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The effect of water extract of sea cucumber *Stichopus variegatus* on rat spinal astrocytes cell lines.

Azim Patar[1], Syed Mohsin Syed Sahil Jamalullail[2], Hasnan Jaafar[3], Jafri Malin Abdullah[1]

[1]Department of Neurosciences, School of Medical Sciences, Universiti, Sains Malaysia, Kota Bharu, Kelantan, Malaysia
[2]Biomedical & Health Research Platform, Universiti Sains Malaysia, Kota, Bharu, Kelantan, MALAYSIA
[3]Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kota Bharu, Kelantan, MALAYSIA

Abstract

Objective of the present study is to evaluate the proliferative activity of water extracts of *Stichopus variegatus* on spinal astrocytes cell lines. To study the doses dependant effect of *Stichopus variegatus* Water Extracts (SVWE) on spinal astrocytes, the extracts were prepared in four different concentrations of 0.1, 1.0, 5.0 and 10.0 µg/ml. The Epidermal Growth Factor (EGF), 10.0ng/ml was used as positive control. The proliferation assay was performed using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) Assay Kit. The proliferation was presented as percentages of surviving spinal astrocytes after 72h treatments. Our study shows that the SVWE has proliferative effect on rat spinal astrocytes in a dose dependant manner. At 10µg/ml SVWE showed a significant increase in the spinal astrocytes proliferation over a period of 72h ($p<0.01$). At the concentration of 5µg/ml, the astrocytes proliferation was seen to increase only at 72h ($p<0.05$). The EC_{50} of SVWE was 5.18 µg/ml. The Malaysian SVWE (EC_{50}=5.18 µg/ml) shows potential as a growth promoting agent to promote proliferation of spinal astrocytes at the concentration of 5.0 and 10.0µg/ml.

Keywords: Stichopus variegatus, Spinal astrocytes, MTS assay, Water extracts

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Introduction

Sea cucumbers from Malaysian Perhentian Islands have both commercial as well as medicinal values besides being used by the locals as an edible food source [1]. Stichopus variegatus (also known as Stichopus hermanii) is a species that is most valued not only as a food source but it also offers a variety of remedies which have been proven in an animal model system [2]. The Stichopus variegatus water extract (SVWE) has been reported to be used for rheumatoid arthritis [3], abdominal pain, liver damage [4] as well as heart ailments [5]. Numerous experiments that have revealed various in vitro effects such as antioxidant [6], anti-angiogenosis [7], anti-microbacterial [1], anti-leshmanial [8], potential cytotoxic agent on T-lymphoblastic cell lines [9] and its proliferation effect on neurosphere [10]. We hypothesized that SVWE may exhibit proliferative effect on spinal astrocytes in vitro.

Astrocytes plays an important structures that provide housekeeping functions necessary to maintain neuronal function, actively shape synaptic function, and act as neural precursors in adult neurogenic regions [11]. Another role of astrocytes is to preserve the host integrity following injury [12]. In spinal cord and brain traumatic injury, astrocytes actively undergoes hypertrophy, prolifertion and migrates to form reactive astrocytes and may induce the inflammatory reaction at the side of injury [13]. Accumulation of reactive astrocytes leads to astrogliosis, a physical and chemical barrier for regeneration during chronic phase of injury [12, 13]. The needs for astrocytes to act as neuroprotector and metabolic supporter after injury are very crucial. Astrocytes contribute to restoring the extracellular ionic environment [14], sequestering extracellular glutamate and producing neurotrophic factors during acute phase of injury [12]. Astrocytes expressed large receptor repertoire for proliferation and differentiation that include G-protein and ion channel that have been intensively reviewed [11, 13].

Epidermal growth factor (EGF), a mitogenic peptide that comes from epidermal growth factor family stimulates DNA synthesis and proliferation of astrocytes in vitro via interaction of EGF-EGF receptor bindings and activates

extracellular signal-regulated kinases (ERK) signalling cascades [15]. EGF stimulates spinal astrocytes proliferation and differentiation in vitro [16, 17]. Other EGF families members including transforming growth factor alpha (TGFα), amphiregulin, heparin binding (HB-EGF), betacelullin (BTC) and epiregulin also plays a vital role in promoting proliferation of astrocytes. Recently, a sea cucumber extract from Stichopodidae family was noted to increase the neural stem/progenitor cells (NSPCs) in vitro [10].

Hence, this study was conducted to determine the proliferative effect of SVWE on spinal astrocytes in vitro. To date there is no scientific report on the effects of SVWE on any central nervous system supporting cells (astrocytes in particular) which play critical roles in the different phases of recovery during brain and spinal cord injury.

