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A Preliminary Study: The Selective Effect of Bee Venom on Inhibition of Cell Migration in Metastatic Breast Cancer Cells Over Normal Cells in Comparison with Cisplatin

Ön Bir Çalışma: Arı Zehrinin Sisplatin ile Karşılaştırıldığında Normal Hücrelere Göre Metastatik Meme Kanseri Hücrelerinin Hücre Göçünün Inhibisyonu Üzerindeki Seçici Etkisi

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Abstract

Bee venom is considered as a candidate alternative medication in the treatment of some diseases. Bee venom has *in vitro* selective cytotoxic effect on some cancer cells examined in previous studies, but the mechanism of action has not been fully elucidated. To the best of knowledge there is no study showing migration rate of metastatic breast cancer cells after bee venom treatment. This study aimed (i) to reveal the metastatic behaviour of metastatic breast cancer cells after bee venom treatment compared to cisplatin treatment, and (ii) also to determine the differences in chemosensitivity between normal and cancer cells in terms of migration after bee venom and cisplatin treatments. Following bee venom treatment, would healing assay was used for revealing the migration rate of cells *in vitro*. Findings showed that bee venom was more effective on selective inhibition of

Özet

Arı zehri bazı hastalıkların tedavisinde etkili bir alternatif ilaç adayı olarakortaya çıkmaktadır. Arı zehri, bazı kanser hücreleri üzerinde *in vitro* seçici sitotoksik etkiye sahiptir, ancak etki mekanizması tam olarak aydınlatılmamıştır. Bilindiği kadarıyla, arı zehri muamelesinden sonra metastatik meme kanseri hücrelerinin göç davranışını (hızını) gösteren bir çalışma yoktur. Bu çalışmada, (i) arı zehri muamelesi sonrası metastatik meme kanseri hücrelerinin sisplatin muamalesine göre metastatik davranışının ortaya çıkarılması ve (ii) arı zehri ve sisplatin muameleleri sonrası migrasyon açısından normal ve kanser hücreleri arasındaki farklılıkların ortaya konması amaçlanmıştır. Arı zehrinden sonra, yara/lezyon iyileştirme (wound healing) deneyi, *in vitro* ortamda hücrelerin göç davranışını ortaya çıkarmak için kullanıldı. Bulgular, arı zehrinin kanser hücresi göçünün

cancer cell migration, but cisplatin-mediated inhibitory effect on metastasis could not be detected as even low concentrations of cisplatin induced high rate of cell death. This study concludes that bee venom has selective anti-metastatic properties than cisplatin in metastatic breast cancer cells. The preliminary findings need to be expanded to cover more cell lines and the mechanism for this effect should be disclosed by detailed molecular methodologies in future.

Keywords: Apitherapy, Cancer, Cell Migration, Wound Healing Assay

seçici inhibisyonunda daha etkili olduğuna işaret etmektedir. Ancak normal hücrelerde sisplatin düşük konsantrasyonlarda dahi yüksek sitotoksisite göstermiştir. Bu yüzden sislatinin hücre göçüne etkisi normal hücrelerde tespit edilemedi. Bu çalışma, arı zehrinin sisplatine göre seçici olarak metastatik meme kanseri hücrelerinde anti-metastatik etki gösterdiğine işaret etmektedir. Bu çalışmadaki öncü bulguların daha çok hücre serisinde denenerek genişletilmesi ve bu etkinin mekanizması da gelecekte yapılacak detaylı moleküler metotlarla açıklanmalıdır.

Anahtar kelimeler: Apiterapi, Kanser, Hücre Göçü, Yara İyileşmesi Deneyi

Abbreviations: BV, Bee venom; MEF, mouse embryonic fibroblasts; WH, wound healing; CIS, cisplatin; a.u; arbitrary unit

1. INTRODUCTION

Bee venom is one of the natural drug candidates and considered as an effective medication for alternative therapies for a range of diseases such as rheumatoid arthritis, pain, cancers and skin diseases (Roy & Bharadvaja, 2020). The effects of bee venom and its components (such Melittin) on cytotoxicity of cancer cells have been shown previously (Duffy et al., 2020; Rady et al., 2017). Ideal cancer therapy is aimed to have a specific cytotoxic effect on cancer cells only with minimum or null effect on normal cells. The design of candidate therapies is also focused on controlling metastatic, invasive and angiogenic characteristics of cancer cells. Uncontrolled cell proliferation, metastasis and angiogenesis are the common hallmarks of cancer cells so that these mechanisms are the targets for drug discovery. Cancer cells tend to migrate to neighboring tissues and distant tissues within the body by the processes called ‘invasion’ and ‘metastasis’. There is a group of genes defined as metastasis-suppressing genes that have the roles in preventing invasion of cells (Horak et al., 2008). The potential of bee venom for prevention of cell migration in cancer has not been fully clarified.

