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Research Article

In vitro investigation on response of cultivated hepatocytes from Klunzinger's mullet, *Liza klunzingeri*, to temperature and fetal bovine serum

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Abstract

The appropriate temperature and growth supplementary factors such as fetal bovine serum (FBS) are the necessities needed for cell growth in culture medium. In this regards, the effect of Leibovitz-15 (L-15) medium containing different concentrations of fetal bovine serum (FBS) was assessed on growth and proliferation of cultivated liver cells of klunzinger's mullet, *Liza klunzingeri*, incubated at various temperatures. Ten fish were dissected and the liver was removed and cut into small pieces. The hepatocytes were then disconnected using collagenase type IV. The cells were cultivated for 2 weeks in L-15 containing 0, 5, 10 and 20% FBS and incubated at 20, 25, 28 and 30^{o}C. According to the results, an increase in temperature up to 30°C led to an increase in growth and number of cultured cells, but temperature rise above 30°C had no significant effect on growth of cultivated liver cells from *L. klunzingeri*. Cells cultivated at 20°C did not attach to the culture plate and died. Growth rate and the number of cultivated hepatocytes increased dose dependently and the maximum amount of growth was recorded in cells treated with 20% FBS at the end of the experiment. In conclusion, the general conditions for primary culture of hepatocytes from *L. klunzingeri* reported in the present study represented a foundation to establish an in vitro system for *L. klunzingeri* liver cell culture.

Keywords: Primary cell culture, Fetal bovine serum, Temperature, *Liza klunzingeri*.

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Introduction

Cell culture is referred to a controlled growth process of prokaryotic and eukaryotic cells in a culture medium. Using cell and tissue culture techniques is a new science that is expanding rapidly in most developed countries or in some developing countries.

Meanwhile, cultivation of aquatic cells (especially freshwater and marine fish) has been considerably respected by researchers (Hernández-García *et al.*, 2014). So far, aquatic cell cultures have been used in various researches, some of which include studies of cell and molecular mechanisms, physiological processes, toxicology and ecotoxicology (Fent, 2001; Castaño *et al.*, 2003; Castaño and Becerril, 2004; Schimer, 2006), virology (Villena, 2003; Parameswaran *et al.*, 2007), cancer biology (Bols *et al.*, 2001; Abdul Majeed *et al.*, 2013; Taju *et al.*, 2013; Cagan *et al.*, 2019), biological effects of radiation (Cassidy *et al.*, 2007; Mothersill *et al.*, 2018), aquaculture, genetic studies (Bols *et al.*, 2005), providing vaccines and compounds such as antibodies, interferon and human hormones (Montero *et al.*, 1996; Bols *et al.*, 2001; Hernández-García *et al.*, 2014).

Specifically, toxicology studies using cell culture methods have several advantages over studies using living organisms in laboratory conditions. These techniques are often easier and more repeatable; therefore, the results of these studies are often more accurate than those obtained from studying animals in laboratory conditions. The most important reason for using cell culture techniques is to reduce using living organisms and entry of toxicants into the environment. On the other hand, results are achieved faster and at lower cost (Fent, 2001; Schlenk and Benson, 2001). In general, the aquatic (especially fish) cell cultures have more benefits than mammalian cell cultures. Fish cells can grow in a wide range of temperatures (20 to 35^{o}C) and can be exposed directly to environmental pollutants with varying osmolarity, which facilitates collection of information related to cytotoxicity evaluation. Also, because fish cells have lower metabolism than mammalian cells, they can be stored longer (Hartmann *et al.*, 1992). Isolated liver cells from fish can be used as a tool to generate initial information on hepatic metabolism of xenobiotics, and on the mechanisms of xenobiotic activation or deactivation. During in vitro culture, fish liver cells express stable levels of phase I and phase II enzymes, such as cytochrome P4501A or glutathione S-transferase, and the cells show an induction of biotransformation enzymes after exposure to xenobiotics (Segner and Cravedi, 2001).

Thermoregulation process is a vital physiological mechanism in organisms and cellular activities, and intracellular reactions take place only at suitable temperatures. Cultivated cells need suitable temperature to grow, and the right growth temperature for different cells varies from species to species. In addition to right temperature, growth

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supplements such as fetal bovine serum (FBS) are required for cell growth in culture medium (Zhou *et al.*, 2005).

