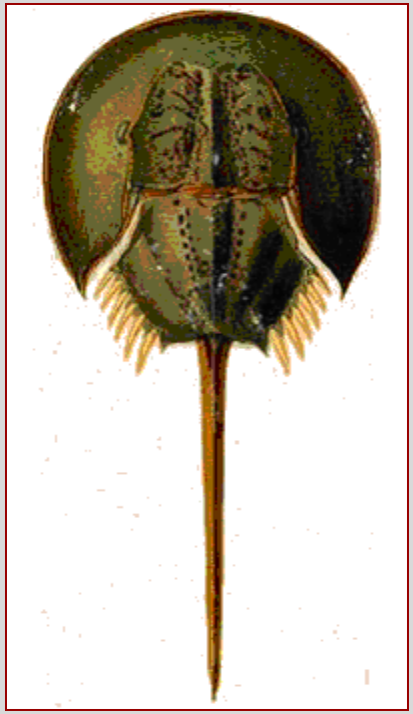
Present and future of horseshoe crab in biomedical research



ANIL CHATTERJI & FAIZAH SHAHAROM

INSTITUTE OF TROPICAL AQUACULTURE

UNIVERSITY MALAYSIA TERENGGANU

[anilch_18@yahoo.co.in]

LAL Milestones

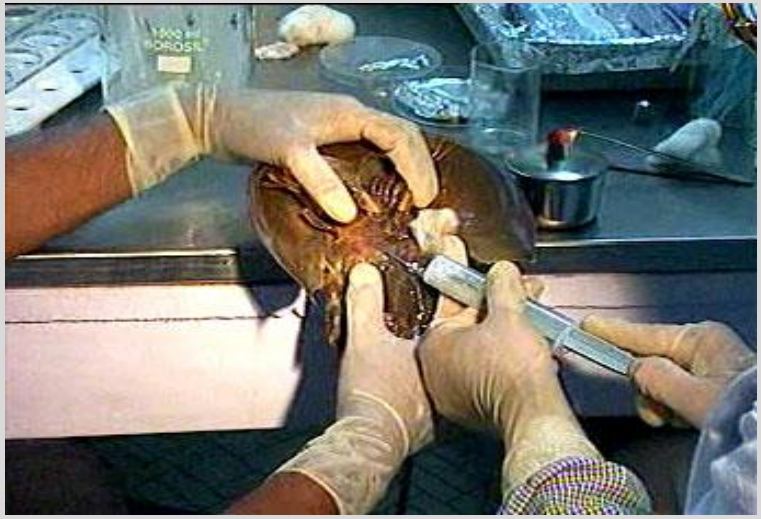
1880	**W. H. Lowell first studies coagulation of *L. polyphemus* blood at Johns Hopkins University.**
1956	**Bang discovers that horseshoe crab blood forms clots when bacteria (Vibrio sp.) are present.**
1964	**Levin and Bang discover that the blood clotting agent in horseshoe crabs is an amoebocyte.**
1968	**Scientists discover that an endotoxin causes blood-clot reaction in horseshoe crabs. The method for preparing LAL from horseshoe crab blood is developed.**
1971	**The standard LAL assay is developed.**
1977	**The FDA replaces the standard rabbit test for endotoxins with the LAL test.**
1983	**The FDA accepts LAL as a standard test for bacterial endotoxin contamination.**
1987	**The FDA establishes guidelines for LAL testing of pharmaceuticals and medical devices.**

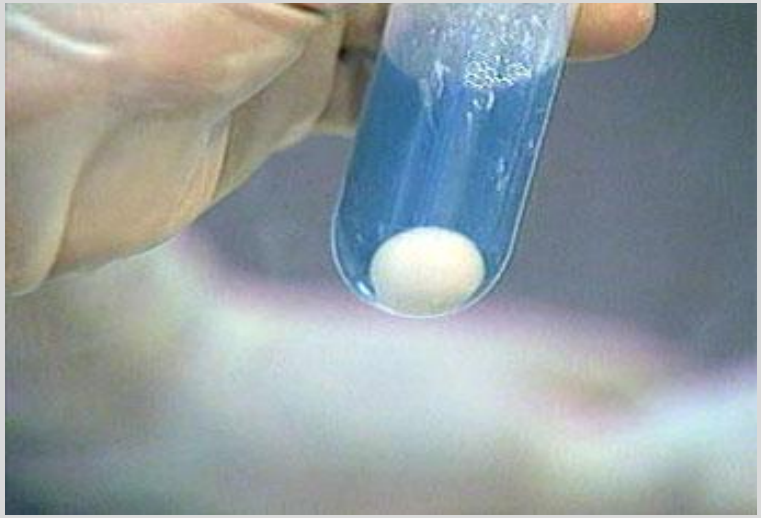
Collection of blood (haemolymph)

Conventional

Companies producing Amoebocyte Lysate
Associates of Cape Cod: http://www.acciusa.com/
Cambrex: http://www.cambrex.com/default.asp
Charles River Endosafe:
http://www.criver.com/products/endotoxin/index.html

New technique





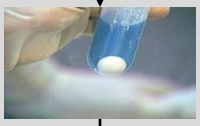
[Indian Patent NO. 192,300 (2004)]

USES OF AMOEBOCYTE LYSATE

- LAL provides annual revenue of $60 m
- It generates a social welfare benefit over $150 m, yearly

Horseshoe crab

Extraction of blood



Separation of amoebocytes

Amoebocyte Lysate (LAL)

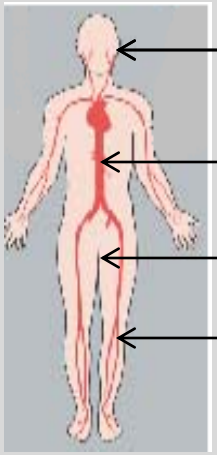
PHARMACEUTICAL PRODUCTS

[Injectable, intravenous drugs and for screening prosthetic devices like heart valves or hip replacements (FDA)]

