

REVIEW ON IN-VITRO AMEBOCYTE CULTURE—A LESSON LEARNED FROM PAST

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Abstract

Limulus Amebocyte Lysate (LAL/TAL) assay is the only standard test approved by the Food and Drug Administration (FDA) to quantify bacterial endotoxin in injectable drugs and implantable medical devices. At present, horseshoe crabs are the sole source of LAL/TAL. Biomedical companies harvest and bleed horseshoe crabs which lead to $\leq 30\%$ post bleeding mortality. The continuity of this practices will eventually deplete wild stock and threat horseshoe crab's population. Though, many alternative biosensors were developed to quantify endotoxins, they are not without limitations and some of them even need LAL/TAL as source detectors. Hence, the LAL/TAL industry in its current form has ethical, ecological, commercial and technical issues that make it unideal standard test. An alternative method of culturing amebocyte in tissue culture medium has been addressed in literature since last 4 decades. This paper will address the issues in amebocyte culture in-vitro based on the published literatures. The paper will also suggest the best source of amebocyte cells (gill, gill flaps, blood), culture mode (monolayer or suspension), culture media (Grace's Insect Medium, Leibovitz's L-15 Medium, Modified Essential medium, Shields and Sang insect medium, TNM-FH Medium, RPMI 1640 medium) and best culture conditions (pH, temperature, osmolarity). This review will also emphasize on key elements in establishing a cell line for the continuous harvesting of amebocyte in-vitro.

Keywords: LAL/TAL, Horseshoe crab, bleeding, endotoxin, cell culture

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1.0 INTRODUCTION

The hemolymph of horseshoe crab contains amebocyte cells that initiate a cascade mechanism when come in contact with Lipopolysaccharide (LPS)

or endotoxins that result in formation of a clot (gel) [1]. Now, pharmaceuticals companies utilize endotoxin detection assays in order to verify the sterility of medical devices and injectable drugs, which backs up the efficacy of sterilization or disapproves it [2]. The LAL

assay is based on *Limulus* Amebocyte Lysate (LAL) of American horseshoe crab (*Limulus polyphemus*) isolated from their blood and contains clotting factors. The assay is widely considered as the gold standard for endotoxin detection as it can detect an endotoxin amount as little as one quadrillionth (10^{-15}) of a gram [3, 4].

However, LAL assay has ethical, ecological, commercial and technical issues that must be addressed before enforcing it as a golden standard. Bleeding horseshoe crab lead to mortality rate as high as 30% when combined with other stress factors such as transportation of horseshoe crabs to and from blood collection centers [5, 6] and decreased physiological and immunological functions [7]. The second issue with LAL assay is the seasonal availability of horseshoe crabs which affects supply of horseshoe crabs to pharmaceuticals industries [8]. Thirdly, there is huge quantitative and qualitative variation between various manufacturers as well as batches [8, 9].

There are many alternatives to *Limulus* Amebocyte Lysate (LAL) assay. For instance, there is the USP rabbit-pyrogen test, but it is 3000 times less sensitivity compared to LAL assay [4] and it involves animal ethics as well. Biosensors are extremely sensitive and can detect as little as 0.0005 EU/mL endotoxin but use of lyophilized extract of *Limulus Polyphemus* amebocyte which brings back the issue of horseshoe crab conservation [10]. Another alternative; in-vitro cell culture, was previously exploited and numerous attempts were made along the years to culture amebocyte cells. Amebocyte cells were successfully cultured in-vitro from different sources and via different methods [8, 11, 12, 13, 14, 15, 16, 17]. This indicates that cell culture techniques can be the solution to stop horseshoe crab bleeding practices and save this living fossil. All these attempts have meet success and failure in different ways pointing to difficulties within possibility. The main issues faced by previous attempts were the near absence of mitosis, irreproducibility of results and short term cell viability [8]. Nevertheless, literatures have presented possible solutions for these problems. For example, mitogens could increase mitosis [12], improved media formulations extended cell viability for months [15] and explanations were given for the discrepancies in results [12].

