Alam et al., Cell Dev Biol 2015, 4:3
DOI: 10.4172/2168-9296.1000162

Cell & Developmental Biology

Research Article Open Access

Horseshoe Crab Peri-Vitelline Fluid Triggers the Human Bone Marrow Stem Cell Differentiation into Cardiomyocyte *In Vitro*

Huma Alam[1], Chinnari Sumedha[1], Siddhartha Pati[1,2]*, Bisnu P Dash[2] and Anil Chatterji[1]

[1]*Malkolak Institute of Marine Studies, Dona Paula, Goa-403 004, India*

[2]*Department of Bioscience and Biotechnology, Fakir Mohan University, Balasore-756020, India*

Abstract

In the developing eggs of the horseshoe crab (*Tachypleus gigas*, Muller), the peri-vitelline fluid (PVF) occurs at the 21st stage of embryogenesis. Besides maintaining the homeostasis balance inside the developing eggs it also helps in organogenesis. Considering PVF assisting cell differentiation and proliferation during embryonic development, we made an attempt to examine its activity in triggering human bone marrow stem cells transforming into cardiomyocyte. Out of ten-peak PVF profile obtained from FPLC analysis, only the eighth fraction (PVF-8) showed highest activity on the differentiation of human bone marrow stem cell into myocyte differentiation. By FACS analysis, the optimum dose of PVF-8 was 0.1 mg/ml where maximum rate of bone marrow stem cells differentiation into myocyte observed. Since PVF-8 was showing the highest myocyte differentiation activity it was further analysed for its purity on SDS-PAGE where a single band of 29 kDa molecular weight was obtained. The protein sequencing of the PVF-8 showed the presence of 122 amino acids. In order to identify the myocytes present in the colonies formed after the long-termed culture of bone marrow stem cells, cells expressing myosin were identified both by immunohistochemical and FACS analysis. In immunehistochemical analysis, PVF-8 was found to induce a great expression of myosin in some adherent's cells incubated with it as compared to control cells cultured in presence of VEGF or b-FGF. Moreover, in presence of PVF-8, peroxidase activity in the exposed cells surpassed those in the control group. These results confirmed that PVF-8 effectively improved the rate of bone marrow stem cell differentiation.

Keywords: Horseshoe crab; Peri-vitelline fluid; Cardiomyocyte

Introduction

New advances in cardiomyocyte regeneration are being made in human embryonic stem cell research. Because of their ability to differentiate into any cell type in the adult body, embryonic stem cells are another possible source for cardiac repair cells. The first step in this application was taken by Itskovitz-Eldor et al. [1] who demonstrated that human embryonic stem cells can reproducibly differentiate in culture into embryoid bodies made up of cell types of three embryonic germ layers. The cardiomyocytes showed cellular markers consistent with heart cells and demonstrated contractile activity similar to cardiomyocytes when observed under the microscope. However, there is an unsolved controversy about the role of endogenous progenitor cells in cardiac homeostasis and myocardial regeneration [2].

During early developmental stages of the horseshoe crab embryo, as the inner egg membrane is being freshly formed, the space between the inner egg membrane and embryo (called peri-vitelline space) is filled with a fluid which is known as the peri-vitelline fluid [3]. The PVF of the fertilized eggs of the Indian horseshoe crab contains peptide(s) capable of positively influencing differentiations of specific organs [4,5]. Such peptides are likely to be present in minute quantities and may be in addition to the four major proteins reported in the Japanese horseshoe crab [6].

In this study we demonstrated that PVF-8, a fraction of PVF obtained from the fertilized eggs of the horseshoe crab helped in increasing the differentiation of CD 34^+ stem cells into myocytes in comparison to the other known growth factors. Therefore, it is suggested that PVF-8 contains an active component enhancing the differentiation of stem cells in myocytes, which may have therapeutic application in ischemic cardiopathy and vascular diseases.

