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Isolation and Culturing of Earthworm (*Eudrilus eugeniae*) Coelomocytes

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ABSTRACT

Coelomocytes, the immune cells present in the coelomic fluid of *Eudrilus eugeniae* were isolated using electric, cold and heat shocks in two different media, phosphate buffer saline (PBS) and Hank's balanced salt solution (HBSS) for demonstrating the ideal treatment for isolation of invertebrate cells and for their further multiplication in the given media. Results of all the methods showed concurrent cell density from the time of isolation to subculturing. Cell viability was measured using hemocytometer and trypan blue exclusion method. It was found that all the three treatments had higher viability in HBSS media compared to PBS media. For isolation of coelomocytes, the most appropriate method was found to be by using cold shock treatment in HBSS media, indicating least cell damage with good recovery of cells. The present study can serve as a useful aid in further immunocytochemical studies.

Keywords: cell viability, extraction methods, Hank's balanced salt solution, phosphate buffer saline, subculture
Abbreviations: FCS, fetal calf serum; **HBSS**, Hank's balanced salt solution; **PBS**, phosphate buffer saline

INTRODUCTION

Coelomocytes play a remarkable role in the functioning of earthworm immune system and are involved in phagocytosis and release of lytic factors which are characteristic of innate immunity. They are present in the coelomic cavity of earthworm. Earthworms have pores that connect the coelomic cavity to the exterior, through which cells are extruded following stress. These cells are considered the immune cells of lower coelomate animals (annelida, mollusca, arthropoda), which are types of leukocytes that have long been considered to constitute the major innate (unspecific) immune defense system of these animals (Hostetter *et al.* 1972; Engelmann *et al*. 2005). These cell types are studied because they provide information about mechanisms governing innate immunity. Coelomocytes from various sources have shown to be capable of phagocytosis and thus performs functions of macrophages, have natural killer cell features, mediate lytic reactions against several targets and also secrete antimicrobial peptides (Porchet-Hennere *et al.* 1992; Cooper *et al*. 1995; Cossarizza *et al.* 1996; Cooper *et al*. 2002; Koros *et al.* 2002). The earthworm model was for several reasons chosen for analysis. They have a simple system, in that they lack adaptive immunity (Fischer and Horvath 1977) and they display an innate immune response (Dhainaut and Scaps 2001). They have a well-studied life cycle and their coelomocytes tend to be of two types- small (cytotoxic) and large (phagocytic) (Engelmann *et al.* 2005).

Eisenia fetida is the most commonly studied earthworm for immunological studies. The coelomocytes of this annelid were classified into four major categories based on cytomorphology and cytochemistry – acidophils, basophils, chloragocyte cells and neutrophils (Valembois *et al.* 1992). Chloragocytes, also called chloragogen cells or eleocytes, constitute a subpopulation of phagocytic coelomocytes. These cells contain characteristic granules, called chloragosomes which are thought to be involved in the protection of cells and organisms against foreign substances (Murav'ev *et al*. 1994; Adamowicz 2005) and have been associated with lytic activities (Peeters-Joris 2000; Kauschke *et al.* 2001; Koenig *et al*. 2003).

Since not much work has been carried out on similar lines, *Eudrilus eugeniae*, a tropical epigeic earthworm has been chosen for the present study. This study focused mainly on isolation and culturing of coelomocytes.

MATERIALS AND METHODS

Hank's balanced salt solution (HBSS), Phosphate buffer saline (PBS), Laminar air flow, water jacketed automatic CO_2 incubator, Inverted phase contrast microscope, trypan blue dye, hemocytometer, micropipettes, earthworm (*E. eugeniae*), ice cubes, electric wires connected to battery to get a current flow of 3V, hot plate, pressure cooker, microwave oven, autoclave, pH meter were used for the study.

Preparation of media

Hank's balanced salt solution and Phosphate buffer saline were prepared as per the method of Freshney (2006).

Collection of earthworms

E. eugeniae was maintained in cement structures (1.8 × 0.9 × 0.6 m) for converting the garden waste of the college campus along with cow dung slurry.

Earthworms were collected from these bins. They were placed on ordinary wet filter paper in plastic boxes (25 × 15 × 12 cm) with lids having fine pin holes. To avoid contamination, after 48 hrs, when the gut was cleared of organic matter due to feeding of earthworms on filter paper, they were removed for surface cleaning. They were thoroughly washed in running tap water before rinsing in glass distilled water and then in PBS and HBSS media. The surface-cleaned earthworms were placed in a sterile Petri dish (9 cm diameter) and used for extraction of coelomocytes. Care was taken not to disturb them too much to avoid any excretion of gut contents. Any excreted material found in the Petri dish was discarded.

