
**UNIVERSITI PUTRA MALAYSIA**

# *In Vitro AND In Vivo ASSESSMENT OF WOUND HEALING PROPERTIES OF PERI-VITELLINE FLUID EXTRACTED FROM FERTILIZED EGGS OF ASIAN HORSESHOE CRAB, Tachypleus gigas (Muller 1785)*

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By

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy**

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Abstract of thesis present to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**_In Vitro_ AND _In Vivo_ ASSESSMENT OF WOUND HEALING PROPERTIES OF PERI-VITELLINE FLUID EXTRACTED FROM FERTILIZED EGGS OF ASIAN HORSESHOE CRAB, _Tachypleus gigas_ (Muller 1785)**

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**December 2017**

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Horseshoe crab is an ancient species existed 475 million years ago. The discovery on the precious compound of its blood, the amebocyte lysate, has benefited human kind contributing to the development of an endotoxin detection kit. Ever since then various studies have been carried out on this species, particularly in its life cycle how it impacted other life forms. This study focuses on the less studied aspect, the peri-vitelline fluid (PVF) of its eggs. Void of parental care, the horseshoe crab embryo developed with nourishment solely from PVF. This observation suggests that this PVF may contain beneficial property towards tissue regeneration. Hence, PVF of a local horseshoe crab, *Tachypleus gigas* was investigated for its wound healing ability.

The first part of this study was conducted to screen the biochemical composition through proximate analysis (protein, fat, carbohydrate, ash, moisture and energy), minerals contains (arsenic, antimony, cadmium, lead, mercury, stanum, copper, zinc, calcium, iron, potassium, magnesium, manganese, sodium, phosphorus, selenium, chromium and nickel) in PVF and embryo at 4$^{th}$ embryonic stage. While protein screening was carried on PVF extracted from 3$^{rd}$ and 4$^{th}$ embryonic stages.

The next part was on the *in vitro* study to determine the viability of 3T3 (mouse skin fibroblast cell) cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium and migration assay. Experiments were conducted using eight concentrations (1.56 to 200 µg/mL) of PVF for 72 hours. Then, *in vivo* study was carried out on brine shrimp and *Sprague dawley* rats. Toxicity tests on brine shrimps were using PVF with concentration of 1.85 to 1000 µg/mL. Experiment on rats was conducted with treatment consisted of 0.9% saline, petroleum jelly, Solcoseryl jelly 10%, PVF of 3$^{rd}$ embryonic stage

100 and 200 mg/g, PVF of 4th embryonic stage 100 and 200mg/g. Rats inflicted with wound then treated with the above treatments for 15 days. Rats were then euthanized and sacrificed, skin, liver and kidneys collected for histological study. Proximate analysis on the PVF of 4th embryonic stage for protein, fat, carbohydrate, ash, moisture and energy were 0.5 mL/100 mL, 0 mL/100 mL, 0.3 mL/100 mL, 3.0 mL/100 mL, 96.2 mL/100 mL and 1.4 Kcal/100g, respectively, while for the embryo of 4th embryonic stage 59.2 mL/100 mL, 12.1 mL/100 mL, 12.9 mL/100 mL, 9.4 mL/100 mL, 6.4 mL/100 mL 397 Kcal/100g, respectively. Protein screening on the PVF samples showed that the 3rd embryonic stage has more protein spots compared to the 4th stage. The pattern of the protein spots also differs between these two embryonic stages.

Based on the *in vitro* study, PVF was found to be non-toxic to 3T3 cells with 80% viability for all the tested concentrations. In the migration assay, PVF dosages of 100 and 200 µg/ mL for the two embryonic stages (3rd and 4th) showed 100% migration of 3T3 cells after 24 hours as compared to positive control (0.5% silver sulfadiazine). Toxicity test showed non-toxic effects on the brine shrimps for all the PVF dosages (1.95 to 1000 µg/mL). The rats treated with PVF from the 3rd embryonic stage at 100 mg/g and 4th embryonic stage at 200 mg/g showed accelerated healing, earlier by 3 days as compared to positive control (Solcoseryl gel 10%). While, the wounds treated with PVF at 200 mg/g from the 3rd and 4th embryonic stages healed well internally without adverse effect on the livers and kidneys. The finding of this study showed that the PVF extracted from the *T. gigas* eggs has beneficial property to support the rapid healing of wound without detrimental effect on the rat. It can be concluded that PVF of *T. gigas* has potential to be used as treatment to accelerate healing of wound particularly in patients with slow recovery.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Doktor Falsafah

