In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

Samia Ahmed Kamal
Virology Department, Animal Health Research Institute (AHRI), Dokki, Giza 99999, Egypt

Abstract: Bee venom (BV) was used from long time ago in the medical field as treatment of chronic joint affections. In the recent decades, the screening process of new sources of antimicrobials discovers its high advantageous characteristics for combating various types of microbes, as well as trials to discover its anti-cancer medicinal fields. Lumpy skin disease virus (LSDV) causes disease in cattle of economic importance, and this work aimed to find treatment as well as alternative inactivant for LSDV. The use of bee venom as antiviral was experimented in this work and exhibited satisfied inhibitory effects on LSDV, meanwhile, the antigenic properties was still intact. The viability of virus was tested in tissue culture cells lines and in embryonated chicken eggs. According to doses and time of exposure, the cell lines of Hep-2 (human larynx carcinoma) and MCF7 (breast carcinoma cell line) were treated with different concentrations of BV and examined after 24 h post-inoculation. The Hep-2 and MCF7 cell lines were treated with various concentrations of BV in descending doses as follow: 25, 20, 15, 10, 5 and 0.5 ug/mL of BV. Then bee venom pathological effects on Hep-2 cells and MCF7 cells were observed, such as apoptosis, retarded growths and cytolysis. The results indicate the possibilities of using bee venom as anti-neoplastic and antiviral.

Key words: Bee venom, lumpy skin disease virus, anticancer, Hep-2 cells, MCF7, antiviral natural substances.

1. Introduction

Lumpy skin disease (LSD) acquired its name from the skin lesions on the infected cattle. It is characterized by formation of hard nodules on skin and mucous membranes accompanied by fever, lesions on the internal organs, general deterioration of health with emaciation, enlarged lymph nodes, edema of the skin, and sometimes death. LSD in cattle usually was complicated by secondary infections (bacteria, screw worm, other contaminant viruses “orphan viruses”), which affected the productivity of dairy farms and may cause deaths from complications of secondary infections or from primary infections by more pathogens endemic in the environment [1-4]. Lumpy skin disease virus (LSDV) (genus Capripoxvirus, family Poxviridae) is stable, survives at ambient temperature for long periods and is highly resistant to inactivation, surviving in necrotic skin nodules for up to 33 d or longer, in desiccated crusts for up to 35 d and at least 18 d in air-dried hides. So, it can remain viable for long periods in the environment [5]. The virus is susceptible to sunlight and detergents containing lipid solvents, but in dark environmental conditions, such as contaminated animal sheds, it can persist for many months. The virus is susceptible to 55 °C for 2 h and 65 °C for 30 min, but can be recovered from skin nodules kept at -80 °C for 10 years and from infected tissue culture fluid stored at 4 °C for six months. It is affected by higher alkalinity or acidity, and killed by ether (20%), chloroform, formalin (1%) and some detergents, e.g., sodium dodecyl sulphate [1, 6]. There is no specific antiviral treatment available for LSDV-infected cattle. Two vaccines, however, Neethling and Kenya sheep and goat pox virus, have been used widely in Africa with success [1]. Diagnosis of LSD usually depends on its unique and characteristic clinical signs and its specific macroscopic and microscopic histopathologic appearance that never share resemblance with other diseases. However, mild and subclinical forms require
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

125

rapid and reliable laboratory testing to confirm diagnosis. Laboratory diagnosis of LSDV could be achieved by different techniques, as electron microscopy, egg inoculation, cell cultures, fluorescent antibody test and serological tests. However, polymerase chain reaction (PCR) was applied for routine diagnosis, because it is more sensitive and specific, and for rapid detection of LSDV in field specimens [7, 8]. Hep-2 and MCF7 cell lines were derived from cancer lesions and used in vitro in research work. Bee venom (BV) from the sting of honeybee (Apis mellifera L.) is traditionally used in China, Korea and Japan for arthritis, tendinitis, bursitis and other chronic conditions. It is reported to have pro-inflammatory [9] and anti-inflammatory effects [10-14]. Effective control of LSD requires solutions focused on combating the LSDV by finding remedy and safe vaccine.

