Optimization of macrophage isolation from the Persian sturgeon and the Caspian kutum fish: a comparative study

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Abstract The aim of this research was a comparative study on the isolation and culture of head kidney macrophages derived from *Acipenser persicus* and *Rutilus frisii kutum* as teleost and chondrostei species of fish. The macrophages were isolated by density gradient sedimentation, followed by adherence to a plastic surface. They exhibited strong phagocytic activity against bacteria. The effect of cell density, incubation time, FBS percentage, pH and temperatures on the cell number and viability were determined and compared. Also, the effect of light/dark regimen on viability, adherence, release of reactive oxygen species (ROS) and nitric oxide (NO) in the macrophages was determined. The results showed that the

Caspian kutum macrophages were more sensitive to FBS percentage and cell density whereas the Persian sturgeon macrophages were more sensitive to pH of the cell culture media. The adherence and viability of the macrophages from both fish species firstly increased ($P < 0.05$) after exposure to a light/dark regimen, but then significantly decreased as did ROS and NO productions. For the first time, this study has determined the optimal conditions for primary culture of macrophages derived from sturgeons, and shows the unique effect of light on the biology of fish immune cells.

Keywords Fish macrophages · Light exposure · Primary culture

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Introduction

Fish, as a diverse group of vertebrates, can be used extensively as a biological model system. Fish cells are easy to obtain and consequently its culture can be used for studies of fish health or to better understand similar conditions for mammalian cells in biomedical research (Cossins and Crawford 2005). There are several advantages of using fish primary cell cultures over cell lines and in vivo experiments. Unlike cell line, the primary cells are heterogeneous and still represent parent cell types and specific properties of the tissue (Badhe and Nandanpawar 1991). The

primary cells can be reorganized into a monolayer that is easier to study compared to the complex tissue structures (Qiu et al. 2016) and may represent a good model for tissues in vivo (Freshney 1994). Moreover, primary culture of fish cells is relatively easy and has a low cost compared to using living fish that require large aquaculture equipment, consumption of a large amount of water and killing of fish. This is especially important for large or endangered fish species. Compared to mammalian and avian cell culture, primary culture of fish cells is quite flexible and the cells can grow within a broad range of incubation temperatures (Lannan 1994). Furthermore, fish cell culture does not require short term changes (feeding) in growth medium (Nicholson 1989).

Macrophages are major phagocytic cells in fish (Garduno and Kay 1994) and play a crucial role in fish immunity via antimicrobial activity (Norum et al. 2005), production of cytokines, regulation of immune effector cells (Gordon et al. 1992) and keeping homeostasis (Gordon et al. 1992; Murray and Wynn 2011). Thus, primary culture of fish macrophages is one of the most useful models for in vitro immunity research (Fierro-Castro et al. 2013; Qiu et al. 2016). The culture methods for macrophages from fish head kidney have been established since the early 1990s (Braun-Nesje et al. 1982; Sharp and Secombes 1993) and used for many aspects of biological studies. However, most of these studies have varied culture conditions to consider all factors that may affect primary culture of fish macrophages from particular fish species. Therefore, the use of these routine cell culture methods along with consideration of factors affecting macrophage culture, and comparison of these factors between different types of bony fish will help clarify many unknown aspects of fish cell culture.

Sturgeon fish survived over 200 million years ago (Bemis et al. 1997). They are harvested for food, and the eggs of sturgeon (caviar) increase their importance considerably worldwide. The most important species of sturgeon is *Acipenser persicus* living in the Caspian Sea, Iran (Bahmani et al. 2001). Since the sturgeons have a long life, they presumably must survive against many microbial attacks (e.g., viruses and parasites) over their lifespan and have immune system extensively depending on leucocytes (Fänge 1986). Establishment of sturgeon macrophage cultures in comparison to bony fish macrophage cultures will allow studies of these cells in their immune responses,

and provides an in vitro model for applied and evolutionary studies.

In this study, the method of isolation and culture of macrophage from either the head kidney of Persian sturgeon as a chondrosteian fish model or Caspian kutum as teleost fish model was optimized, and compared between the two fish. The both fish are endemic and economically valuable species in the Caspian Sea. The effects of different factors, including pH, temperatures, cell density, percentage of FBS, light exposure and adhesion time, on the yield and survival of primary macrophage culture from the two fish species were investigated. The optimum culture condition for each fish species was also determined in terms of the viability assay, morphology and phagocytic characteristics of the cells.

