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Mini Review: Protein Components of Perivitelline Fluid (PVF) of Horseshoe Crabs & Its Applications in Medical Research

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Abstract: *Horseshoe crabs have been identified as one of the marine species that contribute greatly to scientific advances especially in haematological studies. Besides the haemocytes of these creatures, they are also well known for their protein contents in the perivitelline fluid (PVF). Due to this, over the years, there has been increasing interests in the scientific community to explore the function of proteins in the PVF of horseshoe crab for applications in various biomedical areas such as immunology, embryology and tissue or cell engineering. This article will review the two main protein components found in the PVF of the horseshoe crab namely, lectins, and hemocyanins with regards to its applications in medical research.*
Keywords: *Horseshoe crab, perivitelline fluid (PVF), lectin, hemocyanin*

I. Introduction

Marine bioresources which include marine cyanobacteria, algae, invertebrate animals and fishes provide a great variety of specific and potent bioactive molecules such as natural organic compounds which include fatty acids, polysaccharides, polyether, peptides, proteins and enzymes. Many researchers have reviewed and described the potent therapeutic agents derived from marine resources and their medicinal applications (Burja et al., 2001; Smith et al., 2010; Vo and Kim, 2010).

A very versatile marine species which provide valuable sources to some of the most vital biomedical testing is horseshoe crabs. These pre-historic creatures first appeared 475 million years ago in the Ordovician period and can be classified into four distinct species. Three of its species, Tachypleus tridentatus, Tachypleus gigas and Carcinoscorpius rotundicauda are distributed in Southeast Asia, while the fourth, Limulus polyphemus, can be found along the Atlantic coast of North America (IUCN, 2013). These anthropods not only have been studied extensively particularly in elements in its haemocytes that have contributed to many haematological related research but also with regards to the constituents in its perivitelline fluid.

The perivitelline fluid (PVF) refers to the fluid that fills the space formed between the blastula (embryo) and the egg shell (Punzo, 2000). There is still dearth of information on the individual components in the PVF due to the variability in the different species of horseshoe crab which could contribute to the variation in the detected constituents.

II. Protein Components In The Pvf Of Horseshoe Crabs

Among the desirable components in PVF which are extracted for the purpose of medical research are the proteins. The proteins that are present in PVF include hemaglutinins and hemocyanins, which may have an important role during embryogenesis (Sugita and Sekiguchi, 1979; Shishikura and Sekiguchi, 1984a). The protein components in the PVF of a Japanese horseshoe crab, T. tridentatus were classified into two proteins and two protein groups; a group known as hemocyanin (H proteins) and the unidentified proteins represented as B-1 protein, B-2 protein and the "rest proteins". The concentrations of these proteins differ at different stages of embryo development (Sugita and Sekiguchi, 1979). Additionally, Shishikura and Sekiguchi (1984b) have reported the isolation of glycoproteins (known as ABS-I, -II, and –III) with potent agglutinin-binding activity from the PVF of T. gigas embryo. These proteins were found to have high contents of aspartic acid, glutamic acid and glycine in common. Similar to the Japanese horseshoe crabs, the peptides in the PVF of Indian horseshoe crabs are also likely to be present in small quantities. However, these peptides may be represented as different kinds of proteins, in addition to the four major proteins stated earlier in the Japanese horseshoe crabs (Ghaskadbi et al., 2006).

III. Lectins

One of the bioresources, lectins are considered as the promising candidates for useful therapeutic agents because of the carbohydrate structures (proteoglycans, glycoproteins and glycolipids) that have been

implicated in certain cell types and also due to their physiological and pathological functions including host-pathogen interactions and cell-cell communications (Ogawa et al., 2011). Lectins refer to groups of sugar-binding proteins with the exception of antibodies and enzymes that recognize specific carbohydrate structures, resulting in the regulation of various cells via glycoconjugates. Thus, they are able to identify the types of cell and stages of cell development including embryonic stem (ES) and induced pluripotent stem (iPS) cells through histochemical applications, flow cytometry and lectin microarrays (Toyoda et al., 2011). This is because cells often show altered surface glycoproteins and glycolipids depending on the physiological and pathological conditions.