Wound healing (WH) assay (scratch assay) is commonly used to show healing properties of skin defects (Han et al., 2011) so that it is a conventional method to assess the healing effect(s) of candidate agents designed especially for cosmetic purposes (Maeng et al., 2018; Poulose et al., 2020). This assay can also be used for understanding the invasive movement of cancer cells *in vitro* (Grada et al., 2017). Although the effect of bee venom on healing of diabetes-induced wound was shown by WH assay (Badr et al., 2016; Hozzeinab et al., 2018), it has not been used in cancer cells treated with bee venom. There is a limited data on the variation of the composition of bee venom in seasonal and regional environment (Junior et al., 2010) whereas the composition of other bee products such pollen, honey and propolis highly depend on

environmental conditions (Souza et al., 2016, Valencia et al., 2012). This makes bee venom a more advantageous agent to standardize its significant biological effect in biomedical and pharmacological studies.

2. MATERIALS AND METHODS

2.1. Cell Culture

Cells used were i) MDA-MB-231 metastatic breast cancer cells and ii) primary mouse embryonic fibroblasts (MEFs). MDA-MB-231 and MEF cells were cultured in RPMI and in DMEM media, respectively, including 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C incubator humidified with 5% CO_2. Cells were seeded into 6-well plates (200.000 and 400.000 cells per well for MDA-MB-231 and MEF cells, respectively). Cells were orbitally shaked well upon seeding to allow cells equally dispense alongwith the surface before settling down. Cells used were at passages 8-9.

2.2. Wound Formation

After cells reached at full confluency in culture, cells were wounded using a sterile ruler and a sterile 100p pipette tip through the midline of well. Microscopy images were taken immediately after cells were wounded (these images recorded as 0h incubation). The sizes of wounds in microscopy images (as centimeters) were representatively shown by measurement tool of Microsoft power point.

2.3. Bee Venom/Cisplatin Treatment

Bee venom (BV) was obtained from *Apis mellifera* in Black Sea region of Turkey by Zonguldak Beekeepers Association to Prof Sevgi Kolaylı (Department of Biochemistry, Karadeniz Technical University). BV was weighed and dissolved in 0.9% NaCl at 5 mg/ml as a main stock. Cells were treated with bee venom (8, 12, 16, 18 and 20µg/ml) or cisplatin (from Koçak Farma 10 mg/20mL) (final concentrations in media as 8, 12, 25, 50 and 100 µg/ml) for 3, 24, 48 or 96h after cells were wounded (these concentrations are final concentrations prepared within the culture media). Control cells were untreated for each agent (0 µg/ml). The concentrations were selected according to our previous study (unpublished yet) that focused on the determination of cytotoxic doses of BV which included 8, 12, 25, 50 and 100 µg/ml (unpublished data). The previous findings declared the IC50 value of BV for metastatic breast cancer cell line which was 8 µg/ml. But in this study, lower concentration intervals (8-20 µg/ml) were used to keep cells alive much possible to understand their movement to heal the wound.

2.4. Microscopic Evaluation and Area Calculation for Wound Healing (WH)

Images of the cells cultured at each concentration of bee venom or cisplatin for each incubation time point were captured using 5x objective of AxioVert A1 inverted microscope (Zeiss, Germany). Two different regions were captured alongside the wound for each treatment to realize

the variability on the wound area. Wound area represented by arbitrary units (a.u) was calculated using Image J software (NIH, US). All experiments were performed at least three times independently. This should be noted that the cell viability was not analyzed after wound formation.

2.5. Statistical Analysis

Area (represented by a.u) was analyzed using Post-Hoc test of UNIANOVA (Univariate analysis of variance) analysis by SPSS Software (version 23). Standard error of the mean (+/-) was calculated for each treatment to understand variability between independent replicates. Significance levels used were $p<0.0001$ (****), $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*).

3. RESULTS AND DISCUSSION

3.1. Comparison of the Effects of Bee Venom and Cisplatin on Wound Healing of Cancer Cells

The wound was almost healed at 48h in untreated (dose 0 µg/ml) MDA-MB-231 cells **(Figure 1)**. Significant healing was induced after bee venom (18 µg/ml) treatment for 24h, healing process continued at 48h **(Figure 1A)**. However, all the cells after cisplatin (at 50 and 100µg/ml) were not viable resulting in no measurable wound area. After 48h incubation, cells treated with BV was still in the healing process **(Figure 1A)**. But cisplatin (25µg/ml) after 48h was not conclusive for healing as it resulted in cell death. Nevertheless, MDA-MB-231 cells had a more tendency to heal the wound after bee venom at the common dose used for cisplatin as well (12µg/ml) for 48h **(Figure 1B**, see also **Figure 7)**. Figure 2 shows representative images of wound healing properties in MDA-MB-231 cells after bee venom. Significant wound healing was started at 24h and continued at 48h (by 12µg/ml) **(Figure 2)**. There was a technical obstacle to analyze cells after cisplatin at 25 µg/ml as majority of cells appeared in rounded (epithelial-like) cytoplasmic morphology (shown by dashed arrow in **Figure 3**) indicating activated cell death following cell detachment. (Both cells used in this study are in fibroblastic morphology).(Flake et al., 2013). Therefore, it was not possible to access "wound healing" properties of cisplatin on cancer cells. This suggested that cisplatin was more effectively cytotoxic on cancer cells (but also healthy cells, see below) than bee venom was. One of the hallmarks of cancer cells is uncontrolled proliferation of the cells and another hallmark is to gain ability to invade to other tissues. Controlling proliferation is possible with the use of cytotoxic agents, but a challenge stands on that almost all chemotherapeutics also have cytotoxic effect on normal cells. In cancer therapy, metastatic potential of cancer cells appears to be the main problem. To deal with invasiveness of cancer cells, a group of drugs are designed to prevent metastasis and these drugs are called as "migrastatics" (Gandalovicova et al., 2017). Findings pointed out that bee venom is of great importance to be thought as a migrastatic.

