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Investigation of long-term effect of Black Sea bee's venom on the cytotoxicity of pancreatic cancer cells

Karadeniz arısı zehrinin pankreas kanseri üzerinde uzun vadede sitotoksik etkisinin araştırılması

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Abstract

Bee venom is one of the potential natural mixtures to be used in therapies of a range of diseases. The cytotoxic effect of bee venom on a number of cancer cells has been revealed by different research groups. These *in vitro* studies revealing cytotoxicity of bee venomare designed to include serial concentrations of bee venom under various incubation times, followed by the application of cytotoxicity assays. Common approach in the field is to determine the most effective cytotoxic conditions within those experienced. Differentially, in this study it wasaimed to understand whether the cytotoxic effect of venom obtained from Black sea beehas been remained even if bee venom was removed after a while of incubation. For this aim, the conditions (including doses between 8-100 µg/ml and 24h incubation time) were determined in AR42J pancreatic cancer cells and the prolonged effect of bee venom under the determined concentrations was investigated. The findings showed that the cytotoxic effect of bee venom still continued in cells treated with bee venom once. This suggests that bee venom-induced cell death can be memorized by the cells somehow or/and bee venom may provoke a cascade of apoptosis occurring in a long time. This however

Özet

Arı zehri, çeşitli hastalıkların tedavisinde kullanılan doğal ürünlerden biridir. Arı zehrinin çok sayıda kanser hücresi üzerindeki sitotoksik etkisi, farklı araştırma grupları tarafından ortaya çıkarılmıştır. Arı zehrinin sitotoksik etkisini ortaya çıkaran bu *in vitro* çalışmalar, prensip olarak çeşitli inkübasyon sürelerinde farklı arı zehri konsantrasyonlarını kullanarak sitotoksisite analizlerinin uygulandığı çalışmalardır. Alandaki ortak yaklaşım, bu uygulanan şartlar içinde en etkili sitotoksik koşulları belirlemektir. Farklı olarak, bu çalışmada Karadeniz ırkı *Apis mellifera*'dan elde edilen zehrin sitotoksik etkisinin, arı zehri ile bir süre inkübasyondan sonra hücrelerden uzaklaştırılması halinde bile sitotoksik etkisinin devam edip etmediği sorusu üzerine odaklanılmıştır. Bu amaç doğrultusunda AR42J pankreas kanseri hücrelerinde en etkin koşullar (8-100µg/ml dozaralığıve 24 saatlik inkübasyon) belirlendi ve arı zehrinin belirlenen bu konsantrasyonlarda ortamda olmadan uzun sürelietkisinin varlığı araştırıldı. Bulgular, arı zehrinin sitotoksik etkisinin arı zehri ile bir kere muamele edilen hücrelerde dahi devam ettiğini göstermiştir. Bulgularımız arı zehri kaynaklı hücre ölümünün, popülasyondaki hücreler tarafından bir şekilde hafızaya alınabileceğini ya

needs further investigations.

da arı zehrinin uzun bir süre boyunca meydana gelen apoptozis yolağını tetikleyebileceğini göstermektedir. Ancak bunun sebebini tespit etmek için ileri araştırmalara ihtiyaç vardır.

Keywords: Bee venom, cytotoxicity, cancer, AR42J cells

Anahtar kelimeler:arı zehri, sitotoksisite, kanser, AR42J hücreleri

Abbreviations: BeeVenom, BV

1. INTRODUCTION

Alternative medicine is one of interests in the therapy of numerous diseases, and apitherapyis one of the alternative therapies referring the use of all the bee products such as pollens, honey, propolis and venom. In the development of new drug candidates, bee venom appears to be a valuable drug candidateespecially in cancer therapy. There is a line of evidence suggesting the significant effect of bee venom on the cytotoxicity of cancer cells (Jo et al., 2012; Park et al., 2011;Zheng et al., 2015).The current disadvantage of chemotherapeutics is to be lack of sensitivity during the therapy as having side effects on normal cells. These effects can unfortunately include death of healthy cells as well as cancerous ones or provocation of normal cells to be transformed that would participate in carcinogenesis later. Bee venom has been shown to kill the cancer cells selectivelythan normal cells (Tu, Wu, Hsieh, Chen, & Hsu, 2008; Zheng et al., 2015). But, to the best of knowledge, there has been no study investigating the cytotoxicity of bee venom on pancreatic cancer cells (AR42J cell), and also no study aiming to understand the long-lasting effect of bee venom on pancreatic cancer cells. In the present study, we first aimed to reveal the effective conditions of bee venom in terms of cytotoxicity of AR42J cells, then to reveal whether cell death pattern remained the similar or not after bee venom removed. The findings showed that bee venom induced dose-dependent cell death in AR42J cells, and the death trend maintained even after bee venom cells reached confluency. removed from the culture environment. This confirms that bee venom is the potent as a cancer therapeutic, and suggests that prolonged effect of bee venom can add pharmaceutic value to its potential.