Materials and method

Animals

The Persian sturgeon (*Acipenser persicus*) and the Caspian Kutum (*Rutilus frisii kutum*) weighing 40–50 g and 30–40 g, respectively, were obtained from Eslami farm and Shahid Rajai Fish Proliferation and Culture Center (Sari, Mazandaran Province, Iran). Before the experiment, the fish were acclimated to dechlorinated tap water at room temperature under a natural light cycle for at least 2 weeks prior to use. The fish were fed with commercial fish food twice a day.

Cell suspension preparation

The fish were anesthetized by exposure to 0.1% 2-phenoxyethanol (Sigma Aldrich, Gillingham, UK) and hilled. The fish body was then disinfected with 70% ethanol. Blood was first collected from the caudal vein (for removing extra blood) using sterile syringes. Then, the head kidney was removed aseptically and transferred into tubes containing cold L-15 medium (Sigma-Aldrich) containing 10 U/mL heparin (Sigma Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL gentamicin (Gibco, Paisley, UK). The tissue was initially cut into small pieces using sterile scissor, then passed through a 100 µm sterile nylon mesh. The cell suspension from *C. kutum* and *P. sturgeon* was gently layered on 51% and 54% v/v Percoll (Sigma-Aldrich.UK) (percoll density

gradient was previously prepared, and the most number of macrophages, confirmed by non-specific esterase and Giemsa staining, were obtained from these two densities), then centrifuged at 400g for 25 min, according to the method described by Secombes (1990). Cells at the medium-Percoll[®] interface were transferred into clean tubes and washed twice by centrifugation at 200g for 10 min at 4 °C. Cells were resuspended in incomplete L-15 medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin (GibcoTM) and 1% fetal bovine serum (FBS). After incubation overnight, non-adherent cells were removed by washing the cultures with phosphate buffered saline (PBS) three times. The cells were then cultured in L-15 complete medium containing different concentrations of FBS.

Cell viability

The cell viability was measured by hemocytometer counting after trypan blue exclusion test at different time points (0, 24, 48, and 96 h) and under different culture conditions (as outlined below).

Cell morphology and adherence

An inverted light microscope (Nikon Eclipse TS100, Tokyo, Japan) was used to observe morphology and adherence of the cells, and then imaged.

Phagocytosis assays

For phagocytosis assay, *E. coli* bacteria (CGMCC 1.2389) obtained from Microbiology Lab in Babol Medical University, were cultured for 24 h and the bacterial suspension was prepared according to Seeley et al. (1990) with some modifications. Briefly, bacteria were fixed in 10% formalin, washed 3 times with PBS, and resuspended (10^5 cell/mL) in PBS. Macrophages were exposed to the bacteria for 1 h and the cells were stained with Giemsa (Talaye Teb Azma, Tehran, Iran) and observed under phase contrast microscope (Nikon Eclipse TS100).

Culture conditions

Optimization of cell density

The number of cells, derived from each fish, were counted by haemocytometer, and densities were adjusted to 2.5×10^5 , 5×10^5 , 1×10^6 and 1.5×10^6 cells/mL, and plated in flat-bottomed 96-well tissue culture plates. After overnight incubation at 23 °C, monolayers of adherent cells in the micro plates were washed twice to remove unattached cells, and the number of adherent cells were counted according to the method described by Qiu et al. (2016). Briefly after the different incubation time, the unattached cells were washed off, and the number of adherent cells were counted per well by using the following formula:

The number of adherent cells per well = (the number of adherent cells in one figure) $\times$ (the bottom area of one well/one figure area) (Qiu et al. 2016). At least 50 figures were included for each group. Three replicates were used for each group.

Optimization of adhesion time

Cells (1×10^6) were suspended in L-15 medium supplemented with 1% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin (Gibco) and 10 U/mL heparin (Sigma-Aldrich). Then, the cells were incubated in 96-well tissue culture plates at 23 °C for 0, 2, 6, 12, 48, and 96 h. After the different incubation time, the unattached cells were washed off, and the number of adherent cells were counted per well using Qiu et al. (2016) method. Three replicates were used for each group.

