Potential applications of horseshoe crab in biomedical research

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Abstract **-** Horseshoe crab is one of the oldest existing living fossils comprising four main species today. Of these, *Limulus Polyphemus* is found in North America and the other three species, *Tachypleus tridentatus, Tachypleus gigas* and *Carcinoscorpius rotundicauda* are found in Southeast Asia. Horseshoe crabs play important roles in the regulation of the coastal ecology communities whereby the eggs serve as the main diet of shorebird species during the migrating season. Horseshoe crab is also seen as a versatile organism, useful in the biomedicine field particularly, as its blue blood has been widely integrated to be used for endotoxin tester in vaccines, drugs and injectables. Researchers have explored a material called perivitelline fluid (PVF) from the egg of a fertilized horseshoe crab which is rich in important proteins and amino acids that are crucial for embryogenesis. Previous studies have shown that PVF has the ability to enhance cell growth and differentiation as well as in promoting generation of certain organs. Testing of PVF on many types of cells has shown positive results and hence, it is suggested that PVF could be used as a supplement to support cell growth in future. Highlighting the horseshoe crab as a living fossil, this review brings out the relevance of the blue blood and PVF of the horseshoe crab as sources benefitting molecular research.

Keywords **- Horseshoe crab, perivitelline fluid, embryogenesis, cell differentiation, biomedicine**

Received 14 July 2019.
Revised 01 Sept 2019.
Accepted 10 Nov 2019.
Published Online 13 Dec 2019

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1 INTRODUCTION

One of the most interesting materials that has the potential to support growth, regeneration as well as differentiation of cells is the Perivitelline Fluid (PVF) from horseshoe crab. PVF is a fluid present in the space between a fertilized oocyte of the horseshoe crab and the outer membrane surrounding the oocyte called zona pellucida [1, 2]. This fluid has been often extracted in many previous studies as it contains many biologically active components that are said to be beneficial towards the growth and development of cells. This review traces of the evolution of horseshoe crab and then discusses the versatility of horseshoe crab in providing various valuable biologically active compounds that are used in the biomedical industry.

The oceans and seas serve as a precious habitat for many living creatures. Marine organisms hold crucial roles in biogeochemical processes whereby they sustain and give balance to the biosphere. They also provide humankind with a tremendous variety of natural products that are essential and functional for various drugs development in treating diseases and even cancers [3]. Generally, there are five main kingdoms (Monera, Protista, Fungi, Plantae and Animalia) in the marine world. Vertebrates that populate the marine account for another subphylum with a total of seven phyla as tabulated in Table 1. The marine world offers endless number of natural resources that are useful for many health and medical applications nowadays. Horseshoe crabs belong to the phylum Arthropoda which is characterized by their jointed limbs and their hard-outer shell which molt off as they mature through life.

One of the famous arthropods “living fossil” is the horseshoe crab which is classified as merostomes (Table 1). Horseshoe crab is often referred as the “living fossil” as the unique shape of its archaic body has survived through 350 million years and the shape remains unchanged phenotypically [4]. It can withstand harsh situations from the estuarine and coastal shallow habitats. Its body system has evolved to tolerate a wide range of salinity, temperature, desiccation and submergence [5]. This is due to the different

types of environment that a horseshoe crab will go throughout its entire life. Generally, horseshoe crab prefers to live in a calm sea or an estuary with muddy sand bottom [6]. However, during mating season, they migrate towards the seashore to breed and lay eggs. Therefore, during these times, they are subjected to face the abrupt changes of environmental conditions whereby they will experience lower salinity than the littoral zone [5]. In fact, as soon as the eggs of horseshoe crab hatches, even the juveniles are naturally forced to experience the low saline conditions of the shore zone to significantly higher saline conditions of the littoral zone as they migrate towards the sea. Therefore, due to its ability to weather the fluctuating habitats' environment throughout its entire life has made horseshoe crab population to survive until this day.