Scientists usually screen the nature to find new sources of antimicrobials, and cationic peptides are major adventure in this field, while BV contains cationic peptides beside many other valuable substances that have synergistic effects. The aim of this study was to study the pathologic effects of BV on cell lines for safety concerns and the suitable doses, in other hand, to study the effects of safe doses of BV on LSDV as a step towards applications as antiviral.

2. Materials and Methods

2.1 Preparation of BV Stock Solution

The colonization is conducted at the apiaries of Plants Protection Institute (PPI), Beekeeping Department, Agriculture Research Center (ARC), Egypt.

The electrical shock method has been used to stimulate the bees to sting. The collector frame was placed at the entrance of the hive and connected to a device which supplies electrical impulses; when bees receive a mild electric shock, they sting the surface of the collector sheet, as they see this to be the source of danger. The deposited venom between the glass and the protective material was dried, then later scrapped off by a razor blade and collected in a dark bottle in a powder form. According to Mraz [15], BV powder (crude) of dose 2 mg was dissolved in 2 mL tissue culture media—minimum essential medium eagle (MEME), and then filtered by 0.22 μm syringe filter. That final concentration of the BV stock was 1,000 ug/mL (i.e., 1 μL = 1 ug) and kept at -20 °C. Preparation of BV solution was performed according to Kamal et al. [16].

2.2 LSDV and Hyperimmune Sera

The field strain of LSDV and hyperimmune serum were kindly provided by Dr. Mohamed Hussein from Animal Health Research Institute (AHRI). It was used for cell culture adaptation and propagation [17].

Recent local isolate of LSDV from natural outbreak in Damenhour city in 2014, was tested by AHRI and confirmed by immunofluorescent test and RT-PCR.

2.3 Tissue Culture Cell Lines

Maiden-Darby bovine kidney cells (MDBK), Hep-2 and MCF7 were prepared and provided by Egyptian Company for Production of Vaccines, Sera & Drugs (VACSERA), Egypt. The MDBK cells were used for adaptation of LSDV, virus titration as well as serum neutralization test (SNT). Hep-2 and MCF7 cell lines were used for studying histopathological effects of BV on living cell. The cell lines of Hep-2 and MCF7 were treated with different concentrations of BV and examined after 24 h post-inoculation. The Hep-2 and MCF7 cell lines were treated with various concentrations of BV in descending doses as follow: 25, 20, 15, 10, 5 and 0.5 ug/mL of BV [16].

2.4 LSDV Titration and Determination of Infectious Dose (ID50)

MDBK cell lines were used for determination of virus’ infectivity titer. The 50% tissue culture infectious dose of a virus (TCID50) was carried out by traditional methods of virus quantification. Viral
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

infectivity titer was evaluated according to the method adopted by Reed and Muench [18].

2.5 Incubation of LSDV with BV

The stock mixture of inactivated LSDV was prepared by adding BV to LSDV and left for 8 h at room temperature (21 °C), then kept in refrigerator at -20 °C [16].

2.6 LSDV Isolation from Natural Outbreak

Skin biopsies, comprising epidermis and dermis of the nodular skin lesions, were collected from local cattle in Al Behera Governorate during an LSDV outbreak in 2014. Biopsy specimens were taken aseptically and the incisions were sutured. Samples were collected in 15 mL sterile tubes and stored at -20 °C until use. The procedures were performed according to Payment and Trudel [19].

2.7 Embryonated Chicken Eggs Inoculation: Testing BV Inactivation Ability of LSDV

Five groups of 11-day-old embryonated chicken eggs were used to test the viability of LSDV. The inoculation was performed via the chorio-allantoic membrane (CAM) route. The dose of all samples was 0.1 mL/egg. The first group was infected with LSDV from natural samples, the second embryonated chicken eggs group infected with LSDV isolated in tissue culture (Egyptian strain), the third group (positive control) injected with BV stock solution and the fourth group (test group) injected with LSDV mixed with BV. The fifth group was kept as control negative without injection. All groups were incubated at 36 °C in CO2 incubators [19].

2.8 Virus Neutralization Test

 Infective tissue culture material containing disintegrated cells and maintenance medium was centrifuged at 1,500 rpm for 10 min. Serial 10 fold dilutions of the supernatant were made in Hanks saline and mixed with equal volumes of undiluted serum. Parallel dilutions were mixed with normal serum. Contact was allowed to take place for 1 h at 37 °C, and after that, the mixture were inoculated onto established monolayers in culture tubes, five tubes being used per dilution. All tubes were examined daily for cytopathologic effects (CPE) [19].