Optimization of FBS concentration

After overnight incubation of the cells (1×10^6) derived from the Caspian kutum and the Persian sturgeon, the macrophages were washed with L-15 medium and cultured in complete L-15 medium containing 5, 10, 15 and 20% FBS. The cells from each FBS treatment group were detached using trypsin/EDTA (0.05% trypsin, 0.02% EDTA) (Sigma Aldrich) every day for 4 days and the cell number was measured with a hemocytometer. Three replicates were used for each group.

Optimization of culture temperature

Macrophages (1×10^6) from each fish were cultured in 24-well plates containing 1 mL of L-15 complete medium at 18, 20, 23, 28 and 31 °C. Cell number and viability were evaluated at 1, 3 and 5 days after seeding. Three replicates were used for each group.

Optimization of pH

The cells with density of 1×10^6 cells/mL were seeded in a 24-well plate. After overnight culture, unattached cells were removed. The macrophages from each fish were then cultured in L-15 complete medium at 23 °C with pH values of 7.1, 7.2, 7.3 and 7.4. The cell number and viability were measured at 1, 3, and 5 days after seeding. Three replicates were used for each group.

Effect of light exposure

Macrophages were seeded (1×10^6 cells/mL) into 24-well plates with 1 mL L-15 complete medium and incubated at 23 °C for 1, 3, and 4 days. One group was incubated in an incubator supplied with a natural light cycle (12 h light/12 h dark) and a second group was incubated in the dark. At the end of the experiment, cell morphology, cell number and viability were evaluated and ROS production in the medium was measured using the nitroblue tetrazolium (NBT, Sigma-Aldrich) assay. Briefly, 1 mg/mL NBT and 1 µM LPS (Sigma-Aldrich) were added to a 96-well of culture plate. After 3 h incubation, the plate was washed once with 70% methanol to remove extracellular formazan, then, 2 M KOH (120 µL) and DMSO (140 µL) were added. The plates were read at 630 nm in an ELISA reader (Sarmiento et al. 2004). The macrophages were stimulated with LPS (1 µM), and nitric oxide production was calculated (Green et al. 1982) after 72 h of stimulation. Three replicates were used for each group.

Statistical analysis

Data were expressed as the mean $\pm$ SD and analyzed using Student's *t* test and one-way ANOVA, followed by the Student–Newman Keuls test to determine differences between groups ($P < 0.05$). SigmaPlot software (ver.11.0) was used for the analysis.

Fig. 1 The morphology of head kidney macrophages from the Caspian Kutum (a) and the Persian sturgeon (b) after 24 h of culture. Formation of giant cells in the Caspian kutum (c) and the Persian sturgeon (d) after 5 days of culture. *E. coli* was phagocytized by macrophages from Caspian kutum (e), and Persian sturgeon (f). Aggregations around debris (mass of dead cell) by macrophages from Caspian Kutum (g) and Persian sturgeon (h) in the culture medium. Giemsa staining

Results

Behavior and morphology of macrophages

In both fish, cultured cells adhered to the polystyrene cell culture plate after 1–2 h and were spread after 4–24 h. They had a bean-shaped nucleus, surrounded by a relatively large amount of cytoplasm (Fig. 1a, b), and were 7–15 µm in diameter. Some contaminating non-adherent small cells; presumably lymphocytes were observed in the macrophage culture but vanished after washing the cultured macrophages. Also cells with strongly basophilic cytoplasm and lobulated nuclei were sometimes observable. After 24 h, more than 80% of the cultured cells were macrophages that had strong phagocytic activity (Fig. 1e, f) for fixed *E. coli*. Macrophages from the head kidney of the Persian sturgeon were smaller than that of the Caspian kutum (Fig. 1b). These cells were kept in the culture for 2 weeks. However, decrease in cell numbers or detached cells and refractile inclusions in the macrophage cytoplasm usually appeared after 7 days. After 4 days, some adherent cells from the Persian sturgeon started to fuse (Fig. 1c). These multinucleated cells (giant cells) maintained their phagocytic potential. In the Caspian kutum, multinucleated cells (giant cells) were formed after 48 h but were not able to phagocytose (Fig. 1d).

