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Evaluation of anticoagulant- and hemocyte-maintaining solutions for the study of hemolymph components in the spiny lobster *Panulirus argus* (Latreille, 1804) (Decapoda: Achelata: Palinuridae)

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ABSTRACT

Functional studies on humoral or cellular responses in the hemolymph ofcrustaceans require the selection of suitable anticoagulant and hemocyteworking solutions.Although preventing plasma-clotting could be straightforward, avoiding activation and clotting of hemocytes is a challengethat varies even among related species. We studied the suitability of several anticoagulant- and hemocyte-maintaining solutions in the spiny lobster *Panulirus argus* (Latreille, 1804), with emphasis in the preservation of hemocyte number and viability. It was found that the modified Alsever solution was the ideal anticoagulant, while modified L-15 medium and *Panulirus argus* saline (PAS) were the best hemocyte-maintaining solutions. Hemolymph volume average in *P. argus* was 10.5% of fresh body weight (more than 50 ml per adult individual), which makes this species an attractive model for functional studies of hemolymph components in crustaceans.

Key Words:coagulation, clotting, hemocytes

INTRODUCTION

The hemocytes from the hemolymph of arthropods are the main players in both cellular and humoral immune response (Jiravanichpaisal *et al.*, 2006). While the current model of coagulation in crustaceans is useful, significant departures from the model occur in different groups, mainly at the initiation stage of the process that involves the hemocytes (Perdomo-Morales *et al.*, 2019). The ability to control the coagulation of plasma and hemocytes in crustacean species has fundamental and applied relevance.

Plasma coagulation and the activation, clumping, and lysis of hemocytes should be properly avoided before attempting studies on the humoral and cellular components of hemolymph and their biological functions. Prevention of clotting, for example, may impairthe determination of hormone titers in the hemolymph of the freshwater crayfish *Procambarusclarkii*(Girard, 1852) (Kwok & Tobe, 2006).An increased dilution of hemolymph may delay the coagulation reaction, ultimately producing a less firm clot, while diluting hemolymph with the same volume of isotonic solution is not enough to prevent coagulation (Durliat&Vranckx, 1981).These procedures lead to extreme dilutions of components of the clotting system far from physiological concentrations. The use of a suitable anticoagulant solution is therefore mandatory.

Hemolymph clotting in crustaceans is mainly due to the polymerization of plasmatic clotting protein promoted by the hemocyte-derived transglutaminase enzyme (TGase) (Perdomo-Morales *et al.*, 2019). Anticoagulant solutions are mostly based either in direct inhibition of TGase, or in the chelation of calcium ions required for TGase activity. Many known solutions are able to prevent plasma clotting and others could be rationally designed. There are, however, few studies comparing different anticoagulants for crustacean hemolymph and their implications for the assessment of humoral and cellular response.

We evaluated several solutions for handling the hemocytes *in vitro* using *P. argus* as the model species. While most anticoagulant solution tested were efficient preventing plasma clotting, we found strong differences in their performance in term of both preservation of cell number and viability. We recommended an anticoagulant solution for this and perhaps other lobster species, but also highlight that differences in anticoagulant effectiveness can be found even among closely related species, thus their suitability should be evaluated in a case-by-case basis.

MATERIALS AND METHODS

Spiny lobsterswere collected in the Gulf of Batabanó, Cuba (21°39.0431´N, 83°09.8436´W; 21°41.0015´N, 83°092463´W; 21°40.1016´N, 83°11.0297´W)by scuba diving. The collection was undertakenunder permission of the Fisheries Regulator Department from the Ministry of the Fishing Industry of Cuba (Rodríguez-Viera *et al.*, 2014). Lobsters were kept in captivity in tanks with circulating seawater that flow-through a biological filter, with constant aeration and photoperiod of 12 h light: 12 h darkness. Water quality was monitored twice a week: ~26 °C, pH~8.0, salinity 36 psu, oxygen ~6.0 mg^{-l}, and ammonia-N~0.07 mg^{-l}(Rodríguez-Viera *et al.*, 2017). Only intermolt individuals were used (see Lyle & MacDonald, 1983).