Materials and Methods

Harvesting and processing of SV

Sea cucumbers were freshly harvested from Pulau Perhentian Kecil and Perhentian Besar, Kuala Terengganu, Malaysia. The harvesting was conducted in June 2009. This study was approved by Ministry of Natural Resources & Environment, Malaysia. Stichopus variegatus (SV) were harvested at the depth of approximately 30 metres below the sea level at two different fixed sites: (Point A: $5^0$54'59"N $102^0$43'26"E) (Point B: $5^0$54'9"N $102^0$45'19"E) as reported in previous work [25]. The SV was identified according to method described by Forbes et al., (1999)[18]. The body of SV was bilaterally cut at the dorsal midline. The internal organs were removed and the body walls were cleansed under running tap water at least six times. The body walls were cut into small pieces (1 x 3cm) and dried in an oven at 50^0C. The dried body tissues were blended using standard laboratory blender (ScienceLab,USA) into a powder form, placed in a plastic container and stored at 4^0C prior to extraction as described by previous authors [1, 19, 20].

Stichopus variegatus water extraction

The extraction was based on the method published by Fredalina et al., (1999) [20]. Homogenized tissue (20g) was mixed with 100ml of distilled water. The mixture was shaken at 80 strokes / min at room temperature for 3h using a Incubator-Shaker (Newbrunswick Scientific, USA). Thereafter, the mixtures were centrifuged at 1238 G for 20 min using Beckman AccuSpin (Beckman Coulter, USA). The supernatant was collected and the residue was mixed with another 50ml of distilled water. The process was repeated followed by centrifugation and the final residue was mixed with 25ml distilled water. The supernatants were combined in round bottom flask before for freeze drying process. The supernatant was freeze dried at -50^0C under a vacuum pump at 60 kPa for 36h using standard laboratory freeze dryer(Heto LL3000, Denmark)

Sterilization

The lyophilised extracts were subjected to sterilization using high electron beam rays according to method described by Furuta et al., (2002)[21] using Linear Accelerator (Siemens Primus, Germany). The extracts were kept in plastic containers, submerged in a 5cm depth of distilled water and exposed to gamma rays at 50 Gray(Gy) for 10min with as described by Furuta et al.,[21].

Astrocytes cell lines and treatment

Spinal astrocytes cell line (ScienceCell Research Laboratories,USA) was cultured in Dulbecco's Modified Eagle Medium (Invitrogen,USA) supplemented with 10% fetal bovine serum (Invitrogen,USA) and 1% penicillin-streptomycin antibiotic (Biosource,USA). Epidermal Growth factor (Sigma Adrich,Malaysia) was used as positive control and the untreated cell lines was used as a negative control. The SVWE was prepared to achieve the final concentration at 0.1, 1.0, 5.0, 10.0 µg/ml. The extracts were stored at 4^0C under sterile conditions prior to investigation.

The [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfophenyl)-2H-tetrazolium] (MTS) Assay

Confluent spinal astrocytes were counted and seeded in 96 well plates at a cell density of 1 x 10^4 cells per well. The extracts and EGF were added into cells grown in monolayer and incubated at 37^0C with 5 % CO_2 atmosphere for 24, 48 and 72 h respectively. The proliferation assay was performed using MTS assay kit (Promega, USA) [22]. Viability of the cells was measured in triplicate using 20µl of MTS solutions followed by incubation for 2 hours. The absorbance was read at 570nm using a standard spectrophotometer (Biochrom, United Kingdom) and the 50% effective concentration dose (EC_{50}) was determined.

Statistical analysis

The differences were analysed by Student-t test. The values of relative proliferation were expressed as percentage mean ± standard error means (S.E.M). A p value of <0.05 were considered statistically significant.

Results

MTS Assays

Figure 1 and 2 showed the effects of SVWE and EGF on spinal astrocytes cultures in vitro. In brief, the proliferative responses of rat spinal astrocytes were noted to be in linear dose- and time-dependent manners upon subjecting to SVWE. Higher SVWE concentrations of 5.0 and 10.0ug/ml were observed to significantly increases ($p<0.05$) cell proliferation over 72 h of treatment period

of compared to lower SVWE concentrations of 0.1 and 1.0 µg/ml. Similarly, a significant enhance ($p<0.05$) of relative proliferation for EGF treated cells was recorded at 24, 48 and 72 h of incubation periods. In particular, the relative percentage of cell proliferation at 72 h for this group was calculated as high as 187.2%