Cell density and adhesion time

In both fish species, the number of viable/adherent cells were counted after 24-h incubation at different cell densities (Fig. 2). The results showed that increasing the number of cultured cells increased the number of adherent cells ($P < 0.05$). Furthermore, in macrophage culture of the Persian sturgeon, the highest number of adherent cells was observed after 2 h-incubation while for the Caspian kutum it was obtained after 12 h-incubation (Fig. 3).

Fig. 2 Primary culture of macrophages from the Persian sturgeon and the Caspian Kutum. Optimum cell density for achieving maximal cell adhesion rate and cell survival. Uppercase (*Rutilus frisii kutum*) and lowercase (*Acipenser persicus*) letters indicate significant difference (mean $\pm$ SD)

Fig. 3 The effect of incubation time prior to washing on viable cell in primary macrophages derived from the Persian sturgeon and the Caspian Kutum. Uppercase (*Rutilus frisii kutum*) and lowercase (*Acipenser persicus*) letters indicate significant difference in number of viable cells

Fig. 4 The number of viable primary macrophages derived from the Persian sturgeon and the Caspian Kutum, cultured with different concentrations of FBS at 23 °C for 1–4 days.

Fig. 5 The number of viable adherent primary macrophages derived from the Persian sturgeon and the Caspian Kutum cultured at different temperatures (mean $\pm$ SD) for 24-h. Lowercase (*Rutilus frisii kutum*) and uppercase (*Acipenser persicus*) letters indicate significant difference

Effect of FBS concentration, temperature and pH on the cell survival

Results in both fish species showed that the macrophage number was improved with increasing FBS concentration. The optimum value for the Persian sturgeon was 15–20% FBS (Fig. 4) while for the Caspian kutum, 10–15% FBS typically gave the best survival.

Macrophages derived from the Persian sturgeon and the Caspian kutum exhibited no significant difference in survival ($P > 0.05$) at different incubation temperatures ranging from 23 to 28 °C. The cell viability for both fish significantly decreased at temperatures higher than 31 °C and at temperature lower than 21 °C for the Caspian kutum (Fig. 5).

Uppercase letters indicate significant difference between groups and lowercase letters indicate significant difference between days (mean $\pm$ SD)

Fig. 6 The number of viable adherent primary macrophages derived from the Persian sturgeon and the Caspian Kutum, cultured at different pH values for 1–4 days. Uppercase letters

indicate significant difference between groups and lowercase letters indicate significant difference between days (mean $\pm$ SD)

Fig. 7 The effect of natural light cycle on viability and cell adherence of primary macrophages derived from the Persian sturgeon and the Caspian Kutum. Lowercase letters indicate

significant difference between days in light/dark regime and uppercase letters indicate significant difference in dark regime (mean $\pm$ SD)

Macrophages derived from both fish species were highly sensitive to culture pH. The maximum survival was observed at pH 7.2 (Fig. 6) but higher pH was detrimental and few cells were alive at pH 7.4 after 2 days or at pH 7.3 after 3 days for Persian sturgeon. The result showed that Caspian kutum macrophage could tolerate pH 7.3 better than Persian sturgeon macrophage (Fig. 6).

Light exposure

While exposure to natural light cycle (12 h light/12 h dark) initially increased the adhesion potential and survival of macrophages ($P < 0.05$) (Figs. 7, 8), the number of viable adherent cells decreased dramatically after 2 days ($P < 0.05$), such that by day 3, few cells were alive and mostly were spread and elongated. These cells were cross-connected by cytoplasmic

extensions forming bridges and huge giant cells (Fig. 8). After 4 days, most of these cells were dead and detached. The only difference between two species was that the Caspian kutum cells were still highly viable 2 days after exposure to the natural light cycle (12 h light/12 h dark) (Fig. 9).

ROS and Nitric oxide production after natural light cycle

Figure 10 shows stimulatory effect of 1 μ M of LPS on the Persian sturgeon and the Caspian kutum macrophages in vitro after exposure to the natural light cycle. In both cases, ROS and NO production was the highest on day 2 and the lowest on day 4.