Hemolymph was extracted from the coxa of the fourth walking leg using 10ml pyrogen-free disposable syringes containing a pre-cooled anticoagulant solution. Allanticoagulants solutions were used at a 1:1 hemolymph:anticoagulant ratio except in N-ethylmaleimid(NEM), which was 4:1. Glassware used was depyrogenated at 250 °C for 30 min, plastic materials were endotoxin-free certified, and we usedMilli-Q ultrapure waterto avoid microorganism and their cell-wall derived elicitors to induce cell activation, coagulation, and lysis (Levin, 1967). Hemocytes were handled with care as they are extremely labile and prone to become activated (Söderhäll &Cerenius, 1992).

The anticoagulant-and hemocyte-maintaining solutions (HMS)are detailed in Table 1. All solutions were sterile-filtered through 0.22 µm using a Sartolab® RF system (Sartorius Stedim Biotech, Goettingen, Germany) and stored at 4 °C until used (less than a week). HMSlack anticoagulant activity, but should preserve cell viability

and number. They are intended to be used for preparing hemocyte lysates and/or to assess hemocyte response in *in vitro* studies.

<Table 1>

The separation of plasma and hemocyte fractions is required for the study of humoral and cellular components of the hemolymph. Such fractionation is usually accomplished through low-speed centrifugation, which is also used to washout plasma residues from hemocyte fractions. Centrifugation, however, imposes an additional stressful situation to the *ex vivo*hemocytes

The outcomes of our study are based on the assumption that the more suitable anticoagulant solutions are those that prevent plasma clotting while enablinga minimum of cell clotting and lysis upon centrifugation. We found that the removal of plasma residues from hemocytes is accomplished following at least two washings.

The performance of each solution was assessed in the initial mixture of hemolymph-anticoagulant and after each of two successive washings. Assessment involved placing2 ml of hemolymph in anticoagulant in a 2 mlEppendorf Biopurcentrifuge tube (Eppendorf, Hamburg, Germany) and centrifuging at 800 × g for 10 min at 4 °C. The supernatant was discarded, the hemocyte pellet gently resuspended in 1 ml of the corresponding fresh, cold anticoagulant solution or HMS. Washing was repeated once and the hemocytes resuspended in either anticoagulant or HMS. An improved Neubauerhaemocytometer and a phase-contrast microscope under 40 × magnification showed that total hemocyte counts (THC) were obtained in the initial mixture of hemolymph-anticoagulant and in hemocyte suspensions after each centrifugation stepGranulocytes (GC) were readily differentiated from hyalinocytes (HC) and semi-granulocytes (SGC), so that in addition to THC, partial differential hemocyte counts (GC and SGC+HC) were also documented. Cell viability was assayed

by the trypan blue exclusion technique (Mascotti *et al.,* 2000).

The hemocytes from *P. argus*were characterized by Li & Shields (2007). It was found that GC readily differentiate morphologically from HC and SGC, with GC accounting for the lower proportion of the total circulating cells in the hemolymph. GC are also particularly sensitive to the *in vitro* environment, exhibiting the shortest survival rates (Li & Shields, 2007).

RESULTS AND DISCUSSION

NEM was identified as the ideal anticoagulant preventing cell aggregation in the lobster *Homarusgammarus*(Linnaeus, 1758) (as *H. vulgaris*) and the spiny lobsters *Jasuslalendei* (H. Milne Edwards, 1837), *J.paulensis*(Heller, 1862), *J. frontalis*(H. Milne Edwards, 1837), *Panulirus regius*De Brito Capello, 1864, and *P.mauritensis*Gruvel, 1911 (as *P. vulgaris*), (Durliat&Vranckx, 1981). NEM was also found as a suitable anticoagulant in the shrimp *Sicyoniaingentis*(Burkenroad, 1938) (Martin *et al.,* 1991), the crayfish *Pentastacusleptodactylus*(Eschscholtz, 1823) (as*Astacusleptodactylus*), and the lobster *H.gammarus*(as *H.vulgaris*)(Durliat&Vranckx, 1989), in which oxalate, sodium citrate, and cysteine hydrochloride were poor anticoagulants causing rapid damage of hemocytes.

