In vitro primary culturing of cells and explant tissue of Conus cumingii venom duct: Cytotoxicity assessment of their culture supernatant on HEK 293T

M.S. Viswanathan1, Mootapally Chandra Shekar1,2, Sabapathi Arularasan1, Neelam M. Nathani3, Sathishkumar Arumugam1, Amrutlal K. Patel4, Indra R. Gadhvi2 & Chaitanya G. Joshi5

1Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608 502, India
2Department of Marine Science, Maharaja Krishnakumarsinhji Bhavnagar University, Bhavnagar -364002, India
3Department of Life Sciences, Maharaja Krishnakumarsinhji Bhavnagar University, Bhavnagar -364002, India
4Hester Biosciences Ltd., Ahmedabad - 380006, India
5Department of Animal Biotechnology, College of Veterinary Sciences & A. H., Anand Agricultural University, Anand - 388001, India

*E-mail: vtpviswa@gmail.com

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In present study, we attempted to establish primary culture of cells in suspension and tissue explants from the venom duct of Conus cumingii for ample production of conopeptides. Venom secretory cells migrated from tissue explant within a day and the primary culture cells were successfully maintained up to 20 days. Further, the culture supernatant from both the cell suspension and explant was treated with HEK 293T cells for determining its cytotoxic effects which revealed significant reduction of cell viability giving a prompt evidence for the successful conopeptides production by the primary culture in the growth medium.

Keywords: Marine cone snail; Venom duct; Cell suspension and Explant tissue; Primary culture; Secretory cells; Cytotoxicity

Introduction

Mini creatures residing in the ocean including fishes, worms and other mollusks are often affected by the neurotoxin producing animals especially cone snails that selectively target the different ion-channels and nerve cell receptors of the central nervous system, thereby causing paralysis to the victim. These marine predatory cone snails are greatly distinguished from other sea animals by their slow moving nature, attractive shell features and the potential of secreting distinct arrays of over 100 toxic substances in their sophisticated venom gland as an adaptation to capture the prey and escape from other hunting animals. Hence, the venom manufacturing system is generally considered as an obvious bedrock for the deadly venomous animals like bees, puffer fish, spider and snake. A long elongated tube like organization called venom duct is the central venue for the complex nature of the venom apparatus. Interestingly, a number of these divergent conopeptides are biosynthesized in the secretory epithelial cells of distal part of this venom duct region. Overall, mixture of low molecular weight peptides are the predominant constituents in the venom of each Conus species with specific functions. Consequently, these small bioactive molecules are valuable marine derived neuro toxic compounds for use as potential therapeutic agents. For example, Ziconotide, the synthetic version of ω-conopeptide MVIIA derived from the marine snail Conus magus has been employed as a pain reliever in the treatment of severe chronic diseases. Moreover, δ-conotoxin and χ-conopeptide MrIA were isolated from C. amadis and C. marmoreus respectively and screened as nerve system affecting compounds.

Based on the available literature, it is well known that the tissue composed by epithelial cells is surprisingly a major source for the production of toxic substances in the venom duct region of marine cone snail. Relatively few studies describe the cultivation of marine invertebrate cells as another possibility for manufacturing more quantity of the desired molecules. Apart from that, the production of venom substances by snake primary cell culture form the venom duct tissue culture is also developing as a potent strategy. Our prior investigation also
suggested that the cell culture supernatant from the venom duct of *Conus biliosus* in the primary culture suspension medium depicts cytotoxic effect on human embryonic kidney cell line. The predatory sea snails of genus *Conus* are less commonly found in deeper waters. The rocky sub-littoral epipelagic zone of Saurashtra coast along the Arabian Sea with some sandy patches stands for different macrofauna species including *Conus cumingii*. Accordingly, the present study aimed for the first time to develop primary cell as well as explant culture of venom duct region of *C. cumingii* to produce conopeptides in the culture medium, followed by the analysis of the cytotoxic effect of these culture supernatant on the Human Embryonic Kidney 293T cell line.

**Materials and Methods**

Live specimens of *Conus* species were collected randomly from Porbandar coastal line of Gujarat, India. The live specimens were transported to laboratory and maintained in an artificial tank of sea water before being processed for further purpose. The collected samples were rinsed with distilled water to remove salt and other debris for identification based on standard shell morphology characteristics and color pattern as described earlier to confirm the species *C. cumingii*.