Fig. 8 Photomicrograph of primary macrophages derived from the Persian sturgeon exposed to a natural light cycle for different times. Day 1: Most of the cells were oval shaped, spread and with strong ability to phagocytose. Day 2: the shape was the

same as on day 1 but the numbers had decreased. Day 3: cell number and ability to phagocytose significantly decreased and macrophages made formed giant cells. Day 4: large numbers of detached cells and dead cells are seen

Discussion

In the present study, the best way to isolate and cultivate head kidney macrophages derived from two types of bony fish, chondrostei and teleost, which are endemic to the Caspian Sea, were compared. Primary culture of macrophages derived from these two groups is important to allow study of comparative evolutionary biology and functional immune responses of these particular fish species.

Initial cell seeding is an important factor for macrophage primary culture (Qiu et al. 2016) because inoculum volume size and cell density directly affect cell survival (Rodriguez et al. 2001). This study showed that in both fish increasing cell density resulted in enhanced macrophage adherence. In the Persian sturgeon, cell density did not affect viability

significantly (data not shown). In the Caspian kutum, maximum cell viability and adherence were achieved using 1×10^6 cells/well at seeding. Increasing the initial cell seeding number further to 1.5×10^6 cells/well significantly decreased viability and adherence in the Caspian kutum macrophage culture. This may be due to rapid consumption of essential nutrients (FBS) and accumulation of harmful toxic products in the medium with increasing cell seeding number (Rodriguez et al. 2001). The more important role of FBS in the cell viability and adherence in the Caspian kutum macrophage compared to Persian sturgeon macrophage was also shown in Fig. 4, and can be considered as a result for confirmation of this hypothesis. Teleost fish macrophages might need a more complex growth condition and nutrients compared to chondrostei fish. Therefore, teleost macrophages have higher

Fig. 9 Photomicrograph of the Caspian kutum macrophages exposed to a light/dark regime for different times. Day 1: cells seem healthy and adherent with high phagocytic potential. Day 2: macrophages formed giant cells, spread with high potential of

phagocytosis and the cell number decreased. Day 3: cell number significantly decreased and the macrophages formed pseudopodia. Day 4: most of the cells were detached and had many pseudopodia

Fig. 10 Reactive oxygen species (ROS) (a) and nitrite production (b) by cultured macrophages derived from the Persian sturgeon and the Caspian Kutum after stimulation with bacterial LPS. The cells were exposed to a natural light cycle for different

times. Uppercase (*Rutilus frisii kutum*) and lowercase (*Acipenser persicus*) letters indicate significant difference (mean $\pm$ SD)

sensitivity to variation of nutrients in the medium and the initial cell seeding density. According to the results, maximum adherence of macrophages from the

Persian sturgeon was observed 2 h after seeding, and afterwards the cell attachment potential slowly decreased and most of the cells would be practically

dead and detached after 48–72 h. On the other hand, the maximum attachment of the macrophages from the Caspian kutum was observed 24 h after seeding, then the cell viability and attachment sharply declined, and few living cells were in poor condition after 72 h. This event can be explainable by the fact that the macrophages were initially enriched using Percoll. Several previous studies indicated that Percoll contamination might have cytotoxic effects, and proportionally decrease cell viability and adherence during primary incubation (Ribas et al. 2014; Qiu et al. 2016). The present data suggest that macrophages from the Persian sturgeon were more sensitive to Percoll than those from the Caspian kutum. Furthermore, macrophages of the Persian sturgeon may have faster adaptation to in vitro conditions than those of the caspian kutum and consequently, cell attachment time is shorter for the Persian sturgeon macrophages.

The results also showed that the survival of the Persian sturgeon macrophages did not significantly change in the medium containing different amounts of FBS after 4 days. Surprisingly, macrophages from the Caspian kutum were more sensitive to variations in FBS concentration.

In this study, macrophages showed better survival at temperatures lower than 31 °C (Fig. 4). Optimum temperature for macrophages from the Caspian kutum was 21–28 °C whereas for the Persian sturgeon was 18–28 °C. Regarding conformity of fish body temperature to the environment, changes in environmental temperature can change acyl chain conformation and orientation (Hardie et al. 1994) and consequently affect cell membranes by rigid or gelling formation of membrane lipid in variant temperatures (Hazel 1984). Moreover, this change can occur in macrophage membrane and may affect its proliferation, adherence and immune response. Several previous studies showed higher macrophage activity in *Cyprinus carpio* and *Tinca tinca* adapted to low temperature (Collazos et al. 1994; Le Morvan et al. 1997). Hardie et al. (1994) reported that MAF (Macrophage Activation Factor) production in rainbow trout macrophages depends on temperature in vitro and in vivo. Since the increase of temperature higher than optimum range in both fish (31 °C) may change membrane viscosity and function of this cell (Clem et al. 1991), it can lead to decreases in cell attachment and their numbers. The Persian sturgeon usually lives better in colder water (Chebanov and Galich 2011) than the Caspian kutum

(Ahmadian et al. 2015) and therefore their macrophages survived better at lower temperature.

