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Maintenance of haemocytes of edible oyster, *Crassostrea madrasensis* (Preston) in artificial media

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ABSTRACT

The haemocytes of *Crassostrea madrasensis* were maintained in Hank's balanced salt solution (HBSS), HBSS with 10% foetal calf serum (FCS), tissue culture media M 199, and M 199 with 10% FCS. Total counts of viable haemocytes were taken at 0 hr, 24 hrs, 48 hrs, and 72 hrs. HBSS and HBSS with 10% FCS were found to maintain cell viability upto 72 hrs without much reduction in the count of viable cells. In M199 and M199 with 10% FCS, it was found that a substantial increase of small hyalinocytes occurred at 24 hrs and 48 hrs. This was followed by a steep fall in the number of viable cells at 72 hrs in both the media. The HBSS, HBSS with 10% FCS, M199 and M199 with 10% FCS were found to be good for maintaining the cells in viable state. However, only M 199 and M 199 with 10% FCS indicated multiplication of haemocytes.

Introduction

In bivalves, haemocytes constitute the first line of defense against potential pathogens. In order to study the functional characteristics of these cells *in vitro,* they have to be suspended in a medium, in which, they can function normally. Maintenance of the haemocytes in media is essential for studies such as density gradient centrifugation and cell separation, phagocytosis, chemotaxis, cell aggregation, adhesion, etc.

According to Bachere *et al.* (1995), a modified Alsever's solution is efficient in preventing cell degradation and maintaining hemocytes in a quiescent state. They have suggested a medium, which contain calcium and magnesium, where the haemocytes can recover their attachment and spreading behaviour. Bayne *et al.* (1979) has used 0.1% N-ethyl maleimide in millipore filtered seawater for cytological and cytochemical examination of haemocytes of *Mytilus californianus.* Tissue culture media M199 and Bge media in 1:1 proportion were used by Noda and Loker (1989) for suspending haemocytes of *Biomphalaria glabrata,* whereas, M199 with foetal calf serum was used for haemocytes of *Crassostrea virginica* by Chen and Rudo (1976). Chen (1996) used

calcium magnesium tris buffered saline for the maintenance of haemocytes of abalone, *Haliotis diversicolor.* Kent *et al.* (1989) used EDTA buffer and Friebal and Renwrantz (1995) used Mytilus ringer solution for the maintenance of haemocytes *of Mytilus edulis.*

In all these cases, the suitability of the media for the maintenance of haemocytes and its effects on the functions of haemocytes have not been studied except for M199, which has been tested and found suitable for the haemocytes of *B. glabrata* and *C. virginica.*

In the present study, an attempt was made for the maintenance of haemocytes of *Crassostrea madrasensis* in different media so as to find their suitability to support these cells.

Materials and methods

C. madrasensis specimens of shell length 4 to 6 cm were obtained from Vypeen Island, Cochin and maintained in filtered and aerated sea water of 25 ppt salinity, in 10L basins with 5 numbers in each basin at room temperature. They were fed with *Chaetoceros* sp. *ad libitum* every morning, after the removal of waste. About 50% of the water was exchanged with fresh seawater every alternate day.

Four types of media were used for the study. They were, 1) Hank's balanced salt solution (HBSS) 2) HBSS with 10% foetal calf serum (FCS) 3) Tissue Culture media M 199 and 4) M 199 with 10% FCS. The HBSS, FCS and M199 were obtained from HIMEDIA, Mumbai, India. The media were prepared as follows: One vial (9.76gm of HBSS 9.6 gm of M199) of entire dehydrated media was added to 900 ml of double glass distilled water and stirred until it was dissolved. 0.35g and 2.2g of sodium bicarbonate were added to HBSS and M 199 respectively. 5 units of heparin and lOOug of streptomycin were added to each of the media. The pH was adjusted to 7.4 and made up to 1000 ml using double distilled water. The media 2 and 4 were prepared by adding 10% FCS under sterile conditions. The media were sterilized by filtration through millipore 0.22u membrane filter and stored in dark at 4°C.

Haemolymph was withdrawn from the posterior adductor muscle of the oyster using a sterile hypodermic syringe. 0.5ml of haemolymph was drawn into the syringe containing 0.5ml media and transferred into sterile tubes in three replicates. The tubes were incubated at 18°C.