3.2. Comparison of the Effects of Bee Venom and Cisplatin on Healing of Normal Cells

Wound in the culture of untreated normal cells (MEFs) was fully healed at 48h, but MEF cells treated with BV at lower concentrations (8 or 12µg/ml) for 48h started to significantly heal **(Figure 4A)**. At 96h, healing was not complete (data not shown). Wound was not healed at 48-hour incubation with cisplatin **(Figure 4B)**, but the cells did look to undergo senescence or apoptosis. This suggests that cisplatin is more effective on cytotoxicity of normal cells than cancer cells. In our unpublished data, it was shown that normal cells rescued from cell death after bee venom treatment more likely than cisplatin treatment suggesting that bee venom has a higher cytotoxic selectivity for cancer cells. Melittin within bee venom was shown to induce selective cell death for cancer cells before (Rady et al., 2017). One of the mechanisms for melittin-induced selective cytotoxicity was driven by the inhibition of HER2 (human epidermal growth factor receptor 2) and EGFR (epidermal growth factor receptor) (Duffy et al., 2020). The significant increase in HER2 is common in metastatic breast cancer cells so that this is a fashionable target for drug discovery in cancer therapy. However, the current study did not examine the anti-invasive effects of individual components of bee venom but as a whole mixture.

The results are quite consistent with the previous findings that showed that even low concentration of BV (8µg/ml) was cytotoxic for MDA-MB-231 cells but was cytotoxic after 50µg/ml in normal cells (unpublished data). This suggests that the selective cytotoxic effect of bee venom on metastatic breast cancer cells can be also observed for its significant effect on preventing cell invasion and metastasis. On the other hand, the previous study showed that lower concentration (8µg/ml) of cisplatin was toxic for normal cells as well as for MDA-MB-231 cells (12µg/ml) (unpublished data from the thesis) (Tetikoglu, 2020). The highly toxic property of cisplatin resulted in a trouble while optimizing concentrations for WH assay.

The statistical comparisons of wound area (by a.u) between cancer cells and normal cells after bee venom and cisplatin are given in **Tables 1 and 2,** respectively. *p* values less than 0.5 were considered as significant. In general, dose and incubation considered as individual or combined (dose + hour) parameters resulted in significant changes in wound area after bee venom or cisplatin. Combined effect of three parameters (incubation/hour, dose, and cell) was significant after cisplatin (**Table 2**, $p<0.0001$) but not after bee venom treatment (**Table 1**, $p>0.05$).

The comparisons between bee venom and cisplatin treatments in MDA-MB-231 and MEF cells are given in **Figure 7**. Wound area was compared after the same doses used for both agents. Untreated MDA-MB-231 and MEF cells behaved similarly for wound healing ('cell + hour' not significant in **Table 3**), but cells treated with 8 µg/ml bee venom or cisplatin did show significantly different behaviour on wound healing ('cell + agent + hour' $p<0.0001$ in **Table 4**). However, the considerable difference was

observed after the treatments with 12 µg/ml agents (right panel, **Figure 7**). After 12 µg/ml of bee venom or cisplatin treatment for 48h, gap area was significantly open in cancer cells (**Table 5**, parameters 'cell + hour' $p<0.0001$), which was consistent with that there was no considerable difference in the healed area of wound between bee venom and cisplatin treatments in cancer cells (**Table 5**, 'agent + hour', $p>0.05$).

Table 1. *p* values between parameters effecting wound area after bee venom in MDA-MB-231 metastatic breast cancer cells and normal MEF cells (R Squared = 0.773, Adjusted R Squared = 0.744)

Individual or combined parameters	*p* value (Significance level)
Dose (µg/ml)	0.000 (****)
Incubation (hour)	0.000 (****)
Cell line (type)	0.000 (****)
Dose+incubation (hour)	0.000 (****)
Dose+cell type	0.007 (**)
Incubation (hour)+cell type	0.000 (****)
Dose+Incubation (hour)+cell type	0.216 (not sig)

$p<0.0001$ (****), $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*)

This suggests the similar anti-invasive effect of bee venom and cisplatin on both cancer cells. The wound area observed in MEF cells was significantly healed after bee venom treatment (12 µg/ml at 48h) **(Figure 5)** but wound healing assay could not be conclusive in normal cells as they were induced to apoptotic morphology after cisplatin (12 µg/ml at 48h) **(Figure 6)**, and not available cells found to be measured. Together with the previous findings of this study and literature, all findings conclude that bee venom is a cytotoxic agent as effective on cancer cells as cisplatin, and that has also considerable anti-metastatic potential. Therefore, bee venom appears to be more advantageous for selective anti-proliferative and anti-migrative functions on cancer cells over normal cells, whereas cisplatin does have a low level of or null selectivity.