Regarding pH, fish blood pH varies from 7.2 to 7.5 (Steiro et al. 1998) and optimal macrophage culture occurs in this range. The results showed that macrophages from the Persian sturgeon are more sensitive to alteration of pH than those from the Caspian kutum and died immediately after 72 h and 96 h when pH was increased to 7.4 and 7.3, respectively. The change in pH can have conflicting effects on immune system parameters (Uribe et al. 2011), including growth and proliferation of macrophages. Wang et al. (1995) showed that rising pH to 7.4 inhibited growth of a macrophage cell line from gold fish.

Macrophages (from both fish) exposed to light/dark (12 h light + 12 h dark) regime showed better adhesion and higher viability than cells exposed only to dark. However, this phenomenon was not permanent after 24 h (in the Persian sturgeon macrophage) and 48 h (in the Caspian kutum macrophage) natural light cycle exposure. Pervious study showed that the loss of circadian rhythms in mice macrophages can cause greater cell adhesion and migration (Wang et al. 2016) but whether this phenomenon is true for other orders of vertebrates, specially primitive vertebrates, has remained unknown. Just like other peripheral clocks, reaction of immune cells to the light condition resulting from local circadian clock causes that the animal can better adapt to the requirements of individual habitats (Kornmann et al. 2007). The morphology of macrophages changed and tended to form colonies and undergo multinucleated giant cell formation (MGF). Unlike giant cells from the Persian sturgeon, these cells from the Caspian kutum were not able to phagocytose *E.coli*. Probably light induced fusogenic proteins after 24–48 h and consequently MGF formation. According to a previous study, macrophage cells from gilthead seabream died shortly after forming MGF (Couso et al. 2002). The authors suggested that MGF formation induces death via apoptosis as nucleus fragmentation and cellular shrinkage occurs.

The results also showed that NO and ROS levels significantly increased after 24–48 h of light exposure in both fish but were significantly decreased after 72 h of exposure. The possible cellular mechanisms of the visible light effect are very complex. Duan et al. (2001) reported that Laser light increases the respiratory burst of neutrophils in cows (Duan et al. 2001).

Many previous studies also reported that visible-light irradiation causes formation of ROS as a result of the activation of endogenous photosensitizers (Karu 1999; Lubart et al. 1992; Zan-Bar et al. 2005). Such endogenous photosensitizers include mitochondrial cytochromes (Pottier and Truscott 1986), porphyrins (Fraikin et al. 1996), pyridine cofactors, NADPH/NADH (Cunningham et al. 1985), riboflavin and tryptophan (Edwards and Silva 2001) and Fe-S clusters (Kim and Jung 1992). Sometimes ROS fluxes can stimulate vital cell processes (Suzuki et al. 1997), thus, the initial production of ROS by macrophages is able to act as a messenger to activate cell processes such as proliferation and adherence. In this study, light exposure affected cell shape and survival, probably due to ROS production. However, recognizing the early stages of apoptosis or necrosis resulting from ROS production in the cells using viability test is not possible and needs more sensitive methods, and is suggested for future research.

Conclusion

The present study successfully optimized macrophage primary culture for two fish species from two different bony fish orders, and these methods can be used for future study on these cells and fish immune system. The results showed that the Caspian kutum macrophages were more influenced by seeding cell density, adherence, incubation time and culture temperature whereas the macrophages from the Persian sturgeon were more sensitive to pH of the culture medium. Both fish macrophages responded to the natural light cycle by increasing ROS and NO. The finding related to the effect of visible light on fish macrophages is novel and may be useful for studies on how light may affect fish biological systems especially the immune system.

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Compliance with ethical standards

Conflict of interest There are no potential or actual conflicts of interest.