Lectins can be found in all taxa from microbial organisms, plants and animals and they are involved in various cellular processes depending on their specific recognition of complex carbohydrates. Animal lectins can be classified into several categories based on the structural similarity of carbohydrate recognition domain (CRD) and their characteristics: C-type lectins (CTLs), galectins, I-type lectins, pentraxins, P-type lectins, tachylectins and so forth (Gabius, 1997).

Even though various lectins can be found in the haemocytes of the horseshoe, these components also can be extracted from PVF. Isolation and characterization of lectin proteins from the PVF of horse shoe crabs including; T. tridentatus (Nagai et al., 1999) and T. gigas (Ghaskbadi et al., 2008) has been reported over the years. These lectins were proposed to have essential role in the completion of early stage of embryogenesis by interacting with endogenous glycoproteins or N-acetylhexosamines. Sub-types of lectins can be identified from different species of horseshoe crabs.

An example of lectins is tachylectins-related proteins. Tachylectins or known as non-self-recognizing lectins have been identified in the Japanese horseshoe crabs (T. tridentatus) from haemocyte (tachylectins-1 to 4) and plasma (tachylectins-5A to 5B) (Beck et al., 2001). The tachylectins can also be found in the PVF of the horseshoe crabs, namely tachylectins-P (TL-P) presented as 27-kDa lectin. The sequence of amino acid of TL-P is 99% identical to that of haemocyte-derived TL-1 but both these lectins are significantly different in biological and biochemical characteristics (Nagai et al., 1999).

IV. Hemocyanins

In body fluid of many anthropods, hemocyanins, a copper-containing proteins act as an agent for oxygen transport. These proteins evolved early in the arthropod stem lineage from the phenoloxidases, which are the oxygen-consuming enzymes involved in the melanin pathway (Burmester, 2002). Other members of the arthropod hemocyanin superfamily have lost the ability to bind copper and thus oxygen leading to the production of the non-respiratory pseudo-hemocyanins (cryptocyanins) which serve as storage proteins in decapod crustaceans and the hexamerins in hexapods (Markl et al., 1992; Burmester, 2002; Pick and Burmester, 2009).

Hemocyanins refer to large proteins that may consist of complexes up to several million Daltons. Within all arthropod hemocyanins, the main structure of a hexamer of six similar/ identical subunits in the 75-kDa range is conserved, although in a lot of cases, the hexamers associate to produce quaternary structures with up to 8 × 6 subunits (Markl and Decker, 1992; van Holde and Miller, 1995). Every subunit of anthropod hemocyanins comprises of three domains namely, domain I (5 to 6 α-helices), domain II (a 4 α-helix bundle encompassing the di-copper centre) and domain III (7 stranded anti-parallel β-barrel) (van Holde at al., 2001; Jaenicke et al., 2012; Rehm et al., 2012).

V. Medical Applications Of Lectins And Hemocyanins In The Horseshoe Crab

Many researchers have shown the function of lectins as recognition molecules in cell–molecule and cell–cell interactions in a variety of biological systems (Axford and Kieda, 1998; Wigglesworth-Cooksey and Cooksey, 2005; Singh et al., 2011). In a greater sense, the foregoing discussion suggested the ability of lectins to act as recognition molecules inside cells, on cell surfaces and in physiological fluids. Lectins such as galectins have been reported to be capable of inducing cell proliferation among other functions like cell arrest and apoptosis. These lectins have been implicated in organ morphogenesis, tumor cell metastasis, leukocyte trafficking, response of the immune system, inflammation and recognition of extracellular matrix (Sharon and Lis, 2004).