Venom glands were dissected out from the identified study animal in sterile laboratory conditions. Primary culture of cell and tissue explant from venom duct of *C. cumingii* was performed based on modified protocol described previously for primary culturing of mammalian salivary glands. The cone snails were thoroughly wiped with 70% ethanol. The snail was further cracked using hammer to dissect out the whole body tissue. Immediately, body tissues were transferred under laminar air flow chamber in a petri dish containing PBS and antibiotics (Penicillin: 50IU/ml; Streptomycin: 100µg/ml; Gentamicin: 5µg/ml). Afterwards, the venom glands were split from their connective tissue using sterile blade, needle and scissors to carry out further dissection of venom duct in animal cell culture laboratory conditions. Excised venom glands were transferred into Class II laminar air flow chamber in a petri dish containing PBS and antibiotics. Subsequently the venom ducts were removed from venom gland and washed thoroughly with only PBS followed by Medium 199.

The venom duct tissues were minced to slurry in basal medium M-199. Using enriched basic medium M-199 (50 IU/mL pencillin, 100µg/mL streptomycin, 5 µg/mL gentamycin) and subsequently digested with 0.25% trypsin (Sigma) for 30 min at room temperature (24°C to 28°C) with inversion every 10 min to remove the toxin substance present inside the venom duct. Dissociated cells medium was filtered drop by drop by cell strainer (70 µm in size) to remove all the cell debris present in dissociated cells medium. 5 ml of washing medium M-199 along with 2ml of 5% FBS was added to the filtrate and mixed well using serological pipette to inhibit the trypsin enzyme activity. The mixture (washing medium + BSA + Filtrate) was then centrifuged at 1500 rpm for 10 min and the supernatant was gently removed using vacuum pump to obtain cell pellet from the mixture. Cell pellet was resuspended in 5ml of culture medium M-199 and mixed well to obtain single cell suspension. Dissociated cells were seeded in T-25 culture flask by using serological pipette, grown at room temperature 24˚C-28˚C and regularly observed using inverted phase contrast microscope.

Excised venom duct was washed thoroughly in PBS plus antibiotics (50 IU/mL pencillin, 100µg/mL streptomycin, 5 µg/mL gentamycin) followed by mincing the tissue into 1mm pieces (explant) with sterile scalpel. These explants were washed three times in PBS plus antibiotics solution. Sterilized explants were inoculated in T-25 culture flask. 100 µl of fetal bovine serum was added to each explant and allowed to attach in the substratum of the flask. Then, 1-1.3 ml of sterilized M-199 culture medium with supplemented 10% serum was aseptically dispensed into tissue culture flask, then incubated at room temperature 24˚C to 28˚C and regularly observed under an inverted phase contrast microscope.

Both cell and explant culture medium was changed every 1-2 day interval as alternative days and the periodicity of medium change was decided on the basis of condition of cells and tissue explants. Half of the supernatant (crude) of venom duct cell culture medium was collected once in 2-3 days interval and stored at -20°C for further confirmation about venom production by cytotoxicity analysis. All the steps were carried out at 25°C. In parallel, Human Embryonic Kidney 293T cells (HEK 293T) were cultivated in DMEM F-12 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin for cytotoxicity analysis of venom duct cell culture supernatant to confirm the production of venom from
cultured venom gland cells. Cultured cells from venom duct and HEK 293T were maintained at 37˚C in 5% CO₂ incubator.

Initially, histological observation to study the organization of venom secretory cells in cone snail of the resected *Conus* venom duct was performed by fixing it in 10% formaldehyde. After fixation, moisture content was removed using isopropyl alcohol and then tissue block was infiltrated by paraffin, allowed to solidify in a mold and embedded with a small cube of paraffin. Tissue sectioning was accomplished by using microtome and each section was mounted on microscopic slides. Clearing process was applied to remove the paraffin and then histological section was stained using Hematoxylin and Eosin (H&E). Stained slides were observed under an inverted phase contrast microscope. Cells in both primary cell and explant culture were observed and photographed by using inverted microscope, followed by their collection separately, stained with H&E and compared for histology analysis.