Materials and Method

The fertilized eggs of the horseshoe crab were collected from the nests located on the sandy beach at Balramgari (Orissa) in India. The eggs were incubated at a constant temperature (37 $\pm$ 1°C) in artificial incubators until all eggs became transparent indicating that these developing eggs reached to stage-21 and taking external fluids. At this time, the embryonic PVF was collected using a sterilized 22-gauze needle (under sterilized conditions). While some PVF was aliquoted and stored at -20°C for further use, most of the PVF samples were freeze-dried (Model Edward-England) and re-suspended in sterilized distilled water for purification.

The PVF was sub-fractionated on a Fast Pressure Liquid Chromatography (Amersham Bioscience) using Superose 12 Hr 10/30 column at room temperature (Figure 1). The fractions were eluted in phosphate buffered saline (pH 7.4) with 1.45 mM of NaCl and 0.5 ml fractions were collected and stored at -20°C. To identify the most effective PVF fraction (Figure 2), 8 culture flasks were incubated with medium, cell suspension, 5% Foetal Calf Serum and different fractions of PVF. This was followed by FACS studies which were performed using rabbit anti-human myoglobin antibody. The SDS-PAGE analysis of the most active fraction (PVF-8) was done using 15% gel with Coomassie Brilliant Blue [7]. The amino acid sequencing of PVF-8 was carried out at Applied Bioscience, Mass Spectrometry Laboratory, New Delhi (India) for identification of different proteins.

The bone marrow was collected from the Haematology Division

***Corresponding author:** Siddhartha Pati, Department of Bioscience and Biotechnology, Fakir Mohan University, Balasore-756020, India, E-mail: patisiddhartha@gmail.com

Received October 31, 2015; **Accepted** November 19, 2015; **Published** November 27, 2015

Citation: Alam H, Sumedha C, Pati S, Dash BP, Chatterji A (2015) Horseshoe Crab Peri-Vitelline Fluid Triggers the Human Bone Marrow Stem Cell Differentiation into Cardiomyocyte *In Vitro*. Cell Dev Biol 4: 162. doi:10.4172/2168-9296.1000162

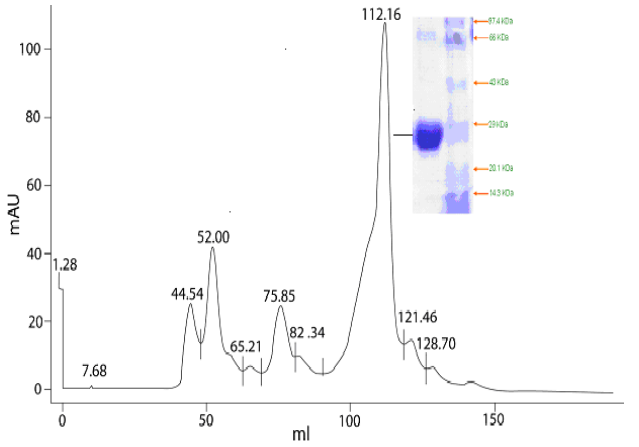

Figure 1: FPLC and gel profiles of peri-vitelline fluid (Buffer: 7.4; Sample load 500 µg; Column: Superose 12.2 column; AUFS: 0.1; Fraction size: 0.5 ml; arrow shows PVF-8).

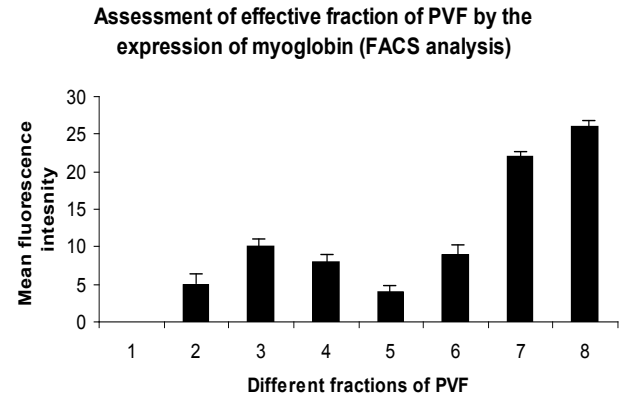

Figure 2: Assessment of most effective fraction of PVF.