2. MATERIALS AND METHODS

2.1. Cell culture: AR42J cells (Cat No 93100618) were purchased from ECACC (European Collection of Authenticated Cell Cultures). Cells were cultured in RPMI 1640 media (Sigma Aldrich, Cat No R8758) including 2mM glutamine, 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Diagnovum), and incubated at 37°C with 5% CO_2 humidification.

2.2. Collection of bee venom: Venom was collected from bees using electro-shock method. This method is more advantageous than surgical method since it does not harm bees. The electro-shock is applied in the low ampere that is enough for the bees leaving the venom. Attention is required to protect bee venom from air as it can undergo crystallization very quickly. Collections were immediately stocked in -20°C till use.

2.3. Bee venom treatment: Main stock of bee venom was prepared in 0.9% NaCl at 5mg/ml, sterile filtered and stocked at -20°C.Cells were treated with bee venom at 8, 12, 25, 50 and 100µg/ml for 24 hours at 37°C with 5% CO_2 after Some cells were untreated. Cells were visualized using the camera of AxioVert A1 inverted microscope (Zeiss, Germany).

2.4. Cell viability by trypan blue exclusion method: Cells were collected via 2000 rpm centrifugation after bee venom treatment for 24h. Supernatant removed, and cells were washed once with 1x PBS (phosphate-buffered saline) (Wisent, 311-010-CL) then were resuspended in 1ml of sterile 1x PBS. 10µl of cell suspension was mixed with 10µl of 0.4% trypan blue dye (Biological Industries, B103-102-1B) and incubated for almost 10 minutes at room temperature. Rest of the cells was cultured again in a fresh media without bee venom. The cell and dye mixture was loaded to the slide of the instrument and cell viability was detected using Countess FL II Automated cell counter (Thermofisher). The standard errors of the viabilities (S.E. +/-1) were calculated using SPSS software. All experiments were performed as at least three independent replicates.

The morphology of cells was altered with bee venom dose **(Figure 1)**. Untreated cells were rounded-shape and tend to grow as clusters. But BV treatment induced smaller cell size, and location of cells apart from each other.This could be also because of that a proportion of cells died and already detached from the surface until the images were taken. The evaluation of cell viabilities revealed that bee venom induceda dose-dependent cell death after 24h incubation **(Figure 2A)**. Afterthis period, BV was removed from the cells,washed and cultured again in fresh media without BV.Cells were collected and viabilities were detected after 24h **(Figure 2B)**, 48h **(Figure 2C)** and 72h **(Figure 2D)**.