References

- Ahmadian E, Malekzadeh-Viayeh R, Zahmatkesh A (2015) Caspian whitefish, *Rutilus frisii kutum* Kamensky, 1901 a potential aquaculture candidate: study on the cumulative effects of salinity and temperature on culture performance. Iran J Fish Sci 14:623–633
- Badhe MR, Nandanpawar PC (1991) An overview of cell culture in fish. Aquatic Fish Database est. <http://aquafind.com/articles/Cell-Culture-In-Fish.php>
- Bahmani M, Kazemi R, Donskaya P (2001) A comparative study of some hematological features in young reared sturgeons (*Acipenser persicus* and *Huso huso*). Fish Physiol Biochem 24:135–140
- Bemis WE, Findeis EK, Grande L (1997) An overview of Acipenseriformes. Environ Biol Fish 48:25–71
- Braun-Nesje R, Kaplan G, Seljelid R (1982) Rainbow trout macrophages in vitro: morphology and phagocytic activity. Dev Comp Immunol 6:281–291
- Chebanov MS, Galich EV (2011) Sturgeon hatchery manual, FAO Fisheries and Aquaculture Technical Paper no 558, XXXII, 303 pp (ISBN 978-92-5-106823-6)
- Clem LW, Miller NW, Bly JE (1991) Evolution of lymphocyte subpopulations, their interactions, and temperature sensitivities. In: Warr GW, Cohen N (eds) Phylogenesis of immune functions, pp 191–213
- Collazos ME, Ortega E, Barriga C (1994) Effect of temperature on the immune system of a cyprinid fish (*Tinca tinca*, L.). Blood phagocyte function at low temperature. Fish Shellfish Immunol 4:231–238
- Cossins AR, Crawford DL (2005) Fish as models for environmental genomics. Nat Rev Genet 6:324–333
- Couso N, Castro R, Noya M, Obach A, Lamas J (2002) Formation of short-lived multinucleated giant cells (MGCS) from cultured gilthead seabream macrophages. Anat Rec 267:204–212
- Cunningham ML, Krinsky NI, Giovanazzi SM, Peak MJ (1985) Superoxide anion is generated from cellular metabolites by solar radiation and its components. J Free Radic Biol Med 1:381–385
- Duan R, Liu TCY, Li Y, Guo H, Yao LB (2001) Signal transduction pathways involved in low intensity He–Ne laser-induced respiratory burst in bovine neutrophils: a potential mechanism of low intensity laser biostimulation. Lasers Surg Med 29:174–178
- Edwards AM, Silva E (2001) Effect of visible light on selected enzymes, vitamins and amino acids. J Photochem Photobiol B 63:126–131
- Elkamel AA, Hawke JP, Henk WG, Thune RL (2003) Photobacterium damsela subsp. piscicida is capable of replicating in hybrid striped bass macrophages. J Aquat Anim Health 15:175–183
- Fänge R (1986) Lymphoid organs in sturgeons (Acipenseridae). Vet Immunol Immunopathol 12:153–161
- Fierro-Castro C, Barrioluengo L, López-Fierro P, Razquin B, Villena A (2013) Fish cell cultures as in vitro models of inflammatory responses elicited by immunostimulants. Expression of regulatory genes of the innate immune response. Fish Shellfish Immunol 35:979–987