of the Hospital Hotel Dieu Paris 4 in France and Ficill Centrifugation Technique was immediately used to separate the mononuclear cells from the bone marrow. The cell layer present in the supernatant was collected whereas all pellets were discarded. The cells were washed with PBS (1X) (Dulbecco's Phosphate Buffered Saline 10X by PAA Laboratories, Austria: Cat No H15-01) and centrifuged again at 1200 rpm for 10 minutes. The cell pellets were suspended in RPMI-640 containing 10% Foetal Calf Serum (Promo Cell). This was followed by one more centrifugation of the material at 1200 rpm for 10 minutes for the separation of CD 34$^+$ cells. The separation of CD 34$^+$ cells from the mixed population of the cells was done using Immunomagnetic Beads following the protocol as described by Easy Sep Stem Cell Technologies Inc. U.S.A.

The isolated CD 34$^+$ cells were suspended in EBM-MV2 and their number adjusted at 4 x 10^5 cells/ml. Then 5 ml of cell suspension was transferred into a culture flask that was previously coated with gelatin (1X). Nine flasks were used for this study. To study the enhancement of colonization by PVF-8, the CD 34$^+$ cells were suspended in EBM MV2 medium containing 10% FCS and enriched with different growth factors (VEGF, b-FGF and PVF-8) according to the requirement of different experiments. All culture flasks were kept for three weeks in an incubator at 37°C injected with 5% CO_2 to obtain 90% cell confluence.

The colony forming assay was adopted from Hill et al. [8]. The isolated CD 34+ cells were re-suspended at 2.5 × 10^6 cells/ml in culture medium in two different sets, i.e., Set A: EBM MV2 medium+cell suspension+5% Fœtal Calf Serum+VEGF and b-FGF (10 µl, 0.25 µg/500 µl). Set B: EBM MV2 medium+cell suspension+PVF (0.1 mg/ml) +5% Fœtal Calf Serum. This was followed by plating the cells at 2 ml/well in fibronectin-coated 6-well plates (Becton Dickinson, UK) and incubated for twelve days at 37°C with 5% CO_2 and 95% humidity. After twelve days, the non-adherent cells were removed, counted and re-suspended at 0.5 to 1 × 10^6 cells/ml in fresh medium in two different sets as described above for further observation.

The following two labtek sets were prepared to observe peroxidase staining in the present investigation:

Set A: EBM MV2 medium+cell suspension+5% Fœtal Calf Serum

Set B: EBM MV2 medium+cell suspension+PVF-8 (0.1 mg/ml) + 5% Fœtal Calf Serum.

Both the labteck sets were incubated under 5% CO_2 for three weeks at 37°C to obtain 90% cell confluence. For the identification peroxidase activity of the cells of colonies, the cells cultured in the wells of labteks were fixed by 4% paraformaldehyde for 5 min at 4°C and then permeabilized with Triton (0.1%) for 20 min. The cells were then washed with PBS 1X and then the peroxidase was detected by incubating cells for 20 min at room temperature with freshly prepared 100 µl of DAB (0.5 mg/ml+H_2O_2 0.1% final concentration). After an incubation period of 20 min at room temperature, the wells of the labteks were washed three times with PBS (1X). The slides from the labteks were detached and permanent slides were prepared with fluroprep. The slides were analyzed using light microscope and photographs were taken of both the labteks.