If cell culture method is to replace bleeding practices, a proper review of literature is needed to gather successful and failed attempts and pinpoint key elements. This paper is a review of more than a dozen experiments done over the last four decades which will help in suggesting a good study design that is likely to succeed.

This review looked into papers, patents and Intellectual properties (IP) published between 1975 and June 01, 2015. Key words such as bleeding horseshoe crab, amebocyte, amebocyte lysate, culturing amebocyte and amebocyte cell culture were used.

A total of 25 research papers and 5 patents were selected and reviewed.

3.0 RESULTS AND DISCUSSION

Horseshoe Crab Background, Body & Blood

Horseshoe crab is a marine animal that descends from Arthropoda's phylum, classified under the class Merostomata and order of Xiphosurida [18, 19]. Horseshoe crabs are more closely related to spiders and insects than to crabs [20] and called "living fossils" as they have been around for 445 million years [21, 22]. The body of horseshoe crab consists of three parts, the front part is Prosoma, a middle part called Opisthosoma and Telson (tail-like). The horseshoe crab obtained their name from their external shell (carapace) which is hard and shaped like a horseshoe [18]. There are four species of horseshoe crab that are classified under three different genera and assigned to two families. There is the *Limulus polyphemus* that belongs to Limulidae family, and *Tachypleus tridentatus*, *T. gigas*, *Carcinoscorpius rotundicauda* under Tachypleinae family [22]. In the International Union for Conservation of Nature (IUCN) red list for threatened species, *Limulus polyphemus* is classified as "Near Threatened (NT)", while *Tachypleus tridentatus*, *T. gigas*, *Carcinoscorpius rotundicauda* are all categorized as "Data Deficient (DD)" [23, 24].

The body of arthropods contain hemolymph which is similar to blood in higher species circulates in the body providing oxygen and immunity. The hemolymph is a liquid plasma that contains mainly hemocyte and proteins [25]. In horseshoe crab, the hemolymph contains amebocytes (blood cells), proteins, sugar (e.g. glucose) salts (sodium, potassium, chloride, calcium, magnesium and phosphorus), enzymes (amylase, lipase, alkaline phosphatase, aspartate aminotransferase, gamma glutamyl transferase others (creatinine, cholesterol and triglycerides) [26]. Previous studies identified few types of hemocytes that circulate in the body of horseshoe crabs. Earlier studies carried out on horseshoe crab's hemolymph indicated the presence of a single cell type which is ovoid (egg-shape) [27]. The cell was labeled as motile in different morphological states as well as on various surfaces [28]. Decades later, it was proven that there are two types of hemocyte in the body of horseshoe crab: amebocyte (granulocyte) and plasmatocyte [29]. Back then, it was believed that the amebocytes makes up 97% of all circulating blood cells, however, recent studies reported amebocyte cells to be 99% of all cells [30, 31]. The amebocytes have also been classified further to L-granule (1.5 μm) and D-granule (0.6 μm) with the larger L-granules containing 3 clotting factors and one antimicrobial factor. The D-granule on the other hand contained only one more antimicrobial factor [31]. In terms of morphology, the amebocyte cells is motile and comes in one of 3 shapes; contracted (round), granular flattened (irregular/stretched circle) and degranulated flattened (irregular flat shape) [8,30,32].

Amebocyte Cell Culture Methods

Cell culture; also known as “tissue culture”, is a century old science that involves plenty of techniques and instruments. It is the science of growing a cell/tissue in-vitro out of its natural environment and into a controlled environment. One of the main advantages of cell culture is the controlled environment which gives reproducible results, an important aspect that LAL assay lacks. Cells can be grown attached to a surface, or in suspension, and the choice of method depends on the nature of the target cell. Moreover, cell culture requires sterile environment, a wide range of nutrients (e.g. Amino acids, carbon sources), certain conditions (e.g. temperature, pH) and number of instruments and tools [33]. In order to choose growth media and conditions, a great deal of literature reviewing is required. However, most of media components and growth conditions depends on the nature of the studied cell, and often selected to mimic natural conditions.