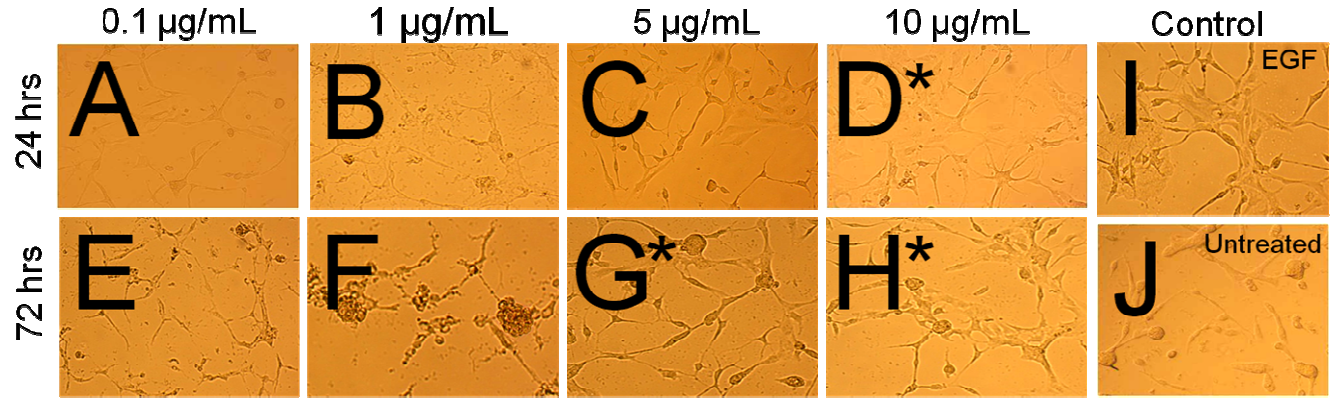

Figure 1. *The images of spinal astrocytes culture in vitro.*
*A-D: The 24 hours of astrocytes images following treatment with different concentrations of SWVE (0.1, 1.0, 5.0 and 10.0 µg/ml). E-H: The 72h of astrocytes images following treatment with different concentrations of SVWE. I : EGF treated cells, J: Untreated cells. At 72 h, there were increased in number of astrocytes after treated with 5 and 10 µg/ml compared to untreated cells. All images were captured under magnifications of 10X (Scale bar= 87µm). The *p<0.05, is considered significant when compared to untreated cells.*