We found that NEM prevented plasma clotting in *P. argus,* but it led to considerable cell aggregation, which resulted in a voluminous white, tissue-like mass of hemocytes containing high amounts of embedded plasma proteins (e.g. hemocyanin). Vigorous homogenization methods were required to disrupt the clump and lyse the hemocytes, still resulting in ahomogenate containing both intracellular and plasma proteins. The clump was composed mainly of GC and SGC, which remained apparently unharmed within the hemocyte mass for several days. We also found that hemolymph

may gel with time even at cold temperatures (4 °C) in the presence of NEM at final concentrations below 50 mM, which indicates that NEM is a poor inhibitor of TGase under the conditions studied. Despite NEM being unsuitable for avoiding the clumping of hemocytes, it has been found to be useful for other applications like the study of plasma proteins such as hemocyanin (Perdomo-Morales *et al.*, 2007, 2008), probably because hemocyanin requires calcium for structure stabilization and activity (Perdomo-Morales *et al.*, 2008). The suitability of an anticoagulant should therefore be also assessed in relation with the aims of the investigation.

NEM is an alkylating agent of sulfhydryl groups that inhibit TGase of vertebrates, mostly in the presence of calcium (Folk & Cole, 1966). NEM proved to be a good anticoagulant also in the presence of chelating agents (i.e. in the absent of calcium), suggesting different enzymatic properties between TGase from vertebrates and crustaceans. NEM was used as anticoagulant for the first time in the horseshoe crab *Limulus Polyphemus* (Linnaeus, 1758), where it prevents amebocyte aggregation and preserves their integrity, probably due to a possible role of the sulfhydryl groups in amebocyte clotting (Bryan *et al.*, 1964).

We also evaluated the suitability of other anticoagulant solutions previously described for crustaceans, particularly for lobsters.

We found that hemocytes clump after the first washing, when cysteine and sodium citrate anticoagulants were used. Since it was not possible to disaggregate clumped hemocytes, these solutions were discarded for further studies. Sodium citrate, as well as EDTA and the TGase inhibitors monodansylcadaverine and glycine methyl ester, inhibit plasma coagulation but not cell agglutination and lysis in spiny lobsters (Durliat&Vranckx, 1981). Cysteine-based anticoagulantsareneverthelessuitable in the spiny lobster *Panulirusjaponicus*(von Siebold, 1824) Aono *et al.*, 1993), which suggest

differences of unknown origin in anticoagulant performance among close-related species.In cysteine seems to promote the binding of the clotting protein to certain component in the hemocytes of *P. leptodactylus*, thus contaminating the hemocyte lysate. The same drawback was observed with potassium oxalate (Durliat&Vranckx, 1989).

In the case of HEPES-EDTA pH 7.3 and caffeine, the number of hemocytes decreased after the second washing (Fig. 1A), although the viability of remaining hemocytes was high (Fig. 1B). The HEPES-EDTA pH 7.3 solution has been used as anticoagulant in the spiny lobsters *P. interruptus* (Randall, 1840) (Hernández-López*et al.*, 2003) and *P. argus* (Pascual Jiménez *et al.*, 2012). We also found that the GC population tends to be the most affected during handling with each anticoagulant solution (Fig. 1C).