**PENILAIAN *In Vitro* DAN *In Vivo* DALAM SIFAT PENYEMBUHAN LUKA DARIPADA EKSTRAK CECAIR PERI-VITELLINE TELUR BELANGKAS ASIAN YANG DISENYAWAKAN, *Tachypleus gigas* (Muller 1785)**

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Belangkas ialah spesies pra-sejarah yang telah wujud sejak 475 juta tahun dahulu. Penemuan kompaun yang berharga pada darahnya seperti amebocyte lysate telah memberi sumbangan positif kepada manusia di dalam membangunkan sebuah kit pengesan endotoksin. Sejak dari itu pelbagai kajian telah dilakukan terhadap spesies ini, terutamanya dalam kitaran hidupnya bagaimana ianya mempengaruhi bentuk kehidupan yang lain. Kajian ini memberi tumpuan kepada aspek yang kurang dipelajari iaitu cecair peri-vitellin (PVF) telurnya. Tanpa sebarang penjagaan dari induk, embrio belangkas dibesarkan bergantung semata-mata pada PVF. Pemerhatian ini menunjukkan bahawa PVF ini mungkin mengandungi ciri-ciri yang berguna di dalam pertumbuhan semula tisu. Oleh itu, PVF belangkas, *Tachypleus gigas* di kaji kerana mempunyai kemampuan di dalam penyembuhan luka.

Bahagian pertama kajian ini dijalankan untuk memeriksa komposisi biokimia melalui analisa proksimat (protein, lemak, karbohidrat, abu, kelembapan dan tenaga), mengandungi mineral (arsenik, antimoni, kadmium, plumbum, merkuri, stanum, kuprum, zink, kalsium , besi, kalium, magnesium, mangan, sodium, fosforus, selenium, kromium dan nikel) di dalam PVF dan embrio pada peringkat embrionik ke-4. Manakala pemeriksaan protein PVF yang diekstrak dijalankan ke atas peringkat embrionik ke-3 dan 4.

Bahagian seterusnya adalah kajian *in vitro* untuk menentukan keupayaan sel 3T3 (sel fibroblas kulit tikus) menggunakan MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromida) tetrazolium dan migrasi . Eksperimen-eksperiment ini menggunakan lapan kepekatan PVF (1.56 hingga 200 µg / mL) yang dijalankan selama 72 jam. Kemudian, kajian *in vivo* dijalankan terhadap udang marin dan tikus *Sprague dawley*. Kajian ketoksikan pada udang marin

menggunakan kepekatan PVF 1.85 hingga 1000 µg / mL. Kajian ke atas tikus telah dijalankan dengan rawatan terdiri daripada 0.9% saline, gel petroleum, Solcoseryl gel 10%, PVF peringkat embrionik ke-3 (100 dan 200 mg / g), PVF peringkat embrionik ke-4 (100 dan 200 mg / g). Tikus-tikus dikenakan luka dan kemudian dirawat dengan rawatan di atas selama 15 hari. Tikus-tikus kemudian dibius dan dikorbankan untuk mengumpul sampel kulit, hati dan ginjal untuk kajian histologi.

Analisis proksimat pada peringkat embrionik ke-4 PVF untuk protein, lemak, karbohidrat, abu, kelembapan dan tenaga ialah masing-masing 0.5 mL / 100 mL, 0 mL / 100 mL, 0.3 mL / 100 mL, 3.0 mL / 100 mL, 96.2 mL / 100 mL dan 1.4 Kcal / 100g, manakala untuk embrio peringkat embrionik ke-4 ialah masing-masing 59.2 mL / 100 mL, 12.1 mL / 100 mL, 12.9 mL / 100 mL, 9.4 mL / 100 mL, 6.4 mL / 100 mL 397 Kcal /100g. Pemeriksaan protein pada sampel PVF menunjukkan bahawa peringkat embrionik ke-3 mempunyai lebih banyak tompokan protein berbanding dengan peringkat ke-4. Corak tompokan protein juga berbeza di antara dua peringkat embrio ini.