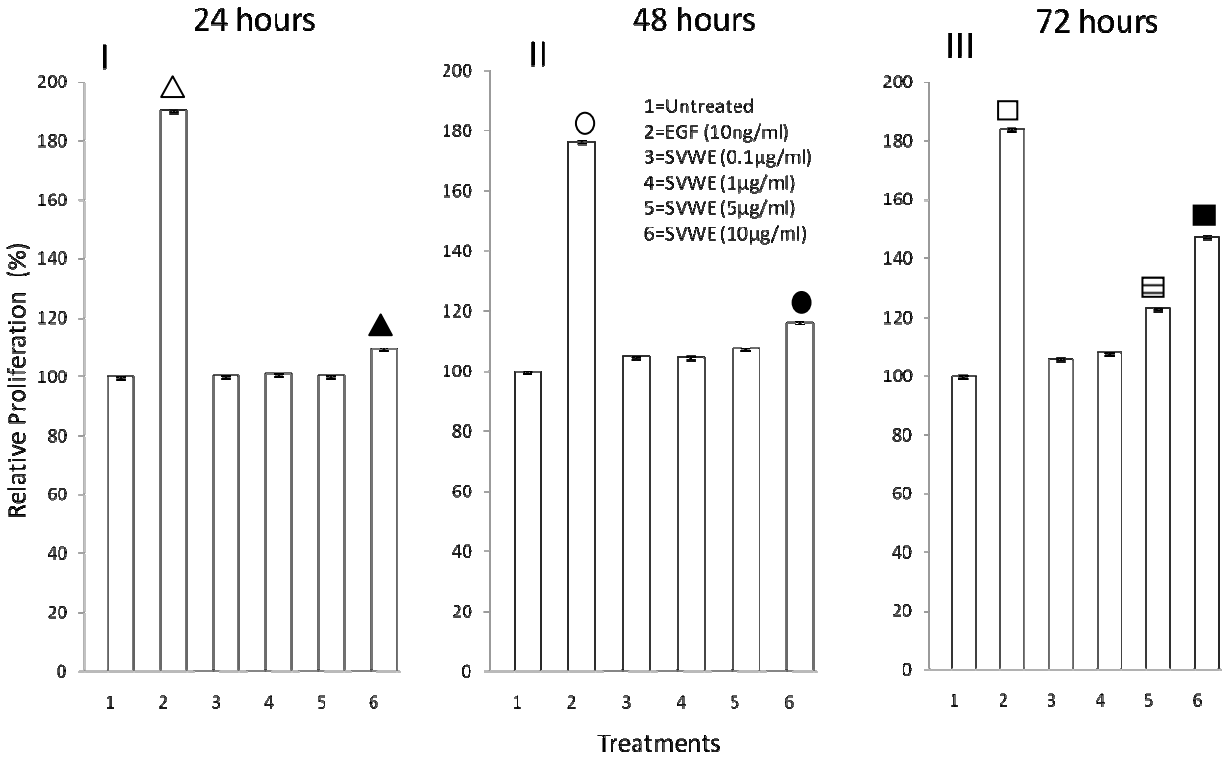

Figure 2. *The effect of SVWE and EGF treatments on spinal astrocytes culture in vitro*
Each value is the average ± standards error of mean (S.E.M). Bars indicate S.E.M. Significance of differences, was evaluated by Student's t-test; Panel I = treated Vs 1△ , $p<0.01$, ▲ $p<0.05$. Panel II = treated Vs 1: ○, $p<0.01$, ●, $p<0.05$. Panel III = treated Vs 1: , □ $p<0.01$, ■ , ▤ $p<0.05$.

When the cultures were treated with 10µg/ml of SVWE, there was significant increment in MTS incorporated into the astrocytes over 72h. At the SVWE concentration of 5µg/ml, the rate of proliferation was significantly in-

creased at 72h when compared to the control group. There was no significant proliferative activity seen at 0.1µg/ml and 1.0µg/ml concentrations of SVWE administration on astrocytes cultures when compared to untreated astrocytes (Figure 1).

EC_{50} value of SVWE

The EC_{50} value is concentration of the compound necessary to increase in the cell density by 50% in the period of 72h. EC_{50} was determined using OriginPro 8 SR4 (OriginLab Coorporation,USA). In this study, the effective concentration (EC_{50}) of SVWE was determined as 5.18 µg/ml.

Discussion

The findings of present experiment showed that the proliferation of spinal astrocytes increased at 5.0 and 10.0 µg/ml of SVWE over 72h. This result suggests that treatment with SVWE induce proliferation and differentiation of spinal astrocytes in a dose dependent manner. Our results are therefore in the same line to the other in vitro and in vivo reports [1, 3, 5, 19, 23, 24]

We propose that the proliferation of spinal astrocytes in this study could be associated with the bioactive compounds present within SVWE. We performed Gas Chromatography Mass Spectrometry (GCMS) to identify possible active compounds that may be involved in promoting cell growth in our recent work[25]. Our data on GCMS shows that protein components contributed the highest chemical composition of total SVWE(37%), followed by hydrocarbon(21%), ester(16%), carboxylic acid compounds(9%), phenol (7%) and others (10%). However, as our current interest is to study the effect of the crude SVWE, an isolation of single biocompound was not performed. A significance increase in relative cell proliferation of spinal astrocytes in our current experiment might be due to presence of glycosaminoglycan (GAG) which can be commonly found in marine invertebrae [24, 35]. Previously, GAG has been also reported for its ability to promote the proliferation of blood vessel in vitro [25].

The potential of SVWE as growth promoter in vitro demands a further analysis of the effects of SVWE on spinal astrocytes proliferative in vitro. Several interesting reports shows proliferative effects of Stichopus variegatus extract on fibroblast cell lines [26], neurite growth [27], blood vessels in vitro [28] and neural stem/progenitor cells [29].The use of spinal astrocytes cell lines in this study was due to the fact that astrocytes constitutes the main glial cell population of the spinal cord in which the astrocytes play a major role in nutritional support and neuron development [11]. It is well described that astrocytes together with smaller triangular, fusiform and multipolar shaped cells are the most common cells reported in rat spinal cord cytoarchitecture [30]. In pathological cases like spinal cord injury, proliferating reactive astrocytes are proven essential for early regeneration process, provide the protective functions for neurons and oligodendrocytes and preserve motor function after acute injury [31]

In this study, epidermal growth factor (EGF) was used as mitogenic agent so as to stimulate the proliferation of astrocytes in culture. Our data derived from EGF-treated cell culture was found to be comparable with the findings reported on the effects of EGF to stimulate astrocytes proliferation and differentiation in vitro over 72 hours [16, 17, 32]. The EGF acts on the astrocytes via receptor interaction and activates ERK signalling cascades and subsequently proliferative genes activates ERK signalling cascades [15]. The other possible reason for astrocytes proliferation following treatment with EGF is through the activation of Rgr-1 that has been intensively reviewed as early growth response gene proliferation and growth [32-34].

In conclusion, this current study demonstrated that the Malaysian SVWE promote proliferation of astrocytes cell lines at concentration of 5µg/ml and 10µg/ml. The mechanism that mimics EGF needs to be further studied in more details.

Conflict of interest statement

The author(s) declare that they have no competing interests

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Correspondence to:

Jafri Malin Abdullah
Department of Neurosciences
School of Medical Sciences
Universiti Sains Malaysia,
16150 Kota Bharu, Kelantan
Malaysia