Table 2. *p* values between parameters effecting wound area after cisplatin in MDA-MB--231 metastatic breast cancer cells and normal MEF cells (R Squared = 0.939, Adjusted R Squared = 0.924)

Individual or combined parameters	*p* value (Significance level)
Dose (µg/ml)	0.000 (****)
Incubation (hour)	0.000 (****)
Cell line (type)	0.000 (****)
Dose+incubation (hour)	0.000 (****)
Dose+cell type	0.000 (****)
Incubation (hour)+cell type	0.000 (****)
Dose+Incubation (hour)+cell type	0.000 (****)

$p<0.0001$ (****), $p<0.001$ (***), $p<0.01$ (**) and $p<0.05$ (*)

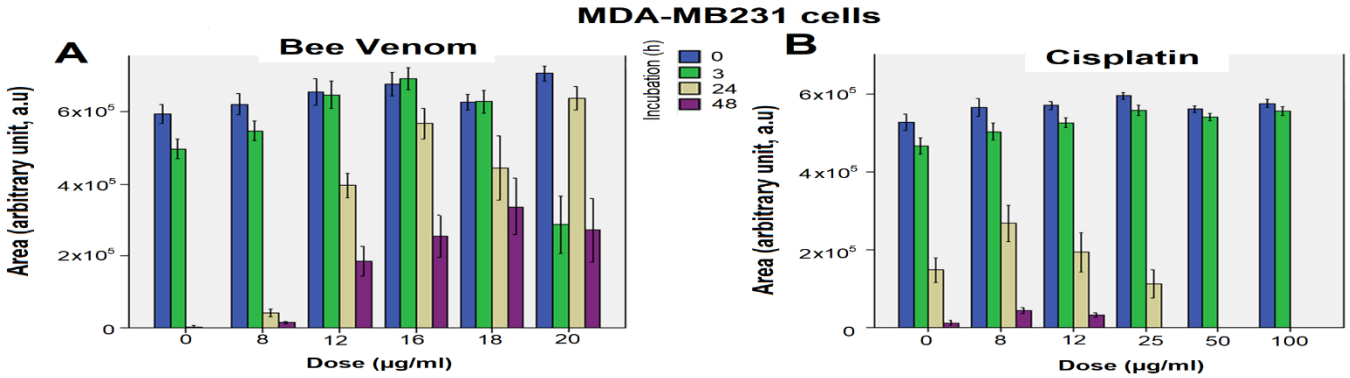

Figure 1. Wound area (arbitrary unit, a.u) after bee venom (0, 8, 12, 16, 18 and 20µg/ml at 0, 3, 24 and 48h) **(A)** and cisplatin (0, 8, 12, 25, 50 and 50µg/ml at 0, 3, 24 and 48h) **(B)** in MDA-MB-231 metastatic breast cancer cells. Standard error of the mean represented by +/- s.e. UNIANOVA test was used for statistical analysis.