Pertaining to its function in immunology, the lectins' pathway is identified as one of the activation pathways for the mammalian complement system, besides two other pathways namely, classical and alternative. These pathways merge at the essential event which is the proteolytic activation of the central component C3 to C3b that covalently tags foreign microorganisms for engulfment by phagocytes. The deposit of C3b also acts as catalyst for lytic pathway where the membrane attack complex (MAC) is formed leading to the lysis of the pathogen (Fujita et al., 2004: Nonaka and Yoshizaki, 2004). Zhu et al. (2005) reported that a functional homolog of vertebrate complement 3, CrC3, which was isolated from C. rotundicauda together with several plasma lectins (tachylectin 5a and 5b, represented as TL5a and TL5b and known as carcinolectins – CL5s) binds to a

wide range of microbes which resulted in the formation of the frontline innate immune defense system. Moreover, TPL (Tachypleus plasma lectin)-1 and TPL-2 which have different ligand specificities can be applied in the detection and discrimination of bacteria and endotoxins removal (Kuo et al., 2006).

It is hypothesized that these proteins extracted from the PVF of horseshoe crab produce proliferation-enhancer activity (Ghaskadbi et al., 2008; Srijaya et al., 2013). Srijaya et al. (2013) have reported the enhancement in the gonadal maturity in red tilapia upon the treatment with PVF from C. rotundicauda. The lectins from horseshoe crabs also potentially produce positive effect in the other type of cell's development and proliferation. For example, the lectin isolated from, T. gigas with a relative molecular mass of 2.7 x 10^4 which consists of 221 amino acid residues, were found to support the cardiac development activity in the chick embryo (Ghaskbadi et al., 2008). This indicated that the isolated lectin is capable of stimulating various aspects of chick embryo's embryonic development and specific organ enhancement (brain and heart), thus suggesting the presence of molecules (peptides) that stimulate growth and differentiation of specific organs. It was postulated that the peptides may be present in small concentration as a specific protein (Nagai et al., 1999). The presence of stimulant in PVF also could be derived as explained by Yatskievich et al. (1997) whereby, the stimulation of cardiac myogenesis in posterior region of chick pregastrula epiblast requires a sufficient activin or an activin-like molecule. A more recent discovery has shown that a 27 kDa lectin from Indian horseshoe crab embryos promoted increase in the number and diameter of blood vessels in chick embryos after treatment with PVF which indicates their pro-angiogenic effect through upregulation of VEGF and VEGFR-2/kinase domain receptor genes expression (Surekha et al., 2013).

Another component of proteins in horseshoe crabs, hemocyanin also serves as valuable source for medical research. For example, hemocyanin which binds at the scaffold consists of the chitin coated with antimicrobial peptides resulting in phenoloxidase activity which appears to function as a trigger for the wound healing of exoskeleton (Nagai et al., 2001). The conversion of hemocyanin into a phenoloxidase-like enzyme occurs through two major activation modes 1) proteolytic treatment with proteases (e.g. trypsin or microbe-derived) or 2) physical disruption of protein conformation (using agents such as detergents, solvents, salts and/or phospholipids). Hemocyanins also have also been suggested to act as a precursor of antibacterial and antifungal peptides (Coates and Nairn, 2014). Based on these studies, it is imperative to assume the essential role of hemocyanins as integral component in invertebrate's innate immunity.

VI. Conclusion And Future Directions For Research On PVF

Substantial evidences have been published on the proliferative effect of the PVF of horseshoe crabs. This might serve as a solid foundation for the production of growth factors or supplements which contained the elements in the PVF to be used in the areas of embryology and cell/ tissue culture. 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 particularly very useful in the dentistry and medical fields (eg. for bone cancer treatment). Together with the advancement in stem cell research, PVF also may be studied as a valuable source to support organogenesis.

It is also important that the researchers acknowledge the fact that horseshoe crabs are considered to be one of the endangered species. The conservation of these marine creatures must be promoted so that the positive outcomes of the research related to the PVF of horseshoe crabs could be reaped. This should lead to better breeding programs thus maintaining a sustainable population of these priceless anthropods.

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