Bee venom induced the interaction of phosphorylated histone variant, γH2AX, and intracellular location of beta actin in cancer cells

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Research Article

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Abstract

Bee venom is a natural compound and candidate anti-cancer agent with selective cytotoxic effect on some cancer cells. However, the cellular mechanisms of how bee venom selectively targets cancer cells remain elusive. The aim of this study was to reveal the genotoxic effect of bee venom in concordance with the location of β-actin protein throughout nucleus or/and cytoplasm. For this aim, the level of H2AX phosphorylation (γH2AX) and intracellular location of β-actin were assessed by immunofluorescence in liver (HEPG2) and metastatic breast (MDA-MB-231) cancer cell lines compared to normal fibroblasts (NIH3T3) after bee venom. Co-localisation of γH2AX and β-actin were also analysed. The results showed that the levels of γH2AX staining decreased in normal cells but increased in cancer cells. Majority of β-actin was localised within the cytoplasm of normal cells after bee venom, but it was mostly accumulated within the nucleus in cancer cells. Co-localisation of β-actin and γH2AX both in nucleus and cytoplasm was induced in each cell by different patterns. The results showed that normal and cancerous cells had different responses against bee venom, and suggested that bee venom induced a cellular response by the interaction between γH2AX and β-actin.

1. Introduction

Alternative medicine, also called complementary medicine, is defined as the use of natural plant- or animal-derived products for the treatment of various diseases. Apitherapy is one of the alternative medicine approaches that uses bee products *e.g.* bee venom (BV) for therapeutic purposes. A range of *in vitro* and *in vivo* studies have revealed that BV has analgesic, anti-inflammatory, anti-microbial, anti-viral and anti-cancer activities (Son et al., 2007; Uddin et al., 2016). Its anti-cancer activity has been shown in various cancer cells including prostate, breast, lung, liver, skin and bladder cancers by induction of cell death and growth arrest (Orsolic, 2012; Tu, Wu, Hsieh, Chen, & Hsu, 2008). BV is composed of a range of enzymes, peptides and amines including melittin and phospholipase A2, apamin, histamine, dopamine, norepinephrine, adolapine, and mast cell-degrading peptides (Huh et al., 2010; Lariviere & Melzack, 1996; Son et al., 2007). These components of bee venom can affect cancer cells with different cellular pathways. For example, it has been shown that bee venom induced cell death in glioblastoma cells by suppressing the effect of matrix metalloprotease-2 enzyme (Sisakht, 2017). In addition, phospholipase A2 found in bee venom (Doery & Pearson, 1964; Putz et al., 2007; Sobotka et al., 1976) selectively acts on cancer cells with damaged membranes, but cannot penetrate normal cells with intact membranes (Putz et al., 2007). Melittin, the major component of bee venom and a lytic peptide, can integrate to the hydrophobic layer of the membrane and target the membrane (Leuschner & Hansel, 2004). BV inhibited the proliferation of cancer cells by stimulating the cellular immune response in the lymph nodes (Liu, Chen, Xie, & Zhang, 2002; Orsolic et al., 2003). While bee venom induced apoptosis in human leukemia cells; it did not cause cytotoxicity in healthy bone marrow cells through some mechanisms (Hong et al., 2005; Jang et al., 2003; Moon et al., 2006) suggesting that bee venom is a promising agent for selective cytotoxicity on cancer cells.

Cancer is a disease defined by uncontrolled cell proliferation, and cell invasion to other tissues while normal cells maintain their orginal locations and the balance between cell proliferation and cell death by strict cellular signals. Although the main focus of cancer treatments is to selectively induce cell death in malignant cells, many of current chemotherapeutics are the lack of cytotoxic selectivity for cancer cells. A part of investigations have targeted to find natural or synthetic new therapeutics that have non-cytotoxic effects on normal cells but cytotoxic effects on cancer cells (Tu et al., 2008; Zheng et al., 2015), and bee venom can be one of the candidate for this purpose (Hong et al., 2005; Ip et al., 2008; Jang et al., 2003; Jeong et al., 2014; Jo et al., 2012). Although the cytotoxic effect of bee venom has been shown in various cancer cells (Moga, Dimienescu, Arvatescu, Ifteni, & Ples, 2018), the mechanisms of its selectivity for cancer cells remain unclear. In our previous study, the selective effect of bee venom on cancer cells has been confirmed in metastatic breast cancer and hepatocellular cancer cell lines and the study also showed that cells undergo different patterns of epigenetic reprogramming after bee venom (Uzuner, Birinci, Tetikoglu, Birinci, & Kolayli, 2021). However, to the best of knowledge, there is no understanding on the cellular mechanisms of bee venom for this selectivity in terms of inducing DNA damage by double strand breaks (DSBs) and altering cytoskeleton localisation, in cancer cells compared to normal cells.

This study aims to reveal the response of cells against bee venom in terms of inducing DNA damage and repair by H2AX phosphorylation and β-actin location within the cells. The findings suggest that bee venom induces DNA damage mediated by H2AX phosphorylation and intra-cellular mobility of β-actin in cancer cells compared to normal cells.

2. Materials And Methods

2.1 Bee venom collection and preparation

Bee venom used was collected as stated previously and SDS-page analyses were also done (Uzuner et al., 2021). Bee venom main stock was prepared in 0.9% NaCl and stocked as 5mg/ml aliquots at -20°C.