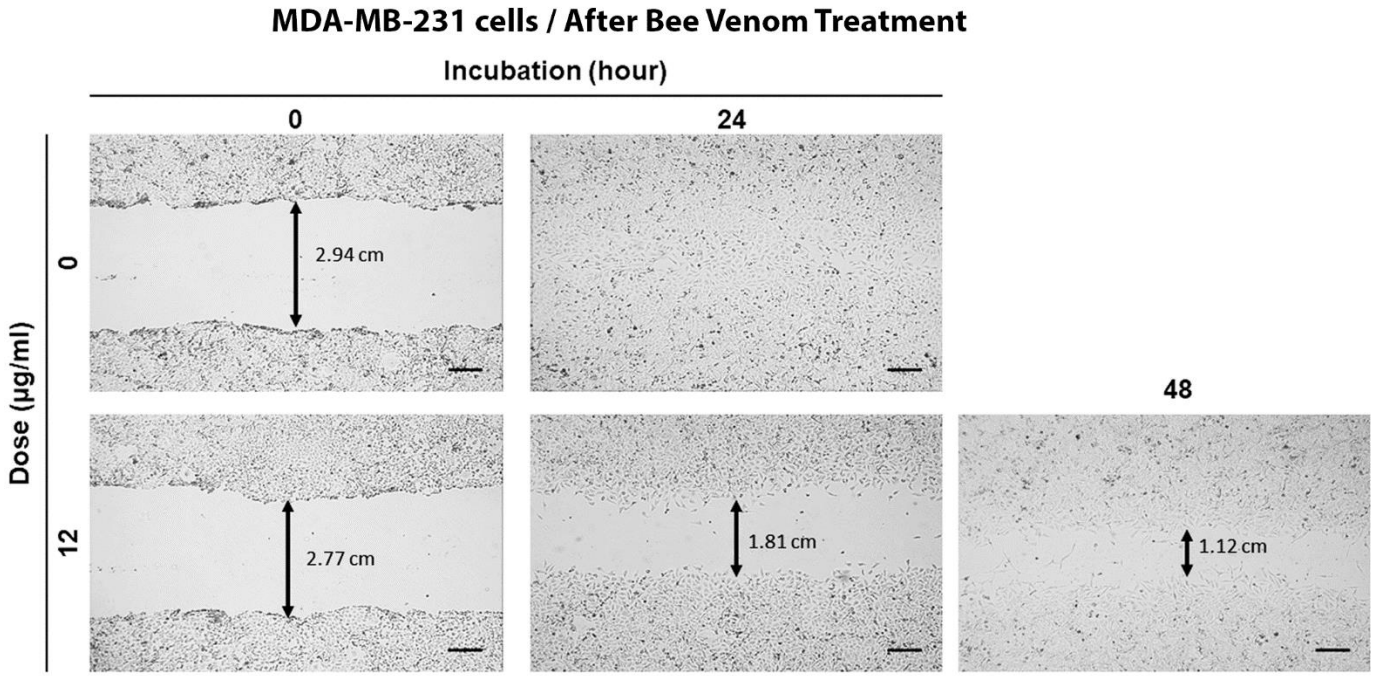

Figure 2. Representative microscopic images of MDA-MB-231 cells (with representative areas of wounds) after bee venom treatment (12µg/ml) compared to untreated cells at 0, 24 and 48 h. For untreated cells (0µg/ml), image was not shown at 48h as cells at 48h were the same as they were at 24h. Scale bar is 50 micron.

