Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Isolation and characterization of head kidney derived macrophages of *Labeo rohita*

Abhishek Awasthi[1], Gaurav Rathore[2]*, P.K. Pradhan[1], S.C. Rebello[1], M.Y. Khan[3] and W.S. Lakra[2]

[1]National Bureau of Fish Genetic Resources, Lucknow-226 002, India
[2]Central Institute of Fisheries Education, Mumbai-400 061, India
[3]Baba Saheb Bhimrao Ambedkar University, Lucknow-226 025, India
*Corresponding Authors Email : grathore69@gmail.com

Publication Info

Paper received:
24 May 2013

Revised received:
05 September 2013

Accepted:
12 September 2013

Abstract

Macrophages play a significant role in non-specific defense mechanisms of all vertebrates against pathogens. One critical element in the area of fish immunology is the unavailability of *in-vitro* model of immune cells. Therefore, it is essential to develop methods for harvesting and culture of macrophages for assessing innate immune functions of rohu, *Labeo rohita*, an important culture fish of India. Head kidney leukocytes from were isolated by density gradient sedimentation, so as to exclude other cells. Among isolated leukocytes, only macrophages showed the unique property of sustained adherence on plastic surfaces. These cells exhibited optimum growth at 28 ºC in L-15 containing 20% FBS. Cultured head kidney macrophages (HKM) demonstrated the property of phagocytosis as evidenced by engulfment of yeast cells. Bacterial lipopolysaccharide (20µg/ml) resulted in functional activation of macrophages as seen by enhanced reactive oxygen and nitrite production; and lysosomal enzyme activity. These results show that *in-vitro* model of HKM cells can be used to study the role of macrophages in innate immune responses against various immunomodulators.

Key words

Head kidney, *Labeo rohita,* Macrophage, Phagocytosis

Introduction

Macrophages in teleosts play a crucial role in the innate and acquired defense against pathogens. They can be activated to possess enhanced antibacterial activities (Norum *et al.,* 2005). Fish macrophages are highly phagocytic and have potent intracellular antimicrobial defenses (Rieger *et al*., 2011). In fish, head kidney (HK) has both hematopoietic and endocrine functions and head kidney macrophages (HKM) are vital components of the innate immune system of fish (Datta *et al*., 2009, Fishelson *et al*., 2005). The relative ease of dissection of this tissue and the subsequent processing to purify macrophages has made HKM a important *in-vitro* model in fish immunology. This model has provided a extensive immunological data derived from functional assays of macrophages such as the phagocytosis of pathogens, cloning and characterization of novel genes (Husain *et al*., 2012), gene expression studies (Furne *et al*., 2013, Pellizzari *et al*., 2013) and microarray analysis (Lu *et al*., 2013). Head kidney derived macrophages have been used to examine nonspecific immune responses in previous studies (Romeo *et al*., 2000; Datta *et al*., 2009).

The head kidney is known to be a rich source of macrophages for their *in- vitro* culture in several fish species like *Cyprinus carpio* (Verburg-Van *et al*., 1995; Joerink *et al*., 2006), *Dicentrarchus labrax* (Bennani, *et al*., 1995) goldfish (Wang *et al*.; 1995), *Gadus morhua* (Sorensen *et al*., 1997) and rainbow trout (Stafford *et al*., 2001). Number of continuous cell lines derived from HKM have been described, including a goldfish macrophage cell line (Neumann *et al*., 1998) and several trout and salmon cell lines (Brubacher *et al*., 2000; Neumann *et al*., 2001; Rolland *et al*., 2005). In India, HKM of *Clarias batrachus* has been used as *in-vitro* model for assessment of arsenic toxicity (Datta *et al.,* 2009) and cytotoxicity induced by *Aeromonas hydrophila* (Banerjee *et al.,* 2012). The aim of this study was isolation and characterization of head kidney macrophages from rohu, *Labeo rohita*. The cells

were characterized on the basis of important functional properties such as phagocytosis, reactive oxygen species production, nitrite production and lysosomal enzyme activity.