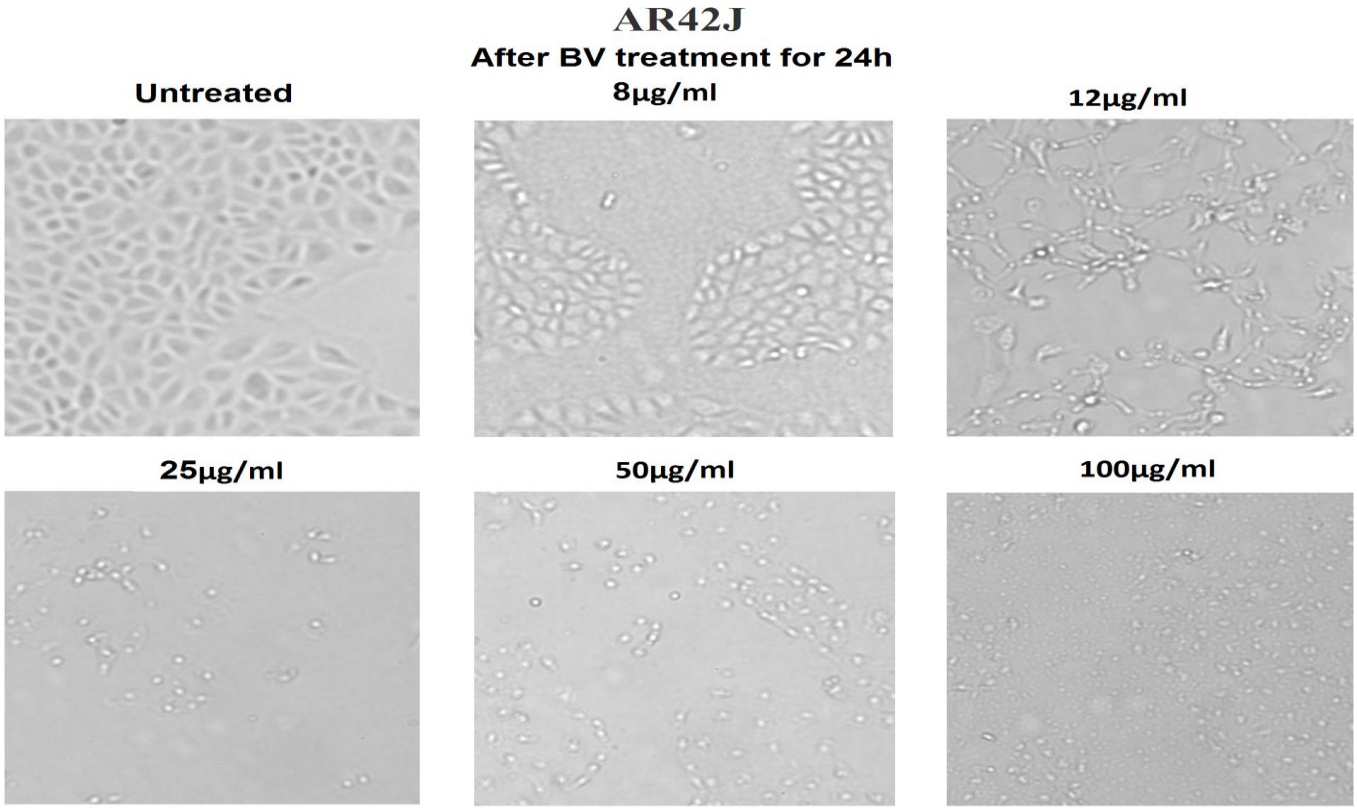

Figure 1. Morphological assessment of AR42J cells after bee venom

The cells during extended periods without bee venom incubation showed a similar cell death pattern **Figure 2B-D),** and cellular morphology maintained as after BV. Cells after 72h were shown as representatives **(Figure 3)**.

Bee products are important supplements to enhanceimmune system in adults and children. In addition to facilitate self-defense of the body, these are of interest in research with the potential use for therapies of some diseases, such cancer. Although the favorite ones are honey and propolis,bee venom is also a compoundthat can be used in the therapy with the rising importance.