Currently, horseshoe crabs are represented by four species with distinctive morphology which are *Limulus polyphemus* and *Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda* [4, 7]. Rudkin and colleagues discovered a new fossil horseshoe crab, *Lunataspis aurora* in Manitoba (Canada), characterized by fusion of opisthosomal tergites into two sclerites [8].

Human beings have been utilizing horseshoe crabs for many purposes for the past several decades [9]. Some of them used the tail spines as spear tips and the body after grinding as fertilizers for fields and ponds [4]. Ancient remedy was also practiced traditionally in India by pricking the forehead with the horseshoe crab tailpiece as a pain reliever for various types of pains [4]. Besides that, populations in India, Singapore, Malaysia even enjoy horseshoe crab meat, eggs and even appendages as a local delicacy [7].

2 THE VALUABLE BLUE BLOOD OF HORSESHOE CRAB

Researchers have found the blood of horseshoe crab is useful in many pharmaceutical applications. *T. gigas* are well known for its blue blood which contains amoebocytes [9]. The amoebocytes are packed with huge refractile granules which contain many blood clotting factors. Typically, the amoebocytes in horseshoe crab blood solidifies when it comes in contact with endotoxins thus making the horseshoe crab blue blood as a great endotoxin tester for foods and drugs industries. Therefore, the amoebocytes lysate of horseshoe crab termed as Limulus Amoebocyte Lysate (LAL) has been actively extracted and purified to be used as an endotoxin tester in food, drug and pharmaceutical industries [4, 7]. The vital benefits of horseshoe crab are dependent on a crude LAL test manufacturing process which involves capturing the horseshoe crabs, bleeding them and centrifuging to concentrate the amebocytes. This is followed by addition of water to the packed amebocytes which results in lysing and releasing the coagulation proteins [10]. LAL secretes a number of blood clotting factors and later activates the blood clotting mechanism when it is exposed to small amount of endotoxin or bacterial pyrogens [11]. Degranulation of amoebocytes as well as the release of the coagulogens immediately takes place thus forming a gel or clot when even minute amount of endotoxin is present [12].

An important serum protein called lectin is also found in the haemolymph of horseshoe crab that is very useful in the detection of Gram-positive bacteria families such as Pseudomonadaceae, Enterobacteriaceae (*Escherichia coli*), Bacteroidaceae and Neisseriaceae [7]. Pyrogen testing by lysate has become a more preferred method than the classic rabbit pyrogen test as it is more sensitive to small amount of endotoxins apart from giving quicker diagnostic result in the food, drug and pharmaceutical industries [4, 7]. The purified LAL has the capability of detecting one millionth of a billionth of a gram of endotoxin in less than 1 hour [7]. Therefore, LAL was already patented and recognized by drug regulatory authorities and industry in most countries and are currently used as an end-product testing method for endotoxin associated in human, animal injectables and drug products.

3 CHITIN AND CHITOSAN – TURNING WASTE INTO SUSTAINABLE PRODUCT

Due to the huge consumptions of crustaceans mainly comprising crabs, significant amount of waste calls for an effective management of disposal or reuse through cost-effective means. In the past decades, huge amounts of research have been conducted in the discovery of high value chemicals found in the crustacean waste [13]. The outer shell of a horseshoe crab (*Limulus*

polyphemus) is made primarily of chitin (26.4 % on a dry weight basis), protein, and other mineral matters [14]. Chitin and its origin called chitosan is a naturally abundant derived substance that is made up of sugar compounds. It is largely found in the shells of exoskeletons of animals such as shrimps, crabs and lobsters, and wings of insects [15, 16].