Materials and Methods

Sampling : Healthy *Labeo rohita* (300-500 g) obtained locally were maintained in fibre reinforced plastic tanks and provided pelleted fish feed every day. Fish were acclimatized in the laboratory for 15 days prior to experiment. Diseased fish were removed immediately from the tanks.

Isolation of macrophages : Head kidney macrophages were isolated as per method described by Norum *et al.* (2005) with slight modifications. Briefly, rohu were euthanized with MS-222 (Sigma-Aldrich, St. Louis, USA) and head kidney was aseptically removed. Head kidney tissue was homogenized in 10 volumes of phosphate buffered saline (PBS) containing 2X concentration of antibiotic-antimycotic solution (Invitrogen) and gently passed through 100 µm sterile nylon mesh. The resulting cell suspension was layered on Histopaque -1077 (Sigma-Aldrich) and centrifuged at 400×g for 30 min at 24°C without using brakes. Mononuclear cells (MNCs) were collected from interface, diluted with PBS and centrifuged at 250×g for 10 min. Cultures were initiated by seeding the MNC's in a 25 cm^2 culture flasks (Nunc) containing 20 ml of L-15 Leibovitz L-15 medium (Gibco) supplemented with 20% foetal bovine serum (FBS) and antibiotic-antimycotic solution. After 24 hr of incubation at 28 °C, the non-adherent cells were removed by washing the cultures three times. Residual non-adherent cells were removed by washing the cultures again after 2–3 days. After formation of monolayer, the cells were subcultured at regular intervals using Tripsin-EDTA (Invitrogen).

Effect of temperature and FBS on cell growth : The effect of temperature and FBS concentration on cell growth of HKM was studied. Cells at a concentration of 1×10^5 were inoculated in cell-culture flasks and incubated at 28 ^{0}C for 2 hr to allow attachment. Then set of flasks were incubated at different temperatures of 25, 28, 30 and 37° C for growth tests. Every day, duplicate flasks at each temperature were washed with PBS twice and trypsinized with 0.2 ml of 0.25% trypsin -EDTA solution. When the cell rounded up, the cell density was measured by a haemocytometer and the numbers were expressed as cells per mm^2. The experiment was carried out for 5 days in triplicate. The growth response to different concentrations of FBS (5, 10, 15, and 20%) on cell growth was assessed in triplicate 25 cm^2 flasks using the same procedure as mentioned above at 28° C.

Phagocytosis : The phagocytic activity of the cultured head kidney macrophages was tested with baker's yeast (*Saccharomyces cerevisiae*), by the following procedure. The yeast cell suspension was prepared following the protocol of Roy and Rai (2004). Briefly, the yeast cells were heat-killed at 80°C for 15 min and washed thrice with PBS. The pellet was finally suspended in culture medium supplemented with 5% FBS to get a concentration of approximately 10^8 cells ml^{-1}. HKM (1×10^5 cells) were seeded in sterile coverslips kept in a petri plate and allowed to grow for 24 hr in L-15 medium. Next day, 100 µl of yeast suspension was then placed over the cover slips. The plate was then incubated for 90 min in a humid chamber at 28 ºC. After incubation period, the coverslips were washed twice with PBS and fixed with 100% methanol. Cells were stained with Giemsa and observed under phase contrast microscope. As a control, cells from *Epithelioma papulosum cyprini* (EPC) cell line were also seeded on coverslips separately and similar procedure was followed as described above.

Production of reactive oxygen species : ROS production by HKM was examined by reduction of nitroblue tetrazolium (NBT) as described by Wang *et al*. (1995) with slight modifications. Briefly, HKM were plated into each well of 96 well tissue culture plates (Nunc) at a concentration of 1×10^5 cells in 100µl of complete L-15 medium. Macrophages were stimulated with different amounts of LPS (0 to 40 µg ml^{-1}) for 12 hr. In another experiment, macrophages were primed with suitable amount of LPS (20 µg ml^{-1}) for 12 hr and respiratory burst activity was pulsed with different concentrations of PMA (0 to 100 ng ml^{-1}).