**Assessment of cytotoxicity**

In order to determine the cytotoxic effects of culture supernatant of primary venom duct explant culture on HEK 293T cells, the viability was measured by MTT [3-(4, 5-dimethyl thiozol-2-yl-2, 5-diphenyl tetrazolium bromide] assay. The cells were seeded into 96-well culture plates at a density of 1.0 X 10^5 cells/well and subsequently incubated overnight in a humidified 5% CO₂ incubator. The growth medium was removed without disturbing the cell sheet. The monolayer of cells was washed with PBS to remove dead cells and excess fetal bovine serum. The culture medium (M-199) and venom bulb extract served as negative and positive control respectively. Undiluted crude supernatant and each dilution of the crude sample ranged from 1:10 to 1:1000 were added to respective wells of 96 well plates in triplicate. The cells were observed under inverted microscope for significant changes in their morphology. After 48 hours of incubation, cell viability was analyzed by MTT assay. 20µl of MTT (5mg/ml) was added to each well and incubated at 37˚C for 3 to 4 hours. Then, the medium was replaced with 150µl of dimethylsulfoxide and incubated for 45 minutes in CO₂ incubator. A colored formazan crystal was assayed spectrophotometrically at 570 nm using a plate reader. The results were presented as the percentage of cell viability.

**Results**

In the present study, live specimens of *C. cumingii* were confirmed through conventional taxonomical approach. The morphological key characters observed were cone shaped and shell medium to large, moderately solid to solid with dirty white to brown shoulders, heavily covered with curved light brown lines and blotches and shaped angulate to round. The spire was observed to be of low to moderate height with straight to slightly convex outline. The aperture was uniformly wide; outer lip sharp and evenly convex. The cone possessed dull white to yellow body whorl which was heavily covered with broad reddish brown to dark brown streaks. The aperture was bluish white, periostracum thick and brownish in color. They were moderately 30mm - 42mm (Shell length) and 10mm - 2.1mm (Shell width). It was observed to be feeding on worms and sampling was done from the sandy shore of the sea (Fig. 1). The artificial sea water tank and few mollusc species used as feed in this experiment were more sufficient to maintain the study animal in live conditions until further processing of the samples was performed.

The elongated venom duct region, that was the main source for accomplishing the primary culture which was removed from venom gland of *C. cumingii* under proper sterile conditions, was observed to be between 3.2 cm to 4.5 cm in length. Initially, fewer numbers of cells were observed during the culture period (Fig. 2A), however, subsequently cell proliferation initiated within couple of days (Fig. 2B). Later viz. 2nd week onwards, the density of venom secretory cells was found to be more in the culture.

![Fig. 1 — dorsal and ventral view of a typical *Conus cumingii* from Saurashtra coast](image-url)
medium (Fig. 2C). The number of live cells was very less during 3rd week (Fig. 2D) and these cells were non adherent in the suspension culture throughout culture period. For explant culture, the cell migration from the explants as well as proper elongation was achieved successfully (Fig. 3A). A number of cells migrated from the explant during the initial culture period itself. Consequently, all the cells were properly attached in the bottom of the flask after a week (Fig. 3B). During the 13th day, the first sign of cell death was observed (Fig. 3C). Finally, most of the cells died on 24th day and onwards (Fig. 3D).

Venom duct secretory cells were observed for histological properties using H & E staining (Fig. 4A). Two different cell types were predominantly observed in both primary cell (Fig. 4B) and explant culture medium (Fig. 4C). Similar cell types i.e. cuboidal and column-like epithelial cells were confirmed by H&E staining from both the primary cultures (Fig. 4D).

The presence of venom in the cell and explant culture supernatant was tested by MTT assay. The cytotoxic effect was investigated by using various dilutions of supernatant as mentioned previously. The results showed that the 100µl of undiluted cell culture supernatant inhibited the HEK 293T cell proliferation with 52% cell viability (Fig. 5). Similarly, 40% of cell viability was observed in the 100µl of undiluted explant culture supernatant treated HEK 293T cells. For both the culture supernatants, cell viability gradually increased with increased dilutions (Fig. 5). Overall, these two culture supernatant exposure was detrimental to the HEK 293 T cell line.

**Discussion**

The present study was undertaken with the aim of developing in vitro cell and explant culture from the venom duct region of *C. cumingii*. It is quite familiar that developing the animal tissue culture technique is quite a difficult and tedious process. Based on our
previous experience with the conus cell line establishment, use of suitable tissue digesting enzyme, decontamination agent PBS and filtration have yielded more number of cells in the culture medium. The M-199 medium supplemented with FBS proved to be successful for the cultivation of venom duct cells in the optimum temperature range of 24°C to 28°C. The surface area of T-25 culture flask was also beneficial for culturing the venom secretory cells. According to reports on explant culture, the initial attachment of explants in the substratum of culture flask is notably essential for development of primary tissue culture. Therefore, collagen coated culture vessels were used in this experiment, thereby cell migration from the explants as well as proper elongation were achieved successfully.