2.2 Cell culture

Cells used were MDA-MB-231 human metastatic breast cancer cells (American Type Cell Collection ATCC, Cat No. HTB-26, VA, USA), HEPG2 human liver cancer cells (ATCC, Cat. No. HB-8065), and NIH3T3 embryonic mouse fibroblast (ATCC, Cat. No. CRL-1658) cells. MDA-MB-231, HEPG2 and NIH3T3 cells were cultured in RPMI (Wisent Inc. Multicell, USA, Cat. No. 350000CL), EMEM (Cat. No. 320026CL) and DMEM (Cat. No. 319005CL) media, respectively. These media contained 10% (v/v) fetal bovine serum (Sigma-Aldrich Co. St. Louis, US, Cat. No: 12103C) for MDA-MB-231 and HEPG2 cells and 10% (v/v) bovine calf serum (Sigma-Aldrich, Cat. No. 12133C) for NIH3T3 cells, and 1% (v/v) penicillin-streptomycin (Capricorn, Cat. No. PS-B) was added to each media. Cultures were incubated at 37 °C incubator with 5% CO_2.

2.3 Immonuflorescence for DNA Damage (γH2AX) and β-actin

MDA-MB-231, HEPG2 and NIH3T3 cells were cultured in 96 well plates at 5000 cells per well and incubated overnight (O/N). After treatment with bee venom at concentrations of 0, 8, 12, 25, and 50 µg/mL for 24 h, cells were washed and fixed with 4% (w/v) paraformaldehyde (PFA) (ChemSolute 135 Bio, Cat No. 8416. 0500, Germany) for 30 min at room temperature (RT). Cells were treated with 1x PBS containing 0.5% Triton-100 (Biomatik, Cat No. A4025, ON, Canada) for 15 min at RT to permeabilize the cells. Subsequently, cells were blocked with 1x PBS containing 20% (v/v) goat serum (Capricorn, Cat. No. GOA-1B), 200 mg/mL BSA (Sigma, Cat. No. A1470) and 0.01% (v/v) Tween-20 (Sigma, Cat No. P1379) for 2 h at 37°C. Cells were then treated with mouse polyclonal primary antibody □-H2AX (1:200) (Aviva systems biology, Cat. No. OAAN02886) in 1x PBS containing 20 mg/mL BSA and 0.05% triton-100 (v/v) at 4°C overnight (O/N) Control cells were incubated only with secondary antibodies. After incubation, the primary antibody was washed 3 times with 1x PBS then was treated with secondary Goat Anti-Mouse IgG H&L (1:1000) (Texas Red®) (ab6787) antibody in 1x PBS including 20 mg/mL BSA and 0.05% triton-100 (w/v) at 4°C O/N. After incubation it was washed with 1x PBS three times. For β-actin staining, cells were differently treated with the mouse monoclonal primary antibody anti-β-actin (1:250) (BioLegend, Cat. No. 643801) prepared in 1x PBS containing 20 mg/mL BSA and 0.05% triton-100 (w/v) for 1h in the dark followed by the washing with 1x PBS three times. Cells were then treated with secondary antibody (1:1000) Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113) prepared in 1x PBS containing 20 mg/mL BSA and 0.05% triton-100 (v/v) for 1 hour in the dark at RT. Secondary antibody was removed by washing with 1x PBS three times. As the last step, cells were incubated with 4 µg/mL bis Benzimide Hoechst 33342 (Sigma, Cat No. 14533) prepared in 1x PBS to label the DNA. Finally, each well was examined under the inverted fluorescence microscope (AxioVert A1, Zeiss, Germany), and images were taken as at least five different regions per treatment. ImageJ software (NIH, USA) was used to analyse the level of staining for γH2AX. The intra-cellular localisation of β-actin was analysed by grouping cells according to staining pattern as i) in cytoplasm only, ii) in nucleus only, and iii) in both cytoplasm and nucleus, and the cells for each group were counted.

2.4. Statistical Analyses

Statistical analyses were performed with SPSS software (Version 23). The staining level of γH2AX and the number of cells with different β-actin localisation were compared using UNIANOVA (univariate analysis of variance). The Post-Hoc tests of UNIANOVA were performed for pairwise comparisons. $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****) were used for significance values in comparisons.

3. Results

3.1 Detection of DNA Damage

Immunostaining was performed for γH2AX, a DNA damage marker, after 24 hours of BV treatment to understand whether bee venom has a genotoxic potential on NIH3T3, MDA-MB-231 and HEPG2 cells **(Fig. 1)**. The levels of γH2AX staining decreased in normal NIH3T3 cells **(Fig. 1A-1D)**, but increased in HEPG2 **(Fig. 1B-1E)** and MDA-MB-231 **(Fig. 1C-1F)** cells after bee venom. HEPG2 and MDA-MB-231 cells were

highly sensitive to BV at the highest dose (50 µg/mL) **(Fig. 1E-1F)**. The previous study showed that IC50 values for NIH3T3, MDA-MB-231 and HEPG2 cells were 50 µg/mL, 8 µg/mL and 12 µg/mL, respectively (Uzuner et al., 2021).