Nitrite production : Production of Nitrite by HKM was detected following the method described by Wang *et al*. (1995). Briefly, cells were plated into 96-well microtiter plates (1×10^5 per well) with complete medium having different concentrations of LPS (0 to 40 µg ml^{-1}) for 96 hr. In another experiment, macrophages were primed with different concentrations of PMA (0 to 100 ng ml^{-1}) and appropriate concentration of LPS (20 µg ml^{-1}). Cell-free culture supernatants were collected and assayed for the presence of nitrite using a kit (Amresco, Solon, USA; Cat. No. N165 kit). Nitrite concentration was determined by comparing with a standard curve prepared from known concentration of sodium nitrite.

Lysozyme assay : The lysozyme activity of HKM was evaluated using turbitimetric assay (Sankaran *et al*., 1972). Briefly, cells were seeded into 96-well microtiter plates (1×10^5 per well) with complete medium having different concentrations of LPS (0 to 40 µg ml^{-1}) for 24 hr. In another experiment, macrophages were primed with different concentrations of PMA (0 to 100 ng ml^{-1}) and appropriate concentration of LPS (20 µg ml^{-1}). After 24 hr, culture supernatants of HKM cells were collected for determining lysozyme activity. The drop in turbidity (OD_{450}) was measured after 5 min of incubation. Quantification of lysozyme activity was done as per standard definition of one unit of lysozyme. One unit of lysozyme activity corresponds to linear decrease in OD_{450} of 0.001 per minute.

Results and Discussion

Labeo rohita is an important culture fish of India and enhanced understanding of its immune system would lead to

effective management of diseases in aquaculture. Previously, monoclonal antibodies of rohu were developed for their use in monitoring of humoral immune response (Rathore *et al.,* 2008). The present work focused on development of an *in-vitro* model for assessing the innate immune response of rohu against new drugs being used in aquaculture. In fish, HKM play a significant role in innate immune system, they phagocytose and destroy the invading pathogens through ROS generation (Nayak *et al*., 2007) and release of several pro-inflammatory cytokines essential for the initiation of host immune responses (Pressley *et al*., 2005). HKM of *L. rohita* were isolated by density gradient centrifugation of cells harvested from head kidney so as to exclude other cells. For this, opaque ring containing mononuclear cells was obtained from the interface and seeded in 25 cm^2 flasks containing L-15 medium and incubated at 28 °C overnight. A well-known characteristic of macrophages and granulocytes is their capacity to adhere to glass or plastic. Macrophages started to spread over the plastic surface within two hours of culture, while neutrophilic granulocytes adhered, but remained globular after several hours in culture. The non-adherent cells were removed next day by washing the cells with PBS. Adherent cells showed aggregation and multiplication at several places in the flask (Fig. 1A). A complete monolayer of HKM cells was formed in 2 weeks (Fig. 1B). Thereafter, the cells were trypsinized and split in 1:2 ratio. Subcultured cells showed faster growth than primary culture and formed a monolayer in 6 days. HKM cultures were maintained for 5 months and survived upto 20 passages.