FOOD PRODUCTS

DIAGNOSIS / TREATMENTS

Clinical diagnosis

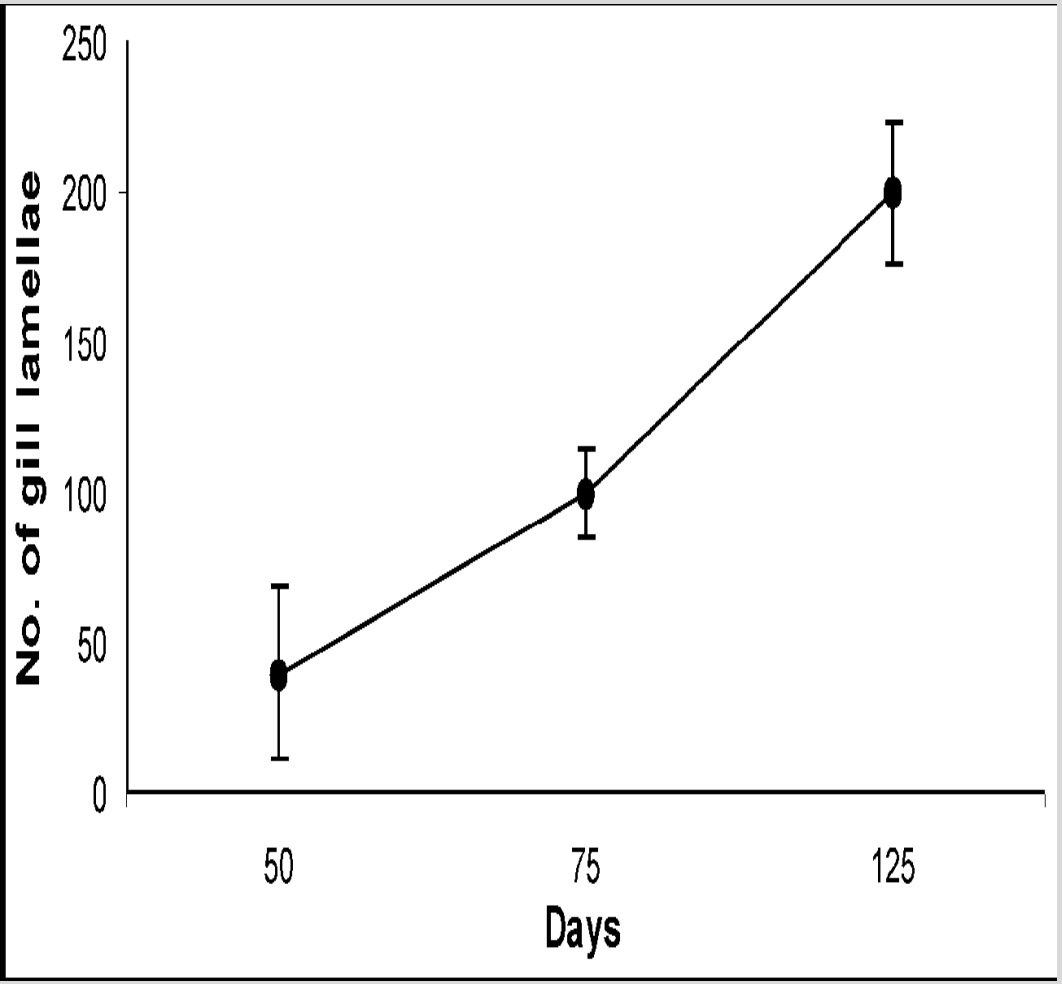


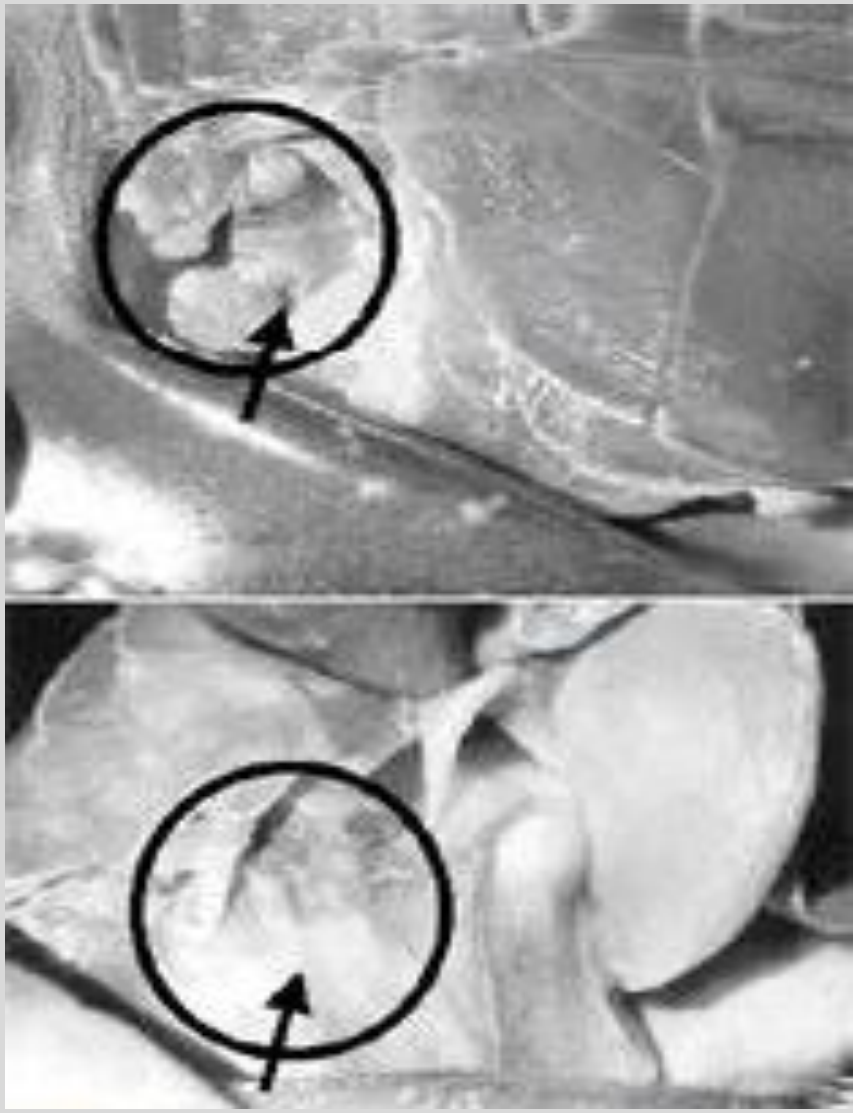
- Eye disease
- Spinal meningitis
- Urinary infections (*Neiserria gonorrhea*)
- Rheumatism

Homeopathic treatments (Limulus)

- Body & mental exhaustion
- Drowsiness after sea bathing
- Gastroentric symptoms
- Pain at right side of body

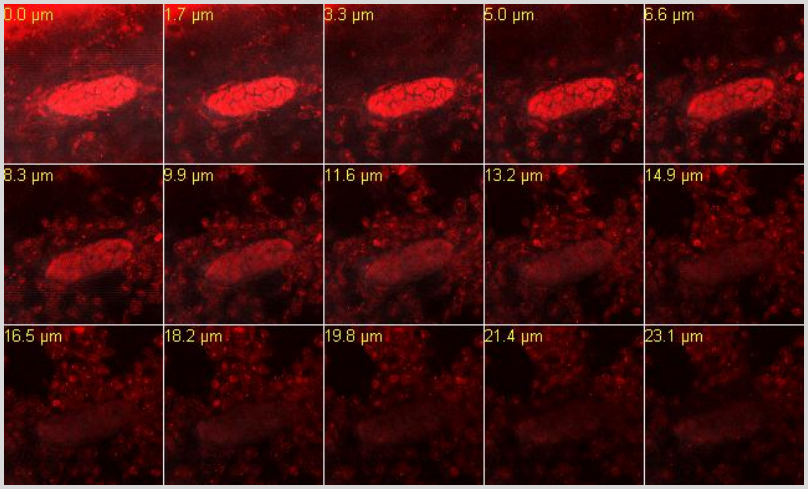
Regeneration of gill lamellae of the Indian horseshoe crab



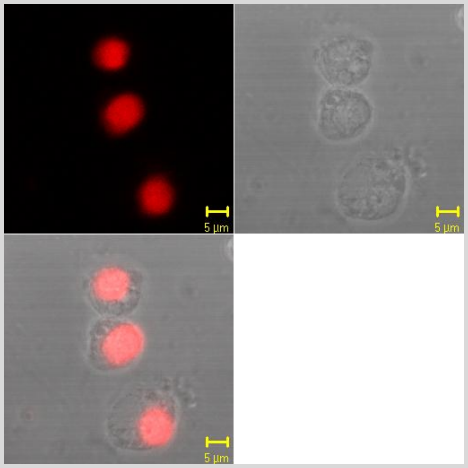


[Chatterji *et al*. (2004), *Current Science, 87* (11), 1511-1512]

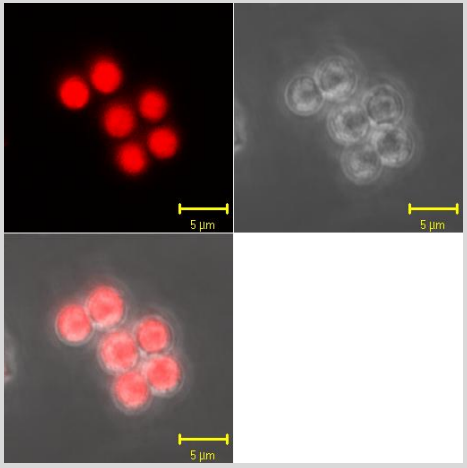
Optical Stack of Gill flaps of *T. gigas*



In vivo



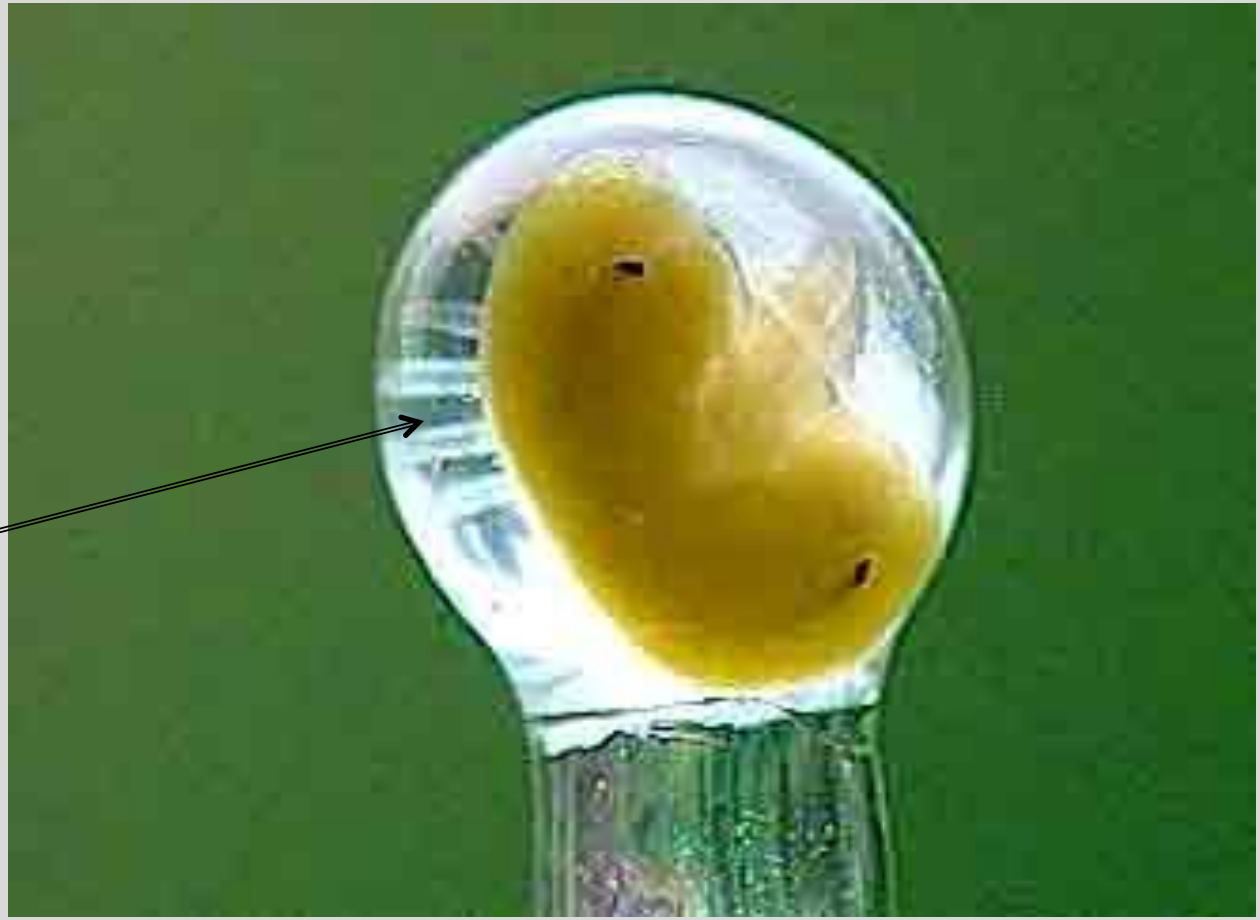
In vitro



[In vitro Cellular & Developmental Biology Animal, 38, 255-257 (2002].