2.9 Agar Gel Precipitation Test (AGPT)

AGPT was performed for detecting the antigenic properties of LSDV under experiment, the treated virus with BV and the original viruses used in the experiment [19].

3. Results and Discussion

3.1 Adaptation and Titration of LSDV in MDBK Cells

The titration results of LSDV in MDBK cells were shown in Table 1. The highest level gives at the 10th passages titre of log_{10}(6.4)/1 mL and MDBK showed satisfactory growth rate for LSDV. The highest infectivity titer was used for the experiment.

3.2 Embryonated Chicken Eggs Inoculation

The results showed that both the control groups (negative control and positive control) were normal and still alive until the end of experiment. The test group showed normal appearance without pathological changes, which indicated the absence of live viral particles. However, the other groups showed pathologic changes in CAM (Fig. 1). This finding indicates that LSDV is completely inactivated by BV. The use of embryonated chicken eggs in testing the

<table>
<thead>
<tr>
<th>Virus passages</th>
<th>Time of CPE appearance (d)</th>
<th>Virus titre Log_{10}(TCID50)/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>5.4</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>6.0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

CPE: cytopathic effect.

TCID50: tissue culture infective dose 50 (this method evaluates an endpoint where 50% of the cell cultures are infected).
viability of LSDV is the most suitable method, however, other methods were used as second choice. The dose of 0.1 mL/egg was determined after testing different sets of embryonated chicken eggs, and the small dose was determined to avoid the direct toxicity of BV on embryonated chicken eggs [17].

3.3 Morphological Examination of Hep-2 and MCF7 Cell Lines Treated with BV

3.3.1 Morphological Changes of Unstained Hep-2 Cell Lines

The unstained Hep-2 cells was treated with different concentrations of BV while still attached to the culture chamber and examined by the inverted microscope. The morphological changes were shown in Fig. 2. The apparent effects were graduated from nearly destructed cells to lesser effects according to concentration. In this experiment, the exposure time was fixed (24 h). The cells were divided into eight groups. In group 1 (control), non-treated cells showed complete and intact sheet of cells (Fig. 2a). In group 2, cells treated with 20 µg/mL BV showed incomplete sheet with destructed cells (Fig. 2b). In group 3, cells treated with 15 µg/mL showed destructed sheet (Fig. 2c). In group 4, cells treated with 10 µg/mL showed incomplete sheet with some normal parts (Fig. 2d), which indicated incomplete destruction of cells by BV. In group 5, cells treated with 5 µg/mL showed moderate changes in the form of slowing cell growth, thus it could be explained as BV acts at this concentration biologically while cells tries to overcome its toxic effects (Fig. 2e). However, with lesser concentrations, the toxic effects disappeared gradually and the biological effects were expressed as changes in the growth rates. These biological changes were observed clearly in the lower concentrations, beginning from cells treated with 2.5 µg/mL, which showed slight changes in the form of slowing in the cell growth (Fig. 2f) (group 6). While cells treated with 1 µg/mL BV showed complete sheet with slight changes (Fig. 2g) (group 7). Consequently, the lesser effects were observed in cells treated with 0.5 µg/mL BV, which showed complete sheet with slight changes (Fig. 2h) (group 8). These findings indicated that the safest dose begins from concentration 0.5 µg/mL.

3.3.2 Morphological Changes of Unstained MCF7 Cell Lines

The inverted microscopy examination results of unstained MCF7 cells treated with different concentrations of BV were shown in Fig. 3. The time of exposure was fixed (24 h) and it was divided into six groups. In group 1, the control untreated cells showed the normal sheet (Fig. 3a). In group 2, cells treated with 20 µg/mL BV showed complete digestion and cell remenants (Fig. 3b). In group 3, cells treated with 15 µg/mL BV showed digestion (Fig. 3c). In group 4, cells treated with 10 µg/mL BV showed smaller and shrinkage cells (Fig. 3d). In group 5, cells treated with 5 µg/mL BV showed some shrinkage (Fig. 3e). In group 6, cells treated with 0.5 ug/mL BV showed normal picture (Fig. 3f).