Cell viability was tested using trypan blue dye exclusion technique. 0.1% trypan blue was used for staining. The cells with stained nuclei were considered dead. Viable cells were counted in a haemocytometer. The total viable haemocyte count was taken at 0 hr, 24 hrs, 48 hrs and 72 hrs.

Results

The haemocyte counts obtained at different intervals in different media are given in Table 1. In HBSS the initial haemocyte count ranged from 63 to 64 x 10^4 cells per ml with a mean of 63.67+1.58 x 10^4 cell's per ml. At 24 hrs, the count was between 64 and 86 x 10^4 cells per ml with a mean of 73 +11.53 x 10^4 cells per ml. The haemocyte count at 48 hrs ranged from 66 to 72 x 10^4cells per ml with a mean of 68.67 + 3.06 x 10^4 cells per ml. On completion of 72 hrs the count varied from 50 to 76 xlO^4 cells per ml with a mean of 65.33 ± 13.61 x IO^4 cellsper ml.

HBSS with 10% FCS contained 63 to 65 x 10^4 cells per ml initially with a mean of 64 + 1 x 10^4 cells per ml. The count was between 59 and 63 x 10^4 cells per ml at 24 hrs and mean was 61.67 + 2.31 x 10^4 cells per ml. The haemocyte count again decreased to a range of 52 to 63 x 10^4 cells per ml with mean of 58.67 + 5.86 x 10^4 cells per ml by 48 hrs. At 72 hrs the count

TABLE 1. *Total haemocyte count (X10^4 cells per ml.)*

Time	HBSS	HBSS with FCS	M199	M199 with FCS
Ohr	64 63 64	63 65 64	85 67 60	71 73 64
24 hr	64 69 86	63 63 59	69 100 53	116 102 98
48 hr	66 72 68	61 52 63	132 158 137	158 140 115
72 hr	70 50 76	49 67 63	64 91 123	120 90 80

ranged from 49 to 67 x I0^4 cells per ml with mean of 59.67 ± 9.45 x 10^4cells per ml.

The tissue culture media M 199 had an initial haemocyte count between 60 and 85 x 10^4 cells per ml with a mean of 70.67 ± 12.9 x 10^4 cells per ml. The count ranged from 53 and 100 x 10^4 cells per ml with a mean value of 74 + 23.9 x 10^4 cells per ml at the end of 24 hrs. At 48 hrs the count almost doubled and ranged from 132 to 158 x 10^4 cells per ml with mean value of 142.33 + 13.8 x 10^4 cells per ml. In all the three replicates small hyalinocytes were abundant. With the completion of 72 hrs, the count was reduced to a range of 64 to 123 x 10^4 cells per ml with mean value 92.67± 29.54 x 10^4 cells per ml.

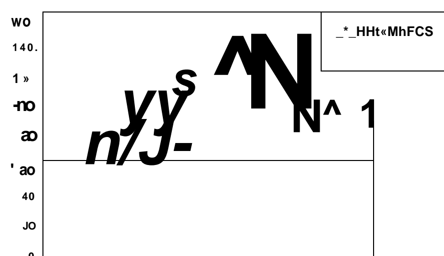

Fig. 1. Variatiom of mean haemocyte count with time in different media (x 10^4 cells per ml.).

M 199 with 10% FCS showed almost similar results as M 199. The initial count varied from 64 to 73 x 10^4 cells per ml with mean value 69.3 ± 4.73 x 10^4 cells per ml. The count increased to a range of 98 to 116 x 10^4 celk per ml with average of 105.3 + 9.45 x 10^4 cells per ml at 24 hrs. At 48 hrs the value doubled to a range of 115 to 158 x 10" cells per ml with a mean of 137.67 + 21.59 x 10^4 cells per ml. In this treatment also small sized hyalinocytes were abundant in all the three replicates. The count again decreased to a range of 80 to 120 x 10^4 cells per ml with mean value of 96.67 + 20.82 x 10^4 cells per ml at 72 hrs.

The variation in mean haemocyte count with time in the four different media is given in Fig. 1. The result was analyzed using analysis of variance technique. The results of analysis show significant difference between the different media and within the media with different time interval at 5% level of significance. The analysis of variance of the data is given in Table 2.