[US Patent No 6,790,659 (2004)]

Recent advances in various researches carried out on PVF



A developing embryo between 39 and 42 day after fertilization

List of main researches on biomedical applications and PVF

- Jorgenson, J. H. and Smith, R. F. (1973) Preparation, sensitivity, and specificity of Limulus lysate for endotoxin assay. ***Appl. Microbiol., 26***:43-48.
- Cohen, E. (1979) *Biomedical Applications of the Horseshoe Crab (Limulidae).* Alan. R. Liss. Inc., New york, 668 pp.
- Cooper, J. F. (1979) Acceptance of the limulus test as alternative pyrogen test for radio pharmaceuticals and interathecal drugs. ***Progress Clin. Biol. Res.*** 29:345.
- Rudloe, A. (1979). *Limulus polyphemus.* A review of the ecologically significant literature. In : Biomedical application of the Horseshoe crab (Limulidae). E. Cohen ecol. Alan R. Liss. Inc., New York, 27-35.
- Ross, V. C. and Bruck, C. W. (1982). *Endotoxin testing of medical devices with LAL:FDA requirements*. In:Endotoxin and their detection with the Limulus Amebocyte Lysate *Test*, Watson, S. W., Levin, J. and Novitsky, T. J. (Eds). ***Alan R. Liss. Inc., NewYork***, 39-48.
- Srimal, S. and Bachhwat, B. K. (1985) Blood Brother-a living fossil helps to save human life. ***Sci. Age***, 51-55.
- Srimal, S., Miyata, T. Kawabata, S. and Iwanaga, S. (1985). The complete amino acid sequence of coagulogen isolated from south-east Asian horseshoe crab, *Carcinoscorpius rotundicauda*. ***J. Biochem***. (Tokyo), ***98***: 305-318.
- Toh, Y., Mizutani, A., Tokunaga, F., Muta, T., and Iwanaga, S. (1991). Morphology of the granular hemocytes of the Japanese horseshoe crab, *Tachypleus tridentatus* and immunocytochemical localization of clotting factors and antimicrobial substances. ***Cell Tissue Res., 266***, 137–147.
- Friber, J. A., Weathers, P.J., Gibson, D.G. III (1992). Culture of amoebocytes in a nutrient mist bioreactor. ***In vitro Cell. Dev. Biol., 28A***(3): 215-217.
- Muta, T., and Iwanaga, S. (1996) The role of hemolymph coagulation in innate immunity ***Curr. Opin. Immunol. 8***, 41–47.
- **Sugita, H. and Sekiguchi, K. (1979) Protein components in the peri-vitelline fiuid of the embryo of the horseshoe crab. *Dev. Biol., 73*, 183-192.**
- **Nagai,T., Kawabata, S. I., Shishikura, F., Sugita, H. (1999). Purification and characterization of an amino acid sequence of an embryonic lactin in peri-vitelline fluid of the embryo of the horseshoe crab. *J. Biol. Chem., 274:*37673-37678.**
- **Parab, P. B., Chatterji. A. (2003) Isolation of peri-vitelline fluid of the fertilized eggs of horseshoe crab and use thereof, US patent #20030017212.**
- **Shishikura, F., Sekiguchi, K. (1984a). Studies on perivitelline fluid of horseshoe crab embryo. Purification and properties of agglutininfrom the perivitelline fluid of *Tachypleus gigas*, embryo. *J. Biol. Chem., 96*, 621-628.**
- **Shishikura, F., and Sekiguchi, K. (1984b). Studies on peri-vitelline fluid of horseshoe crab embryo. II. Purification of agglutinin-binding substance from the peri-vitelline fluid of *Tachypleus gigas* embryo. *J. Biol. Chem. 96* (3):629-636**.

Use of PVF in enhancing early gonad maturation in commercially important fish

Control

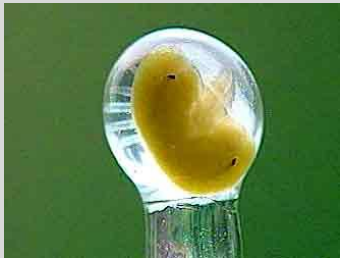
Experimental

Control

Experimental

Testes

Ovary

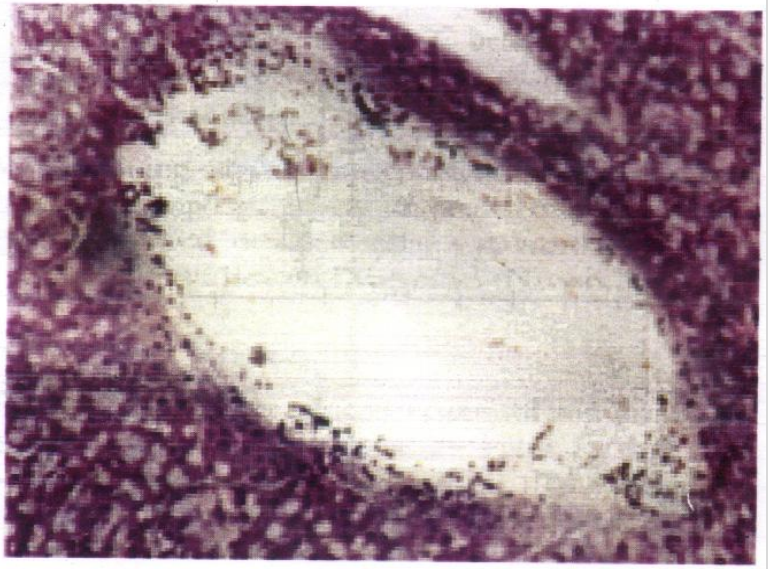


HCG?

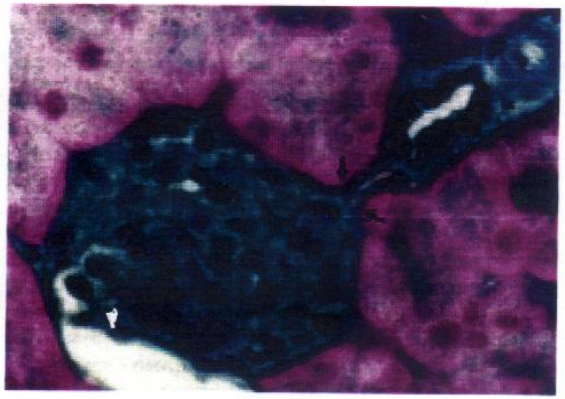
After 7th day of treatment with 100 µl PVF

PVF with proline zinc complex and diabetes

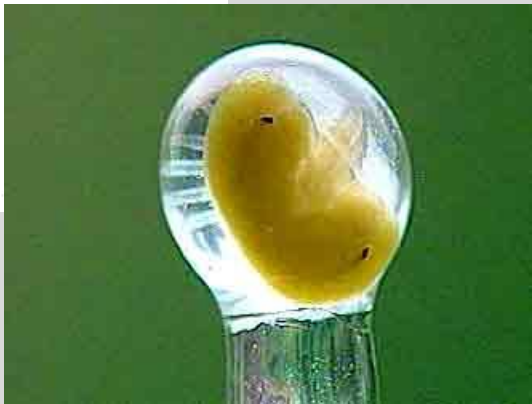
Normal pancreatic tissue



Diabetic pancreatic tissue



Regenerating islets



- National Institute of Oceanography, Goa, India
- National Center for Cell Science, Pune, India

Cardiac promoting effect of PVF

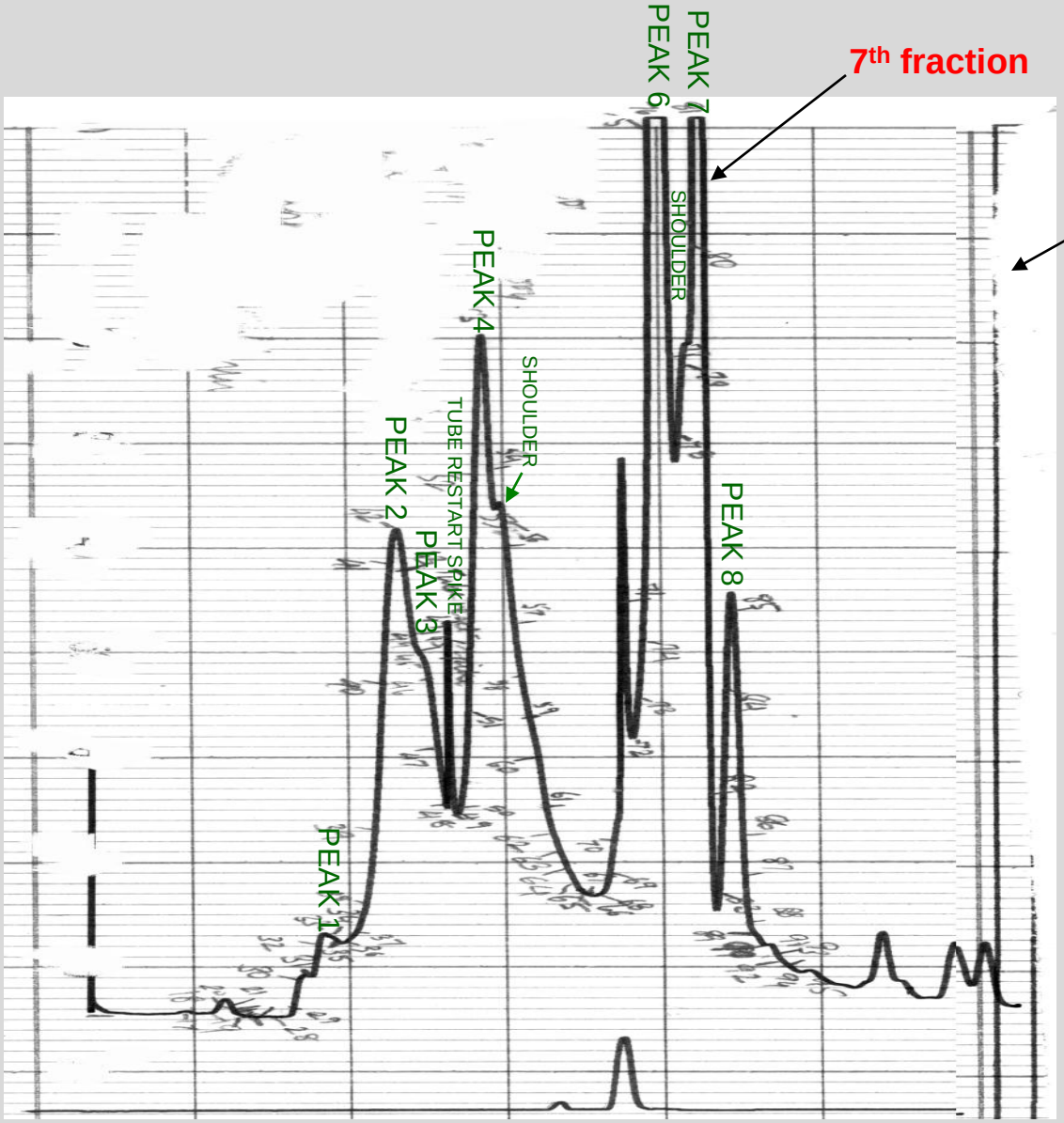
Control

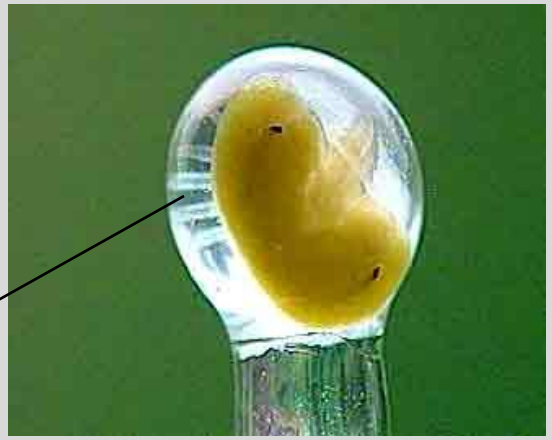
PVF (1:10) treated

[US Patent Nos 20030017212 (2003) & 7,087,429 (2006)]