- Fraikin GY, Strakhovskaya M, Rubin A (1996) The role of membrane-bound porphyrin-type compound as endogenous sensitizer in photodynamic damage to yeast plasma membranes. *J Photochem Photobiol B* 34:129–135
- Freshney RI (1994) Culture of animal cells: a manual of basic technique, 3rd ed. Wiley, New York, pp 71–103
- Garduno RA, Kay WW (1994) Isolation and culture of head kidney macrophages. *Anal Tech* 3:327
- Gordon S, Lawson L, Rabinowitz S, Crocker P, Morris L, Perry V (1992) Antigen markers of macrophage differentiation in murine tissues. In: Russell SW, Gordon S (eds) *Macrophage biology and activation*. Springer, Berlin, pp 1–37
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. *Anal Biochem* 126:131–138
- Hardie L, Fletcher T, Secombes C (1994) Effect of temperature on macrophage activation and the production of macrophage activating factor by rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Dev Comp Immunol* 18:57–66
- Hazel JR (1984) Effects of temperature on the structure and metabolism of cell membranes in fish. *Am J Physiol Regul Integr Comp Physiol* 246:R460–R470
- Karu T (1999) Primary and secondary mechanisms of action of visible to near-IR radiation on cells. *J Photochem Photobiol B* 49:1–17
- Kim CS, Jung J (1992) Iron-sulfur centers as endogenous blue light sensitizers in cells: a study with an artificial non-heme iron protein. *Photochem Photobiol* 56:63–68
- Kornmann B, Schaad O, Reinke H, Saini C, Schibler U (2007) Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators. *Cold Spring Harb Symp Quant Biol* 72:319–330.
- Lannan C (1994) Fish cell culture: a protocol for quality control. *Methods Cell Sci* 16:95–98
- Le Morvan C, Clerton P, Deschaux P, Troutaud D (1997) Effects of environmental temperature on macrophage activities in carp. *Fish Shellfish Immunol* 7:209–212
- Lubart R, Wollman Y, Friedmann H, Rochkind S, Laulicht I (1992) Effects of visible and near-infrared lasers on cell cultures. *J Photochem Photobiol B* 12:305–310
- Murray PJ, Wynn TA (2011) Protective and pathogenic functions of macrophage subsets *Nature reviews immunology* 11:723–737
- Nicholson BL (1989) Fish cell culture: an update. *Adv Cell Cult* 7:1–18
- Norum M, Bøggwald J, Dalmo R (2005) Isolation and characterisation of spotted wolffish (*Anarhichas minor* Olafsen) macrophages. *Fish Shellfish Immunol* 18:381–391
- Pottier R, Truscott T (1986) The photochemistry of haemato-porphyrin and related systems. *Int J Radiat Biol Relat Stud Phys Chem Med* 50:421–452
- Qiu W, Liu S, Chen J, Hu L, Wu M, Yang M (2016) The primary culture of carp (*Cyprinus carpio*) macrophages and the verification of its phagocytosis activity. *In Vitro Cell Dev Biol Anim* 52:10–19
- Ribas JLC et al (2014) Effects of anti-inflammatory drugs in primary kidney cell culture of a freshwater fish. *Fish Shellfish Immunol* 40:296–303
- Rodriguez E, Perez M, Casanova P, Martinez L (2001) Effect of seed cell density on specific growth rate using CHO cells as model. In: Lindner-Olsson E, Chatzissavidou N, Lüllau E (eds) *Animal cell technology: from target to market*. Springer, Dordrecht, pp 434–437
- Sarmiento A, Marques F, Ellis AE, Afonso A (2004) Modulation of the activity of sea bass (*Dicentrarchus labrax*) head-kidney macrophages by macrophage activating factor (s) and lipopolysaccharide. *Fish Shellfish Immunol* 16:79–92
- Secombes C (1990) Fish immunology technical communications. Salmonid macrophages and analysis of their killing activity. SOS Publications, Fair Haven
- Seeley K, Gillespie P, Weeks B (1990) A simple technique for the rapid spectrophotometric determination of phagocytosis by fish macrophages. *Mar Environ Res* 30:37–41
- Sharp G, Secombes C (1993) The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. *Fish Shellfish Immunol* 3:119–129
- Steiro K, Johansen A, Gildberg A, Bøggwald J (1998) Optimising of culture conditions and stimulation of head kidney macrophages from Atlantic cod, *Gadus morhua* L. *J Fish Dis* 21:335–344
- Suzuki YJ, Forman HJ, Sevanian A (1997) Oxidants as stimulators of signal transduction. *Free Radic Biol Med* 22:269–285
- Uribe C, Folch H, Enriquez R, Moran G (2011) Innate and adaptive immunity in teleost fish: a review. *Vet Med* 56:486–503
- Wang R, Neumann N, Shen Q, Belosevic M (1995) Establishment and characterization of a macrophage cell line from the goldfish. *Fish Shellfish Immunol* 5:329–346
- Wang Y et al (2016) Endotoxin disrupts circadian rhythms in macrophages via reactive oxygen species. *PloS one* 11:e0155075
- Zan-Bar T, Bartoov B, Segal R, Yehuda R, Lavi R, Lubart R, Avtalion R (2005) Influence of visible light and ultraviolet irradiation on motility and fertility of mammalian and fish sperm. *Photomed Laser Surg* 23:549–555