

Neuroregenerative property of haruan (*Channa striatus spp.*) traditional extract

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Abstract—

Among key neurorestorative strategy in treating neurodegenerative diseases and brain trauma is neurostimulation using non-endogenous substances, thus improving regeneration and repair potential of neurons. The purpose of this work is to treat PC12 cells, an established neuronal differentiation model, with a traditional extract of haruan, *Channa striatus spp.*, an indigenous riverine fish of Southeast Asian countries, and analyse PC12 cells' morphologic and behavioural changes at different concentrations of the extract. PC12 cells were first cultured at 1×10^3 cells/cm² density in 12 well plate in an incubator (5% CO₂, 37°C), each containing 1ml of complete growth medium (Eagle's Minimum Essential Medium with 10% foetal bovine serum), for 48 hours, to reach a 70% confluency. Media was removed and changed, and treated with 100µl, 200µl, 300µl and 400µl of haruan traditional extract (HTE). PC12 cells were observed under camera-equipped inverted microscope at 24, 48, 72 and 96 hours. Neurite outgrowth as well as growth behaviour were noted in comparison to negative (1ml complete growth medium) and positive (1ml cyclic AMP) controls. 100µl of HTE was demonstrated as the optimum concentration at which multiaxonal and multipolar cells and longest neurite outgrowth were seen. The result is suggesting that haruan traditional extract (HTE) is able to exert desirable effects in morphology and behaviour of PC12 cells and may potentially be useful therapeutically in the genesis and repair of neuron cells.

Keywords—haruan, PC12, neurostimulation, neuroregeneration, regenerative medicine

INTRODUCTION

In recent years, it has become evident that brain cells continuously grow and repair itself through the process of neuroregeneration or generation of new neurons, (Caroni, 1998) (Gould, 2002) from the adult stem cell population residing in the brain. The accumulating evidence of neurogenesis in adult raises the possibility that endogenous stem/progenitor cells could be activated and recruited to generate new functional neurons to repair damage to the CNS. (Palmer, 1999) (Temple, 2001) Several different factors including the endogenous neurotrophic factors, such as nerve growth factor (NGF), have been shown to induce neuroregeneration (Patapoutian, 2001) but their potential administration as medical treatment is limited by their impermeability of the blood brain barrier. (Saito, 2006)

In this study, we studied a natural lipophilic extract with proven anti-inflammatory effect as a substitute to

endogenous neurotrophic factor. The haruan traditional extract (HTE) selected for this purpose is studied for its endogenous neurotrophic factor-like action to induce neuronal differentiation, survival and regeneration. The haruan, *Channa striatus*, is a Southeast Asian indigenous snakehead freshwater fish commonly consumed as medicine for treating wound, especially in post-partum mother, and as healing booster for the elderly and sick. The medicinal properties of haruan as wound healing agent, (Mat Jais, 2007) anti-inflammatory, (Mat Jais, 2004), anti nociceptive (Mat Jais, 2005) and anti-oxidant have been reported previously.

The extract is prepared as an aqueous extraction (Mat Jais, 1997) mimicking what is commonly practiced in traditional Malay society. It is rich in poly-unsaturated fatty acids, especially the omega-3 and omega-6 contents such as docohexaenoic acid (DHA), (Mat Jais, 2006). The omega-3

particularly is an important component of brain, making up to 40% of the organ and hence is significant for neuronal growth, (Wu A, 2007) development of synaptic processing in neuronal cell interaction and in the expression of gene regulating cell differentiation and growth. (Uauy, 2001) (Wainwright, 2002). Polyunsaturated content of haruan, working in concert with its high glycine, aspartic and glutamic acid components, play a major role in the induction of wound healing process. (Mat Jais, 1994) Glycine in particular is thought to have role in memory function (File, 1999), and in halting the spread of brain damage from stroke (Gusev, 2000).