<Fig. 1>

One of the most successful anticoagulants for several species is the citrate-EDTA pH 4.6 buffer Söderhäll & Smith, 1983; Johansson *et al.*, 2000). The availability of this anticoagulant has been crucial for studies on crustacean immunity. Citrate delays the lysis of hemocytes while EDTA inhibits the prophenoloxidase activating reaction and the TGase-mediated coagulation. This low-pH buffer, in combination with citric acid, glucose, and NaCl, seems to provide an optimal medium to maintain hemocytes integrity in freshwater crayfishes, without significant loss of cell viability (Johansson *et al.*, 2000). Suspension of crayfish hemocytes in this anticoagulant are able to perform RNA and protein synthesis *in vitro* for at least 24 h (Johansson & Söderhäll, 1989). In shrimps such as*S. ingentis*, citrate EDTA pH 4.6, as well as sodium citrate and potassium oxalate, failed to avoid lysis, activation and aggregation of hemocytes, but were able to avoid plasma clotting (Martin *et al.*, 1991). In this species, TGase

inhibitors like histamine, serotonin, and dansylcadaverine prevent plasma coagulation, but cause massive lysis of hemocytes. We found that citrate-EDTA pH 4.6 and MAS seem to be the best anticoagulant solutions for *P. argus* (Fig. 1A, B), though GC population were more affected with citrate-EDTA pH 4.6 (Fig. 1C). When the volume of hemolymph increased from the conditions studied (1 ml) to 15 or 50 ml under similar experimental conditions, the hemocytes were often prone to activation and aggregation in HEPES-EDTA pH 7.3 and caffeine, further supporting citrate-EDTA pH 4.6 buffer and MAS as preferred anticoagulants to preserve the hemocytes.

We found, however, that citrate-EDTA pH 4.6 promotes the precipitation of plasmatic proteins, especially at temperatures below 15 °C. The same happens in the shrimp *Penaeus californiensis*Holmes, 1900 (Vargas-Albores *et al.*, 1993). Such precipitation is grainy under the microscope that hinders the observation of unwashed hemocytes. The citrate-EDTA anticoagulant at slightly higher pH (5.4) has been used for hemolymph extraction in *P. argus* (Li & Shields, 2007).

Some experimentsmight require the removal of the anticoagulant from the cells to study cellular responses (i.e. cellular immunity), or for preparing hemocyte lysates in the case of humoral response. Rodriguez *et al.* (1995) described that MAS prevented melanization and kept hemocytes in a quiescent state in the shrimp *P.japonicus*(), thus keeping hemocytes in MAS could be unsuitable to study their response *in vitro*.

Commercial mediahave been assessed for the study of viral influence in total and isolated hemocyte populations in *P. argus* (Li & Shields, 2007). The authors found that L-15, Grace's medium, and ML-15 where suitable for the study of hemocyte response, particularly the optimized ML-15 medium, which keeps a high number of hemocytes viable for longer periods. We found that ML-15 medium provideda higher level of unharmed hemocyte number when subjected to two centrifugation steps (Fig.

2), and the GC population where less impaired (Fig. 2C). L-15 worked fine untilthe first centrifugation, but the total number of hemocytes dropped dramatically after the second one (Fig. 2A). Although these media are suitable for primary culture of hemocytes from *P. argus*, they contain divalent cations and tyrosine, a substrate for phenoloxidase, and thus a lysate made in such medium could undergo spontaneous melanization (Perdomo-Morales *et al.*, 2007). We also evaluated SSS and PAS solutions. The THC and hemocyte viability in PAS were similar to ML-15 (Fig. 2A, B), though the GC number was lower after the second washing (Fig. 2C). THC in SSS, however, diminished after the second centrifugation and yielded the lower level of hemocyte viability (Fig. 2A, B). SSS has been successfully used for the study of hemocyte responses in some crabs (La Peyre& Chu, 1990) and shrimps (Rodriguez *et al.*, 1995). PAS has been used to prepare antennule tissue homogenates in studies related to the olfactory organ in *P. argus* (Johns *et al.*, 2004). A similar solution was used by Mulloney&Selverston(1974) to study the nervous system in *P. argus*. We found that PAS is also suitable to study the hemocytes, which could indicatethat it mimics the saline internal environment of this species.

<Fig. 2>

We observed a linear relationship between cephalothorax length (CL) and hemolymph volume in individuals at intermolt stage (Fig. 3). The fresh body weight (W) was determined from CL using the relationship $W = 0{,}00243 \times CL^{2{,}764}$ developed for *P. argus* (Cruz *et al.*, 1981). We found that hemolymph volume represented around 10.5% of fresh body weight of lobsters, which is in accordance with data obtained in other decapods (Depledge&Bjerregaard, 1989).