3.2 Detection of β-actin

β-actin immunostaining was performed to determine its cellular distribution in normal and cancer cells after bee venom treatment **(Fig. 2)**. In normal cells, majority of β-actin was localised within the nuclei of NIH3T3 cells, but it was distributed along with the cytoplasm in a dose-dependent manner after BV **(Fig. 2A-2D)**. In contrast to normal cells, the β-actin was found both in the cytoplasm and nucleus in untreated HEPG2 cancer cells, while almost all were localized within the nucleus at 25 and 50 µg/mL doses of bee venom **(Fig. 2B-2E)**. The β-actin that generally distributed within the cytoplasm in MDA-MB-231 cancer cells, but it was localized more in the nuclei at 25 and 50 µg/mL suggesting that both cancer cell lines responded against cytotoxic doses of bee venom in a similar way with an increase in nuclear accumulation of β-actin **(Fig. 2C-2F)**. The major intracellular location of β-actin is given in **Table 1**.

3.3. Co-localisation of β-actin and γH2AX

To understand the interaction between β-actin and γH2AX after bee venom treatment, cells were co-stained for both β-actin and γH2AX, and merged profiles were obtained. In MDA-MB-231 cells, β-actin and γH2AX were not co-localised within the cells as β-actin was distributed thoughout the cytoplasm (blue arrow) but γH2AX was located within the nuclei (white arrow) in normal cells **(Fig 3)**. Bee venom treatment resulted in increased co-localisation of two markers within both cytoplasm and nucleus. In HEPG2 cells, β-actin and γH2AX were mostly found co-localised in both untreated and bee venom-treated cells (white and red arrows for nuclear and cytoplasmic colocalisation, respectively) **(Fig 4)**. But in untreated cells, β-actin staining was not co-localised with γH2AX to some extent (blue arrows). With bee venom, co-localisation profiles both in nucleus and cytoplasm were more distinctively observed. In NIH3T3 cells, bee venom induced some cytoplasmic co-existence of β-actin and γH2AX (blue arrows), however nuclear co-localisation also occurred (white arrows) **(Fig 4)**. **Table 2** summarises the co-localisation pattern of β-actin and γH2AX in each cells.

Discussion

Bee venom treatment induced β-actin to localise throughout the nuclei of cancer cells in accordance with an increase in phosphorylation of H2AX, but significantly found in the cytoplasmic form in non-cancerous cells in accordance with a decrease in γH2AX nuclear accumulation. The histone H2AX used is a 14 kDa member of the H2A histone family, which is evolutionarily conserved at the C-terminus in eukaryotes. Serine 139 within this motif is rapidly phosphorylated in response to double-stranded DNA damage and apoptosis, and its phosphorylated form is known as γH2AX. Phosphorylation reaches its maximum level at 1–3 minutes after DNA damage occurs, and hundreds of γH2AX molecules are released during each double-strand DNA break (DSBs) (Schmid, Zlobinskaya, & Multhoff, 2012) and the H2AX phosphorylation is the most fundamental modification involved in DNA damage (Sedelnikova, Pilch, Redon, & Bonner,

2003; Sonoda, Hochegger, Saberi, Taniguchi, & Takeda, 2006; Stucki et al., 2005). Therefore the immunostaining pattern of γH2AX has been used for a marker of DSBs and genomic instability in cancer cells (Hamer et al., 2003; Ji et al., 2017; Nagelkerke & Span, 2016; Redon, Dickey, Bonner, & Sedelnikova, 2009). But, H2AX phosphorylation has been also observed in undamaged cells in human (Meyer et al., 2013). To determine whether bee venom has a genotoxic effect on NIH3T3, MDA-MB-231 and HEPG2 cells, immunostaining was performed for γH2AX after bee venom treatment for 24 h, and γH2AX staining was decreased in normal cells while increased in cancer cells after bee venom. The level of DSBs increases with cellular death, chromosomal aberrations, mutations and initiation of pathological effects such as cancer so that repairing of DSBs is critical and essential to prevent carcinogenesis (Kasparek & Humphrey, 2011). Histone modifications associated with DNA damage allow DNA repair factors to access damaged sites of DNA (Waterman, Haber, & Smolka, 2020). H2AX is mostly found in nuclues, but It has been previously reported that phosphorylated H2AX was also accumulated within the cytoplasm in some DNA damage induced by such overactivation of a nerve growth receptor (Jung & Kim, 2010, 2011). In each cells some of γH2AX protein were found in cytoplasm after bee venom. But, the cellular pathway for γH2AX protein accumulation after bee venom needs further investigation.

Cytoskeleton is structured by various proteins such as microtubules, actin and intermediate filaments (Fletcher & Mullins, 2010). The cytoskeletal proteins have important roles at cell movement, biochemical process of cell, and survival of the cell (Ong et al., 2020). Cancer cells have altered skeletal machinery that causes cell invasion and metastasis (Pawlak & Helfman, 2001). Actins have three isoforms including α-, β- (B-actin), and γ-actin and also they are involved in forming microfilaments in the cell (Kavallaris, 2010; Rao & Li, 2004). While normal cells strictly establish cell architecture, mobility and adhesion with actin crosslinks, cancer cells disrupt the actin cytoskeleton arrangement and change the nuclear:cytoplasmic ratio in cells. This facilitates the metastasis by inducing flexible, mobile and fast movement of cancer cells (Ong et al., 2020; Rao & Li, 2004; Tojkander, Gateva, & Lappalainen, 2012).