Berdasarkan kajian *in vitro*, PVF didapati tidak toksik kepada sel-sel 3T3 dengan kadar percambahan hidup 80% untuk semua kepekatan yang diuji. Dalam assai migarsi, dos-dos PVF pada 100 dan 200 µg / mL untuk kedua-dua peringkat embrionik (ke-3 dan 4) menunjukkan 100% migrasi sel 3T3 selepas 24 jam berbanding dengan kawalan positif (0.5% silver sulfadiazine). Kajian ketoksikan tidak menunjukkan kesan toksik pada udang marin untuk semua kepekatan PVF (1.95 hingga 1000 µg / mL). Tikus-tikus yang dirawat dengan PVF pada peringkat embrionik ke-3 pada 100 mg / g dan peringkat embrionik ke-4 pada 200 mg / g menunjukkan penyembuhan luka yang agak cepat seawal 3 hari berbanding dengan kawalan positif (Solcoseryl gel 10%). Manakala luka yang dirawat dengan PVF pada 200 mg / g dari peringkat embrionik ke-3 dan ke-4 telah sembuh dengan baik secara dalaman tanpa kesan sampingan ke atas hati dan buah pinggang. Hasil kajian ini menunjukkan bahawa PVF yang diekstrak dari telur *T. gigas* mempunyai bahan yang boleh membantu mempercepatkan penyembuhan luka yang cepat tanpa memberi kesan sampingan pada tikus. Ini dapat disimpulkan bahawa PVF *T. gigas* berpotensi untuk digunakan sebagai rawatan dalam mempercepat penyembuhan luka terutama pada pesakit yang mengalami pemulihan luka yang agak perlahan.

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 3T3 | Mouse fibroblast skin cell |
| ATCC | American Type Culture Collection |
| DMEM | Dulbecco's Modified Eagle Medium |
| DTT | Dithiothreitol |
| ECM | extracellular lattice |
| FBS | fetal bovine serum |
| FGFs | fibroblast growth factors |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide |
| PDGF | Platelet-derived growth factor |
| PVF | Peri-vitelline fluid |
| RAS | recirculating aquaculture system |
| RB | rehydration buffer |
| TGF- $\beta$ | ransforming growth factor beta |
| VEGF | Vascular endothelial growth factor |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Horseshoe crab is an ancient organism, existed some 475 million years ago. Today the four remaining species somehow have not change much from their earlier ancestor. The significance of this species is on the various important impacts it has on its surrounding inhabitants. Migratory birds feed on its eggs for survival. Human uses it as bait in fishery activity, agricultural fertilizer (Kreamer and Michels, 2009), source of food delicacy for some of the Southeast Asian countries (Christianus and Saad, 2007), and most outstanding contribution to biomedical industry for the development of endotoxin detection kit (Novitsky, 1984; Mikkelsen, 1988; Berkson and Shuster, 1999). This kit contained active compound called amebocyte lysate, extracted from the blood of horseshoe crab, will coagulate immediately when in contact with microscopic organisms (Levin and Bang, 1968). Ever since this discovery, various studies have been conducted on other parts of the horseshoe crab body such as the carapace, compound eyes, digestive organ, reproductive organ and on the egg and embryo. The eggs of this horseshoe crab develops outside the parent body. Embryo molts and increase in size as it get near to hatching (Sekiguchi, 1988). During this developmental period, the embryo depends only on its perivitelline fluid (PVF). Volume of PVF increases depending on the developmental stage of the embryo (Nagai et al., 1999). Several studies showed that this PVF contains proteins like hemagglutinins and hemocyanins, which are suspected to have a role during embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984a).

## 1.2 Problem statement and justification

In medical term, wound is categorized into two stages, acute and chronic (Australian Wound Management Associate, 2011f). Acute stage will turn into chronic when the wound fails to heal within four to eight weeks during recovery period.

Growth factors play an important role in wound healing process. Two types of protein involved in wound healing are platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β). PDGF plays function during inflammatory phase and is initially release from platelets. While TGF-β is release through macrophages and platelets. TGF-β is activated through fibroblast and stimulating the formation of collagen (Hsu and Mustoe, 2010).

Certain protein extracted from organism have the ability to heal wound. Peri-vitelline and amniotic fluids are two of these examples, having pluripotent lines of brain, livers and bone cells, harmless to embryo. These fluids are capable to induce cells regeneration due to their proliferation capacity, multipotency and immunomodulatory activity. Because of these characteristics, the amniotic fluid was explored for its potential use as treatment for wound healing, and its effectiveness in accelerating the healing process in fetal skin condition and adult wounds. In amniotic fluid, the cytokines contains basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin growth factor-binding protein (IGFBP) as growth factors in wound healing (Skardal, 2014).