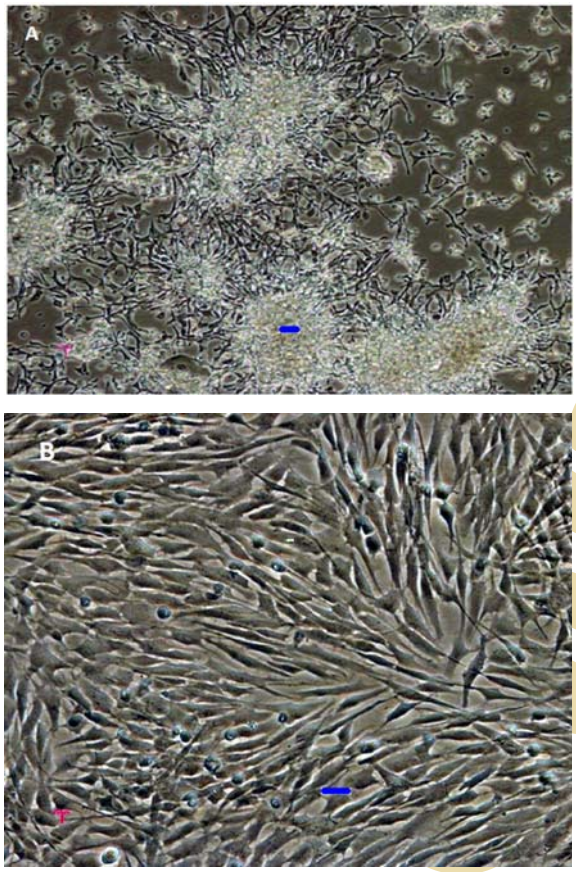

Fig. 1 : Photomicrograph of head kidney macrophages (HKM) cells derived from head kidney leukocytes of *L. rohita*. (A) After 2 day of plating, adherent cells were seen aggregating at some places in the flask; Bar represents 50 µm(B) Monolayer of HKM cells after 14 days; Bar represents 20 µm

HKM cells exhibited different growth rates at different incubation temperature between 25 and 37° C. However, maximum growth was obtained at 28° C (Fig. 2A). No significant growth was observed at 37° C in the cells. The growth rate of macrophages increased as FBS proportion increased from 10 to 20% at 28° C. Cells did not grow well at 5% concentrations of FBS, relatively good growth at 10% but maximum growth occurred at 15% and 20% FBS (Fig. 2B). Phagocytosis is one of the main mechanisms involved in the host protective responses leading to the clearance of pathogens (Alvarez, 2008). The phagocytic ability of the cultured cells was assessed by incubation with yeast. Macrophage cells actively engulfed yeasts (Fig.3) whereas EPC cell line did not show any phagocytic

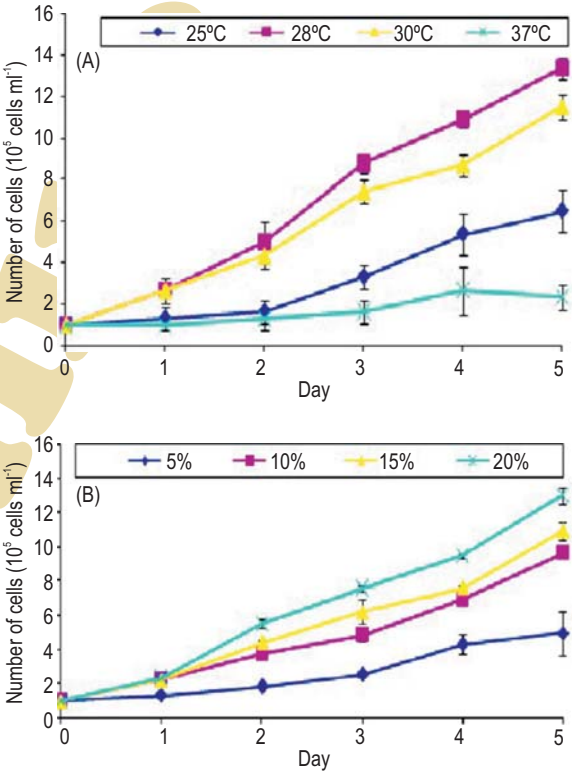

Fig. 2 : Growth responses of head kidney macrophages at different temperatures (A) and at different concentration of foetal bovine serum at 28°C (B)

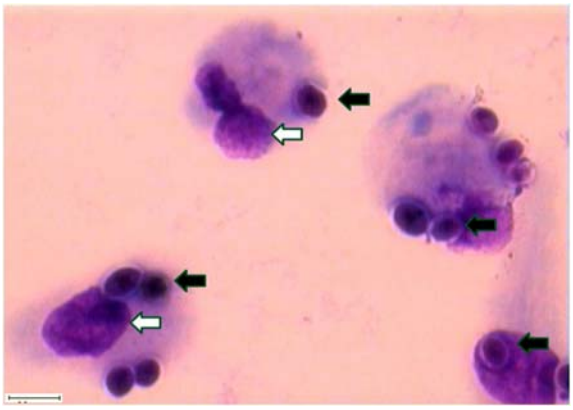

Fig. 3: Demonstration of phagocytic activity of Head Kidney Macrophages (HKM) of *Labeo rohita*. Black arrow shows yeast cells phagocytosed by HKM cells. White arrow shows HKM nucleus. Numerous yeast cells were observed attached to or inside the macrophages. Bar represents 20 µm