Defects in cellular morphogenesis are accompanied by uncontrolled migration, acquisition of invasive features, and genomic instability in carcinogenesis. Cancer cells have abnormalities in each cellular compartment as in nucleus, cytoplasm and membranes. Cytoplasmic rearrangement mediate cell mobility and its abnormalities are one of the key events in cancer cells. For example, actin and tubulin proteins involve in the regulation of intracellular compartments, cell polarity and contractility. During morphogenesis, they determine cell shape and polarity and also promote stable cell-cell and cell-matrix junctions through interactions with cadherin and integrin proteins, respectively (Eden, Rohatgi, Podtelejnikov, Mann, & Kirschner, 2002). Additionally they activate chromosomal segregation and cell division during mitosis. This study showed that β-actin protein is localized both in the cytoplasm and in the nucleus depending on the bee venom response in different cells. The findings support previous studies that have shown the localisation of β-actin within both cytoplasm and nucleus (Caridi et al., 2018; Cerutti, 2019; Schrank et al., 2018). β-actin in the cytoplasm is involved in cell movement, formation of the skeleton and cell division, while β-actin in the nucleus is involved in the G2 checkpoint where especially the repair of DSBs occurs (Schrank et al., 2018). In addition, it has been determined that β-actin has various structural and functional roles in the nuclear structure, such as nuclear matrix assembly,

chromatin remodeling, transcription and mRNA processing (Bohnsack, Stuven, Kuhn, Cordes, & Gorlich, 2006). Mass spectrometry (Bohnsack et al., 2006) and immunoreactivity experiments (Hu, Wu, & Hernandez, 2004) have showed that β-actin is a nuclear isoform associated with heterogeneous nuclear ribonucleoproteins (hnRNPs) and RNA polymerase complexes. It has been also shown that different polymerization states of β-actin coexist in the nucleus (Schoenenberger et al., 2005). Fluorescent staining studies have revealed that approximately 20% of the nuclear actin has a dynamic pattern (McDonald, Carrero, Andrin, de Vries, & Hendzel, 2006). Our results showed that bee venom differentially induced the re-organisation of cytoskeleton but similarly induced DNA damage suggesting that bee venom selectively can stimulate β-actin-mediated DNA repair within the nucleus in cancer cells. Nuclear form of β-actin has been recently shown to mediate re-organisation of nucleolus after DNA repair particularly in ribosomal DNA sequences which is completed after UV-induced damage (Caridi et al., 2018). Another study showed that γH2AX phosphorylation increased in cells after radiation, followed by the binding of NMI (nuclear myosin 1) to ribosomal DNA (rDNA), and subsequent β-actin molecules were recruited into damaged cell nuclei (Cerutti, 2019) after phosphorylated H2AX-mediated binding of NMI to the damaged site (Caridi et al., 2018). NMI and β-actin are required for appropriate rearrangement of the nucleolus after the completion of the DNA repair machinary. Results showed that genotoxicity increased after bee venom in cancer cells but not in normal cells, and β-actin localised within the nuclei in cancer cells. This suggests that β-actin may recruit to damaged site within the nucleus in coordination with γH2AX in the cell nucleus to manage DNA damage. The results in this study indicate that after bee venom treatment, β-actin was accumulated more in the nuclei of cancer cells and but in the cytoplasm of normal cells. Thus, it suggests that bee venom may act by interfering with chromatin changes and mRNA processing of β-actin in cancer cells.

Conclusion

This study attempted to understand i) the genotoxic effect of bee venom in cancer cells by a histone variant, H2AX, phosphorylation and ii) the association of β-actin with this genotoxicity. Results showed that normal and cancerous cells had different responses against bee venom, and suggested that bee venom induced a cellular response by the interaction of γH2AX and β-actin. Overall, this study revealed one of cellular mechanism in cancer cells after bee venom. The results add detailed knowledge for consideration of the use of bee venom as alternative medicine in therapies of some cancers.

Declarations

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Contributions

ST performed experiments. ST and SCU wrote the manuscript. SCU designed the study.

Conflict of interest

The authors declare that there is not conflict of interest.

Compliance with Ethical Standards

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Major localisation of β-actin within the cells before and after bee venom

CELLS	Intracellular location of β-actin	
	Before bee venom	After bee venom
MDA-MB-231	Cytoplasm	Nucleus (after 25μg/ml)
HePG2	Cytoplasm + nucleus	Nucleus (after 25μg/ml)
NIH3T3	Nucleus	Cytoplasm

Table 2. Major colocalisation of β-actin and γH2AX before and after bee venom in three cells

CELLS	Colocalisation	
	Before bee venom	After bee venom
MDA-MB-231	N/A	Cytoplasm + nucleus
HePG2	Cytoplasm + Nucleus	Cytoplasm + nucleus
NIH3T3	Nucleus	Cytoplasm + nucleus