In this study we studied the effect of HTE on NGF-dependent PC-12 cell line, an established cell line often used in neuronal study. PC-12 cell line, which originates from rats phaeochromocytoma, expresses NGF receptors on the cell surface. (Greene, 1976) (Schechter, 1981) Neurite outgrowth in PC12 cells is inducible through the activation of a nitric oxide (NO)-guanosine 3',5'-cyclic monophosphate (cGMP)-cGMP-dependent protein kinase (PKG) pathway followed by extracellular signal-regulated kinase (ERK) activation. (Yamazaki, 2001)

Current report discusses observable changes in PC12 cells related to neurite production and cell behaviour under non-degenerative (serum-rich) assay and should enable future work in determining mechanism of action and blood-brain-barrier permeability of HTE.

MATERIALS AND METHODS

Materials.

Haruan traditional extract, PC-12 cell line (ATCC, CRL-1721), EMEM (Sigma), foetal bovine serum (Gibco), dibutyryl cAMP (Sigma), apo-Transferrin (Sigma), insulin (Sigma), progesterone (Sigma), trypan blue (Sigma).

Preparation of haruan traditional extract (HTE).

The extract is prepared using a traditional, chemical-free method of extraction as described earlier. (Mat Jais, 1997) (Zakaria, 2004).

Cell culture and neurite outgrowth assay.

PC-12 cells were grown in Eagle's minimum essential medium (EMEM) containing 10% (v/v) foetal bovine serum in 5% CO₂, 90% humidified air at 37°C. The cells were seeded onto a 12-well culture plate at a density of 1×10^3 cells/cm². After 48 hours of culture, medium was replaced anew and treatment with haruan traditional extract commenced. Duplicates of wells were treated with 100 µl, 200 µl, 400 µl and 800 µl of HTE, 1mL dibutyryl cAMP (as positive control), and 1mL of complete growth media (as negative control). Cells are observed under an inverted microscope at 72, 96 and 120 hours intervals and images captured using mechanized inverted microscope (AX-80, Nikon).

RESULTS

HTE-induced change in cell behaviour.

PC-12 cells normally grow in growth medium in aggregates, and appear as round-shape cells. Cells cultured in HTE-supplemented culture medium, regardless of the presence of foetal bovine serum (FBS), have significantly different behaviour within 24 hours after HTE was added. In all HTE-treated cells, irrespective of concentration, PC-12 cells started to grow independently of each other and remained so beyond 120 hours.

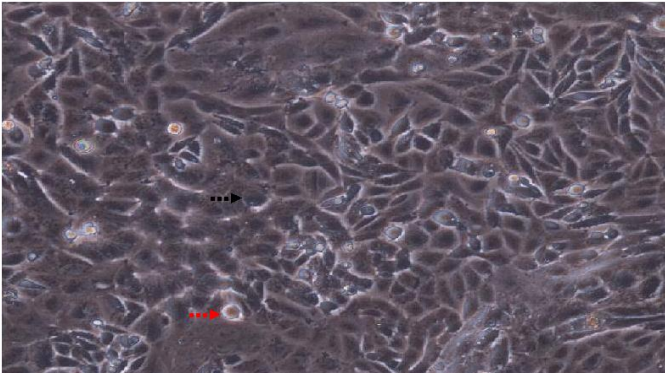


Figure 1: Negative control of PC-12 cells showing aggregating behaviour of PC12. Black arrow showing differentiating cell. Red arrow showing non-differentiating cell.

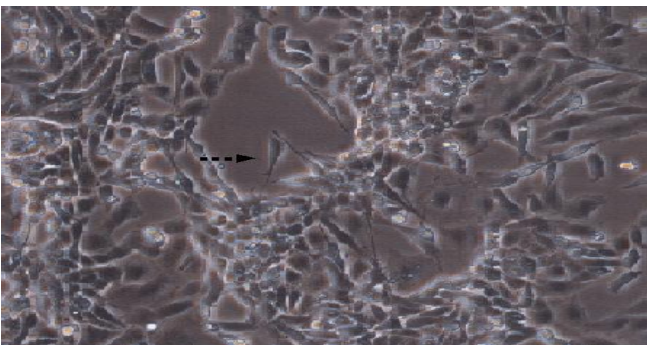


Figure 2: Positive control of PC12 cells in 1ml cAMP showing aggregating behaviour. Arrow showing a cell with neurite extension.