The following two sets were prepared for immuno-fluorescence staining for the present study:

Set A: EBM MV2 medium+cell suspension+5% Fœtal Calf Serum

Set B: EBM MV2 medium+cell suspension+PVF-8 (0.1 mg/ml) +5% Fœtal Calf Serum

The fixation and the permeabilisation of the cells were performed as described above. To avoid unspecific adsorption in the further steps, PBS:BSA (1%) was added in the labteks for 30 min. After washing, the wells were incubated at 4°C for 30 min in presence of rabbit anti-human myoglobin (Sigma Cat No M 8648) fluorescein-labelled anti-rabbit Ig (Amersham Cat no 1034). The two antibodies used were diluted by 1/100 in PBS containing 1% albumin. The labteks were washed three times with PBS (1X). The slides were analysed using both fluorescence and a confocal microscopes. The number of positive cells was counted. For each test, the samples were incubated with fluorescein-labelled anti-rabbit Ig antibody without adding the rabbit anti-human myoglobin antibody and treated as negative controls.

The following four different flasks were prepared for dose assessment activity of PVF-8 for myoglobin expression:

1. EBM MV2 medium+cell suspension+5% Fœtal Calf Serum

2. EBM MV2 medium+cell suspension+PVF- 8 (0.1 mg/ml) +5% Fœtal Calf Serum

3. EBM MV2 medium+cell suspension+PVF-8 (0.01 mg/ml) +5% Fœtal Calf Serum

4. EBM MV2 medium+cell suspension+PVF-8 (0.05 mg/ml) +5% Fœtal Calf Serum

Further the following five sets were prepared for FACS analysis for myoglobin expression in presence of different growth factors:

Set A: EBM MV2 medium+cell suspension+5% Fœtal Calf Serum

Set B: EBM MV2 medium+cell suspension+VEGF (10 µl, 0.25 µg/500 µl) +5% Fœtal Calf Serum.

Set C: EBM MV2 medium+cell suspension+b-FGF (10 µl, 0.25 µg/500 µl) +5% Fœtal Calf Serum.

Set D: EBM MV2 medium+cell suspension+VEGF + b-FGF (10 µl, 0.25 µg/500 µl) +10% serum.

Set E: EBM MV2 medium+cell suspension+PVF-8 (0.1 mg/ml) +5% Fœtal Calf Serum.

For immuno-cytochemical study, FACS analysis was done using a Flow-cytometer (Model: Beckton & Dickenson) following the standard protocol with rabbit anti-human myoglobin antibody (Sigma Cat No M 8648) to observe the expression of myoglobin.

Results and Discussion

A total of 10 peaks were detected in the peri-vitelline fluid of the horseshoe crab on FPLC profile (Figure 1). Initially all fractions collected by this methods were analyzed for the presence of myocyte differentiation activity to select the most effective fraction (Figure 2). Before initiating the experiment, the most effective dose of PVF-8 was also assessed by FACS analysis for myosin expression. It was found that 0.1 mg/ml of PVF-8 showed the maximum expression (Figure 3). Since PVF-8 was showing highest myocyte differentiation activity, it was further analyzed for its purity on SDS-PAGE (15% gel). A single band having 29 kDa molecular weights is a representation of fraction purity (Figure 1). The protein sequencing of PVF-8 showed 122 amino acids (Figure 4).

In order to identify the myocytes present in the colonies formed after long-termed culture of bone marrow stem cells, cells expressing myosin were identified both by immunohistochemical analysis and FACS analysis. In immunohistochemical analysis PVF-8 was found to induce a great expression of myosin in some CD 34⁺ adherents cells incubated with PVF-8 as compared to control cells cultured in the presence of VEGF or b-FGF (Plate 1). Similarly, the mean fluorescence intensity of cells cultured in presence of PVF-8 was also much greater as compared to control cells or cells cultured in presence of VEGF or b-FGF (Figure 5).