Figures

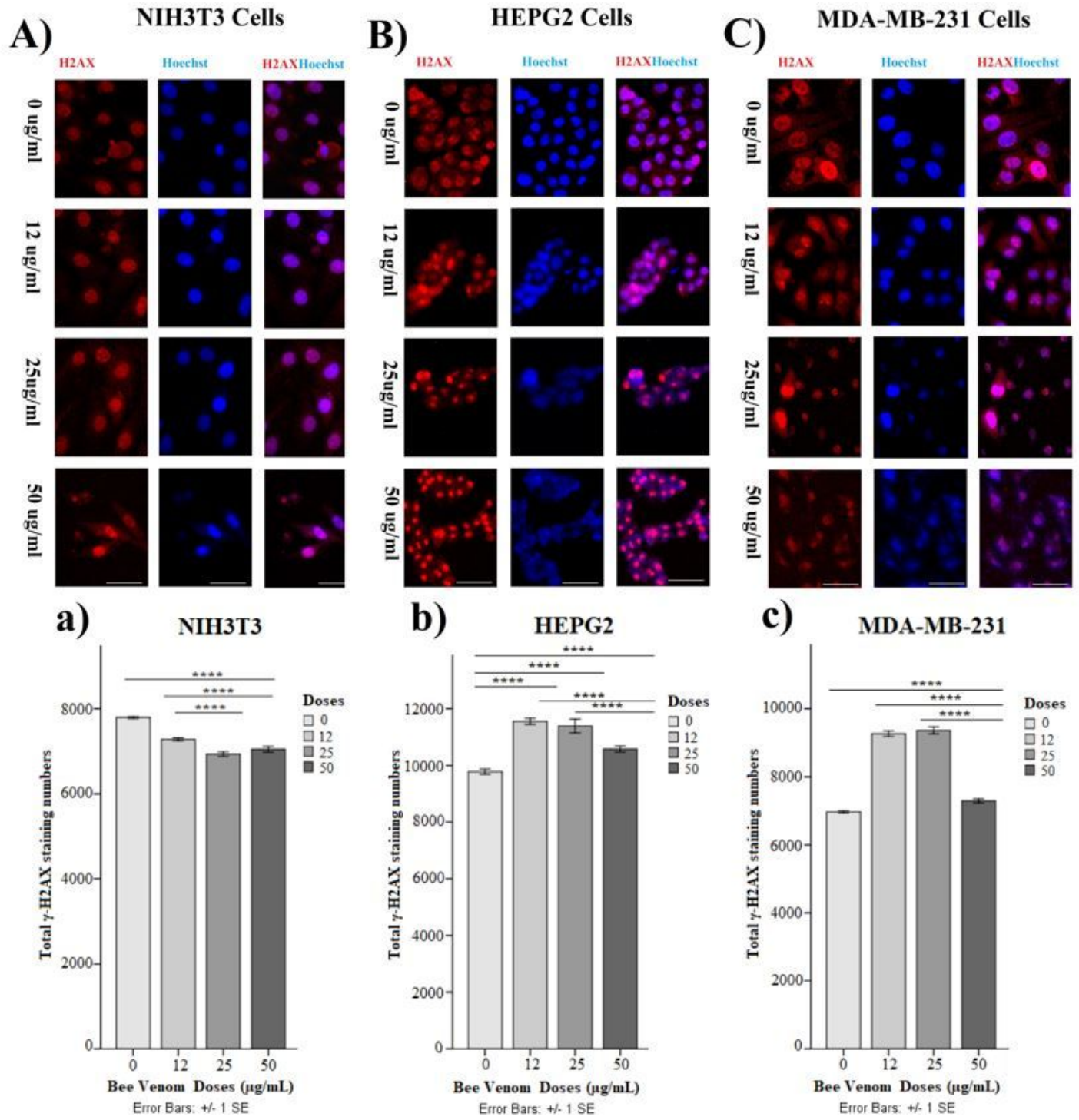

Figure 1

γH2AX staining in normal and cancer cells after bee venom treatment. Representative fluorescence microscopy images of DNA damage by γH2AX (red) immunostaining counterstaied with Hoechst (blue) for **A)** NIH3T3, **B)** HEPG2 and **C)** MDA-MB-231 cells are shown. The levels of γH2AX staining after bee venom are given for **D)** NIH3T3, **E)** HEPG2 and **F)** MDA-MB-231 cells. Statistical comparisons are performed by post-hoc test. Experiments were performed in two independent replicates. The bar graphs show ± standard mean of errors. The scale bar is 10 microns. * = $p < 0.05$, and **** = $p < 0.0001$.