Chitin has been widely reported to be a biodegradable and non-toxic material which is safe for industrial use [17]. Studies have reported the wide range of potential uses of chitin as an antimicrobial agent (insecticides), food additive as tanning remover, food stabilizer, lactose intolerant aid and biomedical and pharmaceutical applications (contact lenses, wound dressings and sutures) [18, 19]. The commonly used practice to extract and produce chitin is by first grinding the shells to homogenize and particulate the size [20]. It is followed by addition of sodium hydroxide and screening, and then washing to remove the proteins attached to the shells. Next, hydrochloric acid is added, followed by screening, washing and pressing to discard any remaining mineral matters. Drying process is then conducted to extract and store the chitin material in powder form [20].

4 PERIVITELLINE FLUID (PVF) - THE NEXT MORPHOGEN

PVF is the fluid that fills the space between the outer envelope and embryo of the horseshoe crab. It is considered to be valuable to many medicinal practices as the PVF contains many types of important primitive proteins which could supplement growth and proliferation of cells [21]. Several studies have been conducted to investigate the use of PVF since the 70s. It was found that PVF contains important proteins, namely, hemagglutinins and hemocyanin that are crucial in the embryogenesis process [22].

Studies have been done previously to demonstrate the positive effect of PVF on the embryogenesis as well as cardiogenesis in vertebrates namely chick embryos [11]. It is reported that the hemagglutinating activity in PVF significantly increased after the third embryonic molting. It is hypothesized that the peptides present in small amounts within the PVF could be the reason for its powerful ability to promote growth of specific organs such as brain and heart. Parab and colleagues [21] demonstrated that the PVF from embryos of the Indian horseshoe crab had promoted embryonic growth in cultured chick embryos and also specifically stimulated the development and differentiation of brain and heart. In the study, gastrulating chick embryos were developed in the presence of various dilutions of PVF of horseshoe crab as growth supplement for several hours. As a whole, positive result in the growth and development of embryos were seen from the development of brain and heart by looking at the stimulation of axis elongation in PVF treated embryos as compared to the controls [21]. Besides that, hemocyanins are also one of the most important biological macromolecules acting as oxygen-transporting glycoproteins among arthropods and molluscs. Based on a proteomic analysis using two-dimensional gel electrophoresis, Nagai et al. [23] reported an increase in the expression of hemocyanin in virus-infected arthropods. The authors subsequently proposed that the compound may play a role in antipathogenic actions against bacterial, fungal, and viral invasions.

Further advancement of the potential of PVF in promoting organ regeneration and cell differentiation was discovered when PVF was found to contain lectin, a compound that promotes cardiac myocytes formation in chick embryos [24]. Treatment with a dose of 30 mg PVF towards the gastrulating chick embryos resulted in 83% enlargement of the heart compared to the controls. Not only that the heart's size was enlarged, the PVF treatment was also effective in promoting the development of a normal functional heart as well [24]. Furthermore, PVF is also able to induce angiogenesis as the enlarged heart chambers were reported to have more extensive blood vessel network which results in a larger blood volume that could be pumped to whole body as compared to the control group [24]. Although the exact mechanism of how the protein lectin in PVF helped in the cell differentiation and organ regeneration in this study is not fully understood, it is well known that lectins are capable of inducing cell agglutination as well as mediating various cellular processes among organisms by recognizing and tagging to the carbohydrate ligand through the basic lock and key mechanism [25]. Lectins are one of the important molecules that made PVF a valuable source of cell growth supplement.