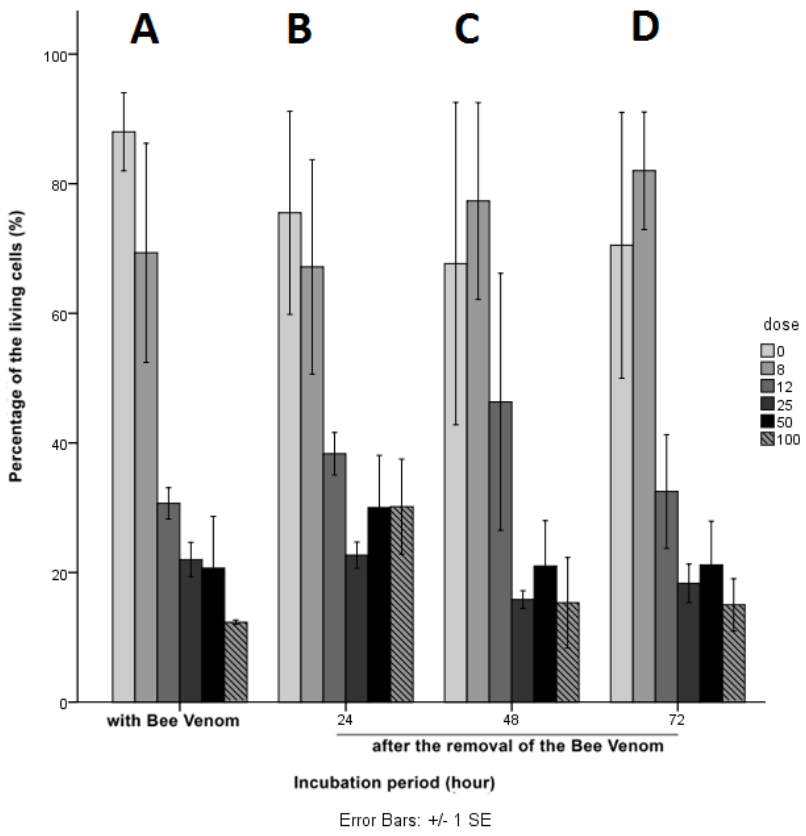

Figure 2. Percentage of live AR42J cells (%) after bee venom for 24h (A), and after another 24h (B), 48h (C) and 72h (D) without bee venom.

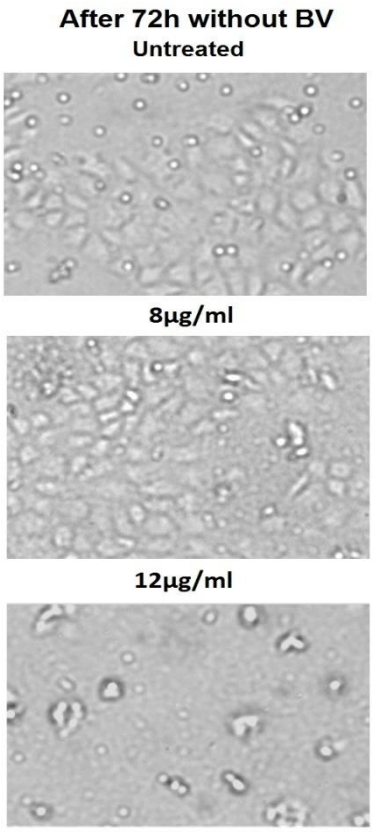

Figure 3. Morhology of AR42J cells after 72h without bee venom

Cancer, the most second cause of death around the world after cardiovascular diseases, is currently treated with synthetic chemotherapeutics. Even if survival rates can improve with the use of these chemotherapeutics depending on the stage of diagnosis etc., these agents have serious side effects on patients. At molecular level, the complication includes the harmful effects of those on normal cells.

Bee venom is a natural compound and its content can vary according to the subspecies of bee, geography the bee lives and seasonal fluctuations (Danneels, Van Vaerenbergh, Debyser, Devreese, & de Graaf, 2015). The major compound of bee venom is melittin and it has been shown to be the major factor inducing cytotoxicity in cancer (Jamasbi et al., 2018; Yang, Ke, Xu, & Peng, 2007). This study does not address which compound of bee venom induced this long-lasting cytotoxic effect on pancreas cancer cells, but a whole compound. This requires a detailed further investigation. However, to the best of knowledge,

this study is the first to report persistent cytotoxic effect of bee venom even after bee venom was removed for 72h. Previous studies revealed cytotoxicity profiles of cells immediately after bee venom (Choi et al., 2014; Ip et al., 2008; Jang et al., 2003; Jo et al., 2012). It has been known that bee venom can induce apoptotic pathways in cancer cells (Jang et al., 2003; Moon et al., 2006; Zheng et al., 2015). But there is no evidence in cellular memory of cell death for further cell populations as cells continue to undergo apoptosis after bee venom introduced once to cells. Removal of bee venom did not change the death profile of pancreatic cancer cells.

Pancreas cancer is one of the common cancers with the reduced survival rate and late diagnosis, and the current study suggests that bee venom can have a potential for the treatment of pancreas cancer. But these preliminary data should be detailed and repeated *in vitro* followed by *in vivo* approaches. In principle, a cell cycle is completed within 24 hours *in vitro*. It means that a cell decides on living as divided to daughter cells or die if any unfixed problems occurred within the cell or/and an external condition may induce cell to undergo apoptosis. However, we have found that cells continued to die up to 72 h after bee venom removed from the cells. These can suggest an additional value of bee venom to maintain the cancer cells at low level after treatment is complete. The results should be compared with a common chemotherapeuticsin use.

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