FPLC Profile of PVF

Fractionation of PVF by FPLC





SPECIFICATIONS:

BUFFER – PBS – pH 7.4

SAMPLE – PVF (BATCH 25.02.2004)

SAMPLE LOAD – 500 µg

COLUMN – SUPEROSE 12, 2 COLUMNS CONNECTED IN SERIES

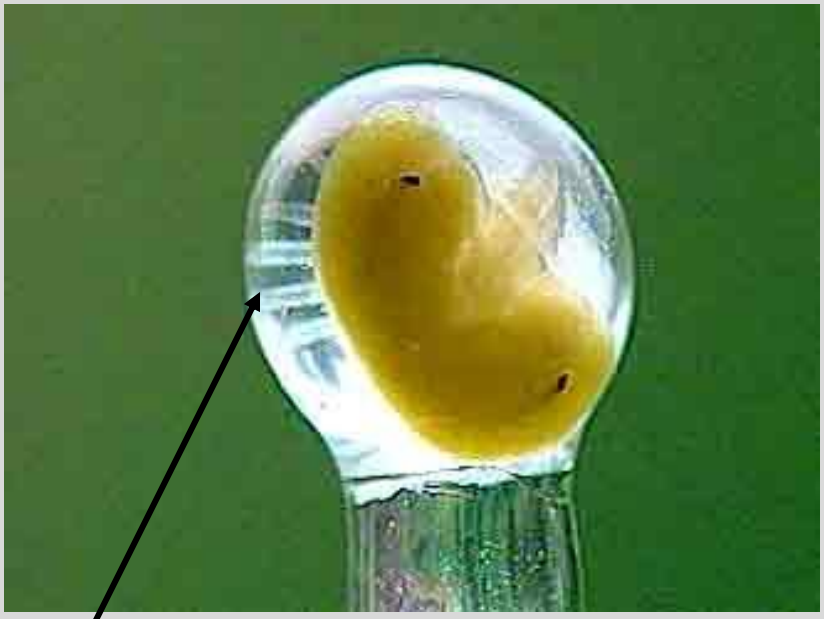
AUFS – 0.1

CHART SPEED – 0.1

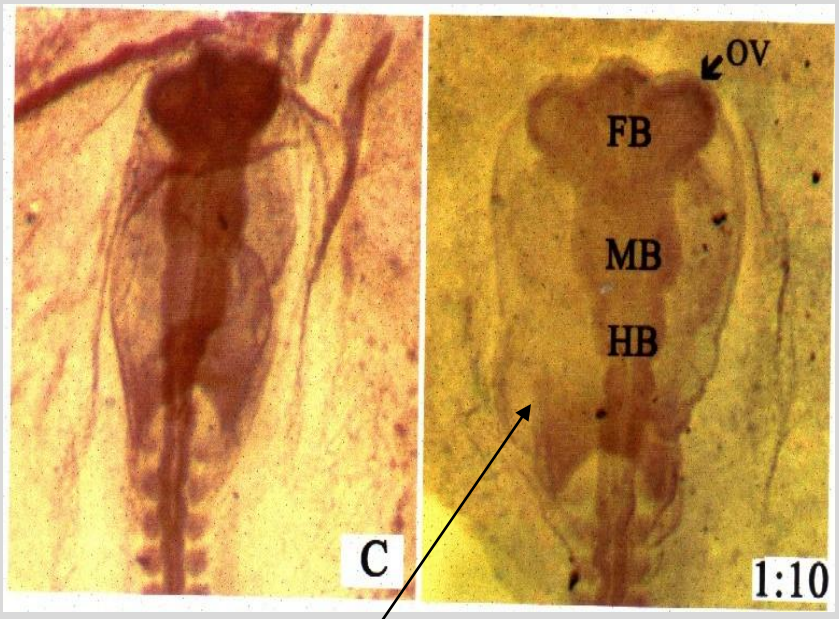
FRACTION SIZE – 0.5ml

Effect of PVF on enhancement of cardiac myocytes cells

- Cardiac myocytes are the first cells to differentiate during developmental process in vertebrate embryo
- Wide variety of molecules take part in this process



PVF

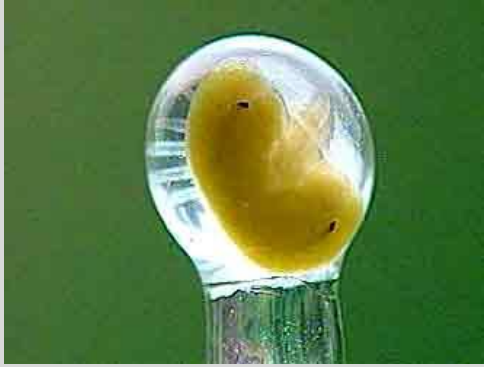


Heart

***{Cellular & Molecular Life Science, 65* (October' 2008)}**

[US Patent No:20040180324 (2004)]

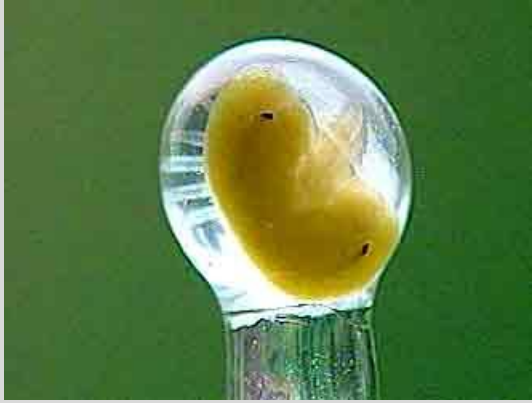
Heart enlargement due to PVF

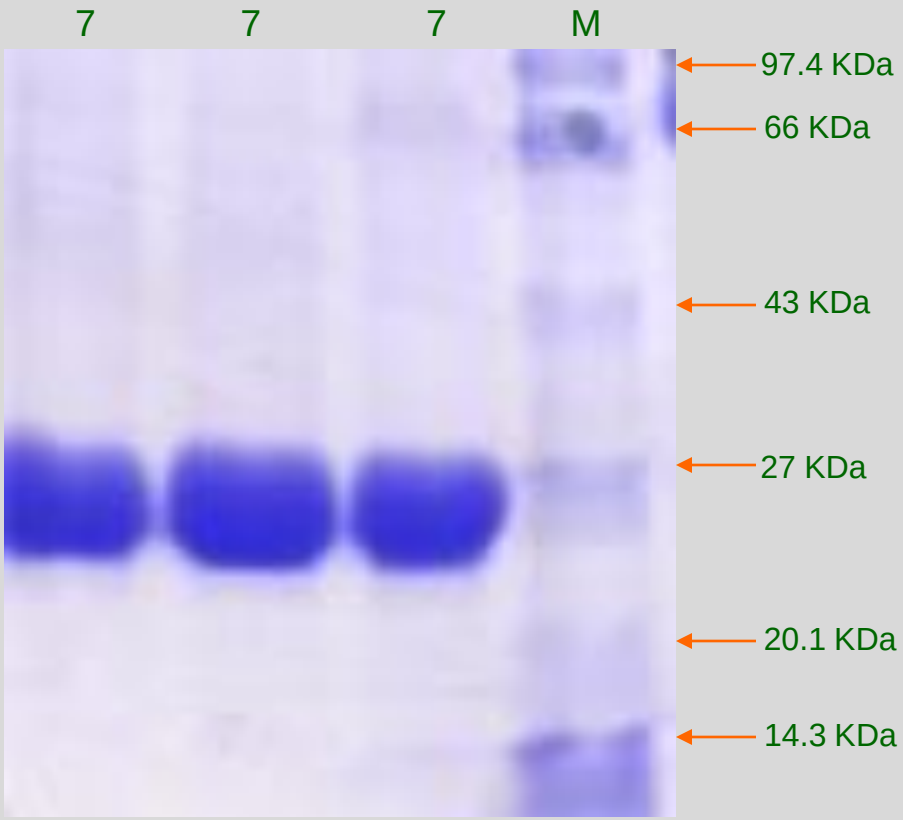


Control

PVF (1:10) treated

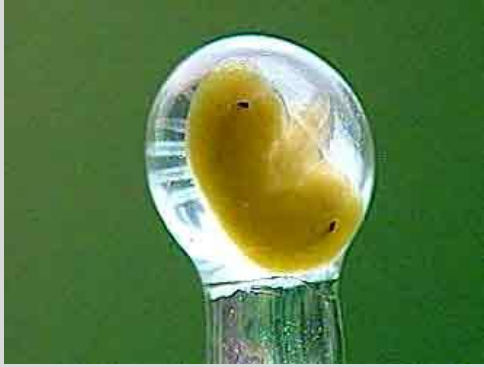
Gel profile of Fraction 7





Legends	
7	Peak 7
M	Protein Standard

[Fraction VII was excised and submitted to W. M. Keck Biomedical Mass Spectrometry Laboratory of Bio-molecular Research Facility (University of Virginia Health System, Charlottesville, USA]



- The sequencing analysis revealed a 221-amino acid protein

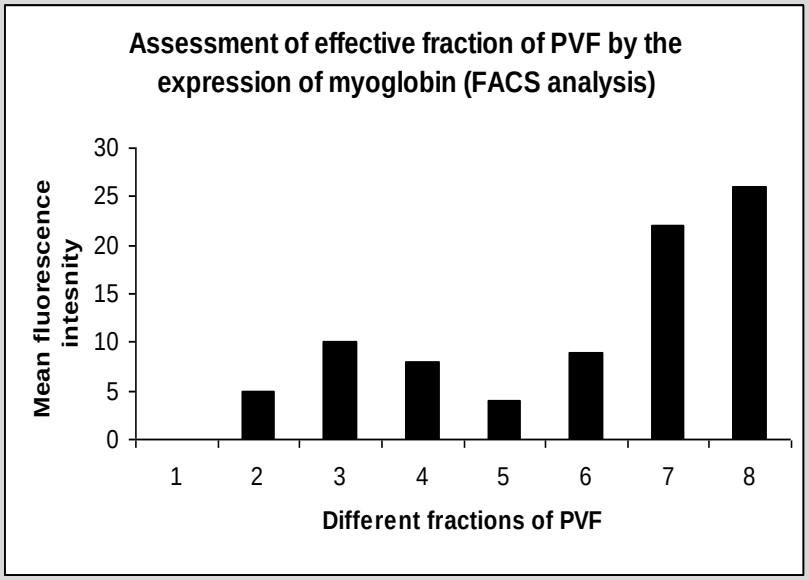
- Homology search using BLAST showed 100% similarity l to Limulus lectin L6, a lipopolysaccharide-binding antibacterial protein from of *T. tridentatus* (Saito *et al*., 1995)