So far, little studies have been conducted on cultivation of cells obtained from fish of Persian Gulf and Gulf of Oman and there is limited information on optimum conditions for cultivation of cells derived from these fish. On the other hand, most studies on aquatic cell culture worldwide are related to freshwater fish. In this regards, the present study aimed to determine optimal conditions, appropriate temperature and FBS amount, for cultivating liver cells from Klunzinger's mullet, *Liza klunzingeri*, a native fish from Persian Gulf and Gulf of Oman. *L. klunzingeri* was selected due to its commercial importance in ROPME (Regional Organization for Protection of Marine Environment) Sea Area (RSA). This fish is a widely distributed native species with high nutritional value which is highly available, and with a growing market demand. Sheibani *et al.* (2020) used ovarian and brain cell culture from *L. klunzingeri*, to assess inhibitory effects of benzo[*a*]pyrene on aromatase activity. Salamat and Derakhshesh (2020) also assessed oxidative stress in mullet, *Liza klunzingeri*, induced by short-term exposure to benzo[a]pyrene and nonylphenol.

Materials and methods

Chemical

The chemicals Leibovitz's L-15 culture medium with L-glutamine, penicillin-streptomycin, fungizone, collagenase Type IV, dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich chemical (USA). Other chemicals, such as ITS (insulin, transferrin, selenium), FBS (fetal bovine serum), and trypsin with EDTA were purchased from Gibco Life Technology (USA).

Experiment design

Ten healthy mature *L. klunzingeri* (45±5 gr mean body weight) were obtained from Institute of Marine Research's field station in Mahshahr (Iran) and moved alive to the laboratory. The fish were maintained in a 300L tank containing aerated, particle-filtered, and UV-treated running seawater (pH: 7.5 and temperature: 30±0.8°C) for a weak. The fish were then moved to a 150L tank containing sterile seawater with 1000 IU ml^{-1} of penicillin and 1000 μg ml^{-1} of streptomycin at room temperature (25 to 28°C) for 24h.

Primary liver cell culture was obtained from *L. klunzingeri*, according to the method described by Xing *et al.* (2015). The fish were sacrificed after euthanizing by 2-phenoxy ethanol (0.35 ml L^{-1}). Liver was then detached and moved to phosphate-buffered saline (PBS, without Ca^{2+}) containing 20 mg/ml of streptomycin, 100IU of penicillin, 10 μg/ml of enrofloxacin and 25 μg/ml amphotericin B for 30 min. Liver tissue was crushed by a razor and moved to PBS solution containing 0.1% collagenase IV for 20 min at room temperature to digest. A triple volume

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of Leibovitz's L-15 culture medium (L-15 medium) containing 20% FBS was added to solution to stop the digestion. The cell suspension was then filtered through a 70 µm nylon mesh and the solution was then centrifuged at 1500 ×g for 7 minutes. The supernatant was removed and the cell pellet was suspended in an equal volume of L-15 medium containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin B. Cell viability test and cell counting were performed using trypan blue exclusion test and a hemocytometer, and cells with > 90% viability were moved into 25 cm^2 tissue culture flasks at a density of 1×10^6 cells/ml. To determine the optimum temperature and appropriate amount of fetal bovine serum (FBS) for liver cell growth, FBS with different concentrations (0, 5, 10 and 20%) were added and the cells were incubated at various temperatures (20, 25, 28 and 30^{o}C) for 15 days. Sampling and cell counting using the trypan blue exclusion test was conducted on days 0, 5, 10 and 15 to determine changes in the number and the shape of cells affected by different amounts of FBS and temperature.

Sub-cultures were obtained using 0.1% collagenase IV/EDTA in PBS (at a ratio of 1:2), when the cells reached confluence. The cells were then centrifuged at 1500 ×g for 5 min. The cell pellet was suspended in fresh L-15 medium supplemented with the same amount of FBS and plated into new plates.

Statistical analysis

Experiments were performed in triplicates. Data were presented as mean±standard error of mean and were tested for normality by Kolmogorov-Smirnov test. Data analysis was carried out using SPSS Version 17. One-way analysis of variance (ANOVA) was used to compare differences in cell numbers among various concentrations of FBS and temperatures in different sampling times. A significant level of $p<0.05$ was accepted.

