]. About 40-50% of the venom peptides is comprised of a basic polypeptide named Melittin, which has the main therapeutic potential of the venom dry matter [14,17]. Wachinger et al have reported inhibitory effect of Melittin on human immuno-deficiency virus 1 replication. Their results indicated that Melittin is able to decrease human immunodeficiency virus 1 mRNAs in a dose-dependent manner [18]. This study aimed to investigate whether dry bee venom containing Melittin would influence release of HBsAg from the PLC/PRF/5 cell line containing integrated viral genome.

Materials and Methods

Preparation of honey bee crowd extract

Genuine bee venom was collected through the sting following mechanical stimulation. Hives containing *Apis Mellifera* colonies were hired from Tabriz province, Iran. Each colony was 'milked' for 5 minutes using a 12-V wet-cell battery in conjunction with a converter (12 V DC to 115 V AC). An electrical timer was used to break the circuit for 4 sec at 3-sec intervals. An average of 20 beehives must be milked in this way to obtain 1 g of venom. 1 µg/ml (1 ppm) dry extract was dissolved in Dimethyl sulfoxide (DMSO) and a serial dilution in the range of 1 to 128 ppm was provided.

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Cell culture of PLC/PRF/5

PLC/PRF/5 was provided from the previous study of our group [19]. A complete medium containing DMSO (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% Pen/Strep (Gibco, USA) antibiotics was prepared for cell culture. A vial stock of the cell line was melted and propagated on T-75 flasks and incubated at 37°C with 5% CO_2 and 10% humidity. After achieving a >90% confluency, cells were harvested and seeded into 96-well plates supplemented with complete medium for cell cytotoxicity assay.

Cell viability and cytotoxicity assay

PLC/PRF/5 cells were seeded either with complete medium or medium containing honey bee crowd extracts. Briefly, 8000-10,000 cells/well were seeded and incubated for 12 hr. After that, supernatants were removed and replaced with either fresh complete medium or extracts. Cell viability was assessed for 12 hr, 48 hr, and 72 hr post-treatment with MTT assay (Sigma, USA). MTT was prepared in 5 mg/ml stocks. After each time-point, wells were washed in phosphate buffered saline (Gibco, USA) and MTT was added (20 µl/well). After reduction of MTT and formation of violet Formazan crystals, DMSO was added to each well (100 µl/well). The plates were read at 570 nm with the enzyme-linked immunosorbent assay (ELISA)-reader (Bio Teck, Lionheart Technologies, Inc., USA). Each test was assessed in triplicate.

Investigation of HBsAg secretion

For HBsAg secretion inhibition, 8000-10,000 cells were seeded into each well in duplicate. 12 hr cells were washed in phosphate buffered saline and replaced with either complete medium or medium containing venom dry extracts. Concentrations below cell cytotoxicity were used for the assay. Supernatants were collected ant kept in -20˚C after 12 hr, 48 hr, and 72 hr post treatment for ELISA. HBsAg Sandwich ELISA kit (PadTan Danesh Co., Tehran, Iran) was used to detect HBsAg in the supernatant according to the manufacture protocol. The plates were read at 450 nm with ELISA-reader (Bio Teck, Lionheart Technologies, Inc., USA). Cut-off value was 0.2 and result was calculated with the following formula:

$$S/Co = \frac{Sample\ OD}{Cut-off\ value}$$

S/Co values more than 1 was considered as positive. Optical densities for each test were also evaluated for assessing HBsAg reduction levels in supernatant. The study was conducted in accordance with Declaration of Helsinki for Human Research and the proposal was approved by the university Ethics Committee at AJA University of Medical Sciences.

Statistical analyzes

Statistical analyses were performed with two-way ANOVA with GraphPad Prism 7. Graphs were drawn by using MS-Excel 2016.

Results

Honey bee dry extracts TCID50

Tissue culture infectious dose 50 (TCID50) was obtained as high as 63.78 at 12 hr post treatment. No significant toxicity was observed at 12 hr post treatment. At higher concentrations, only 64 ppm and 128 ppm had 83% and 86% cytotoxicity, respectively (Fig.

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1). Four concentrations (1 ppm, 2 ppm, 4 ppm, and 8 ppm) with very low cytotoxicity were chosen for investigations HBsAg secretion in PLC/PRF/5 cell line.

However, using lower non-toxic concentra-tions lead to cell propagation. This allows restoration of HBsAg secretion. This is supported by the ELISA report (Fig. 2). As it is illustrated, HBsAg secretion reduces at 12 hr post-treatment. Data showed HBsAg secretion being lowest amount in 8 ppm concentration of dried bee venom. There were no significant differences between concentra-tions 1 ppm and 2 ppm and concentrations 4 ppm and 8 ppm. The selectivity index was ~8 at 12 hr post-treatment.