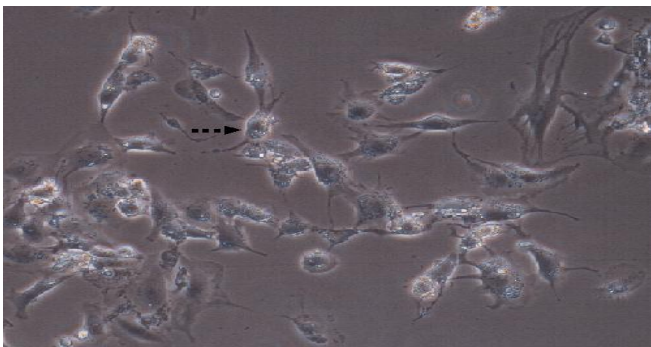


Figure 3: PC12 cells treated with 100 µl of THE showing segregating multipolar cells with axonal extension interacting with each other. Arrow showing a cell with neurite extension.

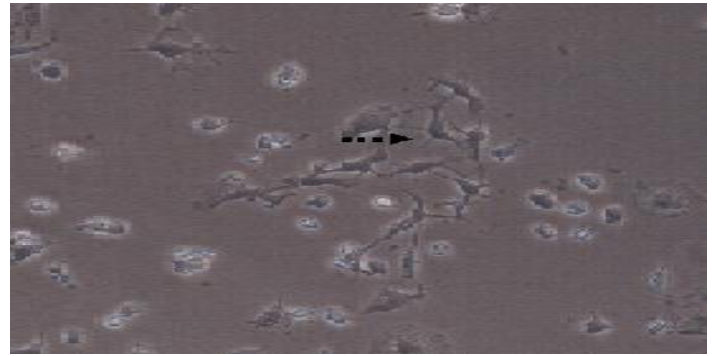


Figure 4: PC12 cells treated with 200 µl HTE showing cells with multipolarity and neurite extension (black arrow).

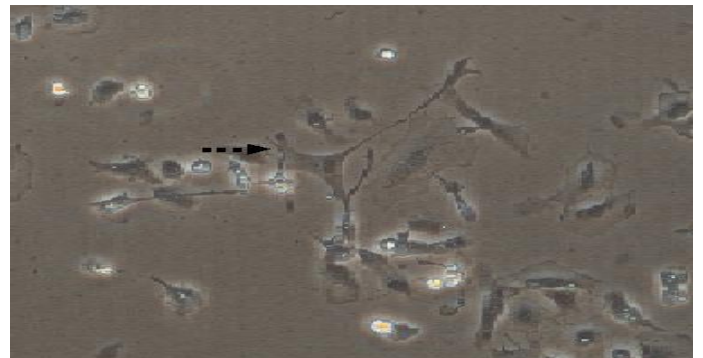


Figure 5: PC12 cells treated with 400 µl HTE showing an increasingly bipolar character. Black arrow showing a bipolar cell.

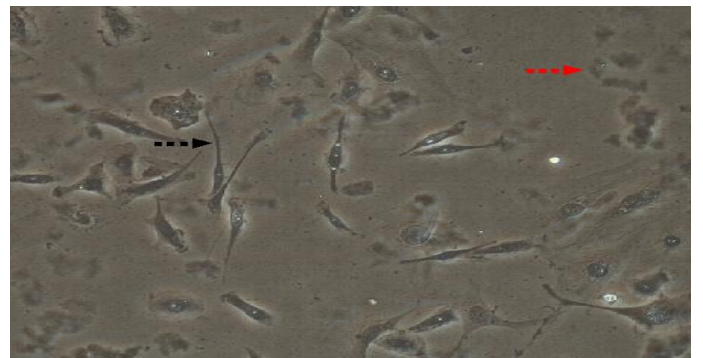


Figure 6: Bipolar PC12 cells treated with 800 µl HTE (black arrow). Red arrow showing debris.