In horseshoe crab, PVF may contained proteins that's contribute to the tissue regeneration for the continuous development of the embryo towards hatching stage. According to Sekiguchi (1988), horseshoe crab PVF contained a primitive protein which is responsible for growth of embryo during embryonic period. Shishikura and Sekiguchi (1984b) reported the presence of three glycoproteins with potent agglutinin-binding activity in the PVF of *T. gigas*. While Parab et al. (2004) managed to use this PVF to induce the proliferation of beta cells (β-cells), responsible for the production of insulin in human. Study by Ghaskadbi et al. (2008) showed that the PVF of horseshoe crab contains peptide(s) capable of inducing the differentiation of specific organs. However, such peptides are likely to be present in minute quantities in the form of proteins (Nagai et al., 1999).

Substantial evidences have been found describing the proliferative effect of the PVF of horseshoe crabs. Taking into consideration the proliferation enhancing activity of PVF, it can be potentially exploited in combination with biomaterials as scaffolds for many different types of cells. This can be very useful in dentistry and medical fields (e.g. for bone cancer treatment). Together with the advancement of stem cell research, PVF can be considered as a valuable source to support organogenesis.

Horseshoe crab also possesses lectins and hemocyanins, and capable to recognise molecules in cell-molecule and cell-cell interactions in a variety of biological systems (Axford and Kieda, 1998; Singh et al., 2011). This suggests the ability of lectins to act as recognition molecules inside the cells, on the cell surfaces and in physiological fluids. Lectins such as galectins have been reported to induce cell proliferation, arrest and apoptosis. These lectins have been used in organ morphogenesis, tumor cell metastasis, leukocyte trafficking, immune system response, inflammation and recognition of extracellular matrix (Sharon and Lis, 2004). Scientific evident showed the cell proliferative effect of horseshoe crab PVF (Parab et al., 2004). This might serve as a solid foundation for the production of growth factors which contain the elements found in the PVF to be used in embryology and cell/tissue culture. Considering the proliferative enhancing ability of PVF, it can be potentially exploited in combination with biomaterials as scaffolds for many different types of cells. The fibroblast cell is the main important structure during skin healing. Fibroblasts in contracting

wounds will increase actin microfilaments (thinnest filaments of the cytoskeleton and are found in the cytoplasm of eukaryotic cells), and these are called myofibroblasts. These myofibroblasts laterally pull the collagen fibres together and closes the wound (Clark, 1988).

Wound healing consists of an efficient movement towards the recovery of the injured tissue (inflammatory, proliferation and remodeling stages). The inflammation stage commences immediately after injury with vasoconstriction to support homeostasis and irritation. The proliferative stage involved in the granulation of tissue expansion shaped essentially by fibroblast and the angiogenesis process. Remodeling stage is described by the reformulations and change in the segments of the collagen fiber that builds the elasticity during the process.

Proliferation for the development of new tissue occurs during angiogenesis, collagen deposition, granulation tissue arrangement, epithelialization, and wound withdrawal (Chang et al., 2004). In angiogenesis, vascular endothelial cells shape new blood vessels (Garg, 2000). In fibroplasia and granulation tissue arrangement, fibroblasts growth creates a provisional extracellular lattice (ECM) by clearing collagen and fibronectin (Chang et al., 2004). Concurrently, re-epithelialization of the epidermis take place, in which epithelial cells multiply and move onto the injured site therefore forming new tissue (Enoch and Price, 2004). In wound compression, myofibroblasts diminish the extent of the injury by grasping the injured edges and smooth the muscle cells. At this point, the cells parts are almost recovered and unwanted cells will apoptosis. During remodeling, maturation and redesigning, collagen is realigned along strain lines, and unwanted cells will undergo apoptosis. Injury recovery process is delicate, vulnerable to intrusion or prompting of the arrangement of non-repairing of endless injuries. Components affecting the non-recuperating constant injuries are diabetes, venous or blood vessel problem, contamination, and metabolic inadequacies due to aging (Australian Wound Management Associate, 2011).

## 1.3 Research objectives

The main objective of this study was to investigate the wound healing properties of tropical horseshoe crab, *Tachypleus gigas* PVF.
Therefore the specific objectives were:

1. To screen and determine the biochemical and protein content PVF extracted from the 4$^{th}$ embryonic stage of *T. gigas* eggs,
2. To determine the cell viability and migration of 3T3 mouse skin fibroblast cell treated with *T. gigas* PVF,
3. To determine the toxicity of *T. gigas* PVF on brine shrimp, and
4. To evaluate the effectiveness of *in vivo* treatment using PVF extracted from *T. gigas* on rat's wound.

## 1.4 Hypothesis of study

$H_O$: Peri-vitelline fluid of *T. gigas* exhibit no *in vitro* and *in vivo* healing ability
$H_A$: Peri-vitelline fluid of *T. gigas* exhibit *in vitro* and *in vivo* healing ability

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