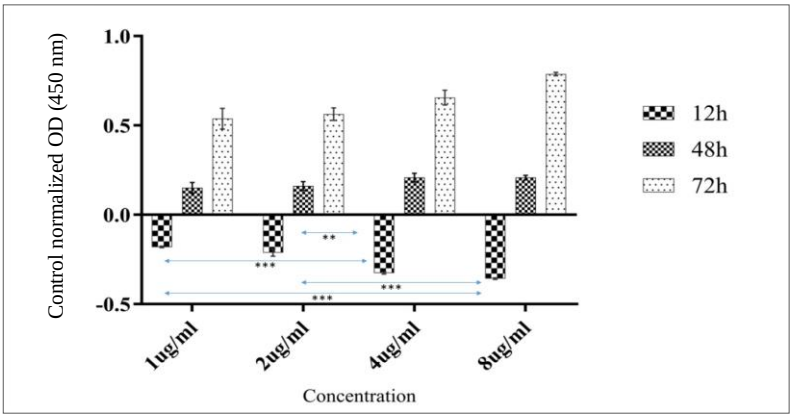

Fig. 1. MTT assay showed not cytotoxicity at low bee venom concentrations.

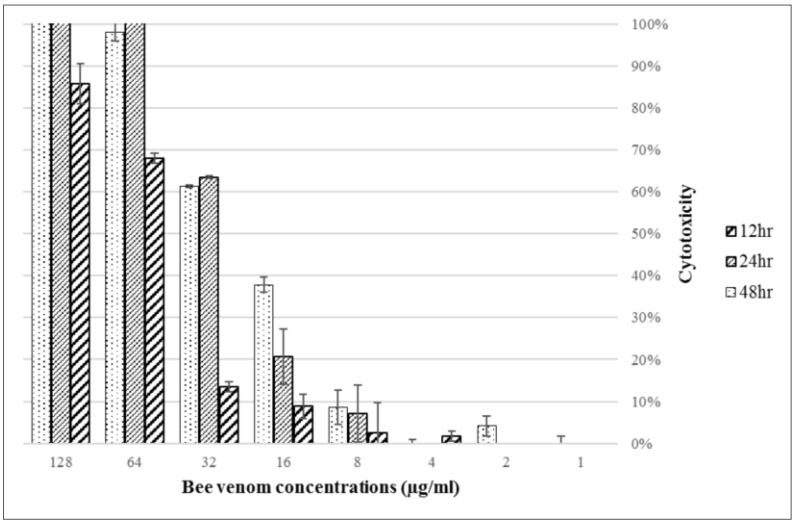

Fig. 2. ELISA assay demonstrated that HBsAg secretion was reduced in a dose-dependent manner at 12 hr post treatment

Discussion

The results indicated that a low concentration of bee venom reduces secretion of HBsAg at early hours of post-treatment.

In our previous study, we have overviewed different classes of HBsAg secretion inhibitors [4]. Here, the effect of dried honey bee venom extract was applied for

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investigating its effect on HBsAg secretion *in vitro*. Melittin is well known to have antiviral activities. Recently, a Melittin-like peptide has been used in the complex of siRNA, ARC-520 for liver targeting [8]. Primary liver carcinoma (PLC)-derived cell line (PLC/PRF/5) was reported containing integrated form of HBV divergent sequence of adw subtype [20, 21]. This cell line contains last fragment of HBV genome and therefore produces and secretes HBsAg but no other known viral protein [22].

Different therapeutic studies have been performed on PLC/PRF/5 to inhibit secretion of HBsAg. Varma et al. showed that herbal medicine, HD-03/ES is able to inhibit HBsAg transcription in PLC/PRF/5 cell line [23]. The presented results indicate the potential anti-HBsAg secretion effect of bee venom dry extract. Further studies are warranted for investigating its possible mechanism of action. In addition, the potential of bee venom active ingredients on HBsAg secretion is needed to be assessed.

Conclusion

Here, it was found that dried extract of bee venom has anti-HBsAg secretion activity. Reduction of HBsAg was dose-dependent and it was in its lowest level at 8 ppm concentration of venom at 12 hr post-treatment. As we have used under-cytotoxic concentration of bee venom, the HBsAg secretion was restored after 24 hr post treatment. Furthermore, mechanism of action of bee venom in reducing HBsAg level need to be investigated in the future.

Conflict of Interest

The authors declare that there are not conflicts of interest.

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