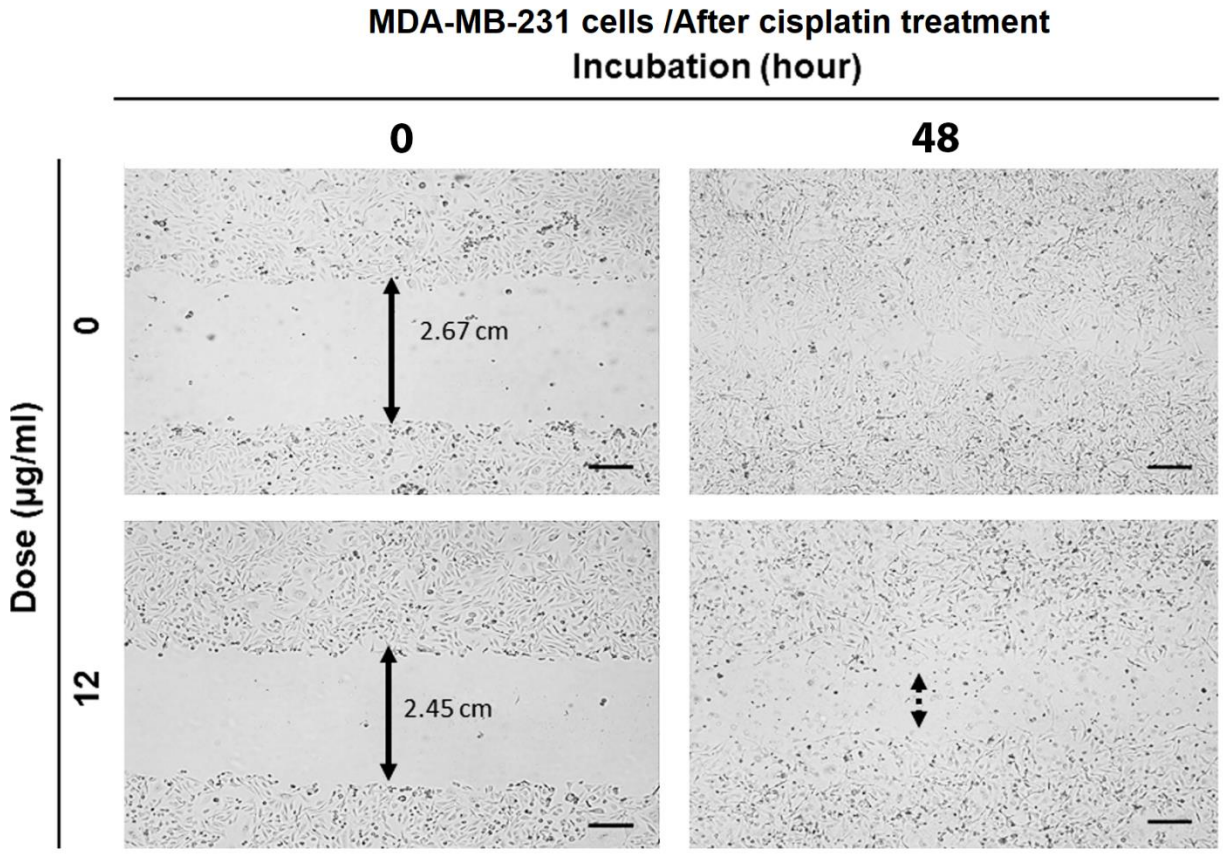

Figure 3. Representative microscopic images of MDA-MB-231 cells (with representative areas of wounds) after cisplatin treatment (12µg/ml) compared to untreated cells at 0 and 48 h. Treated cells showed apoptotic morphology in round shaped at 48h so that calculations for wound healing was not available (shown by dashed arrow). Scale bar is 50 micron.

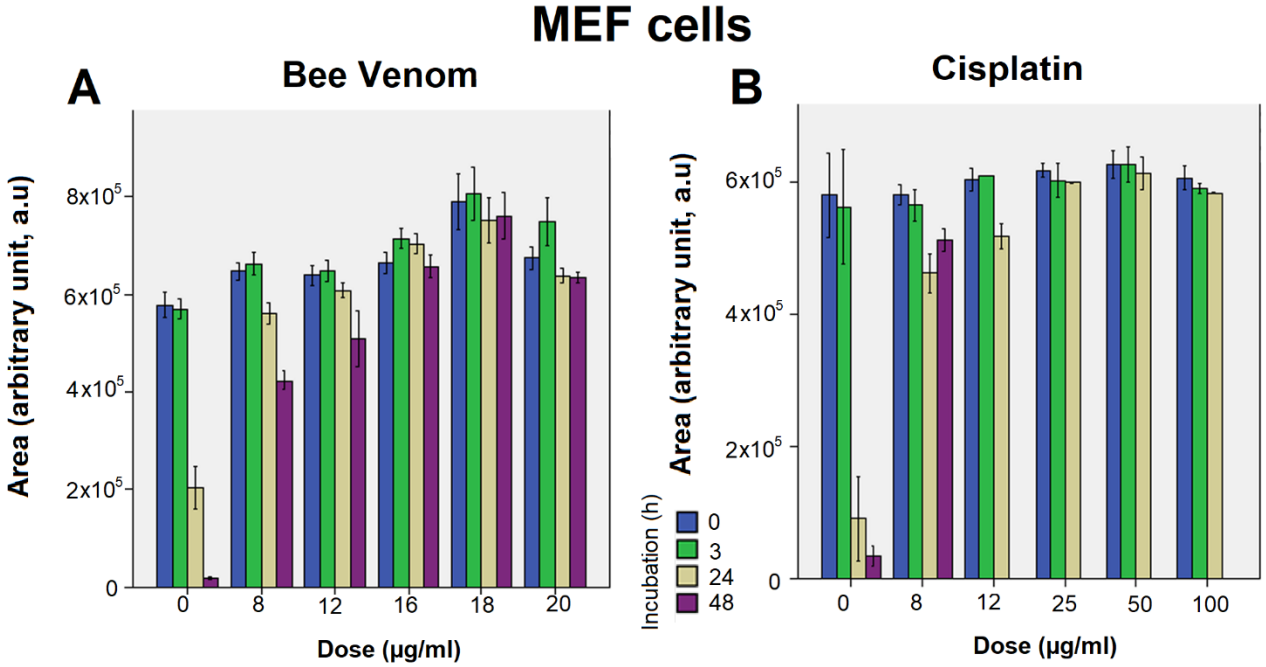

Figure 4. Wound area (arbitrary unit, a.u) after bee venom (0, 8, 12, 16, 18 and 20µg/ml at 0, 3, 24 and 48h) **(A)** and cisplatin (0, 8, 12, 25, 50 and 50µg/ml at 0, 3, 24 and 48h) **(B)** in normal mouse embryonic fibroblast (MEF) cells. Standard error of the mean represented by +/- s.e. UNIANOVA test was used for statistical analysis.

The findings need to be further investigated for extended number of cell lines including the comparisons within the same organism to draw general conclusion. Use of cells from different organisms can lead to misinterpretation as cell ontology differs even if the genomes of organisms (such mouse and human cells used in the present study) are almost identical (Yue et al., 2014).

The main finding of this study suggests that bee venom likely has selective anti-metastatic properties than cisplatin in metastatic breast cancer cells. This is parallel with selective cytotoxic effect of bee venom which was more potent than cisplatin as previously shown in our unpublished data. But one of the main technical problem in this study was that cytotoxic doses for cisplatin can not give a hint about anti-metastatic activity. Therefore, experimental optimization should be designed to determine the most effective dose interval for wound healing with the least cytotoxic activity.