HTE-generated neurite outgrowth

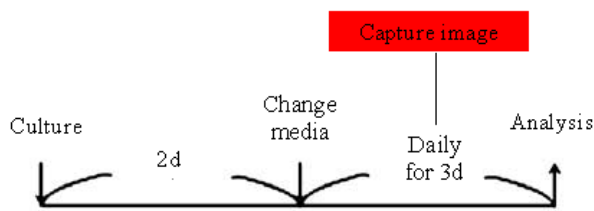


Figure 7: Culture protocol for neurite outgrowth in PC-12 cell line.

Culture of cells is treated with HTE from day 1 and left for 2 days before culture medium is changed with serum-free or maintained as serum-rich medium. For the next 3 days, images of neurite outgrowth would be taken and analysed. Our results show that HTE has an ability to induce neurite outgrowth in a dose-dependent manner.

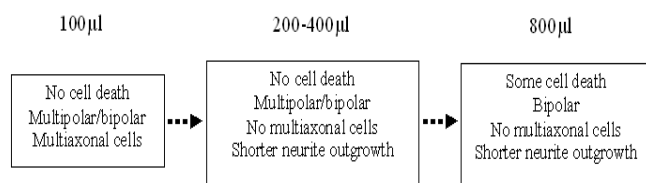


Figure 9: Concentration-dependent change in cell death, morphology and neurite outgrowth in HTE-treated group.

In serum-rich samples, neurite outgrowth is most significant at the lowest dose of 100 µl, especially when not treated with TIP. In several cells, multiple axons phenomenon could be seen. Cells with neurites also appear to interact to each other through axon-cell body contact or neurite-neurite contact. This is suggestive of some cellular cytoplasmic changes, perhaps accumulation or organization of some axonal marker or protein such as tubulin, taking place following signal undoubtedly produced under the influence of HTE.

The length of neurite outgrowth in the 200 µl sample is remarkably shorter than that observed in the 100 µl sample.

Similarly, neurite induction in other samples are not remarkable, with mostly bipolar cells predominates as the concentration gets to 800 µl.

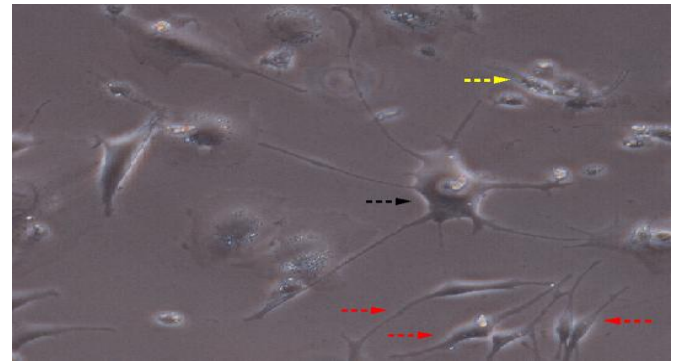


Figure 8: PC12 cells treated with 100 µl HTE showing greatest multiaxonal cell (black arrow). Bipolar cells (red arrow) and dead cells could also be observed (yellow arrow).

DISCUSSION

HTE has been investigated previously as an antioxidant agent and has been shown to contain high numbers of omega-3 fatty acids. We are here interested to investigate is HTE may have possible role in the treatment of brain damage due to neurodegenerative diseases and traumatic injury following for example the use of illicit drugs.

In this study, HTE is shown to be able to exert change in the way PC12 cells behave in culture in serum-rich media. Moreover, HTE is also able to induce neurite outgrowth in these cells at specific concentration.

In this experiment, a positive control treated with membrane-permeable dibutyryl cAMP is used to compare results with HTE-treated samples. cAMP is already an established neurite inducer (Qiu, 2002) and its concentration inside cells are elevated after priming with neurotrophic factors enabling outgrowth and extension of neurite by the activation of protein kinase signaling pathway. (Aglah, 2008)

In our samples, induction of neurite was exhibited in cAMP-treated samples. However there is lesser effect on cells' aggregating behaviour and multipolarity. Most cells in these samples continued to grow in aggregation and remained non-polar. Only a fraction of them were able to grow individually and produce neurite outgrowth.