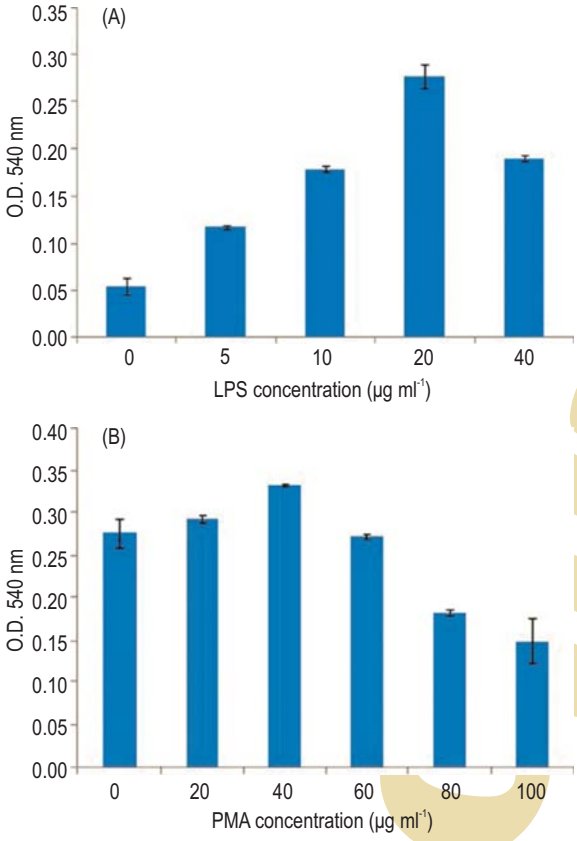

Fig. 4 : Demonstration of reactive oxygen species (ROS) production by HKM cells as detected by NBT reduction assay. Each bar represents mean O.D.±SE of three wells; (A) Effect of different concentrations of lipopolysaccharide (LPS) on ROS production;(B) Effect of different concentrations of phorbol myristate acetate (PMA) with fixed amount of LPS (20 µg ml^{-1}) on ROS production

characteristics. Phagocytosis of yeast cells was earlier reported in catla thymus macrophages (Chaudhary *et al*., 2012).

HKM cells showed ROS production in response to LPS and PMA stimulation. ROS production induced by PMA or LPS only was lower than that triggered by a combination of PMA and LPS. This indicate a combined effect by PMA and LPS for induction of the respiratory burst in fish macrophages. Maximum production of ROS was observed after 12 hr with 20 µg ml^{-1} LPS (Fig.4A). In other experiment, the cells were triggered with varying concentrations of PMA (0 to100 ng ml^{-1}) with appropriate concentration of LPS (20 µg ml^{-1}) and the maximum ROS production was observed at 40 ng ml^{-1} PMA (Fig. 4B). The ROS production of phagocytes was demonstrated in many fish species, including common carp (Marionnet *et al.,* 2006), goldfish (Grayfer *et al*., 2011) and Atlantic salmon (Paredes *et al*., 2013). Nitric oxide production has been shown to have effective antimicrobial mechanism against a number of relevant fish pathogens (Campos-Perez *et al.*, 2000; Hanington *et al.*, 2007; Forlenza *et al.,* 2009). Nitrite production by HKM cells were