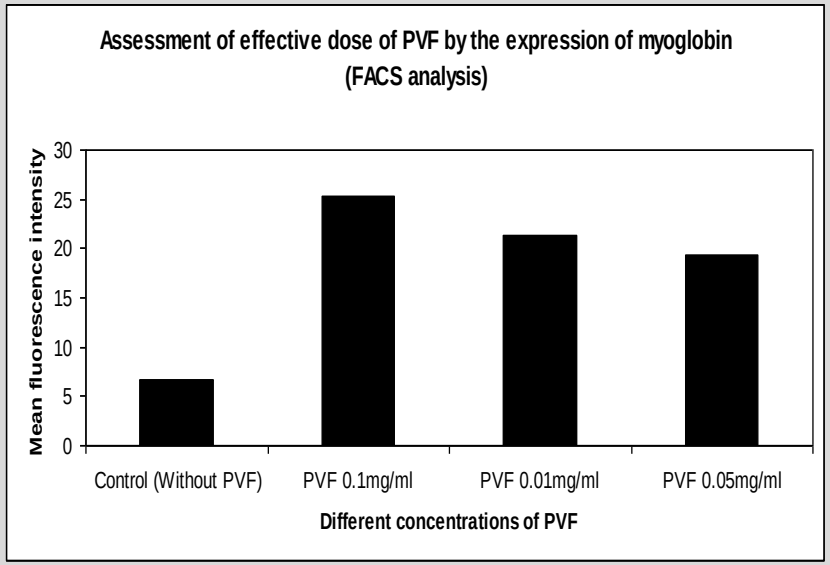
VQWHQIPGKLMHITATPHFLWGVNSNQQIYLCRQPCYDGQWTQISG
SLKQVDADDHEVWGVNRNDDIYKRPVDGSGSWV
RVSGKLKHVSASGYGYIWGVNSNDQIYKCPKPCNGAWTQVNGRLK
QIDGGQSMVYGVNSANAIYRRPVDGSGSWQQISGSLKHITGSGLSE
VFGVNSNDQIYRCTKPCSGQWSLIDG
LKQCDATGNTIVGVNSVDNIYRSG

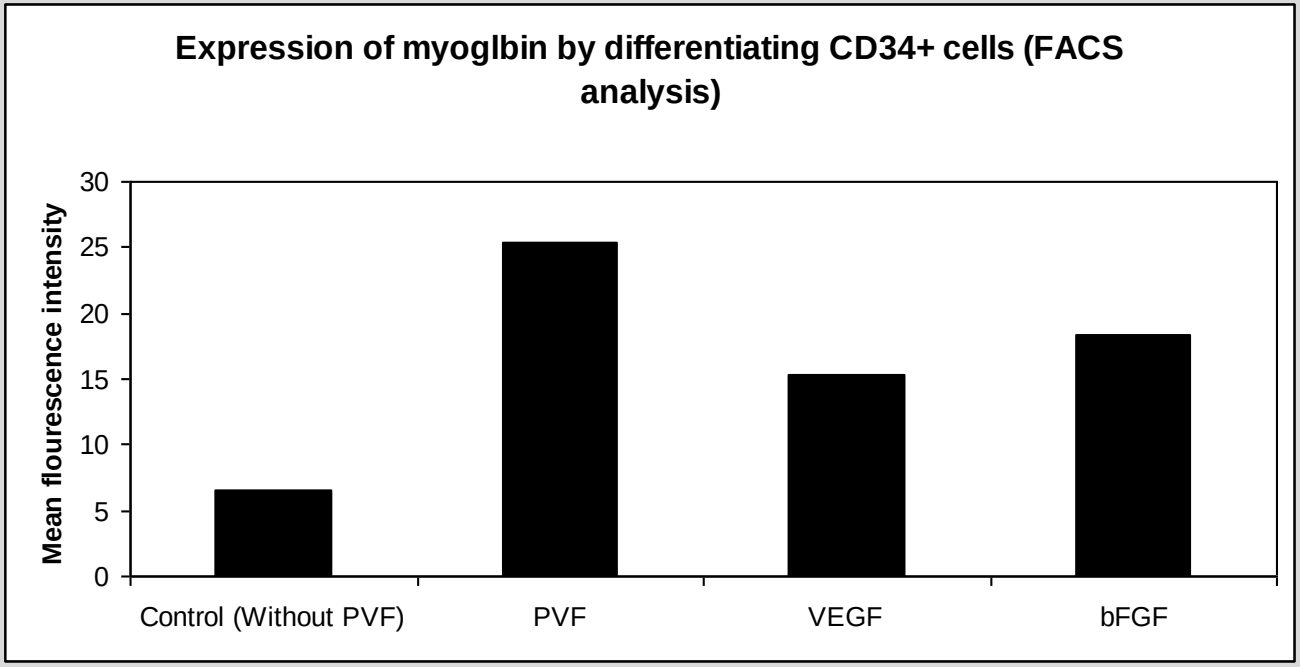
This molecule may prove to be an important tool in the study of cardiomyocyte differentiation

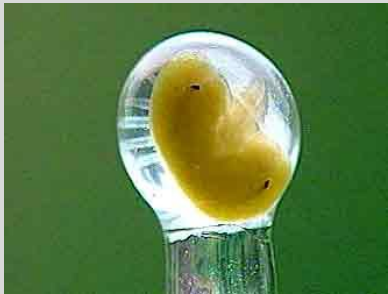
[US Patent Nos 20060210642 (2006) & 20060141055 (2006)]

PVF role in cardiomyocytes differentiation of bone marrow stem cells *in vitro*









NIO, Paris University, UMT

Horseshoe crab (PVF) for culture media & Growth factors

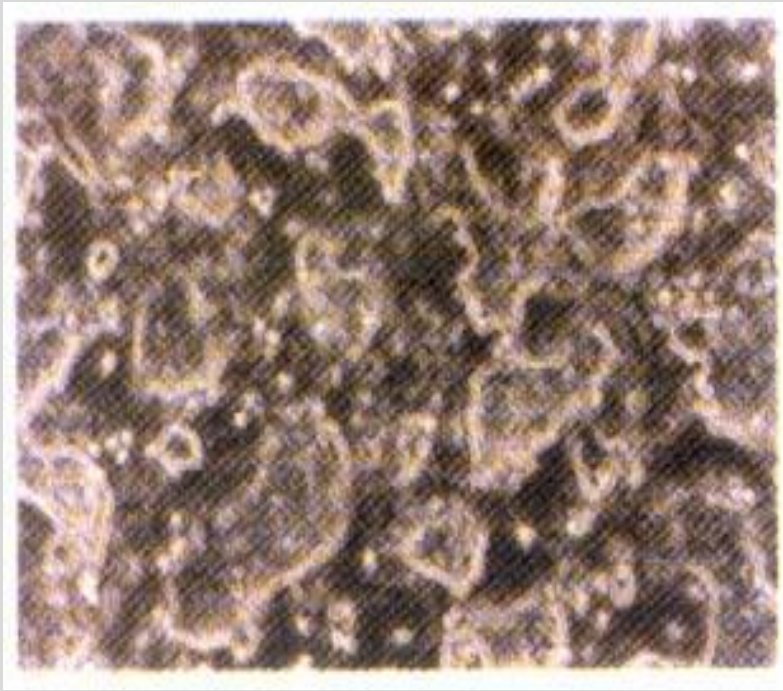
Demands of different growth media in stem cell research

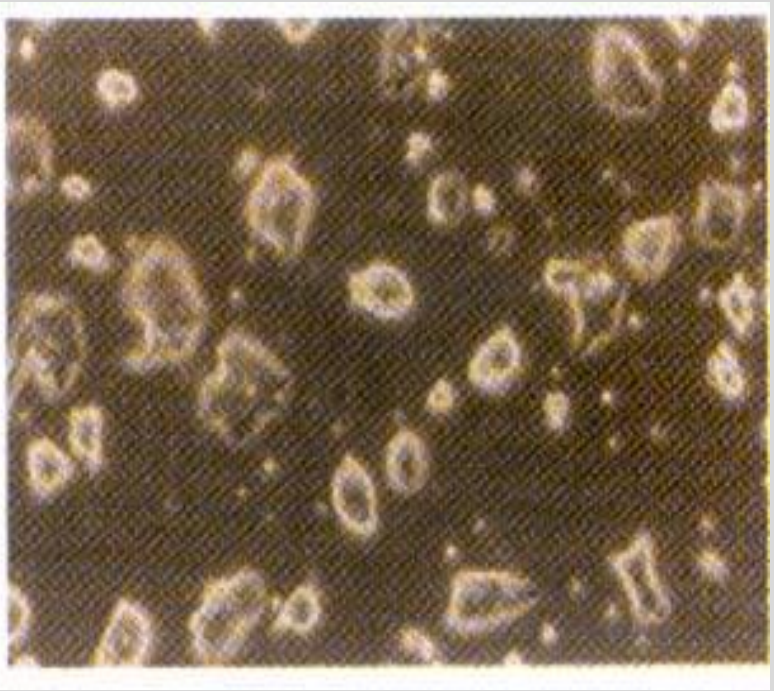
(500,000 litres required per year)

NO	Growth Media	World Market
1	VEGF (Vascular Endothelial Growth Factor) -	US$550 million to $5.3 billion in 2009
2	b-FGF (Basic Fibroblast Growth Factor)	US$ 22 billion by 2012
3	LIF (Leukemia Inhibitory Factor)	US $500 million
4	GM-CSF (Granulocyte Macrophage Colony Stimulating Factor)	US$ 2.2 billion in 2009
5	FCS (Foetal Calf Serum)	US $ 32 billion
6	FBS (Foetal Bovine Serum)	US $ 29 billion