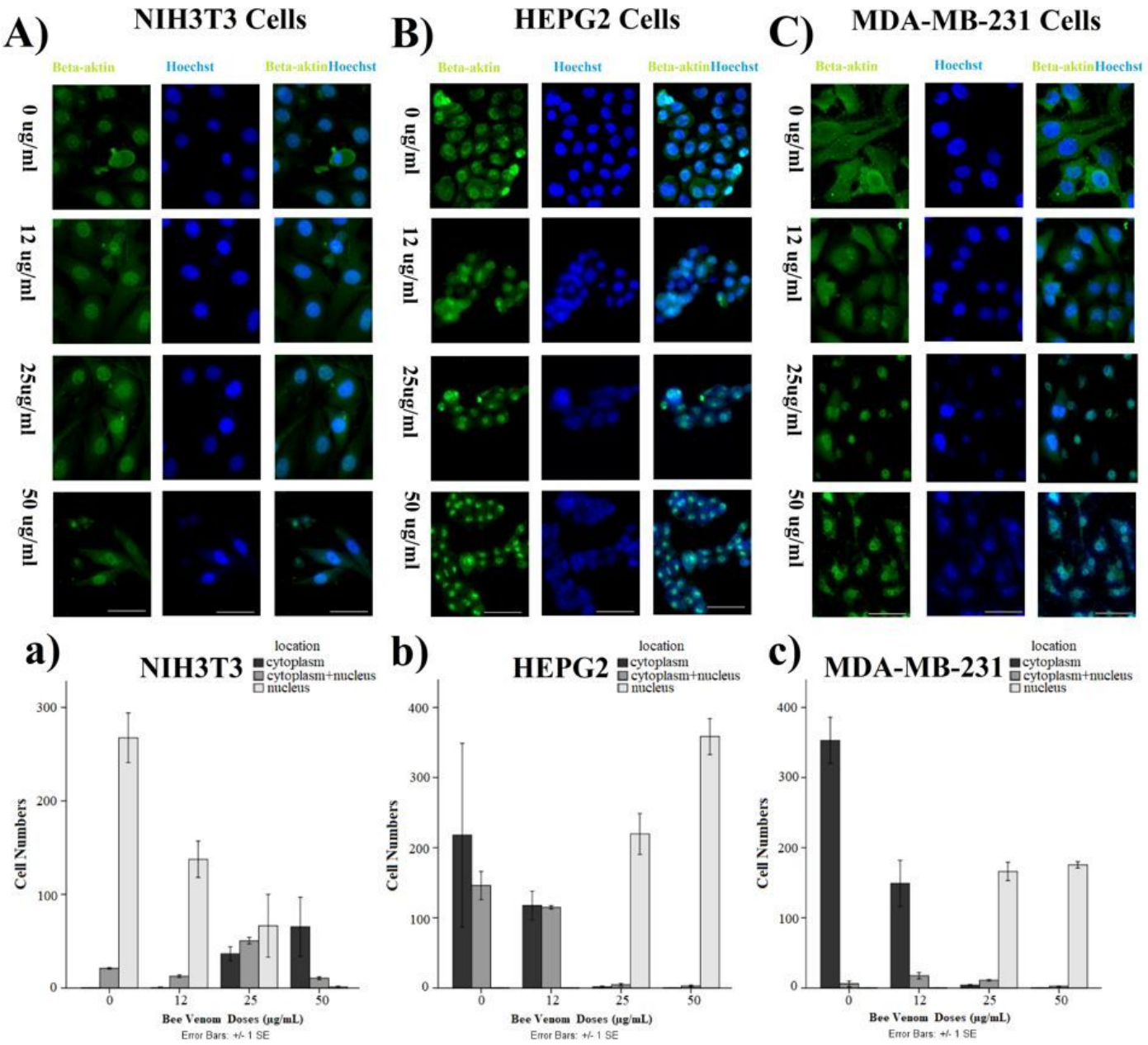

Figure 2

The pattern of β-actin in normal and cancer cells after bee venom treatment. Represented microscopy images for **A)** NIH3T3, **B)** HEPG2 and **C)** MDA-MB-231 cells are shown. β-actin (green) was counterstained with Hoechst (blue) merged images are also given. The bar graphs show cell numbers with β-actin localised in nucleus or cytoplasm or both after bee venom in **D)** NIH3T3, **E)** HEPG2 and **F)** MDA-MB-231 cells. Experiments were performed in two independent replicates. The bar graphs show ± standard mean of errors. The scale bar is 10 microns.