Table 1: Classification of marine organisms

Kingdom	Subkingdom	Phylum	Class	Examples
Monera	Archaebacteria	Thermoacidophiles		Thermobacteria
	Eubacteria	Schizonta		Photobacteria
		Cyanonta		Cyanobacteria
Protista	Protophyta	Pyrrophyta		Dinoflagellates
		Chrysophyta		Diatom
	Protozoa	Sarcodina	Actinopodea	Radiolarians
			Rhizopodea	Foraminiferans
Fungi	Ascomycota			Molds, lichens
Plantae	Seaweeds	Chlorophyta		Green algae Brown algae
		Phaeophyta)		
		Rhodophyta		Red algae
	Tracheophyta	Anthophyta		Flowering plants
Animalia	Parazoa	Porifera	Calcispongiae	Calcareous sponges
			Hyalospongiae	Glass sponges
			Demospongiae	
	Metazoa	Cnidaria	Hydrozoa	Hydrozoans
			Scyphozoa	True jelly fishes Sea anemones
			Anthozoa	Ctenophora Comb jellies
		Ctenophora		
		Platyhelminthes	Turbellaria Cestoda	Planarians Tapeworms
				Ribbon worms
		Nemertea		
		Nematoda		Roundworms
		Priapulida		Priapulid worms
		Mollusca	Polylacophora	Chitons
			Scaphopoda	Tusk shells
			Gastropoda	Snails
			Bivalvia	Clams
			Cephalopoda	Octopuses
		Arthropoda	Merostomata	Horseshoe crabs
			Pycnogonida	Sea spiders
			Crustacea	Crabs, lobsters
			Insecta	Insects
		Pogonophora		Riftia worms
		Brachiopoda		Lampshells
		Echiura		Spoon worms
		Sipunculida		Peanut worms
		Echinodermata	Crinoidea	Sea lilies
			Echinoidea	Sea urchins
			Asteroidea	Sea stars
			Ophiuroidea	Brittle
			Holothuroidea	Sea cucumbers
		Chordata	Urochordata	Tunicates
			Cephalochordata	Lancelets
			Vertebrata	Vertebrates (refer below)
Subphylum	**Superclass**	**Class**	**Order**	**Examples**
Vertebrata	Pieces	Agnatha		Jawless fish
		Chondrichthyes		Cartilaginous fish
		Osteichthyes		Bony fish
	Tetrapoda	Amphibia		Frogs
		Reptilia	Chelonia	Sea turtles
			Squamata	Sea snakes
			Crocodilia	Marine crocodiles
		Aves	Sphenisciformes	Penguins
			Procellariiformes	Tubenoses
			Pelicaniformes	Pelicans
			Charadriiformes	Gulls
		Mammalia	Cetacea	Whales
			Carnivora	Seals, sea lions
			Sirenia	Manatees
			Primata	Humans

Table 2: Summary of potential applications of perivitelline fluid of horseshoe crab in biomedical research

PVF contents	Potential	Reference(s)
Many types of important primitive proteins	Supplement growth and proliferation of cells	[21]
	Enhancing growth, gametogenesis as well as spawning in vertebrate models. Positively influence the early development of gonads in red Tilapia fingerlings by resulting significantly higher gonadal weight and gonado-somatic index (GSI) in the PVF (200 µl) treated tilapia compared to the control	[26]
Haemagglutinins and hemocyanins	Aid in embryogenesis and cardiogenesis in mammals	[21, 22]
	May possess antipathogenic actions against bacterial, fungal, and viral invasions	[23]
Lectin	Stimulate the development of certain organs including brain and heart. Promote organ regeneration and cell differentiation	[24]
	Acts as a cell growth supplement by inducing cell agglutination and helps in mediating various cellular processes among organisms by recognizing and tagging to the carbohydrate ligand through the basic lock and key mechanism	[25]
Crude extract of PVF	PVF was non-genotoxic and safe to be used for further biomedical application. PVF crude extract could be effective in inducing cell viability and cell proliferation	[27]
	PVF-8 has the potential to stimulate and aid cell differentiation by triggering the human bone marrow stem cells into cardiomyocytes	[28]

Besides that, PVF was also reported to give positive influence in the early development of gonads in red tilapia fingerlings by resulting in significantly higher gonadal weight and gonado-somatic index (GSI) in the PVF (200 µl) treated tilapia compared to the control. It was reported that the PVF helped with the gonad maturation in tilapia as the ovaries in PVF treated appeared to be thicker and weighed double than the ones without PVF injection [26]. Thus, it was hypothesized by the authors that PVF contains peptide molecules which are effective in enhancing growth, gametogenesis as well as spawning in vertebrate models.