Horseshoe crab (PVF)-Embryonic stem cell

Influence of PVF in maintenance of undifferentiated state in ES cells



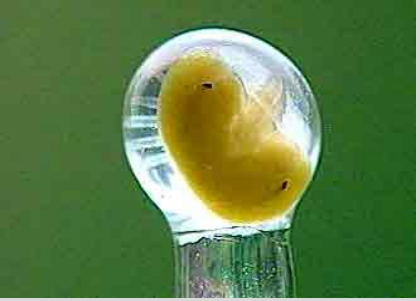


LIF

PVF exhibits more compact morphology

PVF

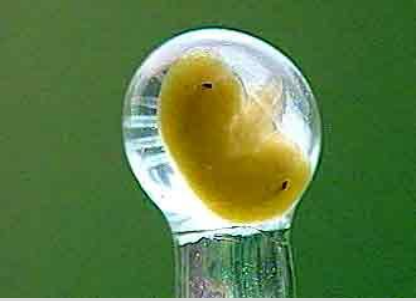
- National Institute of Oceanography, Goa (India)
- National Center for Cell Science, Pune (India)



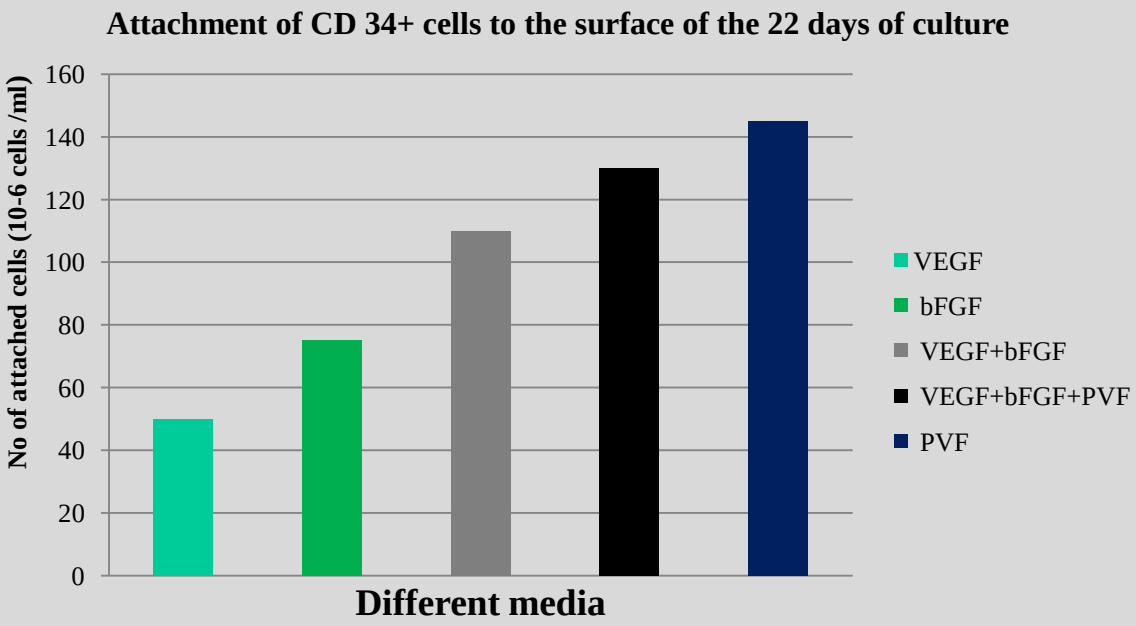
Role of peri-vitelline fluid in maintaining the original phenotype of CD34+ stems cells and colony formation *in vitro*

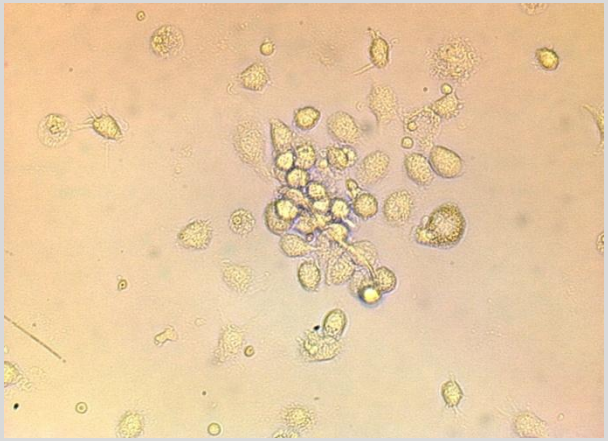
- **Human bone marrow** from healthy volunteers (donors for bone marrow transplantation) was collected from the Haematology Division of the Hospital Hotel Dieu, Paris (France).
- The mononuclear cells from the cells were then immediately separated by **Ficoll centrifugation technique**
- Bone marrow was diluted in 40 ml of L- Glutamin enriched with RPMI 1640 medium.
- **CD 34+ cells differentiated from bone marrow stem cells into endothelial cells in presence of growth factor (VEGF)**
- Mononuclear cell ring was collected and separation of CD 34^{+} cells was carried out **using immune-magnetic beads** as per the protocol of Easy Sep Stem Cell Technologies Inc U.S.A

NIO,Goa, Paris University, UMT

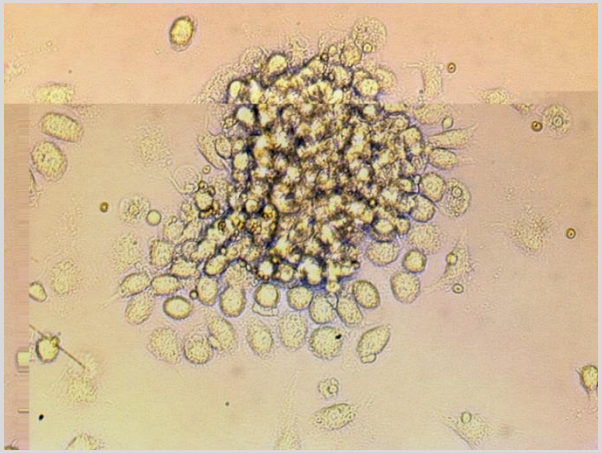


- CD 34+ cells differentiate from bone marrow stem cells into endothelial cells in presence VEGF
- CD 34+ cells remain viable for 3-4 days
- CD 34+ cells do not multiply *in vitro*





Control



PVF treated

Uses:

- Treatment of ischemic and vascular diseases

[US Patent No 0222NF2005, 6/20/2005]

Role of peri-vitelline fluid in promoting vasculogenesis

NIO, Goa, Paris University, UMT

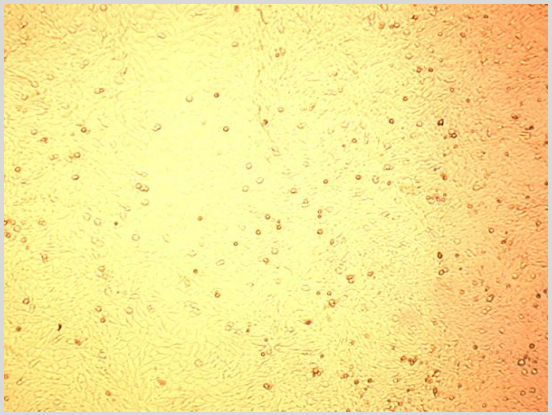
- **Vasculogenesis is a process of differentiation of stem cell endothelial progenitor cells (EPC) into mature endothelial cells**
- **It contributes to new vessel formation and resembles the embryological process**
- **Cellular progenitor population for the generation of EC by a compound may serve as cellular source for therapeutic vasculogenesis**
- **It could represent a novel approach to treat patients with ischemic disease that are not curable with conventional treatments**

[US Patent No 0222NF2005, 6/20/2005]

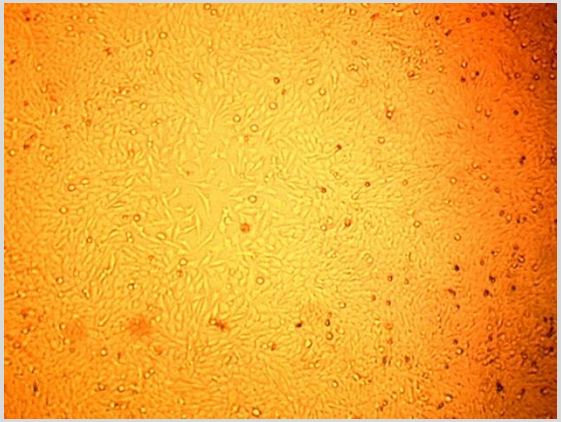
Angiogenesis

Wound healing with PVF

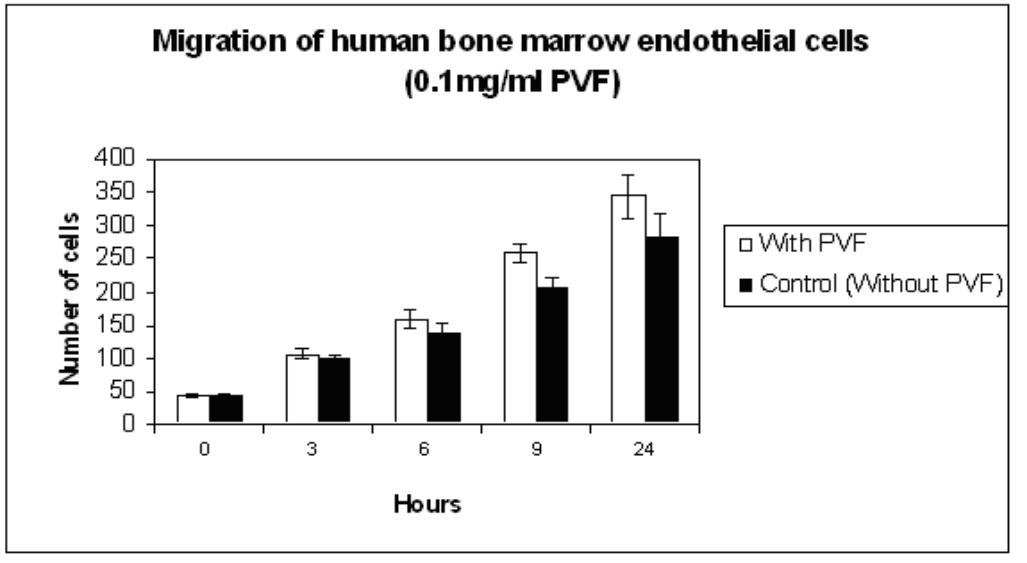
Control



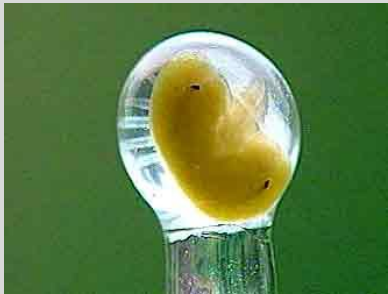
Treated with PVF



(24 hrs)