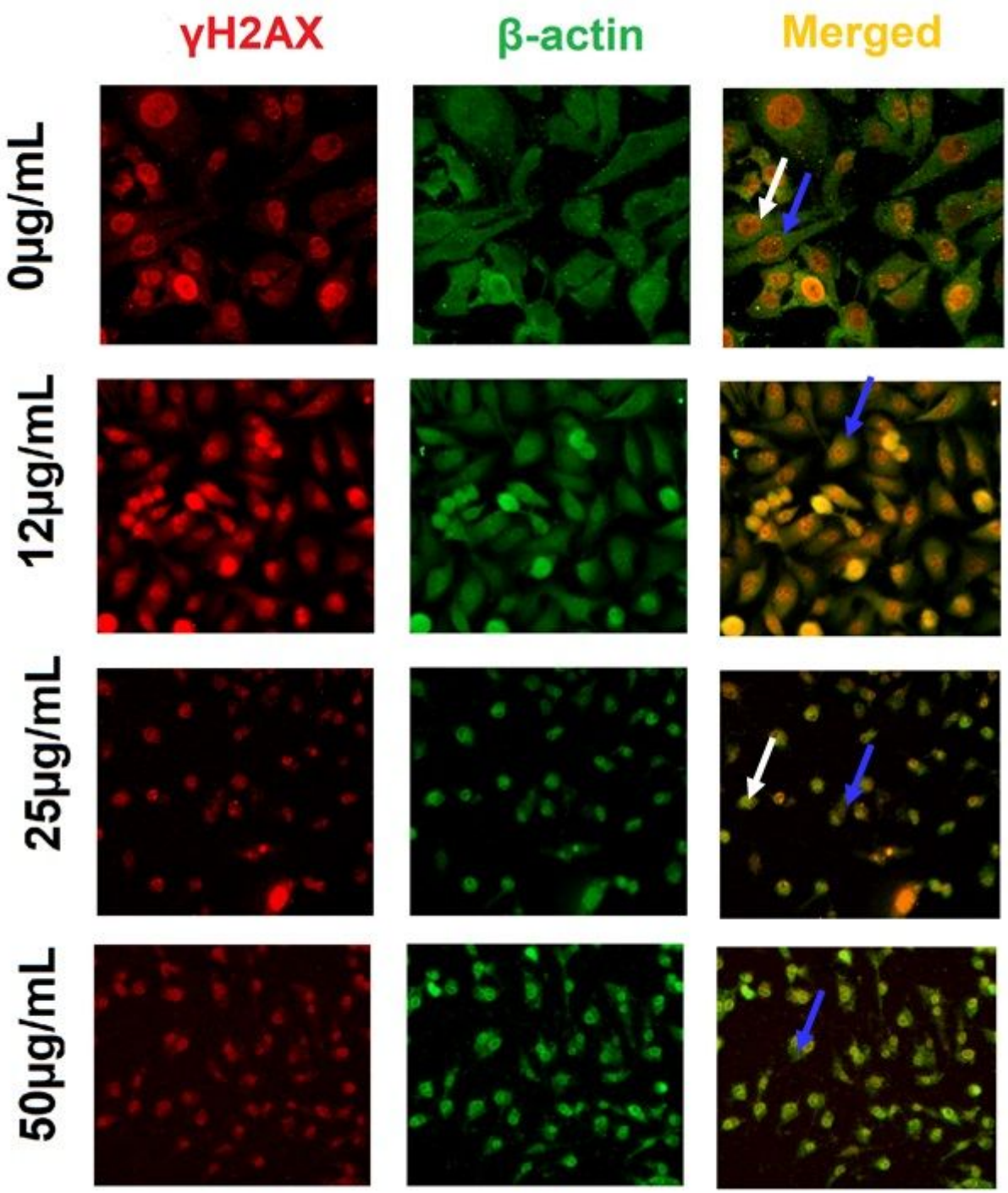

Figure 3

Colocalisation of β-actin and γH2AX before and after bee venom in MDA-MB-231 cells. MDA-MB-231 cells were co-stained for β-actin and γH2AX. Representative microscopy images for each marker and merged images are shown. White arrows show nuclear co-localisation in untreated and in bee venom-treated cells. Blue arrows show cytoplasmic staining for β-actin alone in untreated cells, but co-staining of β-actin with γH2AX in bee-venom treated cells.