Received: 1 April, 2009. Accepted: 15 July, 2009.

Original Research Paper

Isolation of *Eudrilus eugeniae* coelomocytes

Three methods were followed to isolate coelomocytes from the coelomic fluid of earthworms. For isolation, three to four adults, each weighing 0.8-1.2 g were released into sterile Petri dishes for each of the treatment replications.

1. Heat shock treatment

Earthworms, suspended in the HBSS (15 ml) and PBS (15 ml) media respectively were subjected to a temperature of 45°C (1-min treatment, 1 min interval for recovery and repeated 8-10 times) using hot water in a beaker for 15 min (Kale 2006). Due to this external stress condition coelomocytes were extruded into the media which acts as an inoculum for the growth of cells. Fresh media was introduced into these Petri dishes and incubated at 37°C for three days.

2. Cold shock treatment

Earthworms, suspended in the HBSS (15 ml) and PBS (15 ml) media respectively, were subjected to cold shock (4°C, 1-2 mins, 1 min interval for recovery and repeated 8-10 times) with the aid of ice cubes in a beaker for 15 min (Kale 2006). Due to this stress coelomocytes were extruded into the media which acts as an inoculum for the growth of cells. Fresh media was introduced into these Petri dishes and incubated at 37°C for three days.

2. Electric shock

Earthworms, suspended in the HBSS (15 ml) and PBS (15 ml) media respectively, were subjected to electric shock (3V, 30 sec, 1 min interval for recovery and repeated 5-6 times) (Stankiewicz and Plytycz 1998). Due to this shock treatment coelomocytes were synthesized and extruded into the media which acts as an inoculum for further growth and division of cells.

Cell viability

Cell yield and cell viability (Johnstone and Thorpe 1982) were recorded at the time of isolation and after incubation for 3 days using hemocytometric counting and trypan blue cell exclusion assays, respectively.

Statistical analysis of data

Mean of six replications for each of the treatments was considered for statistical analysis. The experiments were run simultaneously for all the treatment trials. Student's *t*-test was applied to look for the level of significance (Gupta 1969).

RESULTS AND DISCUSSION

Different types of coelomocytes were observed in the culture and the same is illustrated in **Fig. 1**, which is still to be characterized based on their morphology.

A gradual increase in the cell density was observed from the time of isolation to subculturing, in PBS (**Table 1; Fig. 2**) and HBSS (**Table 2; Fig. 3**) media in the coelomocytes collected through three different treatments.

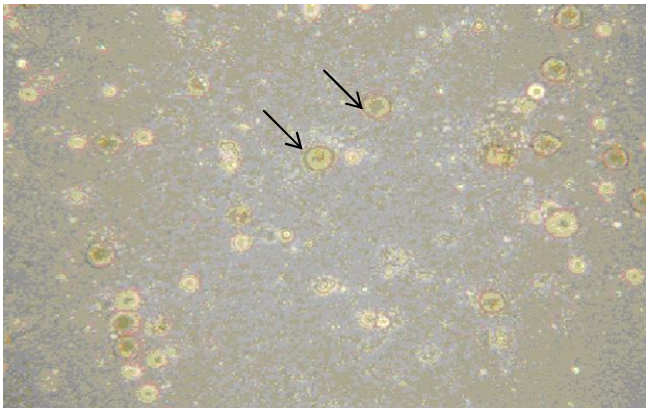

Fig. 1 Different types of coelomocytes (arrows) of earthworm *Eudrilus eugeniae* observed in the culture (original magnification X 40).

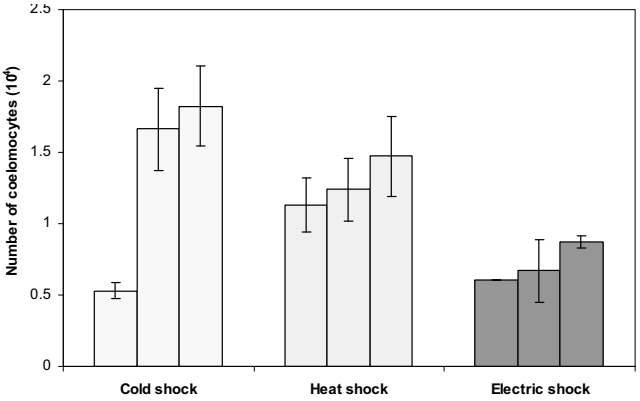

Fig. 2 Coelomocyte density of earthworm *Eudrilus eugeniae* from the time of isolation to subculturing in PBS media on using different isolation methods. Values represent mean ± Standard Error (SE), n=6.