3.4 Histopathological Results

3.4.1 Hep-2 Cells Stained by Hematoxylin and Eosin (H&E) and Examined under Inverted Microscope

The histopathological changes of stained Hep-2 cells treated with different concentrations of BV were shown in Fig. 4. The time of exposure was fixed (24 h)
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

Fig. 2  Morphological changes of Hep-2 cell lines treated with different concentrations of BV (unstained ×20).
and cells were divided into seven groups. In group 1, non-treated cells showed normal pathway of mitotic divisions in various stages; nucleoli were prominent inside large vesicular nucleus and the cytoplasm was abundant (Fig. 4a). In group 2, cells treated with 20 µg/mL BV showed cellular digestion (Fig. 4b). In group 3, cells treated with 15 µg/mL BV showed cellular digestion (Fig. 4c). In group 4, cells treated with 10 µg/mL BV showed cellular digestion (Fig. 4d). In group 5, cells treated with 5 µg/mL BV showed cellular digestion (Fig. 4e). In group 6, cells treated with 2.5 µg/mL BV showed cellular destruction (Fig. 4f). In group 7, cells treated with 1 µg/mL BV showed apoptosis (Fig. 4g).

3.4.2 Stained MCF7 Cells Examined under Inverted Microscopy

The histopathological changes of MCF7 cells treated with different concentrations of BV were shown in Fig. 5. The time of exposure was fixed (24 h) and the cells were divided into four groups. In group 1,
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

Fig. 4  Histopathological changes of stained Hep-2 cells treated with different concentrations of BV (H&E ×40).
non-treated cells showed normal cellular growth (Fig. 5a). In group 2, cells treated with 20 µg/mL BV showed digestion (Fig. 5b). In group 3, cells treated with 15 µg/mL BV showed digestion (Fig. 5c). In group 4, cells treated with 10 µg/mL BV showed digestion (Fig. 5d).

3.5 Cytopathologic Effects of Safe Dose (0.5 ug/mL) on Hep-2 Cell Lines and MCF7 Cell

Hep-2 Cells treated with dose 0.5 ug/mL showed after 24 h vesicle inside the nucleus with irregular edges (Fig. 6), and Hep-2 cells treated with dose 0.5 ug/mL after 48 h showed shrinkage of cells with dense irregular chromatin and distorted nuclear contents (Fig. 7a). Some cells showed abnormal stain affinity of the nucleus with reddish color, and the nucleus showed destructed condensed chromatin inside intact nuclear membrane. The cytoplasm was free from any vacuoles with partially destructed cytoplasmic membrane (Fig. 7b). Some other cells showed dissolved chromatin with intranuclear vesicles formations with completely intact nuclear membrane and two identical intranuclear dark stained inclusion bodies surrounded by hallow zones. The other cell showed large homogenous poorly stained nucleus with intact nuclear membrane and cytoplasm pushed to one side (Fig. 7c). Some cells showed completely dissolved chromatin with completely intact nuclear membrane with the disappearance of the cytoplasm (Fig. 7d).

Fig. 5  Histopathological changes of stained Hep-2 cells treated with different concentrations of BV (H&E ×20).

Fig. 6  Hep-2 cells treated with 0.5 ug/mL after 24 h (H&E ×20).
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

MCF7 cells treated with dose 0.5 ug/mL showed after 24 h vesicular nucleus (Fig. 8), and MCF7 cell lines treated with dose 0.5 ug/mL after 48 h showed shrinkage of cells with dense irregular chromatin and distorted nuclear contents (Fig. 9a). Some cells showed fragmentation (Fig. 9b).

3.6 AGPT Result

AGPT was performed for detecting the antigenic properties of LSDV under experiment, the treated virus with BV and the original viruses used in the experiment. This test showed that the antigenic properties were still intact, which indicated by attaching of BV inactivated LSDV to its specific antibodies and forming well defined and strong line of precipitate (Fig. 10). Other tested viable LSDV was give similar results. However, if BV has destructive effect on LSDV, this antigenic characteristic would diminish, and also the use of safe doses was key to maintain the antigenicity of inactivated LSDV by this method [20].

BV was used from long time ago in the medical field as treatment of chronic joint affections. In the recent decades, the screening process of new sources of antimicrobials discovers its high advantages characteristics for combating various types of microbes, as well as trials to discover its anti-cancer
In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus

Fig. 9  MCF7 cells treated with 0.5 ug/mL after 48 h (H&E ×20).