Crude extract of PVF has also been tested on stem cells derived from human dental pulp to observe its effect in terms of cell proliferation as well as the genotoxicity [27]. This study purposed four PVF crude extract concentrations from the MTT assay which represented 26.887 mg/ml (IC_{50}), 14.093 mg/ml (IC_{25}), 0.278 mg/ml (102% cell viability) and 0.019 mg/ml (102.5% cell viability). They concluded that the PVF produced insignificant proliferative activity on treated dental pulp stem cells (DPSCs) and was non-genotoxic and hence can be considered safe to be used for further biomedical application [27]. From their study, they also found that the cytotoxic effect of PVF was inversely proportional to the viability of DPSCs noting that the PVF crude extract could be one of the agents effective in inducing cell viability and proliferation if used in small specific amount as suggested.

More recently, a study was conducted to test the potential of PVF in stimulating cell differentiation by triggering the human bone marrow stem cells to turn into cardiomyocytes [28]. Based on the fluorescence-activated cell sorting analysis, they suggested that the optimum dose of 0.1 mg/ml from the eighth fraction (PVF-8) showed highest activity on the differentiation of human bone marrow stem cell into myocyte. This study was further strengthened and verified with various tests such as protein sequencing by SDS-PAGE that showed the presence of 122 amino acids, followed by the identification of myocytes in the bone marrow stem cell culture by the expression of myosin using immunohistochemical and FACS analyses. Therefore, this study

confirmed that the PVF-8 treatment on bone marrow stem cells had given a more intense and significantly higher expression of myosin compared to the cells that was cultured with VEGF or b-FGF thus far agreeing with the idea of PVF's potential to supplement cellular differentiation [28]. A summary of the potential applications of PVF of horseshoe crab in biomedical research has been presented in Table 2.

5 CHALLENGES IN UTILIZING INVERTEBRATE PRODUCTS IN BIOMEDICAL RESEARCH

Due to the wide diversity of species of animal in the ocean, it remains a challenge to isolate every phylum that is available, thus resulting in a lack of comparative data between taxa [29]. Consequently, the problem of incorrect taxonomy can lead to complications during peptide isolation from collected samples. Problems such as lack of specificity and sensitivity in terms of chemical and biological compounds between batches could occur as can be seen in the case of developing LAL [30]. Despite a wide range of methods of extraction of the lysate, the traditional clot assay and the ensuing quantitation assays are still weighed down with inconsistencies in terms of sensitivity and specificity.

Another challenge faced for continuous discovery and analysis of natural compounds from marine organisms is the supply of bioactive material that they contain. Minute amounts are available from each organism, thus, the concentrations of active peptides in marine invertebrates are often less than 10−6 % of the wet weight [31]. Due to this issue, stakeholders and companies usually avoid investing in this field since the high cost of production is negated small amounts of product yielded. Besides that, many marine peptides and by-products possess complex structures that are yet to be characterized due to high cost [32].

Next, seasonal and geographical variations, different life stages, age, sex, and physiological state could add to the challenges in coming up with a reproducible invertebrate product for biomedical purposes [33]. Conservation issues also took a toll in enabling the usage of invertebrate products for biomedical implications. Ineffective monitoring and lack of constructive regulation regarding the conservation of invertebrates could bring to serious conservation and sustainability problems [33].

6 CONCLUSIONS

PVF from the horseshoe crab is shown as a natural and rich source of organic compounds and molecules that are proven to support the growth and development of many types of cells. Due to human population expansion, new resources and technological alternatives to modern medical world are warranted in order to improve the treatment methods when dealing with diseases and illnesses that are on the increase. Therefore, further sustained research is imperative to fully explore the potential of PVF and blue blood from horseshoe crab for benefitting biomedical researches relating to tissue engineering and regenerative medicine.

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENT

This review article was supported by Universiti Sains Malaysia Research University Grant (1001/ PPSG/813077).

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