Results

The aim of the present study was to determine the optimum temperature and appropriate amount of FBS for cultivation of liver cells from *L. klunzingeri.*

Figure 1A presents the effect of different temperatures on cultivated liver cells from *L. klunzingeri.* Accordingly, 30°C was found to be the most suitable temperature for growth of liver cells in the culture medium. Increasing temperature to 30°C led to an increase in growth and number of cultured cells, however, increasing temperature above 30°C had no significant effect on growth of liver cells of *L. klunzingeri.* At temperatures below 28°C and also at 32°C growth and number of cultivated cells at the end of the experiment were very low.

The cells incubated at 20 and 25°C grew very slowly (doubling time 15d) than those incubated at 30°C (doubling time 6d), 28°C (doubling time 8d) and 32°C (doubling time 9d). Although

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cells grew and proliferated at 25°C, it could not be considered the optimal temperature, as cells grew and proliferated at 28 and 30°C, about 3 times faster. Cells cultured at 20°C didn't attach to the culture plate and died.

As shown in Figure 1B no considerable change was recorded in the number of cells cultivated in culture medium free of FBS during the experiment. The growth rate and the number of cultivated hepatocytes increased dose dependently and the maximum amount of growth was recorded in cells treated with 20% FBS at the end of the experiment (p<0.05).

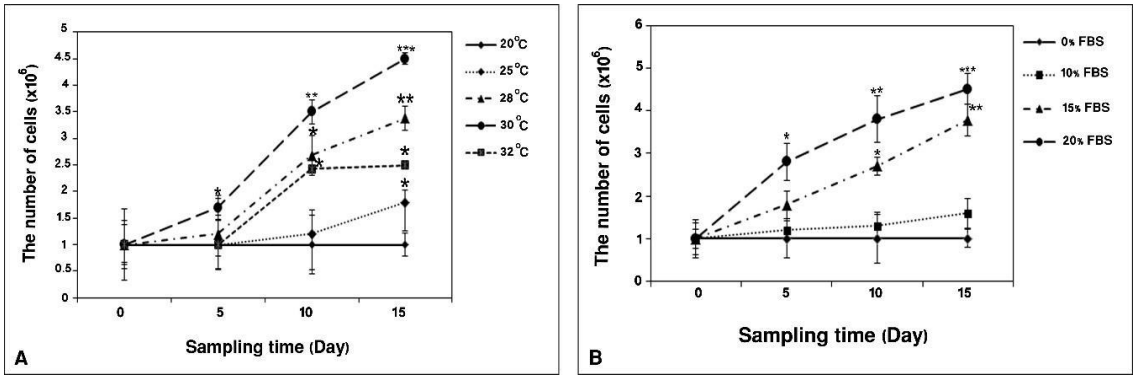


Figure 1: Effect of various incubation temperatures (A) and different concentrations of FBS (B) on proliferation of cultivated hepatocytes from *Liza klunzingeri.* Error bars show standard error.

As represented in Figure 2A the liver cells cultured in a FBS-free medium did not attach to the culture plate and did not grow. Figures 2B and C show cultivated liver cells treated with 5 and 20% FBS (the lowest and highest FBS levels). According to the results, growth rate of cells in the medium containing 5% FBS was very low, while cells exposed to 20% FBS were completely attached to culture plate and appeared in the form of fibroblastic-like cells. Figure 2D represents growth rate and morphology of liver cells exposed to 20% FBS and incubated at optimal temperature (30°C). The growth rate of these cells was significantly higher than that of others during the experiment.