This study aimed to preliminarily reveal whether bee venom treatment inhibits invasive characteristics of metastatic breast cancer cells, and to compare the possible inhibitory effect with normal fibroblast cells. The study also included comparative analysis of cancer/normal cells after treatment with a common chemotherapeutic agent, cisplatin. Findings of this study show that metastatic breast cancer cells healed the wound on culture vessel after bee venom treatment much slowly than cisplatin treatment, and also showed that normal cells migrated more slowly after bee venom but cisplatin-induced healing in normal cells was not conclusive as it was highly cytotoxic to normal cells (at 48h with the same concentration, 12µg/ml) compared to bee venom. These preliminary results suggest that bee venom potently inhibit migration of a highly aggressive form of breast cancer cells, but this needs further investigation for revealing the mechanism of action using high-throughput assays, and the findings should be enhanced using different cell lines derived from cancers and normal tissues.

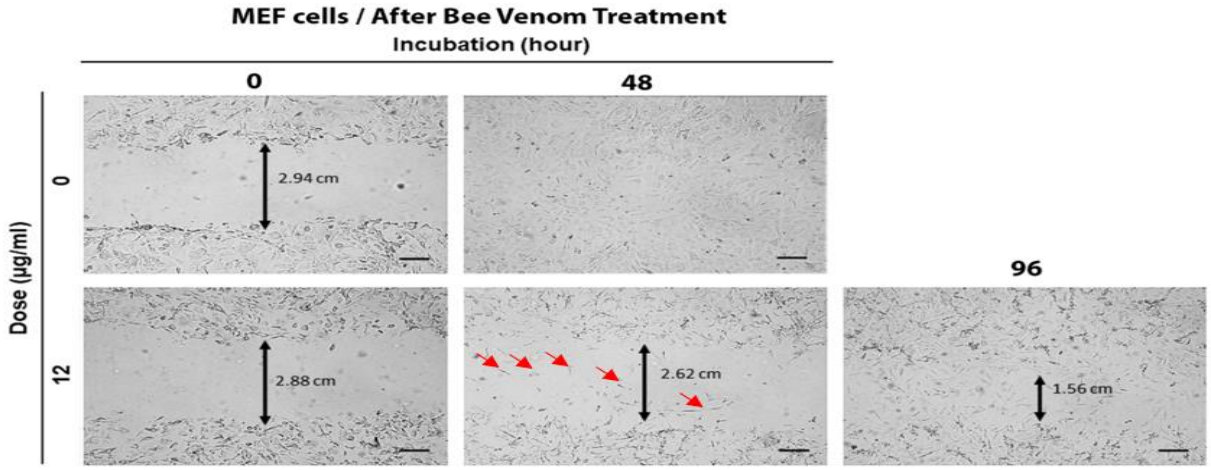

Figure 5. Representative microscopic images of MEF cells (with representative areas of wounds) after bee venom treatment (12µg/ml) compared to untreated cells at 0, 48 and 96 h. Healing was about to complete after 96h incubation with bee venom. Untreated cells (0 µg/ml) at 96h were similar to the cells at 48h (not shown). Scale bar is 50 micron. Red arrows indicate cells moved to midline of the wound area and probably proliferating so that 2.62 cm does not represent the entire area unit.

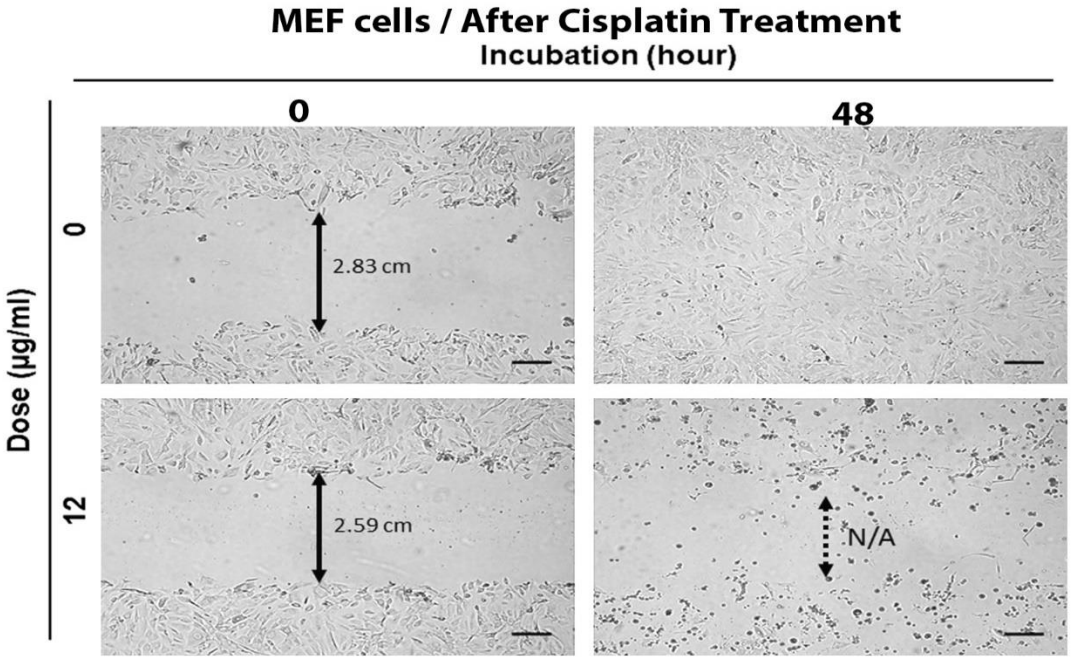

Figure 6. Representative microscopy images of MEF cells (with representative areas of wounds) after cisplatin treatment (12µg/ml) compared to untreated cells at 0 and 48h. Cells at 48 h after cisplatin treatment seemed to undergone senescence or apoptosis so that wound can not be calculated. Scale bar is 50 micron. N/A: not available cells to be detected.