Fig. 10  The positive result of AGPT.

LSDV mixed with BV gave the strong line of precipitate.

medicinal fields. The viability of virus was tested in tissue culture cells lines and in embryonated chicken eggs. According to doses and time of exposure, BV pathological effects on Hep-2 cells (human larynx carcinoma) and MCF7 cells (breast carcinoma cell line) were observed, such as apoptosis, retarded growths and cytolysis. The results indicate the possibilities of using BV in cancer treatment fields and in vaccine preparations against LSDV, also in therapeutic preparations to treat LSD illness.

The control of LSD without vaccination is extremely difficult in endemic areas. In Egypt, cattle vaccinated against the disease by using Kenyan sheep pox culture vaccine could not stop the disease [21]. Accordingly, effective control of LSD requires not only rapid and accurate laboratory diagnosis supported by clinical findings [22], but also real solution focused on combating the LSDV by effective vaccination and treatment. In the present work, BV exhibited satisfied inhibitory effects on LSDV, meanwhile, the antigenic properties was still intact. Embryonated chicken eggs inoculation is considered the best recommended method required for LSDV isolation from the tissue specimens, followed by several passages in the specific tissue cultures [23]. BV contains various peptides, including mellitin, apamin, adolapin and enzymes as well as non-peptide components, such as histamine, lipid and carbohydrates [24-26]. Also, some components of BV can cause inflammation by inducing interleukin (IL)-1β via p38 mitogen activated protein kinase (MAPK), while others act as anti-inflammatory by suppressing inducible nitric oxide synthase (iNOS) and cyclooxigenase (COX)-2 via nuclear factor (NF)-κB. The naturally occurring peptides of whole BV have various pharmacological potencies, however, these components exhibit anti-inflammatory activity in inflammatory cells [27, 28]. The major components of honey BV are mellitin, phospholipase A2 (PLA2) and hyaluronidase [29]. The peptide mellitin comprises approximately 50% of BV [30]. BV has antibacterial, anti-parasitic and antiviral properties [31]. The PLA2 of honey bee and snake venoms have potent anti-human immunodeficiency virus (HIV) activities. These PLA2s block HIV-1 entry into host cells through a mechanism linked to PLA2 binding to cells [32]. The
Inhibitory effects of BV on LSDV could be owned to its enzymatic constituents, besides the cationic peptide mellitin which comprises approximately 50% of BV [30]. However, the PLA2s of honey bee have potent anti-human immunodeficiency virus (HIV) activities. These PLA2s block HIV-1 entry into host cells through a mechanism linked to PLA2 binding to cells [32]. Venoms induce a variety of immune response, including both acute inflammatory responses, such as mast cell degranulation, and adaptive immune responses, such as T helper type 2 responses and IgE production [33]. Venoms of honeybee (hymenoptera species) including the Apis mellifera, cause inflammation and allergy that succumbed to individual variations. BV injected inside vertebrate targets by stings leads to local inflammation in most instance, however the severe allergic response takes place in 3% of the population who are allergic to hymenoptera venoms [34-36]. Therefore, one could emphasize that the whole BV constituents play the synergistic actions role on its therapeutic effects as anti-neoplastic and antimicrobials.

4. Conclusions

From in vitro study and testing BV on cell lines, the safest dose is 0.5 ug/mL. However, the time of exposure is considered as the most important factor in regarding toxic effects of BV on living cells. Moreover, in vitro and in vivo conditions are different in many aspects, because in vitro are restricted to the given materials. While in vivo, the synergistic effects along with many internal factors play major roles that are included in the process of reactions to BV. The biological effects inside the living organism may react with higher doses than that determined in vitro, so theoretically the safe doses in vitro are lower than that could be used in vivo. The inhibitory effect of BV on LSDV was achieved in this work at very lower concentrations (0.5 ug/mL) and the antigenic characteristics were still intact. It is possible to apply BV for disinfecting health hazardous biological laboratory wastes. The results emphasized that BV may be good treatment for LSDV after determining the suitable therapeutic doses.

References

In Vitro Study on the Effect of Bee Venom on Some Cell Lines and Lumpy Skin Disease Virus