Many previous studies explored the possibilities to culture this commercially valuable amebocyte cells cell in-vitro. One of the earliest studies carried out on this matter extracted amebocyte cells from the blood of horseshoe crab (*Limulus Polyphemus*) and was cultivated in a Pearson's amebocyte medium and modified essential medium which contained inorganic salts, amino acids, carbons sources and vitamins [17]. Unique and very interesting method; nutrient mist bioreactor, was also tried to culture amebocyte cells from the gill of horseshoe crab [13]. A different approach exploited previously was harvesting the gill flaps of horseshoe crab; which produces the amebocyte cells, cultivate them in-vitro and later recover amebocyte cells by means of pulsing [14,15]. The study managed to continuously produce amebocyte cells in-vitro from the gill flaps as well of Indian horseshoe crab *Tachypleus gigas* for up to 8 weeks [15]. A recent study screened several media compositions in an attempt to pinpoint the key basic components and essential supplements required to culture amebocyte cells isolated from horseshoe crab's blood [8]. The latest study carried out on this subject successfully maintained amebocyte cells on three different media for a short term but with a very high viability and sensitivity [11]. Cell culture involves setting variable number of parameters and conditions. Besides that, there are abundant literatures available that used various techniques and approaches to culture amebocyte.

First of all, different sources of amebocyte cells have been explored. Few studies isolated amebocytes from the gill and gill flaps of horseshoe crab as it was discovered that amebocyte cells are produced there [14, 15, 16, 28]. The majority of studies though went on isolating the amebocyte cells straight from the blood, where they make up 99% of all circulating blood cells [8, 11, 12, 17, 19, 34, 35, 36]. Extracting amebocytes from gill or gill flaps was attempted long ago when [34] managed to grow amebocyte cells on different

surfaces for a period ranging between few hours and days. A decade later, [14] improved the method and maintained the cells for up to 6 weeks (42 days). Recent studies managed to produce amebocyte cells from gill flaps continuously for 56 days [15] and 90 days [16]. Nonetheless, culturing of amebocyte cells from gill of horseshoe crab requires dealing with gill lamellae as an organ, which makes aseptic techniques and storage more complicated. Moreover, recovering the amebocyte cells from the tissue requires the use of additional chemicals (e.g. Tween-80, copper sulfate) which adds cost, down streaming process and environmental impact to an industrial process that it already laborious [14]. Culturing amebocyte cells from gill or gill flaps is rather tissue/organ culturing than cell culture, and that requires more effort (e.g. aseptic techniques), more complex histology studies to confirm type/characteristics, high inter-sample variation, absence of standard techniques and references, etc. Hence, it's rather easier and more practical to deal with cells (e.g. blood) rather than organ/explant (e.g. gill) culture organs [13].

There are plenty of studies to learn from in which amebocyte cells were extracted from the blood [8, 11, 12, 17, 35, 36]. The second aspect to look at in cell culture is the culture type where a choice is to be made between monolayer (attached) and suspension. Attached growth is not favored as it will be restricted by surface area, or even inhibit growth when cells comes in contact [33]. In previous studies carried out on amebocyte cells, half of these studies grown the cells in suspension which lead to extended growth periods up to 2 months [14, 15, 36]. Moreover, among the three shapes (contracted, granular flattened and degranulated flattened) of amebocyte cells, the most healthy and active state of the horseshoe crab's amebocyte cells are believed to be in the granular (round) form [8, 30, 32]. This granular contracted form of amebocyte cells is best retained in suspension rather than attached culture that flattens the cells and leads to reduced functionality [8, 15, 30, 36]. Therefore, the design of future experiments should consider setting suspension as the culture mode to be employed.

Amebocyte Cell Culture Media

In the previous chapter, the best source of amebocyte cell was established to be the blood and best culture mode was deduced to be the suspension culture. In this chapter, the next and arguably the most important element in cell culture which is the *choice of media* will be addressed. Numerous media and culture conditions have been used such as Grace's Insect Medium (GIM), IPL-41 Insect Medium, Leibovitz's L-15 Medium (L-15), TNM-FH Medium (GMIM), Pearson's Amebocyte medium (PAM), Modified Essential medium (MEM), RPMI 1640 medium and Shields and Sang insect medium (SSIM) [8,11,14,17]. Among all, various formulations of Grace's insect media has been used in the most successful studies [8, 11, 12, 14], with

Leibovitz's L-15 Medium coming second [15, 16, 37]. The results of these studies indicated that Leibovitz's L-15 Medium (L-15) and Grace's Insect Medium (GIM) seem to be the most suitable media that can support the growth of amebocyte cells. The main components of Leibovitz's L-15 medium (L-15) and Grace's Insect Medium (GIM) need to be highlighted to try pinpoint the fundamental nutritional needs of amebocyte cells.