The effect of HTE in these serum-rich samples is different from cAMP as is suggested by the frequency of multipolarity, extension of neurites and multiaxon phenomenon. Induction of neurite in serum-rich samples above 100 µl is less remarkable than in the 100 µl. Many cells with cytoplasmic extension in samples with 200 µl and 400 µl of HTE are bipolar cells and spectacular multiaxonal cells seen in 100 µl sample were not present. HTE at a concentration lower than 100 µl also failed to induce remarkable formation of neurite.

This difference in the effect of HTE may be related to the level of neurite generating compounds and toxicity effect at different HTE dose. The lower dose of HTE is further diluted by the presence of media and hence unable to provide enough stimulation for neurite outgrowth. On the other hand, a higher concentration of HTE may have prevented neurite generation by toxic effect.

The slight contrast between the effect of cAMP and HTE on neurite outgrowth could be due to several reasons. The induction of HTE could have followed the same NGF receptor-Erk activation as cAMP, but with a stronger signal, hence the longer extension of neurite. Another postulation is that HTE may have stimulated neurite outgrowth through a totally different pathway. Other pathways do exist such as those that induce neurite extension via the activation of neurotrophin-Trk receptors (Pollack, 2002) and FGF receptor. (Williams, 1994) (Boscher, 2008) Interestingly too, some neurite inducers have been shown to be able to exert neurite extension independently of cAMP, (Greene,

1979) (Gunning, 1981) in contrast to the normal physiology of neurite outgrowth following activation of the NGF receptors.

It has been suggested by several papers that polarity is determined at the bipolar stage, with one of the two neurites acquiring axonal fates regardless of how many neurites later form. (de Anda, 2008) It could be posited, in view of the result in the serum-rich group of this study, that HTE at the right concentration enable the cells to change into multipolar cells, and then act as a factor that favours their survival. If this could also happen in the brain, perpetuation of this effect would enable cells to become multiaxonal in preparation for more specialized functions.

The result of this study vis-à-vis neurite production in both cAMP and HTE-treated samples supports the possibility of the induction of neurite by HTE, although it is not possible at this juncture to delineate the pathway of mechanism that gives rise to the observable neurite outgrowth. The result also gives an idea of the effect of THE on cells growth behaviour and whether treatment utilizing HTE would be beneficial in correcting functional disability of related diseases and traumatic injuries of the brain.

CONCLUSION

Generation of new neuron is an important step in the recruitment of functional neurons from the neuron stem cell and precursor cell reservoirs in the brain. Factors that could induce morphological change in term of neurite outgrowth in vitro must also be able to penetrate the blood brain barrier, show some neuroregenerative and neuroprotective role and support cell survival, and aid in the formation of neuron function to be of value in term of medical intervention in degenerative diseases and traumatic brain damages. Haruan therapeutic extract (HTE) as observed in this preliminary study seems to demonstrate all of these

characteristics although further study is needed to find more satisfying, conclusive evidence of its ability as neuroregenerative and neuroprotective agent.