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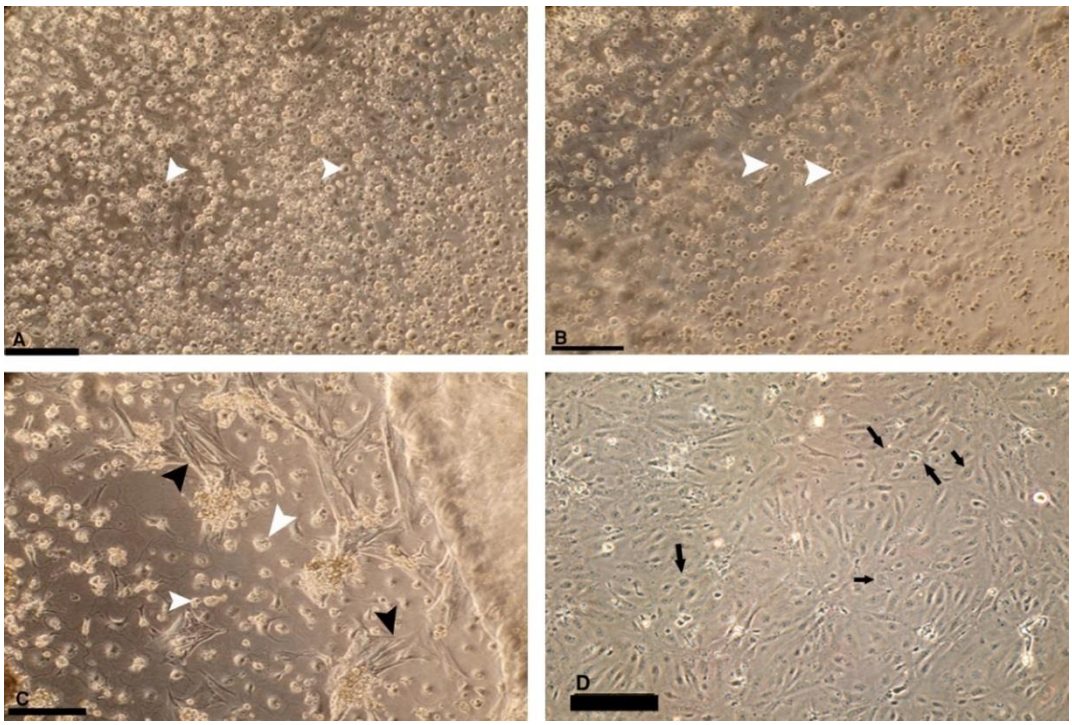


Figure 2: Photomicrographs of cultivated hepatocytes from *Liza klunzingeri*; A. incubated in FBS-free medium at 30°C after 15 days; B. incubated in L-15 medium containing 5% FBS at 30°C after 5 days; C. incubated in L-15 medium containing 20% FBS at 30°C after 5 days; D. incubated in L-15 medium containing 20% FBS at 30°C after 15 days; fibroblast-like cells (black arrowheads), non-attached cells (white arrowheads); A, B, C (Trypan blue; X 3750).

Discussion

So far, little research has been carried out on the cultivation of cells from Liza throughout the world. Up to now, different types of enzymes have been used to digest aquatic tissues and separate the cells. In the present study, collagenase type IV found to be more effective than other enzymes, such as trypsin, in digesting liver of *L. klunzingeri* and separating hepatocytes. In most researches on animal cell culture, collagenase type I and IV have been introduced as more suitable enzymes for hydrolysis of lipid, kidney, liver and islets of Langerhans (Zhou *et al.*, 2006; Søfteland *et al.*, 2009; Patkaew *et al.*, 2014; Ryu *et al.*, 2016). Due to the presence of glycoprotein compounds in liver tissue, proteolytic enzymes such as collagenase are more suitable for digestion of extracellular matrix and isolation of liver cells (Zhou *et al.*, 2006; Patkaew *et al.*, 2014; Ryu *et al.*, 2016).

Maintaining optimum culture temperature is essential for optimal cell growth. Selecting depend on temperature type and origin of cultivated cells. In other words, optimum culture temperature depends mainly on body temperature of the animal from which cells were taken (Freshney, 2010). Several research on aquatic cell culture have shown that best cell growth occur at a temperature

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slightly higher than that of the animal's temperature from which cells were derived (Ott, 2004; Bols *et al.*, 2005). Considering that most tropical fish cell lines grow at a temperature range from 20 to 35^{o}C (Wen *et al.*, 2008), in the present study temperature range was considered to be between 20 and 32^{o}C. This temperature range has been reported in other animal species such *Ctenopharyngodon idella* (Lu *et al.*, 1990) and *Epinephelus awoara* (Lai *et al.*, 2000) as the most suitable temperature range for growth of various cell types in culture medium. In the present study, temperature between 28 to 30°C was recorded as appropriate temperature for primary hepatocyte culture from *L. klunzingeri*. Lakra *et al.* (2010) examined the effects of temperature and concentration of FBS on growth of two new cell lines established from fin (CCF) and heart (CCH) tissue of common carp, *Cyprinus carpio.* Accordingly, the cells grew well in Leibovitz-15 medium supplemented with 10% FBS at the temperature ranged from 24 to 32°C. However, both CCF and CCH cells exhibited maximum growth rate at 28°C. The highest growth rate of various tropical fish cell lines was observed at 28°C (Sathe *et al.*, 1995) and 22-25°C (Tong *et al.*, 1997). A temperature of 35-37°C has been reported to be lethal to many fish cells (Tong *et al.*, 1997).