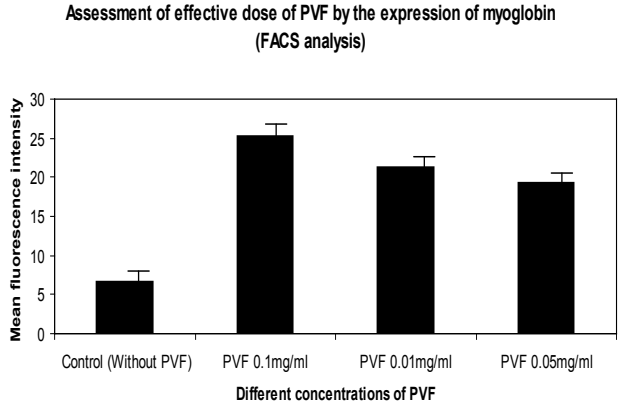

Figure 3: Assessment effective dose of PVF by the expression of Myoglobin.

VQWHQIPGKLMHITATPHFLWGVNSNQQIYLCRQPCYDGQ WTQISGSLKQVDADDHEVWGVNRNDDIYKRPVDGSGSWV RVSGKLKHVSASGYGYIWGVNSNDQIYKCPKPCNGAWTQ VNGRLKQIDGGQSMVYGVNSANAIYRRPVDGSGSWQQIS GSLKHITGSGLSEVFGVNSNDQIYRCTKPCSGQWSLIDG LKQCDATGNTIVGVNSVDNIYRSG

Figure 4: Sequence of fraction 8th of PVF.

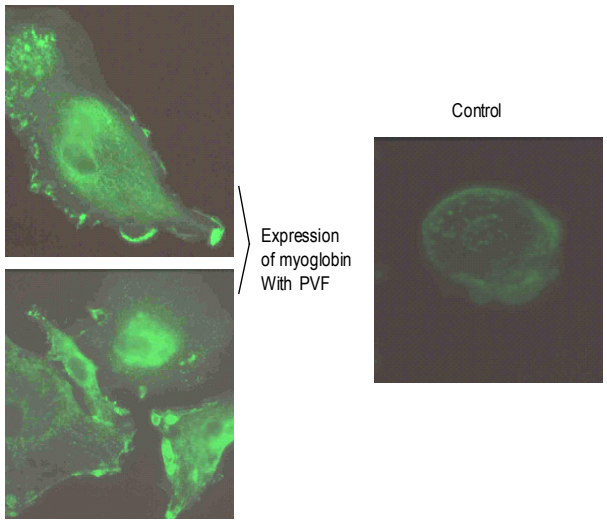

Plate 1: Effect of PVF-8 in the differentiation of CD 34⁺ cells into cardiomyocytes (expression in myocytes).

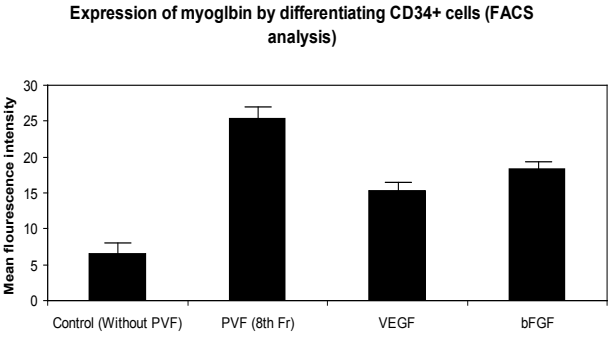

Figure 5: Expression of myoglobin by differentiating CD 34⁺ cells.

Peroxidasic activity was also performed to identify myoglobin of CD 34⁺ adherent cells. It appeared from the results that the number of cells having a peroxidase activity in colonies obtained in presence of PVF-8 was greater than the control experiment (Plate 2). This result was in high correlation with those showing myoglobin detection. Thus PVF-8 helped in improving bone marrow stem cell differentiation into myocyte.