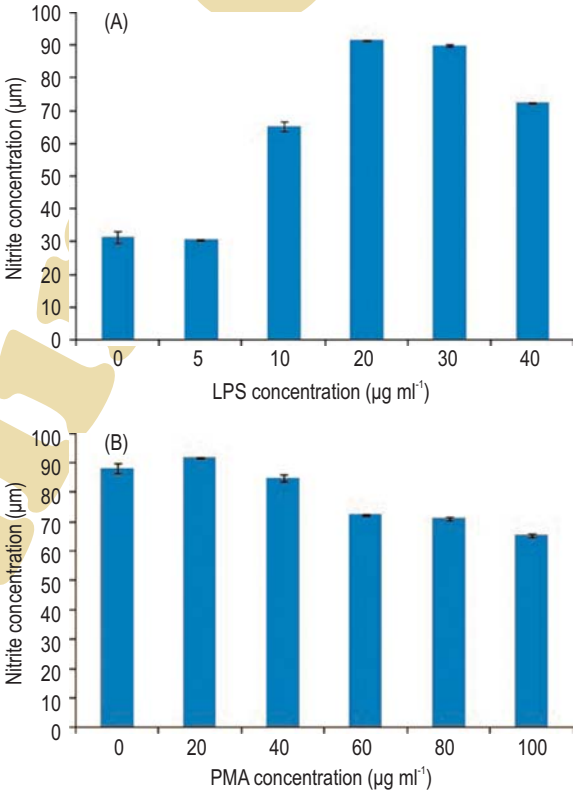

Fig. 5 : (A) Demonstration of nitrite production by HKM cells. Each bar represents mean nitrite concentration of three wells ± SE; (A) Effect of different concentrations of lipopolysaccharide (LPS) on nitrite production; (B) Effect of different concentrations of phorbol myristate acetate (PMA) with fixed amount of LPS (20 µg ml^{-1}) on nitrite production

examined on stimulation with different concentration of LPS and PMA. The cells showed a maximum nitric oxide production in response to 20 µg ml^{-1} LPS (Fig. 5A).In another experiment, the cells were primed with varying concentration of PMA (0 to 100 ng ml^{-1}) with appropriate concentration of LPS (20 µg ml^{-1}). No significant change in nitrite production was observed with addition of PMA (Fig.5B). Culture supernatant from HKM cells collected after 24 hrs of culture showed lysozyme activity. Maximum lysozyme activity was observed in culture supernatant collected from HKM cells primed with 20 µg ml^{-1} LPS (Fig.6A). PMA (20 ng ml^{-1}) along with 20 µg ml^{-1} LPS showed maximum lysozyme activity after 24 hrs of culture (Fig.6B). However, no lysozyme activity was detected in L-15 medium. Lysozyme activity has been previously shown by fish macrophages (Paulsen *et al*., 2001 Chaudhary *et al*., 2012).

The results of our study showed that HKM cells can be used as an *in-vitro* model to assess the role of immunomodulators in evaluating the immunological defense mechanisms and their effects on immune status of rohu. In conclusion, HKM cells were highly phagocytic as evaluated by ability to phagocytose yeasts cells. Cells showed significant activation when stimulated with bacterial LPS and revealed functional properties of macrophages such as ROS production, nitrite production and lysozyme activity.

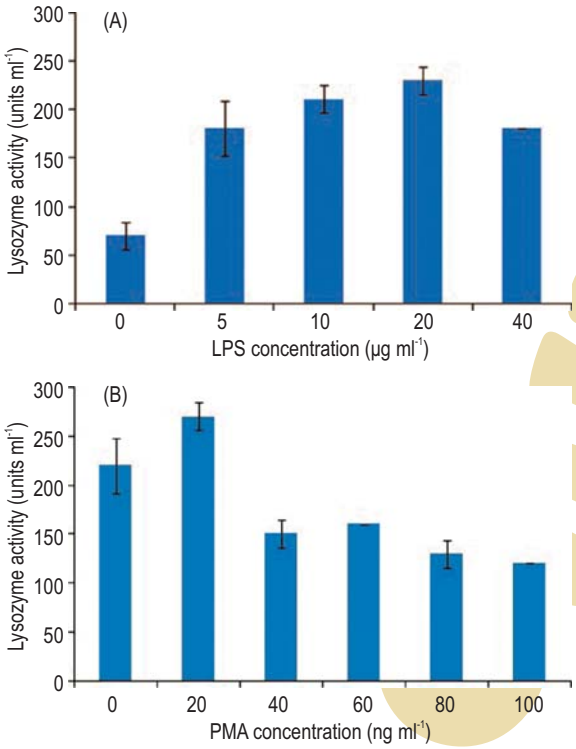

Fig. 6 : Demonstration of lysozyme activity in HKM cells. Each bar represents mean lysozyme activity ±SE of three wells. (A) Effect of different concentrations of lipopolysaccharide (LPS); (B) Effect of different concentrations of phorbol myristate acetate (PMA) with fixed amount of LPS (20 µg ml^{-1}) on lysozyme activity

Acknowledgments

The authors are thankful to Dr. J. K. Jena, Director, NBFGR, Lucknow and Dr. P. Punia, HOD, FHM, Division, NBFGR, Lucknow for their guidance and constant encouragement.