<Fig. 3>

Lobster hemolymph is relatively easy to withdrawn and an adult individual typically provides more than 50 ml (Fig. 3) of hemolymph. Lobsters are manageable

and easy to be keep in captivity (Perera& Simon, 2015), thus they represent an attractive animal model to study the biological function of hemolymph components in decapod crustaceans. It is striking that whereas avoiding plasma clotting is relatively simple to achieve, avoiding lysis and aggregation of hemocytes could be challenging and variable among closely related crustaceans. The reasons are hardly known and might indicate different composition or sensitivity of both membrane-bound and soluble mediators in any of the three types of hemocytes identified among decapod crustaceans. ~~High concentrations of glutamine and glucose apparently help keep viable hemocytes in *P. argus* (Li & Shields, 2007).~~

Summarizing, MAS was the best anticoagulant among those studied in *Panulirus argus*(Fig. 2). The plasma proteins neither coagulated nor precipitated, the hemocyte number and viability were highly preserved even after stressful centrifugation , while GC population was affected to a lesser extent (Fig. 2C).

The ML-15 medium was superior to isosmotic L-15 and Sterile Saline Solution (SSS) media for maintaining hemocyte number and viability. PAS solution could be preferred for obtaining hemocyte lysate in which the melanization reaction want to be avoided. Therefore, for preparing hemocyte suspension in *P. argus,* we suggest to use MAS anticoagulant solution, one washing step with the same anticoagulant for the removal of plasma, and a second washing step with either ML-15 media or PAS solution for final washing and cells resuspension. The material used should be sterile and preferable pyrogen-free, and the working temperature should be kept at around 4 °C.

Finally, we found a linear relationship between cephalothorax length (*CL*) and hemolymph volume in animals at intermolt stage (Fig. 3). The fresh body weight (*W*) was determined from *CL* using the figure $W=0{,}00243\times CL^{2{,}764}$ developed for *P. argus* (Cruz *et al.*, 1981). This way, we found that hemolymph volume represents around

10.5% of fresh body weight of lobsters, which is in accordance with data obtained in other decapods (Depledge&Bjerregaard, 1989). Lobster hemolymph is relatively easy to withdrawn and an adult animal typically provides more than 50 ml (Fig. 3). Lobsters are manageable and easy to be keep in captivity (Perera& Simon, 2015), thus they represent an attractive animal model to study the biological function of hemolymph components in decapod crustaceans.

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Figure 1. Effects of anticoagulant solutions on the hemocytes of the spiny lobster *Panulirus argus*. Total hemocyte count (THC) (**A**); hemocyte viability (%) (**B**);partial differential hemocyte counts (GC and HC+SGC) (**C**). THC and partial differential hemocyte counts were conducted using an improved Neubauerhaemocytometer and a phase contrast microscope under 40 × magnification, with two counts per lobster. Hemocyte viability was assessed by the trypan blue exclusion technique. Three lobsters were used for each experiment. Values represent the mean ± SD.

Figure 2. Effects of hemocyte maintaining solutions (HMS) on the hemocytes of the spiny lobster *Panulirus argus*. Total hemocyte count (THC) (**A**); hemocyte viability (%) (**B**); partial differential hemocyte counts (GC and HC+SGC) (**C**). Solutions assayed: modified L-15 medium (ML-15), L-15 medium,*P. argus* saline (PAS), and sterile saline solution (SSS). THC and partial differential hemocyte counts were conducted using an improved Neubauerhaemocytometer and a phase contrast microscope under 40 × magnification, with two counts per lobster. Hemocyte viability was assessed by the trypan blue exclusion technique. Three lobsters were used for each experiment. Values represent the mean ± SD.

Figure 3. Relationship between length ofcephalothorax and hemolymph volume in the spiny lobster *Panulirus argus*.Fourteen adult spiny lobsters were bled.