Cell viability measurements using trypan blue exclusion method showed an increase in viability at the time of isolation (60-70%) in HBSS media, which was significantly high in heat shock method (**Table 3**).

In the current study, PBS (Torsvik 1995) and HBSS were used for the isolation and culturing of coelomocytes of earthworm *E. eugeniae.* HBSS was used without any mucolytic agents (DiogEme *et al*. 1997). Even in the absence of FCS, which has been used in the previous study along with L-15 medium (Toupin *et al.* 2007), significantly high cell density was seen in HBSS medium in the present study. The reproducibility and ease of preparation of HBSS medium makes it a suitable method for coelomocyte culture.

Earlier studies suggested that contamination and low

Table 1 Coelomocyte density of earthworm *Eudrilus eugeniae* in PBS media using different isolation methods. n=6.

Parameters	Cold shock (mean± S.E) x10^4	Heat shock (mean± S.E) x10^4	Electric shock (mean± S.E) x10^4
Cell count at the time of extraction	0.53 ± 0.06	1.13 ± 0.19	0.60 ± 0.0
Cell proliferation after 3 days of incubation	1.66 ± 0.29	1.24 ± 0.22	0.67 ± 0.22
Growth on subculturing	1.82 ± 0.28*	1.47 ± 0.28	0.87 ± 0.04

The data was analyzed by the student's *t*-test at *p< 0.001 compared to cold shock at the time of extraction in both PBS and HBSS.

Table 2 Coelomocyte density of earthworm *Eudrilus eugeniae* in HBSS media using different isolation methods. n=6.

Parameters	Cold shock (mean± S.E) x10^4	Heat shock (mean± S.E) x10^4	Electric shock (mean± S.E) x10^4
Cell count at the time of extraction	0.53 ± 0.11	1.19 ± 0.33	0.99 ± 0.13
Cell proliferation after 3 days of incubation	1.38 ± 0.34	1.68 ± 0.38	1.39 ± 0.10
Growth on subculturing	2.25 ± 0.34*	1.99 ± 0.20	1.95 ± 0.30#

The data was analyzed by the student's *t*-test at *p< 0.001 compared to cold shock at the time of extraction in both PBS and HBSS.
#p<0.01 compared to electric shock at the time of extraction in HBSS.

Table 3 Measurement of coelomocyte viability of earthworm *Eudrilus eugeniae* in PBS & HBSS media using different isolation methods. n=6

Treatments	Cold shock % ±S.E	Heat shock % ±S.E	Electric shock % ±S.E
PBS	46.75 ± 9.77	64.12 ± 12.7	65.7 ± 10.02
HBSS	66.69 ± 3.36*	70.95 ± 8.25	66.66 ± 10.13

The data was analyzed by the student's *t*-test at *p< 0.1 compared to PBS.

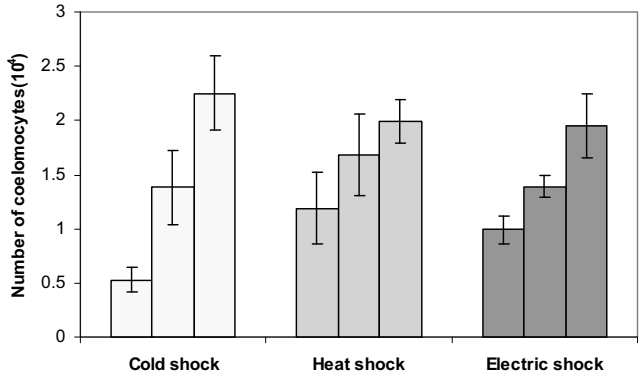

Fig. 3 Coelomocyte density of earthworm *Eudrilus eugeniae* from the time of isolation to subculturing in HBSS media on using different isolation methods. Values represent mean ± Standard Error (SE), n=6