REFERENCES

1. Caroni P, Neuro-regeneration: plasticity for repair and adaptation, Essays in Biochemistry, 1998, 33:53-64
2. Gould E and Gross CG, Neurogenesis in adult mammals: some progress and problems. Journal of Neuroscience, 2002, 22: 619-623 2002
3. Temple S, The development of neural stem cells, Nature, 2001, 414: 112-117
4. Palmer TD et al, Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS, Journal of Neuroscience, 1999, 19: 8487-8497
5. Patapoutian A et al, Trk receptors: mediators of neurotrophin action, Current Opinion in Neurobiology, 2001, 11: 272-280
6. Saito M et al, Neurotrophic factor production in human astrocytoma cells by 2,5,6-tribromogranine via activation of epsilon isoform of protein kinase C, European Journal of Pharmaceutical Science, 2006, 28 (4): 263-271 1a211
7. Mat Jais AM, Pharmacognosy and Pharmacology of Haruan hanna Striatus a medicinal fish with wound healing Properties, BLACPMA, 2007, 6 (3): 52-60
8. Mat Jais Am et al, Effects of Three Local Malaysian Channa spp fish on chronic inflammation, Journal of Oriental Pharmacy and Experimental Medicine, 2004, 5 (1): 91-94
9. Mat Jais AM et al, The effects of L-Arginine, D-Arginine, L-NAME, and methylene blue on haruan Channa Striatus-induced Peripheral Antinociception in Mice, Journal of Pharmacy and Pharmaceutical Sciences, 2005, 8: 199-206
10. Mat Jais et al, Antinociceptive activity of *Channa striatus* (Haruan) extracts in mice, Journal of Ethnopharmacology, 1997, 57: 125-130
11. Mat Jais AM et al, Fatty acid and amino acid composition of three local Malaysian Channa spp Fish, Food Chemistry, 2006, 97: 674-678
12. Wu A, Ying Z and Gomez-Pinilla F. Omega-3 fatty acids supplementation restores mechanisms that maintain brain homeostasis in traumatic brain injury. Journal of Neurotrauma. 2007; 24(10): 1587-1495.
13. Uauy R, Nutrition in brain development and Aging: Role of EFA, Nutrition Reviews, 2001, 64: S24-S33
14. Wainwright PE, DEFA and brain function: a developmental perspective on mechanism, Proceedings of the Nutrition Society, 2002, 61: 61-69
15. Mat Jais AM et al, Fatty acid and amino acid composition in haruan as a potential role in wound healing, General Pharmacology-Vascular Society, 1994, 25(2): 947-950
16. File SE, Fluck E, Fernandes C, Beneficial effects of glycine (Bioglycin) on memory and attention in young and middle-aged adults. Journal of Clinical Psychopharmacology, 1999;19:506-512
17. Gusev EI et al., Neuroprotective effects of glycine for therapy of acute ischaemic stroke. Cerebrovascular Diseases, 2000 (10) : 49-60.
18. Greene LA, Tischler AS, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proceedings of the National Academy of Science, 1976, 73 (7): 2424-2428
19. Schechter AL, Bothwell MA, Nerve growth factor receptors on PC12 cells: Evidence for two classes with differing cytoskeletal association, Cell, 1981, 24 (3): 867-874

20. Yamazaki M et al, Activation of the mitogen-activated protein kinase cascade through nitric oxide synthesis as a mechanism of neuritogenic effect of genipin in PC12h cells, *Journal of Neurochemistry*, 2001, 79:45-54
21. Zakaria et al, Preliminary investigation on the antinociceptive properties of Haruan (*Channa striatus*) fillet extracted with various solvent systems, *Pakistan Journal of Biological Science*, 2004, 7: 1706-1710
22. Qiu J et al, Spinal axon regeneration induced by elevation of cyclic AMP, *Neuron*, 2004, 34: 895-903
23. Aglah C et al, cAMP promotes neurite outgrowth and extension through protein kinase A but independently of Erk activation in cultured rat motoneuron, *Neuropharmacology*, 2008, 55 (1): 8 – 17
24. Pollack Sj and Harper SJ, Trk Neurotrophin receptor activators, *Drug News Perspective* 2002, 15(5): 268
25. Williams EJ et al, Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM and N-cadherin, *Neuron*, 1994, 13 (3) : 583-594
26. Boscher C, and Mege R, Cadherin-11 interacts with the FGF receptor and induces neurite outgrowth through associated downstream signaling, *Cellular Signalling*, 2008, 20 (6): 1061-1072
27. Greene LA et al, Cell culture studies on the mechanism of action of nerve growth factor, *Society of Neuroscience Symposium*, 1979, 4: 153-171
28. Gunning e al, Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells, *Journal of Cell Biology*, 1981, 89: 240-245
29. de Anda et al, Pyramidal neuron polarity axis is defined at the bipolar stage, *Journal of Cell Science*, 2008, 121, 178-185