Discussion

The haemocytes that were maintained

TABLE 2. *Analysis of variance table for the differences within and between media with time interval*

Source	Degrees of freedom	Sum of squares	Mean sum ofsquares	F value
Media	3	14666.0834	4888.695	4.66*
Time interval	3	7723.4167	2574.47	
Interaction	9	9451.749	1050.194	5.1396 *
Error	32	6538.6676	204.333	
Total	47	38379.9167		

- Significant at 5% level at 3 and 9 degrees of freedom.
- Significant at 5% level at 9 and 32 degrees of freedom.

in the four different media gave significant results. The HBSS with and without FCS, though maintained the viable cells, the viability decreased with time. The addition of FCS did not improve the quality of HBSS media. Kent *et al.* (1989) and Freibal and Renwrantz (1995) used balanced isotonic salt solutions like EDTA buffer and ringer solution respectively for maintaining the cells, to study *in vitro* phagocytosis and cytotoxic assay respectively for short duration. They have not examined the effect of the media on the viability of cells. In HBSS there was no increase in the number of cells at 24,48 and 72 hrs. The number of viable cells was more or less maintained upto 72 hrs. This phenomenon is surprising, because in the other media used, there was a steep decline of viable cells at 72 hrs.

Foetal calf serum is supposed to provide colloidal osmotic protection and additional nutrient factors that are available in native plasma. As the different treatment groups already contained some amount of native plasma, FCS may not have any additional benefits in the present experiment.

The medium M 199, not only maintained the cells, but also supported the growth of cells as indicated by the proportional increase in the number of cells at 48 hrs. The increase in the number of cells up to 48 hrs indicated a probable multiplication of the cells in the media. In order to prove the multiplication of the cells, incorporation of radiolabelled thiamidine in the cells has to be tested. In the present study this has not been done. However, the substantial increase in the number of cells in all replicates could not be explained otherwise. There is a fall in the number of cells at 72 hrs. As the media has not been supplemented, the' nutrients in the media must have been exhausted. This, coupled with the high metabolic rate of the dividing cells and accumulation of waste products contributes to the deterioration of the quality of the media. The decrease in the number of viable haemocytes can be explained by the above-described phenomenon.

Cheng and Rudo (1976) and Noda and Loker (1989) have observed the suitability of M 199 as a medium for maintenance of haemocytes. Cheng and Rudo also found that the medium with FCS with or without plasma of oyster supported the viability of cells even up to one week. The results of the present study also support this view, although they have not reported multiplication of cells.

The haemolymph fraction contains immature cells, partially mature cells and mature cells. The hyalinocytes were reported to be immature cells that mature in to granulocytes and the intermediate stage during maturation is the semigranulocytes (Foley and Cheng, 1972). The increase in number of cells at 24 and 48 hrs were mainly contributed by small sized hyalinocytes, and their numbers were proportionately very high. There is a possibility that hyalinocytes under suitable condition had undergone division.

In media M199 when FCS was added, there was an increase in the number of haemocytes at 24 and 48 hrs. Here also there was a fall in the number of cells at 72 hrs. Though there was an increase in number of cells at 24 and 48 hrs, the increase did not vary significantly from those samples maintained in M199 alone. Cheng and Rudo (1976) have studied chemotaxis ofhaemocytes in media M199 with foetal calf serum. They have found that foetal calf serum enhances chemotactic movement ofhaemocytes. The addition of foetal calf serum did not have any beneficial effects on the viability of the cells in the present study. This could be due to the presence of substantial amount of native plasma in the media.

In conclusion, of the four types of media tested, namely, HBSS, HBSS with FCS, M 199 and M 199 with FCS, HBSS and HBSS with FCS maintained cell viability, but did not support the multiplication of the cells. In the media M 199 and M 199 with FCS, there was proportional increase in number of cells that indicated a probable multiplication of the cells in the media. The addition of FCS did not alter the quality of the media as far as the viability of haemocytes are concerned. Among the media tested, M199 appeared to support the multiplication of the haemocytes whereas HBSS maintained the cells. However further detailed studies using 3_H thiadmidine has to be done for the confirmation of multiplication of haemocytes in *vitro.*

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