However, choosing the M-199 supplemented with FBS as appropriate culture medium, regular observation of both cell and explant culture conditions, replacement of the cell growth medium in alternative days and restraint of microbial contamination remained to be a great challenge throughout the culture period. A few earlier studies have successfully developed in vitro culture technique for secretory cells of the venom gland from different poisonous animals including ant and snake. The present study revealed that the multiplication of these secretory cells consistently occurred in the cell suspension culture and remained viable up to three weeks. However, the cells in the tissue explant based culture slowly showed signs of senescence, probable reason being the secretion of venom in the culture medium and were able to survive for around 24 days. Addition of fetal bovine serum in the culture medium may have played an important role in sustaining and prolonged survival of the cells. In spite of several enzymes attempted for the tissue digestion, trypsin was most effective in the disaggregation of cells from the venom duct of the study animal. In addition, snake serum is also mentioned to have many inhibitors involved in the protection of venom secretory cells in culture.

Morphological studies by histology and staining method predominantly revealed the same shapes, confirming the two different types of venom secretory cells from cell suspension and explant culture as cuboidal and column-like epithelial cells, respectively. In a similar study on snake venom secretory cell culture for venom production, columnar or cuboidal cells were also observed in the culture medium. Moreover, other study reported that six different types of secreting epithelial-like cells were obtained in the primary culture of snake venom gland. Another striking condition for difficulty of the cell culture establishments may be the pH variations. It was noted that the pH of freshly collected various snake venom was 5.42 in average, but, the optimum pH for culturing the venom secretory cells in the medium employed was 7.4-7.8.

In this study, the culture supernatant was considered as venom substances and was further employed to detect the venom in the medium by cytotoxic assay. Human Embryonic Kidney 293T cell line was mainly preferred as cell model for the preliminary cytotoxic study due to the stable expression of the potassium ion channel protein in the outer surface of the cell wall by the cell line. Exposure of both cell and explant culture supernatant to HEK 293T cell line revealed considerable decline in cell viability by the MTT assay. Thus, it could be possible that ion channel targeting substances exist in the culture supernatant, there by inducing cell death. But, a few molecular techniques such as ELISA and western blotting employed for the confirmation of toxic components in the medium would provide more evidence about the exact conotoxins that act on the cell viability. In other studies, scorpion venom also revealed in vitro cytotoxic activity, where it was reported that Odontobuthus doriae venom inhibited the growth of human neuroblastoma cells. Our prior studies on the effect of cell culture supernatant of
Conus biliosus and their cytotoxicity on HEK 293T cell line also revealed cell viability similar with outcomes of the current work with another species of Conus predominantly observed in the coastal regions of Saurashtra area. Although our results showed a cytotoxic effect of culture supernatant on HEK 293T cell line, further studies relevant to confirmation of exact venom compounds in the culture supernatant cell line, also revealed cell viability similar with Conus biliosus outcomes of the current work with another species of Saurashtra area Conus. Evidence for the secretion of conopeptides by venom secretory cells, followed by understanding the basic molecular mechanisms behind the demise of HEK 293T cell line upon the exposure of culture supernatant. Therefore, eventual studies in the view of venom secretory cell behavior and production of conotoxin, along with well refined protocol for establishing in vitro method for culturing the venom duct cells are needed for manufacturing the venom for successful pharmacological applications.

Conclusions
The venom duct of C. cumingii was explored with a maiden effort to produce sufficient amount of venom through the secretory cells of primary cell suspension and explant culture, further the potential cytotoxic activities of both cell and explant culture supernatant (crude) against HEK 293T cells supported the venom existence in the medium. These findings open the opportunity in the development of marine invertebrate cell culture technique in order to explore the neuro toxic peptides from the venom duct region of marine cone snails. However, in the present study, a few drawbacks have been raised in proving strong evidence for the secretion of conopeptides by venom secretory cells, followed by understanding the basic molecular mechanisms behind the demise of HEK 293T cell line upon the exposure of culture supernatant. Therefore, eventual studies in the view of venom secretory cell behavior and production of conotoxin, along with well refined protocol for establishing in vitro method for culturing the venom duct cells are needed for manufacturing the venom for successful pharmacological applications.

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References