These media mainly consist of inorganic salts, amino acids, vitamins besides other components. Calcium, potassium, magnesium and sodium based salts were abundantly present in L-15 and GIM media. Numerous types of amino acids such as Alanine, Asparagine, glycine etc were also added to these media. Vitamins and many other components were common in both media such as Riboflavin, folic acid, choline chloride, sugars besides others. Higher inorganic salts concentrations (Calcium, potassium and magnesium) together with addition of lactalbumin and yeastolate have shown remarkable improvement in cell viability [8]. Moreover, addition of horseshoe crab's serum to media (5-10 % v/v) had shown significantly higher cell viability than serum-free media [8, 15, 37]. The role of serum generally is to support cell growth and proliferation by providing growth factors, nutrients, hormones, transport proteins, attachment factors, stabilizing agents, detoxifying agents, etc [38]. As previously mentioned, the serum of horseshoe crab contains proteins, sugar, salts, enzymes, creatinine, cholesterol and triglycerides [26]. The highest cell viability obtained in a serum-free media was 77% after 24 hr in Grace's Insect Medium (GIM) [37]. On the other hand, all long term cultures (up to 90 days) maintained in previous studies were supplemented with horseshoe crab's serum [14, 15, 16, 36] or Fetal bovine serum [12]. This indicated the importance of serum content to the growth of amebocyte cells. However, these studies mostly utilized gill tissue [14, 15, 16] and showed no signs of mitosis, hence, not sustainable source. Moreover, serum is chemically undefined causing qualitative and quantitative variations as well as lead to horseshoe crab mortality; hence its use is discouraged [38]. Therefore, the design of future experiments is not to incorporate the use of horseshoe crab serum. An alternative serum that might be used if needed is Fetal bovine serum (FBS) which previously managed to increase DNA synthesis by up to 4 folds [12].

Amebocyte Cell Culture Conditions

Besides screening media, conditions of culturing such as pH, temperature and osmolarity are also to be screened and set. Generally, pH of media depends on the composition of the media such as the concentration and types of salts, proteins, amino acids etc. From previous studies, the amebocyte cells appeared to favor more neutral pH (7.2-7.8) in gill flaps cultures [13, 14, 15, 28], while cultures from blood grew in wider range (5.17-7.5) [8,11,17,35]. The temperature parameter usually mimicked to that in which

horseshoe crab (mostly *Limulus polyphemus* species) lives in. Various temperature ranges between 18 and 28 °C were used in the reviewed papers, with 20-25 °C being the most suitable [8, 11, 12, 13, 14, 15, 16, 17, 28, 35, 36]. A less studied parameter to look at is the osmolarity, which seems to depend on both, media composition (e.g. organic salts) and osmolarity of the natural environment (e.g. sea water) [12]. In all previous reports, only few measured and mentioned osmolarity. The range of osmolarity that supported the growth of amebocytes ranged between 325 mOsm in L-15 medium [8] and 820 mOsm in SSIM medium [11]. Several studies indicated that the best osmolarity conditions to grow the amebocyte cells are 700-800 mOsm that resemble their natural environment (seawater osmolarity= 720 mOsm) [4, 8, 11, 15, 16, 28]. However, another detailed study suggested to allow horseshoe crabs 3 days to adapt to aquarium water (include 30% seawater) before bleeding. This technique reduced the osmolarity of the horseshoe crab's hemolymph from 706 to 344 mOsm which was close to GIM medium osmolarity of 350 mOsm. The paper basically described that osmolarity is an important factor that inhibit the growth of amebocyte culture, yet it was neglected [12]. Most of these media mentioned above had osmolarity similar to GIM, hence, this approach seems to be another key aspect to consider in future study design and let the horseshoe crabs adapt to lower osmolarity levels.