Comparison of growth and proliferation of cultivated hepatocytes at various temperatures showed that liver cells from *L. klunzingeri* can tolerate a wide range of temperature. According to the results, the cells detached and died at 20^{o}C; while the cell number increased between 25°C (very low proliferation) and 30°C (optimal growth temperature). On the other hand, increasing the temperature above 30°C also decreased growth and proliferation of cultured cells, although growth rate of cells at 32°C was still higher than that below 25°C. Iuchi *et al.* (2020) elucidated that isolated hepatocytes from white-spotted charr could grow in culture at 5-20°C but not 37°C. Bols *et al.* (2005) and Ott (2004) stated that fish cells cultivated at temperatures lower than their optimum temperature showed little or no growth. In addition, liver cells from *L. klunzingeri* exhibited more tolerance to higher temperatures than lower ones. Cells died at 20°C, which was only 8°C lower than optimum temperature for hepatocyte culture.

It seems that FBS is the best supplements to be used in the culture medium, as it is readily available in large volumes and contains many known and unknown growth factors (Lakra *et al.*, 2011). Hayashi and Kumagai (2008) reported that insulin is essential for attachment and spreading of eel hepatocytes. Eel hepatocytes cultured in a serum free medium exhibited normal morphology, but the serum-deficient medium caused an approximately 60% decrease in cell numbers compared to the control. According to Iuchi *et al.* (2020) FBS

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enhance cell growth and growth factors, such as EGF and HGF, are essential for cell growth.

Our other experiments showed the importance and the amount of serum as growth factor in the culture medium. Different concentrations of FBS (0, 5, 10 and 20%) were compared to determine the optimal concentration for growth of liver cells from *L. klunzingeri*. Actually, the hepatocytes cultivated in FBS free medium did not adhere to culture plate, indicating that FBS plays an important role in promoting cell adhesion and proliferation. Zhou *et al.* (2008) reported the same findings with the case of *Acipenser sinensis* fin cell lines. Most cells cultured in basal medium either did not survive at all or were unable to indicate their optimal phenotypic characteristics for any period of time. The cell culture medium needs to be enriched with additional growth and survival factors such as extracellular matrix agents, transport proteins, hormones, and trace elements (Lakra *et al.*, 2010). Wang *et al.* (2012) also reported that, the AGF II cell line (derived from caudal fin tissue of *A. graham*) cultivated in FBS free medium did not adhere to the bottom of the flask, suggesting that FBS may play a key role in promoting cell attachment and proliferation.

Existence of 10 and 20% FBS in culture medium increased the growth and proliferation of cultivated hepatocytes significantly. High and poor growth of cell lines from *Acipenser oxyrinchus oxyrinchus* were recorded in DMEM-15% and DMEM-5% FBS, respectively (Grunow *et al.*, 2011). FBS is essential for survival and optimal growth of cells. Lakra *et al.* (2010) reported that FBS at high concentration (20%) was favorable for cell growth and attachment and cell replication rate increased as FBS concentration is increased from 5 to 20%. There are similar reports on cell lines from other marine fish (Lai *et al.*, 2000, 2003).

However according to Freshney (2010) and Chen and Qin (2011) high concentrations of FBS may prevent cell growth.

In conclusion, the present study reported general conditions for primary culture of hepatocytes from *L. klunzingeri*. Tissue digestion and cell isolation could be applied successfully using collagenase type IV, and cells could be incubated in L-15 medium containing 15 or 20% FBS at optimal temperatures of 28 and 30^{o}C. The results of the present study present a foundation to establish an in vitro system for *L. klunzingeri* liver cell culture.

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