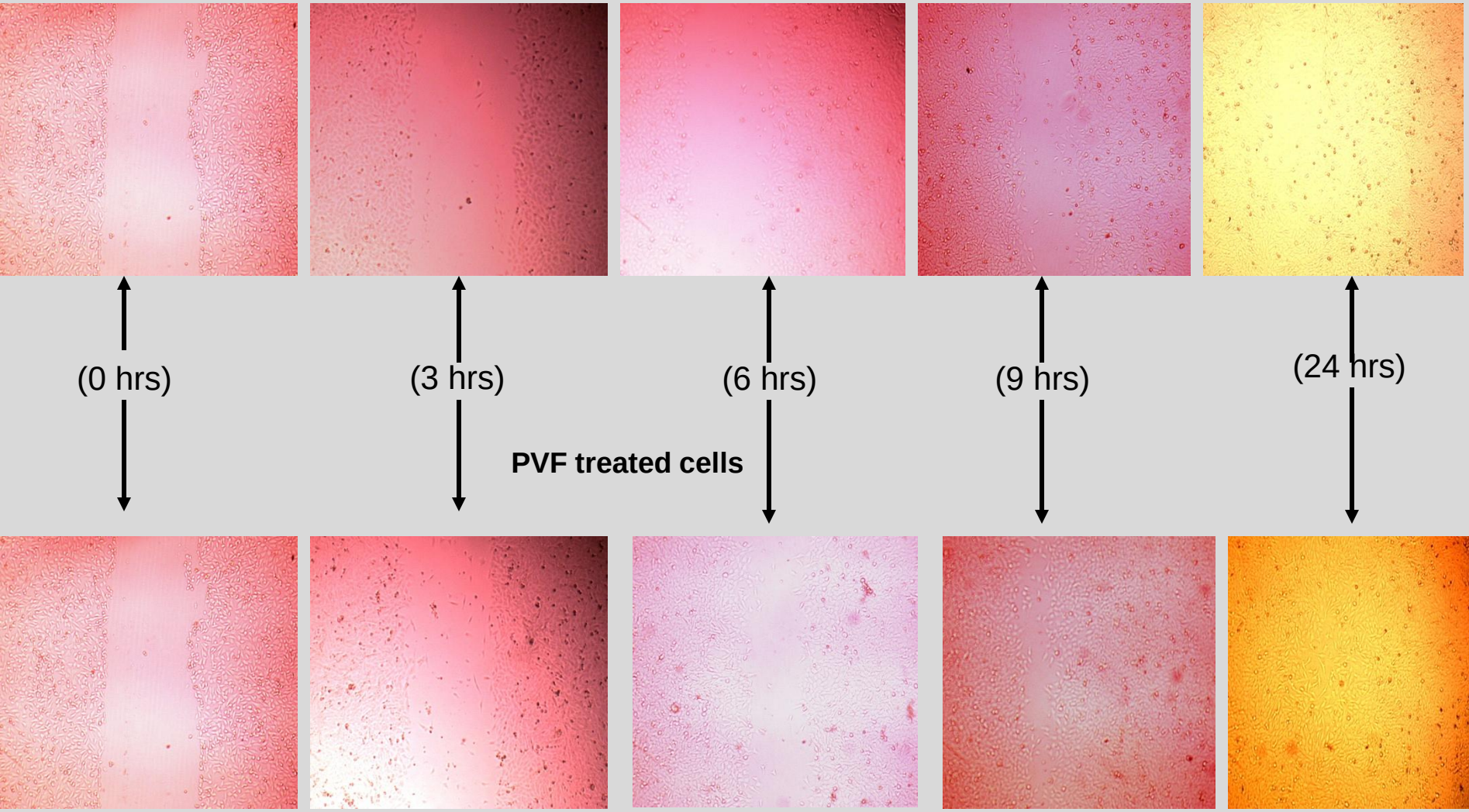


Migration of human bone marrow endothelial cells



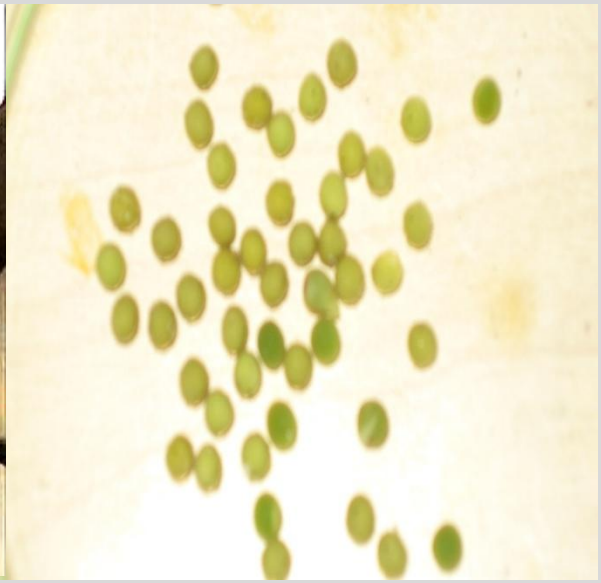
Migration of human bone marrow endothelial cells (0.1 mg/ml of PVF) (Wound healing)

Control

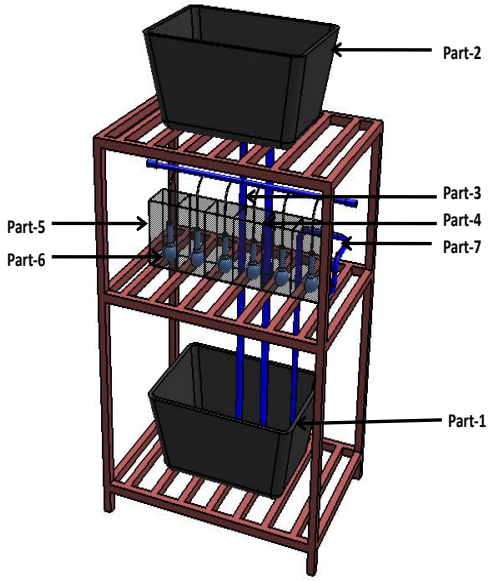


NIO,Goa, Paris University, UMT

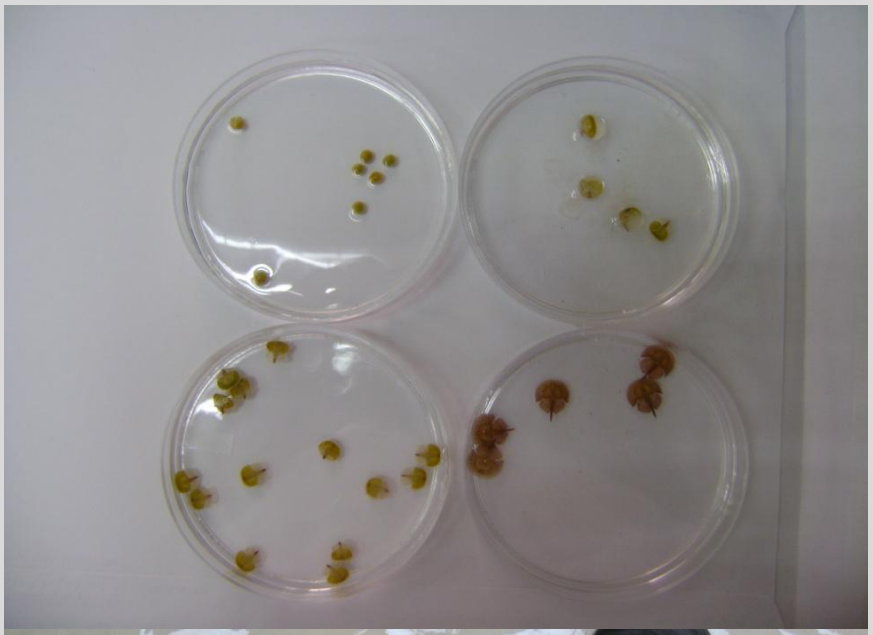
Artificial breeding by electrical stimuli (9 V 600 mA DC current)



Incubation of fertilized eggs in re-circulating type incubator



Collection of PVF and releasing of larvae



Horseshoe crab in Malaysia

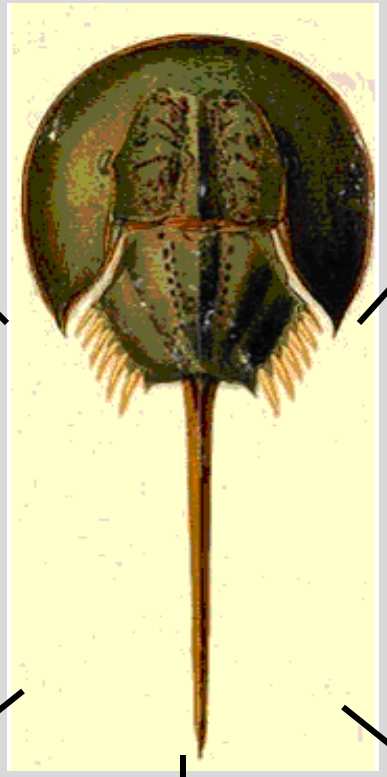
Distribution

***TACHYPLEUS TRIDENTATUS* (POCOCK)**

WESTERN & SOUTHERN JAPAN, TAIWAN, PHILIPPINES & NORTH BORNEO, MALAYSIA

***LIMULUS POLYPHEMUS* (MüLLER)**

ATL ANTIC COAST OF NORTH AMERICA FROM MAINE TO YUCATAM



***TACHYPLEUS GIGAS* (MÜLER)**

BAY OF BENGAL (NORTH -EAST COAST), THAILAND, MALAYSIA, PHILI PPINES, BORNEO & TORRES STRAITS

NEW SPECIES

[Johor Bahru (Malaysia)]