Marine natural products have always been a source of new leads for the treatment of many deadly diseases such as cancer [9,10] and acquired immuno-deficiency syndrome [11]. The pharmacology of marine compounds with anthelminthic, anti-bacterial, anti-coagulant, anti-diabetic, anti-fungal, anti-inflammatory, anti-malarial, anti-

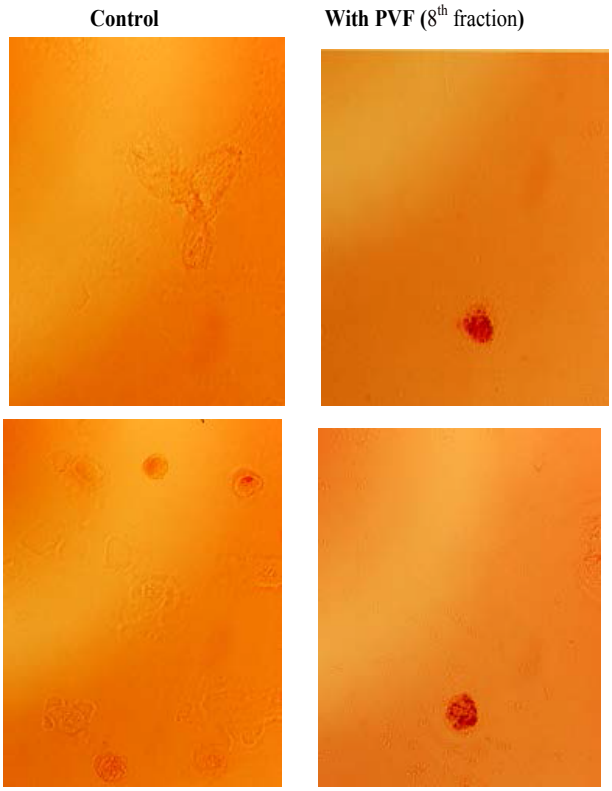

Plate 2: Peroxidase activity in presence of PVF.

platelet, anti-protozoal, anti-tuberculosis and anti-viral activities affecting the cardiovascular and nervous systems have also been reviewed elsewhere [12]. In recent years, a number of marine peptides with potential biological capabilities and promising anti-cancer abilities were isolated from various marine organisms such as microorganisms, algae, fungi, invertebrates and vertebrates. They are either directly or in the form of analogues deduced from structure-activity relationships. Some of them have already been marketed as drugs. Didemnin was the first marine peptide that entered in human clinical trials in USA for the treatment of cancer and other anticancer peptides such as kahalalide F, hemiasterlin, dolastatins, cemadotin, soblidotin, didemnins and aplidine have entered in the clinical trials [13].

In the present study an attempt was made to demonstrate that the purified fraction having molecular weight of 29 kDa and a polypeptide from the PVF of the fertilized eggs of the Indian horseshoe crab improved the differentiation of bone marrow stem cells into myocytes. The present study also indicated that PVF-8 not only induced stem cell differentiation but also helped in colony formation and promoted conversion of bone marrow progenitor cells to myocytes. In another study demonstrated that peri-vitelline fluid stimulated cardiovascular regeneration in an experimental model. These authors have also discussed the possibility of shunting of bone marrow progenitor cells towards cardiomyocyte differentiation [4,5,14].

Several colony-stimulating factors already reported from marine origin [15]. In this study, it has been clearly shown the effect of PVF-8 in the development of myocyte from bone marrow stem cells. However, multilineage developmental capacity of the CD 34$^+$ cells, especially into myocytes and smooth muscle cells is still controversial subject. A series of experiments conducted to prove the hypothesis that vasculogenesis and cardiomyogenesis after myocardial infarction may be dose-dependently enhanvced after CD 34$^+$ cell transplantation [16]. The present study also confirms the improvement in the differentiation of CD 34$^+$ cell to myocyte in presence of PVF-8.