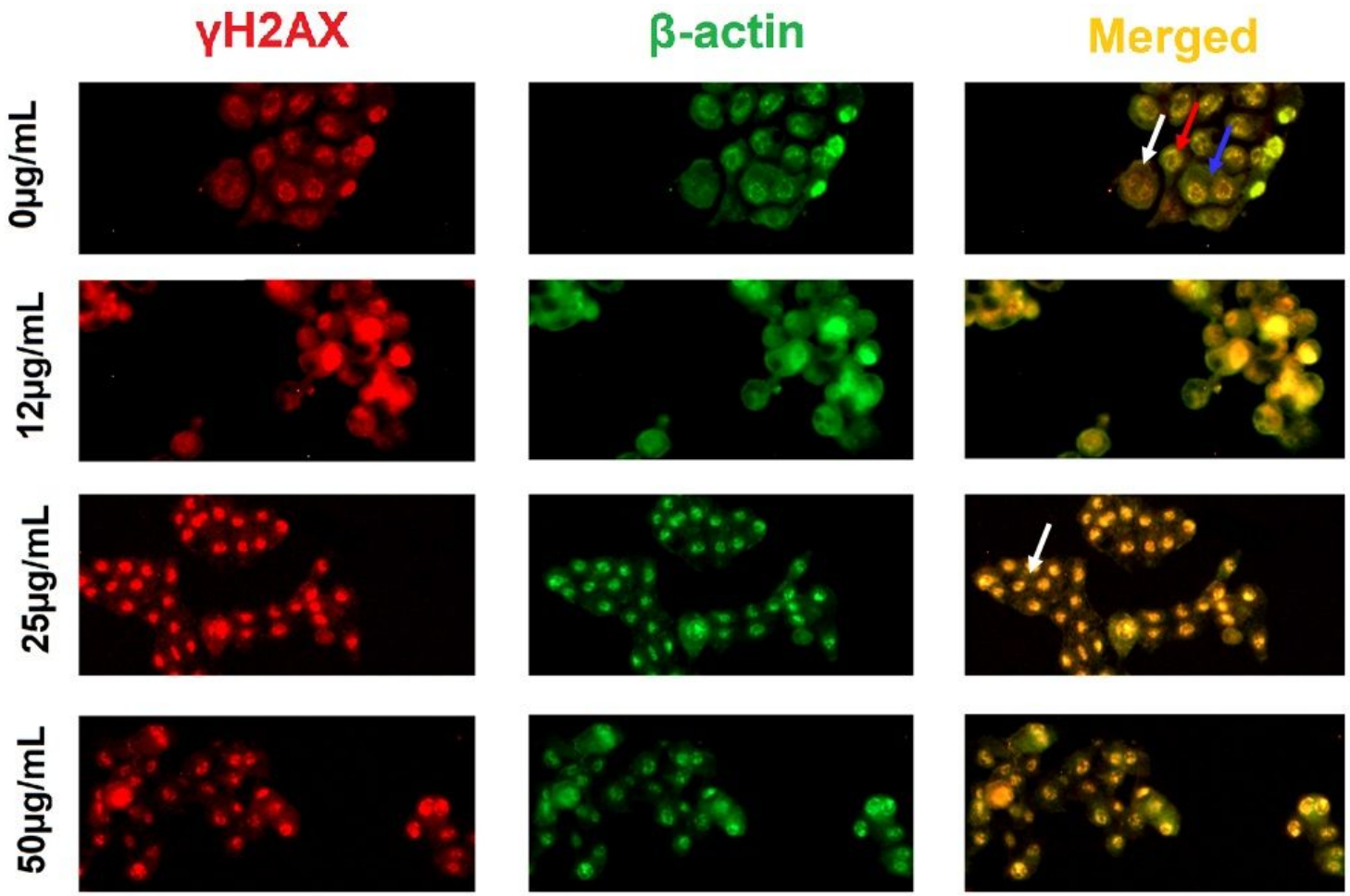

Figure 4

Colocalisation of β-actin and γH2AX before and after bee venom in HePG2 cells. HePG2 cells were co-stained for β-actin and γH2AX. Representative microscopy images for each marker and merged images are shown. White arrows show representative cytoplasmic co-localisation in untreated and in bee venom-treated cells. Blue arrow shows representative cytoplasmic staining for β-actin alone in untreated cells. Red arrow shows representative nuclear co-staining of β-actin with γH2AX.

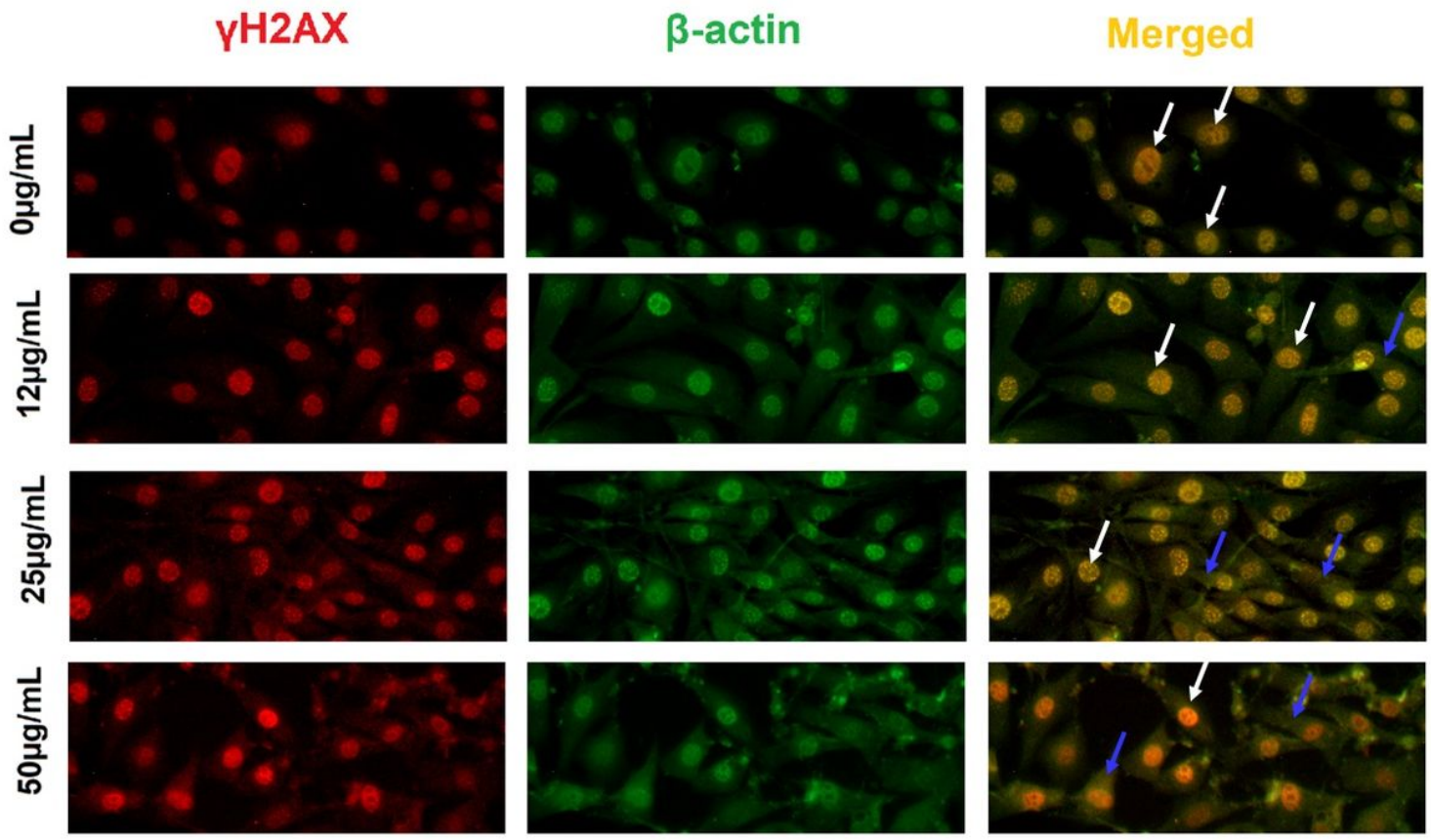

Figure 5

Colocalisation of β-actin and γH2AX before and after bee venom in NIH3T3 cells. NIH3T3 cells were co-stained for β-actin and γH2AX. Representative microscopy images for each marker and merged images are shown. White arrows show representative nuclear co-localisation in untreated and bee venom-treated cells. Blue arrows show representative cytoplasmic co-staining of β-actin with γH2AX.

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