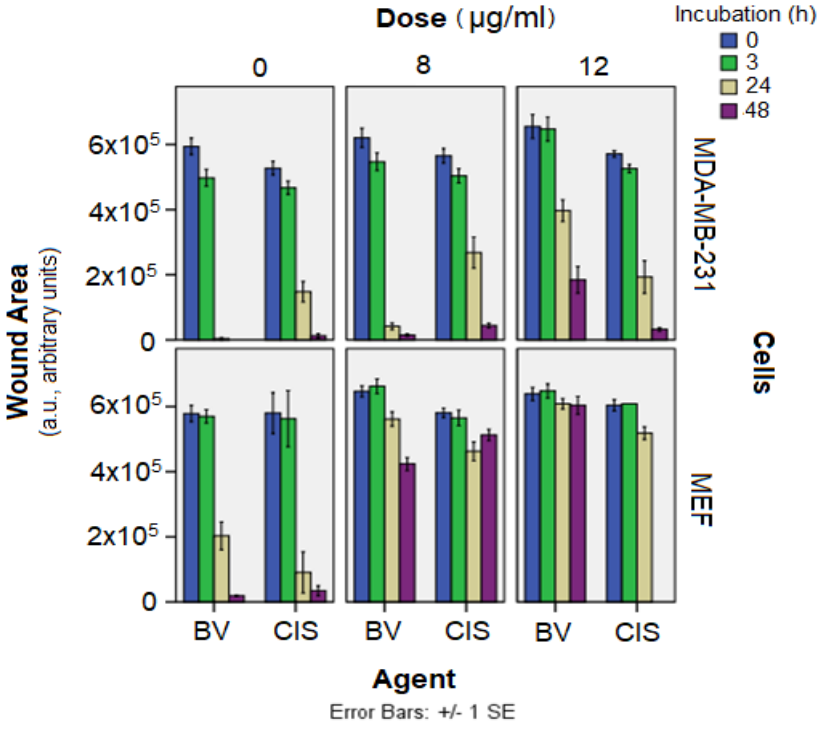

Figure 7. Comparative graphs between wound area (arbitrary unit, a.u) after bee venom (BV) and cisplatin (CIS) at the same doses (0, 8 and 12µg/ml) and at the same incubation times (0, 3, 24 and 48h) within and between MDA-MB-231 metastatic cancer cells and normal mouse embryonic fibroblast (MEF) cells. Standard error of the mean represented by +/- s.e. UNIANOVA test was used for statistical analysis.

Table 3. *p* values between parameters effecting wound area (cell type and incubation time) in untreated control cells (R Squared = 0.920 (Adjusted R Squared = 0.907).

Individual or combined parameters	*p* value (Significance level)
Cell line	0.006 (**)
Incubation (hour)	0.000 (****)
Cell line+incubation (hour)	0.603 (not sig.)

p<0.0001 (****), *p*<0.001 (***), *p*<0.01 (**) and *p*<0.05 (*)

Table 4. *p* values between parameters effecting wound area (cell type, agent type and incubation time) after dose 8 µg/ml (R Squared = 0.956 (Adjusted R Squared = 0.950).

Individual or combined parameters	*p* value (Significance level)
Cell line	0.000 (****)
Agent	0.939 (not sig.)
Incubation (hour)	0.000 (****)
Cell line+Agent	0.004 (**)
Cell line+incubation (hour)	0.000 (****)
Agent+incubation (hour)	0.000 (****)
Cell line+agent+incubation (hour)	0.000 (****)

p<0.0001 (****), *p*<0.001 (***), *p*<0.01 (**) and *p*<0.05 (*)

Table 5. *p* values between parameters effecting wound area (cell type, agent type and incubation time) after dose 12 µg/ml (R Squared = 0.910 (Adjusted R Squared = 0.896).

Individual or combined parameters	*p* value (Significance level)
Cell line	0.000 (****)
Agent	0.000 (****)
Incubation (hour)	0.000 (****)
Cell line + Agent	0.124 (not sig.)
Cell line+incubation (hour)	0.000 (****)
Agent+incubation (hour)	0.418 (not sig.)
Cell line+agent+incubation (hour)	0.862 (not sig.)

p<0.0001 (****), *p*<0.001 (***), *p*<0.01 (**) and *p*<0.05 (*)

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