References

Alvarez-Pellitero, P.: Fish immunity and parasite infections: From innate immunity to immunoprophylactic prospects. *Vet. Immunol. Immunopathol.,* **126**, 171-98 (2008).

Banerjee, C., R. Goswami, G. Verma, M. Datta and S. Mazumder: Aeromonas hydrophila induced head kidney macrophage apoptosis in *Clarias batrachus* involves the activation of calpain and is caspase-3 mediated. *Dev. Comp. Immunol.,* **37**, 323–333. (2012).

Bennani, N., A. Schimid-Alliana and M. Lafaurie: Evaluation of phagocytic activity in a teleost fish, *Dicentrarchus labrax*. *Fish Shellfish Immunol*., **5**, 237-246 (1995).

Brubacher, J.L., C.J. Secombes, J. Zou and N.C. Bols: Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (*Oncorhynchus mykiss*). *Dev. Comp. Immunol*., **24**: 565–574 (2000).

Campos-Perez, J.J., A.E. Ellis and C.J. Secombes: Toxicity of nitric oxide and peroxynitrite to bacterial pathogens of fish. *Dis. Aquat. Organ*., **43**, 109–115 (2000).

Chaudhary, D.K., N. Sood, P.K. Pradhan, A. Singh, P. Punia, N.K. Agarwal and G. Rathore: Establishment of a macrophage cell line from adherent peripheral blood mononuclear cells of *Catla catla*. *In Vitro Cell Dev. Biol. Anim*., **48,** 340-8 (2012).

Datta, S., D. Ghosh, R. Saha, S. Bhattacharya and S. Mazumder: Chronic exposure to low concentration of arsenic is immunotoxic to fish: Role of head kidney macrophages as biomarkers of arsenic toxicity of *Clarias batrachus*. *Aquat. Toxicol*., **92**, 86–94 (2009).

Fishelson, L.: Cytomorphological alterations of the thymus, spleen, head kidney and liver in cardinal fish (*Apogonidae*, Teleostei) as bioindicators of stress. *J. Morphol*., **267**, 57–69 (2005).

Forlenza, M., M. Nakao, I. Wibowo, M. Joerink, J.A. Arts, H.F. Savelkoul and G.F. Wiegertjes: Nitric oxide hinders antibody clearance from the surface of *Trypanoplasma borreli* and increases susceptibility to complement-mediated lysis. *Mol. Immunol*., **46**, 3188–3197 (2009).

Furne, M., E. Holen, P. Araujo, K. K. Lie and M. Moren: Cytokine gene expression and prostaglandin production in head kidney leukocytes isolated from Atlantic cod (*Gadus morhua*) added different levels of arachidonic acid and eicosapentaenoic acid. *Fish Shellfish Immunol.,* **34**, 770–777 (2013).

Grayfer, L., J.W. Hodgkinson, S.J. Hitchen and M. Belosevic: Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin-10. *Mol. Immunol*., **48**, 563-571 (2011).

Hanington, P.C. and M. Belosevic: Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. *Dev. Comp. Immunol.,* **31**, 817–829. (2007).

Husain, M., S. Bird, R. van Zwieten, C.J. Secombes and T. Wang: Cloning of the IL-1β3 gene and IL-1β4 pseudogene in salmonids uncovers a second type of IL-1β gene in teleost fish. *Dev. Com. Immunol*., **38**, 431-46 (2012).

Joerink, M., C.M. Ribeiro, T. Stet, R.J., Hermsen, H.F. Savelkoul and G.F. Wiegertjes: Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *J. Immunol*., **177**, 61-9 (2006).

Lu, X.J., X.Y. Hang, L. Yin, Y.Q. He, J. Chen, Y.H. Shi and C.H. Li: Sequencing of the first ayu (*Plecoglossus altivelis*) macrophage transcriptome and microarray development for investigation the effect of LECT2 on macrophages. *Fish Shellfish Immunol*., **34**,497-504. (2013)**.**

Marionnet, D., P. Deschaux and S Reynaud: Possible implication of macrophages in the regulation of cytochrome P450 activities in carp *(Cyprinus carpio). Fish Shellfish Immunol*., **21**, 80-91 (2006).