Establishing Amebocyte Cell Line

Cell line is defined as "a culture that is subcultured beyond the initial primary culture phase"[39]). Animal cells die or senesce after a number of cell cycles and doublings. Depending on source of cells, senescence will take place after 20 to 50 divisions typically due to shortened telomere [40, 41]. Cell lines are often called "transformed cells" as they have skipped the natural control mechanism incorporated in the genes and operate outside the normal cell cycle. Generally, establishing a new cell line is not encouraged due to time and resources that it requires. However, in many cases; such as horseshoe crab amebocyte cells, there are no cell lines available in the cell bank or other repositories [42]. Horseshoe crab amebocyte has been genetically related to insects [1, 43]. Therefore, their amebocytes have always been cultured in insect's media such as Grace's Insect Medium (GIM) and IPL-41 Insect Medium mentioned previously.

All previous studies reviewed failed to establish a cell line, hence, horseshoe crab bleeding practice is still carried out regularly [8, 11, 12, 13, 14, 15, 16, 17, 34, 35]. Besides optimizing media and culture conditions, the continuous production of amebocyte cells in-vitro will require transforming the cells and establishing an amebocyte cell line.

The fundamental reason to short lived cultures of amebocytes is the near absence of mitosis. The horseshoe crab's amebocyte cells reported to have a very low mitosis rate (1 in 2000 cells) [8]. Hence, the

amount of nutrients the amebocyte cells are provided with is meaningless if the cells are not dividing. The cell cycle goes through four phases, a G₁ (gap) phase where cell decides between remaining in resting phase (G₀), differentiation or DNA synthesis. The next phase is the S-phase in which DNA synthesis takes place. The cell then enters G₂ phase to examine the quality of synthesized DNA and either allow repair if possible or trigger death. If the DNA is of good quality, chromosomes are formed and segregated to form two daughter cells [33]. Cell cycle is regulated via a complex process that involves many genes, growth factors, kinases etc; however, the key element remains to be the synthesis of DNA. As previously mentioned, with the absence of mitosis in amebocyte multiplying the cells in-vitro is just not possible.

Hence, a study was carried out to establish an amebocyte cell line; the only attempt so far to our knowledge, that addressed this issue. The study tested three lymphocyte mitogen: Pokeweed mitogen, Phytohaemagglutinin and concanavalin A. The study managed to increase DNA synthesis by up to 55-folds when Pokeweed mitogen was applied, but no proliferation was recorded. Optimum concentration of Pokeweed mitogen was reported to be 2.5 µg/mL [12]. This suggested that maybe a prolonged treatment with such mitogens can transform the cell and establish the required cell line. The life span of cell also depends on their telomere which shortens with each cell cycle, leading the cell ultimately to death [33, 39]. A study carried out to analyze the telomere sequence of horseshoe crab's amebocyte cells has proved that they contain the (TTAGG)_n telomere sequence of insects [44]. There are numerous cell lines (e.g. sf 9) developed from many types of insects (e.g. *Spodoptera frugiperda*) and applied in various fields (e.g. recombinant protein production) [45]. Therefore, the establishment of a cell line from primary amebocyte cells culture can be achieved; hence, continuous production of amebocyte cells in-vitro is possible.

4.0 CONCLUSION

This paper reviewed previous studies that attempted culturing Horseshoe crabs's amebocyte cells, a number of suggestions can be drawn to direct future researches. First, the horseshoe crabs must be acclimatized to lab aquariums prior to bleeding in order to adjust the osmolarity of their hemolymph. Then amebocyte cells can be extracted from the blood and cultured in suspension as these are the best source and culture mode for highly functional and endotoxin sensitive cells. Grace's Insect modified Medium (TNM-FH) is the best medium till now that can support the growth of amebocyte cells which has an osmolarity of 350 mOsm. The amebocyte cells appeared to favor neutral pH ranges between 7.2 and 7.8 and temperatures between 20-25 °C. An amebocyte cell line could be established with

mitogens such as Pokeweed and if addition of serum is need Fetal bovine serum (FBS) can be used.

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