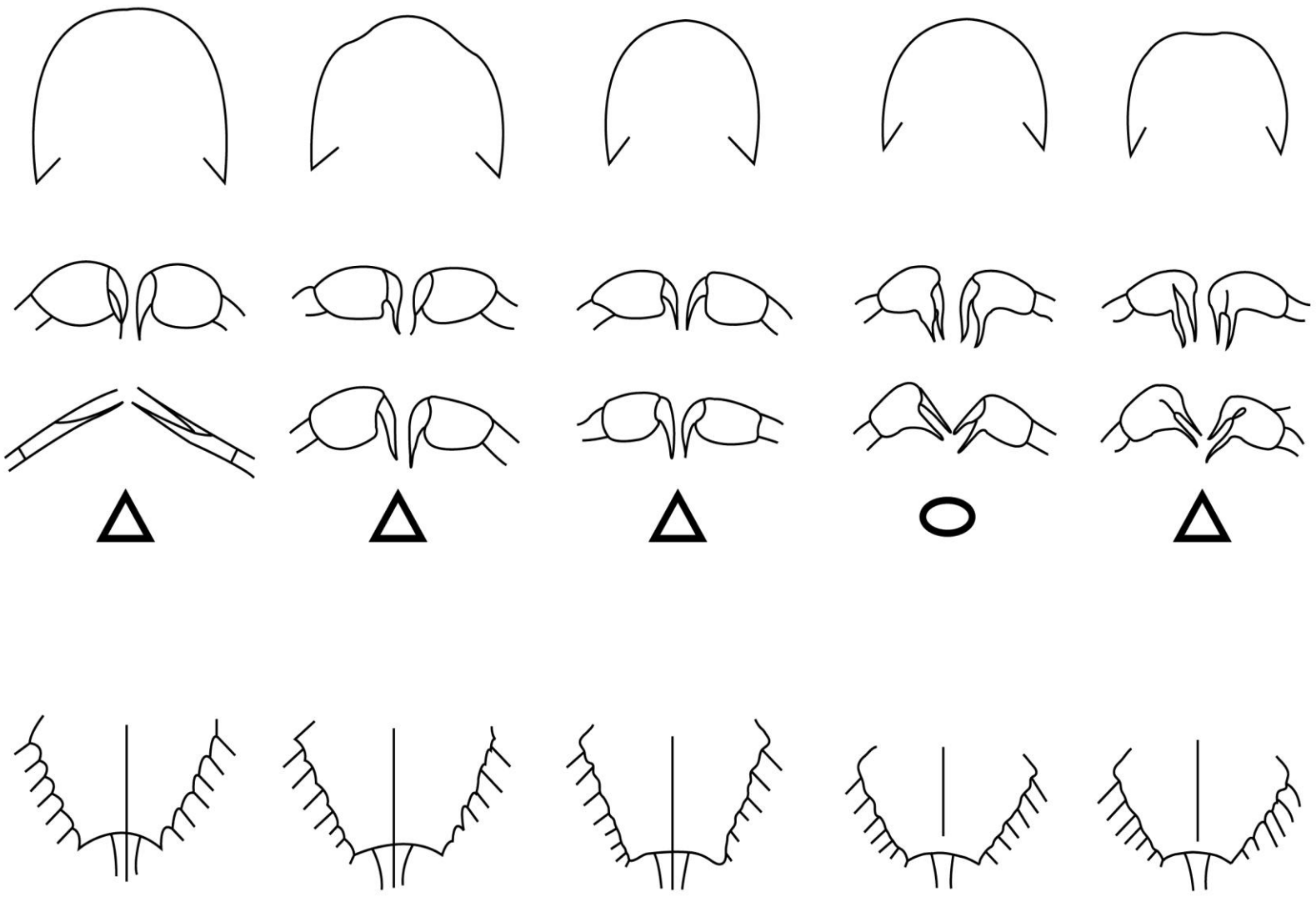
?

***CARCINOSCORPIUS ROTUNDICAUDA* (LATREILLE)**

BAY OF BENGAL, THAILAND, MALAYSIA, PHILIPPINES, BORNEO & TORRES STRAITS

[Chatterji, 1994]

Morphological difference



Limulus polyphemus	*Tachypleus tridentatus*	*Tachypleus gigas*	*Carcinoscorpius rotundicauda*	New species

Tachypleus tridentatus

Weight: **9.5 kg** (a record)

[J. Bombay Natural History Society, 2008]

Spawning ground of *T. gigas* at Balok (Malaysia) in 2009






Spawning ground at Balok (Malaysia) in 2010






Conservational measures suggested to Malaysian Government through WWF [MoU signed on 22 May' 2011]

- **Prevention of over fishing for restoring wild population**
- **Protection of sand dune vegetation on the beaches**
- **Ban on removal of sand gravel from the breeding beaches for construction purpose.**
- **Removal the polluting substances from the beaches and prevention of industrial discharge for protecting breeding beaches**
- **Identifying and protecting the critical habitats**
- **Coordinating and promoting collaborative researches and monitoring law enforcement bodies.**
- **Generating public awareness programmes.**

Location Plan

A first Asian horseshoe crab sanctuary at Kota Bharu in Malaysia

A restaurant of Johor popular only for horseshoe crab's food

INCLUSION OF HORSESHOE CRAB IN SCHEDULE-IV OF THE WILD LIFE PROTECTION ACT -1972 OF INDIA

रजिस्ट्री सं० डी० एल०-33004/99

REGD. NO. D. L.-33004/99

सत्यमेव जयते

भारत का राजपत्र

The Gazette of India

असाधारण

EXTRAORDINARY

भाग II—खण्ड 3—उप-खण्ड (ii)

PART II—Section 3—Sub-section (ii)

प्राधिकार से प्रकाशित

PUBLISHED BY AUTHORITY

सं. 1432] नई दिल्ली, बुधवार, सितम्बर 9, 2009/भाद्र 18, 1931

No. 1432] NEW DELHI, WEDNESDAY, SEPTEMBER 9, 2009/BHADRA 18, 1931

पर्यावरण एवं वन मंत्रालय

अधिसूचना

नई दिल्ली, 4 सितम्बर, 2009

का.आ. 2293(अ).—केन्द्रीय सरकार, वन्य जीव (संरक्षण) अधिनियम, 1972 (1972 का 42) की धारा 61 की उप-धारा (1) द्वारा प्रदत्त शक्तियों का प्रयोग करते हुए, यह समाधान हो जाने पर कि ऐसा करना आवश्यक और समीचीन है, उक्त अधिनियम की अनुसूची 4 में राजपत्र में प्रकाशन की तारीख से प्रवृत्त, निम्नलिखित और संशोधन करती है, अर्थात् :—

2. उक्त अधिनियम की अनुसूची 4 की प्रविष्टि 19 के पश्चात् निम्नलिखित प्रविष्टि अत:स्थापित की जाएगी, अर्थात् :—

"(20) अश्वनाल केंकडा *(ट्चीप्लयस गीगास और कारसिनोस्कोपियस रोटूनडिकाउडा)*;" ।

[फा. सं. 1-15/2008-डब्ल्यूएल-आई]

एम. बी. लाल, अपर महानिदेशक, वन (डब्ल्यूएल)

टिप्पण : वन्य जीव संरक्षण अधिनियम की अनुसूची 4 को समय-समय पर निम्नलिखित सं जे-11012/31/76-एफआरवाई(डब्ल्यूएल) तारीख 29 अगस्त, 1977, सं.-जे-11012/31/76-एफआरवाई (डब्ल्यूएल) 5 अक्तूबर, 1977, सं. आई-28/76 एफआरवाई(डब्ल्यूएल) तारीख 9 सितम्बर, 1980, का.आ. 859(अ) तारीख 24 नवम्बर, 1986, का.आ. 1197(अ) तारीख 5 दिसम्बर, 2001 और सं. का.आ. 1085(अ) तारीख 30 सितम्बर, 2002 द्वारा संशोधित किया गया ।

MINISTRY OF ENVIRONMENT AND FORESTS

NOTIFICATION

New Delhi, the 4th September, 2009

S.O. 2293(E).—In exercise of the powers conferred by sub-section (1) of Section 61 of the Wildlife (Protection) Act, 1972 (53 of 1972), the Central Government being of the opinion that it is expedient so to, hereby makes the following further amendments in Schedule IV of the said Act with effect from the date of publication of this notification in the Official Gazette, namely :—

2. In Schedule IV to the said Act, after entry 19, the following entry shall be inserted, namely :—

"(20) Horeshoe Crabs *(Tachypleaus gigas* and *Carcinoscopius rotundicauda)*.".

[F. No. 1-15/2008 WL-I]

M. B. LAL, Addl. Director General of Forests (WL)

Note : The Schedule IV to the Wild Life (Protection), 1972 were amended from time to time *vide* No. J-11012/31/76-FRY (WL) dated 29th August 1977, No. J-11012/31/76-FRY (WL) dated 5th October 1977, No. 1-28/78-FRY (WL) dated 9th September, 1980 S.O. 859(E) dated 24th November, 1986, S.O. 1197(E) dated 5th December, 2001 and *vide* number S.O. 1085(E) dated 30th September, 2002.

3295 GI/2009

Printed by the Manager, Government of India Press, Ring Road, Mayapuri, New Delhi-110064
and Published by the Controller of Publications, Delhi-110054.

Government of India
Ministry of Environment & Forests
(Wildlife Division)

Paryavaran Bhawan,
CGO Complex, Lodi Road,
New Delhi-110003

F. No. 1-15/2008 WL-I
Dated: 6th October 2009

To

The Chief Wildlife Warden,
All States /Union Territories

Sub: Inclusion of Horseshoe Crab in the Schedule-IV of the Wildlife (Protection) Act, 1972.

Kindly find enclosed a Gazette Notification no. S.O. 2293(E) dated 4th September 2009 regarding inclusion of Horseshoe Crab in the Schedule-IV of the Wildlife (Protection) Act, 1972, for action as appropriate.

Yours faithfully,

(Dr. Anmol Kumar)
Deputy Inspector General (WL)

Encl: As above

Copy to:

1. Pr. Secretary (Forests) All States/UT
2. Director, WII, Dehradun
3. Addl. Director & IGP, WCCB, New Delhi
4. Member Secretary, NTCA, Bikaner House /IGF & Director (PE)/JD (WL)
5. Director, National Institute of Oceanography, Ponda, Goa
6. Director, Central Marine Fisheries Research Institute, Kochi

Director for information
23/11
Dr. Balan Iyer

A.O'S OFFICE
DATE: 1.9.11.2009

DIRECTOR'S SECRETARIAT
23 NOV 2009
Time 4.30 pm
N.I.O. GOA

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1.Growth and morphometric characteristics of the Horseshoe crab, *Carcinoscorpius rotundicauda* (Latreille) from Canning (West Bengal), India. ***Pak. J. Sci. Ind. Res., 31*** (5): 352-353. **Anil Chatterji**, R. Viyayakumar and A. H. Parulekar (1988).

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•Thank you