Nayak, A.S., C.R. Lage and C.R. Kim: Effects of low concentrations of arsenic on the innate immune system of the zebrafish (*Danio rerio*). *Toxicol. Sci*., **98**, 118–124 (2007).

Neumann, N.F., D. Barreda and M. Belosevic: Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. *Dev. Comp. Immunol*., **4**: 417–432. (1998).

Neumann, N.F., J.L. Stafford, D. Barreda, A.J. Ainsworth and M. Belosevic: Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Dev. Comp. Immunol*., **25**, 807–825 (2001).

Norum, M., J. Bogwald and R.A Dalmo: Isolation and characterisation of spotted wolffish (*Anarhichas minor* Olafsen) macrophages. *Fish Shellfish Immunol*., **18**, 381-391 (2005).

Paredes, M., K. Gonzalez, J. and E. Montiel-Eulefi: Immunomodulatory effect of prolactin on Atlantic salmon (*Salmo salar*) macrophage function. *Fish Physiol. Biochem*., **39**, 1215-21 (2013).

Paulsen, S.M., R.E. Engstad and B. Robertsen: Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast beta-glucan and bacterial lipopolysaccharide. *Fish Shellfish Immunol*., **11**, 23-37 (2001).

Pellizzari, C., A. Krasnov, S. Afanasyev, N. Vitulo, R. Franch, S. Pegolo, T. Patarnello and L. Bargelloni: High mortality of juvenile gilthead sea bream (*Sparus aurata*) from photobacteriosis is associated with alternative macrophage activation and anti-inflammatory response: Results of gene expression profiling of early responses in the head kidney. *Fish Shellfish Immunol*., **34**, 1269-1278 (2013).

Pressley, M.E., P.E. Phelan, P.E. Witten, M.T. Mellon and C.H. Kim: Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish. *Dev. Comp. Immunol.,* **29**, 501–513 (2005).

Rathore. G., G. Kumar, N. Sood, D Kapoor, and W.S. Lakra: Development of monoclonal antibodies to rohu [*Labeo rohita*] immunoglobulins for use in immunoassays. *Fish Shellfish Immunol*., **25**, 761-774 (2008).

Rieger, A.M. and D.R. Barreda: Antimicrobial mechanisms of fish leukocytes. *Dev. Comp. Immunol.,* **35**,1238-1245 (2011).

Rolland, JB., D. Bouchard, J. Coll, and J. R Winton: Combined use of the ASK and SHK-1 cell lines toenhance the detection of infectious salmon anemia virus. *J. Vet. Diagn. Invest.*, **17**, 151–157. (2005)

Romeo, M., N. Bennani, M. Gnassia-Barelli, M. Lafaurie and J.P. Girad: Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. *Aquat. Toxicol*., **48**, 185–194 (2000).

Roy, B. and U. Rai: Dual mode of catecholamine action on splenic macrophage phagocytosis in wall lizard, *Hemidactylus flaviviridis*. *Gen. Comp. Endocrino.,* **136**, 180–191(2004).

Sankaran, K. and S. Gurnani: On the variation in the catalytic activity of lysozyme in fishes. *Indian J. Biochem. Biophy.,* **9**, 162–165 (1972).

Sorensen, K.K., B. Sveinbjornsson, R.A. Dalmo, B. Smedsrod and K. Bertheussen: Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *J. Fish Dis.,* **20**, 93-107 (1997).

Stafford, J.L., P.E. McLauchlan, C.J. Secombes, A.E. Ellis and M. Belosevic: Generation of primary monocyte-like cultures from rainbow trout head kidney macrophages. *Dev. Comp. Immunol*., **25**, 447–59 (2001).

Wang, R., N.F Neumann, Q. Shen and M. Belosevic: Establishment and characterization of a macrophage cell line from the goldfish. *Fish Shellfish Immunol.,* **5**, 329–345 (1995).