On the basis of the present results it can be concluded that PVF-8 improved the differentiation of the bone marrow stem cells into myocyte. It is also evidenced that b-FGF and VEGF can also be involved but at a lesser extent than PVF-8 in differentiation of bone marrow stem cells to myocytes. This present study thus opens up entirely new possibilities using PVF-8 for medical application to improve stem cell differentiation into cardiomyocytes on severe ischemic diseases which cannot be improved by classic therapy.

Conclusion

Among all fractions of peri-vitelline fluid of the horseshoe crab (*Tachypleus gigas*; Müller) fraction 8th showed highest biological activity exhibiting differentiation of human bone marrow stem cell into myocyte. The 8th fraction of PVF, showed significant inducement of expression of myosin in adherent's cells as compared to control cells cultured in presence of VEGF or b-FGF. The mean fluorescence intensity of cells cultured in presence of 8th fraction of PVF was also much greater as compared to control cells and cells cultured in presence of VEGF or b-FGF. Our results clearly showed that the number of cells in presence of PVF was greater than that of control experiment.

Acknowledgement

The authors (HA, SC, AC) are thankful to the Chairman, Malkolak Knowledge Center, Hyderabad and (SP, BPD) to the Vice-Chancellor, Fakir Mohan University, Balasore for the facilities and encouragements.

References

1. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, et al. (2000) Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Med 6: 88-95.
2. Anversa P, Leri A, Rota M, Hosoda T, Bearzi C, et al. (2007) Concise review: stem cells, myocardial regeneration, and methodological artifacts. Stem Cells 25:589-601.
3. Sekiguchi K (1988) In: Biology of Horseshoe Crabs (Sekiguchi K, edn) Science House Co, Ltd, Tokyo), 139-181.
4. Parab PB, Ghaskadbi S, Patwardhan V, Mishra GC, Chatterji A, et al. (2004) Cardiac development promoting activity of peri-vitelline fluid of embryos of Indian horseshoe crab, *Tachypleus gigas* (Muller). Patent Number: 0319NF2004.
5. Musa M, Mohd Ali K, Kannan TP,Azlina A, Omar NS, et al. (2015) Effects of Perivitelline Fluid Obtained from Horseshoe Crab on The Proliferation and Genotoxicity of Dental Pulp Stem Cells. Cell J 17: 253-263.
6. Nagai T, Kawabata S, Shishikura F, Sugita H (1999) Purification, characterization, and amino acid sequence of an embryonic lectin in perivitelline fluid of the horseshoe crab. J Biol Chem 274: 37673-37678.
7. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
8. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, et al. (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348: 593-600.
9. Cragg GM, Newman DJ, Yang SS (2006) Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience. J Nat Prod 69: 488-498.
10. Gul W, Hamann MT (2005) Indole alkaloid marine natural products: an established source of cancer drug leads with considerable promise for the control of parasitic, neurological and other diseases. Life Sci 78: 442-453.
11. Tziveleka LA, Vagias C, Roussis V (2003) Natural products with anti-HIV activity from marine organisms. Curr Top Med Chem 3: 1512-1535.
12. Mayer AM, Hamann MT (2004) Marine pharmacology in 2000: marine

compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. Mar Biotechnol (NY) 6: 37-52.

13. Rawat DS, Joshi MC, Joshi P, Atheaya H (2006) Marine peptides and related compounds in clinical trial. Anticancer Agents Med Chem 6: 33-40.

14. Ghaskadbi S, Patwardhan V, Banerjee M, Agarwal S, Lenka N, et al. (2008) Enhancement of vertebrate cardiogenesis by a lectin from perivitelline fluid of horseshoe crab embryo. Cell Mol Life Sci 65: 3312-3324.

15. Misra S, Ghosh A, Varticovski L (1994) Naturally occurring ether-linked phosphatidylcholine activates phosphatidylinositol 3-kinase and stimulates cell growth. J Cell Biochem 55: 146-153.

16. Asahara T, Iwasaki H, Kawamoto A, Ishikawa M, Oyamada A, et al. (2006) Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 113: 1311-1125.