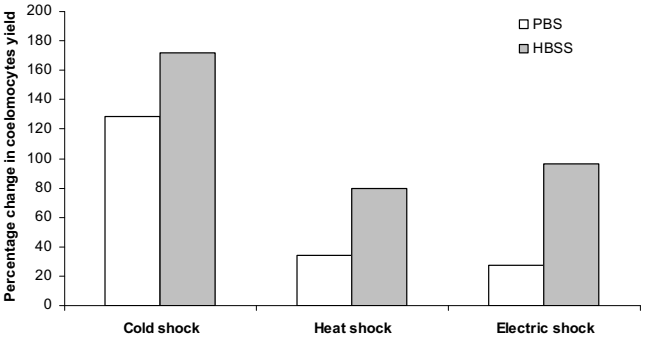

Fig. 4 Comparison of percentage variation in yield of coelomocytes in different extraction methods.

viability of earthworm coelomocytes in tissue culture has delayed *in vitro* studies. Although penicillin, streptomycin, tetracycline and amphotericin B were added, *Lumbricus terrestris* coelomocytes were maintained viable and uncontaminated only for 10 days at 15°C in medium L-15 supplemented with 5 to 10% fetal bovine serum (Toupin *et al.* 2007), Whereas in this study the culture plate with proliferating cells did not exhibit any bacterial or fungal contamination for 15 days at 37°C in HBSS and PBS medium, even though there was no usage of antifungal agents or antibiotics. Although there was protozoan contamination during the isolation stages, the contamination subsided with further culturing and subculturing. Further studies may be carried out to check for the usage of coelomocytes and their metabolites as antifungal agents or antibiotics.

Of the three methods used electric shock (Stankiewicz and Plytycz 1998) is the most commonly used extrusion method and in this study heat shock and cold shock methods for cell extrusion were introduced and compared with the other method.

The results obtained, indicate increase in cell density from the time of isolation to subculturing (**Tables 1, 2; Figs. 2, 3**). In order to find the best method and media for isolation and culturing of coelomocytes, percentage increase or decrease was calculated and the same has been plotted (**Fig. 4**). The cell yield is highest when isolated using cold shock treatment in HBSS media. This may be due to the preservation of the cell wall repair mechanism of earthworm, which could repair the damaged cells using the constituents (phosphates and glucose essential for cellular activities) of HBSS media, which are absent in PBS media. When the earthworms were subjected to cold shock, the essential proteins as well as enzymes were not prone to denaturation as in heat shock and electric shock, which may be the reason for the higher cell density observed.

Since cold shock method shows the highest peak, this method is taken as control to compare heat shock and electric shock methods with cold shock. On comparison, it was found that all showed 40 to 50% decrease in both the media (**Fig. 4**).

On comparison between the cells treated in PBS and HBSS media, it was found that there was an increase in ~40% cell yield obtained by cold shock in HBSS media (**Fig. 4**)

The cell viability using trypan blue exclusion method showed higher viability of cells in all the three treatments in HBSS media compared to PBS media (**Table 3**). This may be due to the presence of all the components in HBSS media which are absent in PBS media. At the time of isolation, heat shock method showed higher cell viability compared to the other two methods but after a week of culturing, cold shock method showed better cell viability as compared to the other two methods. This indicates that, the cells isolated by cold shock had greater recovery compared to the other two methods.

Cell viability in HBSS media using cold shock as the extraction method was more significant than that in PBS media. Even though cell densities were insignificant, the cells extracted and cultured using HBSS media by cold shock method were more intact and viable for further experiments.

Thus, from the following observations it can be concluded that, the best method for isolation of coelomocytes are by using cold shock treatment in HBSS media. The coelomocytes isolated by this procedure can be used for analysis of agglutination property of lectins and also for further immunocytochemical studies.

As pointed out by Venables *et al*. (1992), there is an increasing need to develop new toxicity tests with earthworms in order to assess the contamination of soils. Consequently, much effort has been undertaken towards the development of tests aimed at the evaluation of cellular responses at specific levels and in particular with *L. terrestris* (Rodriguez-Grau *et al*. 1989; Eyambe *et al*. 1991; Goven *et al.* 1994), a widespread endogenous species by comparison with *E. fetida.* An important step in the development of new *in vitro* toxicity tests is the establishment of appropriate and standardized techniques, such as for coelomocytes isolation and maintenance (DiogEme *et al.* 1997). Hence, the current study dealing with isolation and culturing of coelomocytes from *E. eugeniae* could be used to analyze and evaluate sublethal effects of soil pollutants, a much needed study for protection of environment.

ACKNOWLEDGEMENTS

Authors express their thanks to Sister Albina, Principal, Mount Carmel College for providing an opportunity to carry out the research work with the required facilities and Dr. Swayamprabha, Coordinator, Department of Biotechnology, Mount Carmel College for her valuable suggestions and support. University Grants Commission, New Delhi, India, extended the financial support to carry out this research.

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