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Isolation and long-term culture of neural stem cells from *Acipenser persicus* (Borodin, 1897)

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Abstract

In the present study, an *in vitro* brain cell culture was developed from neural cells of Persian sturgeon (*Acipenser persicus*). The tissue samples collected from the anterior, middle and posterior regions of the brain were cultivated separately in DMEM/F12 medium supplemented with 15% fetal bovine serum, antibiotic and antimycotic. The medium was refreshed every 3 days. The cells became confluent after about 3 weeks from the initial time of seeding. The cultured cells from the posterior part of the brain showed high potential of proliferation as they had been passaged 16 times in more than 11 months. To determine optimal temperature, the brain cells were incubated at four temperatures including; 20, 22, 25 and 28°C. The best cultivation temperature was obtained at 25°C. The cultured cells from posterior part of the brain were cryopreserved successfully and the survival rate was 70% after thawing. Immunocytochemistry using antibody against nesting showed that some cells were immunopositive for nesting. Finally, these results suggested that cell cultures from posterior part of the Persian sturgeon brain with high proliferation capacity can be useful for research on brain cells in *A. persicus* in the future.

Keywords: Brain, Cell culture, Immunocytochemistry, Persian sturgeon (*Acipenser persicus*)

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Introduction

Aquaculture and therefore fish culture is rapidly growing because of the increase in world population and consequently the demand for marine proteins. In this regard, sturgeon fish is one of the most valuable fishes in the world. Currently, there are more than 27 species of sturgeon fish 6 of which are present in the Caspian Sea. Persian sturgeon (*Acipenser persicus*, Chondrostei) is one of the most important species in the Caspian Sea that is endangered mainly due to human activities. As a result, intensive culture of this valuable fish is increasingly noteworthy. In this case, limiting the negative factors affecting fish culture including viral and bacterial disease is the main objective. Nonetheless, the health of fish could be threatened by some infectious disease in intensive conditions (Chi *et al.* 2005; Parameswaran *et al.,* 2006). However, simple manageable tools including *in vitro* test systems or ready cell cultures seem necessary for quick and precise assessments. In fact, cell lines and primary cell cultures are essential tools to investigate virology, genotoxicity, environmental toxicology as well as immunology and physiology (Villena, 2003; Schirmer, 2006). For these purposes and some other applications, isolating and preparing of high amounts of cells from various parts of different fish species is of great importance. To date, many cell cultures have been developed from different tissues of teleost fish which have been used to support investigation on virology (Lai *et al.,* 2003; Fan *et al.,* 2010), toxicology (Studincka *et al.,* 1982; Carvan *et al.,* 2000) and many other areas of research (Lakra *et al.,* 2011). However, some researchers refer to the need for developing cell cultures from fish brain (Parameswaran *et al.,* 2006; Cutrin *et al.,* 2007). In fact, basic and applied studies on neural tissues are significant for understanding physiological processes in different fish species especially, those with high commercial value like Persian sturgeon.

The ability of dividing and producing differentiated cells is the classical definition of stem cells (Hinsch and Zupanc, 2006). It seems these cells are present in most tissues including the brain of fish (Kaslin *et al.,* 2008) and fish show indeterminate growth compared to limited growth of mammalian which could be attributed to high stability of proliferative zones (Grandel *et al.,* 2006). As fish grow, new cells are produced in all tissues including neural tissues and the brain continues its growth even in adulthood (Hinsch and Zupanc, 2006). During recent decades, cell cultures from mammalian brain have been used in investigating developmental stages of brain and neurogenesis (Neuhaus and Fedoroff, 1994), neurotoxicology (Veronesi, 1992; Tang *et al.,* 1996), isolation of stem cells (Gregg *et al.,* 2002), response to growth factors (Pitchford *et al.,* 1995) and assessment of drug delivery systems (Rizk *et al.,* 2004). Despite mammalian, preparing cell cultures from fish brain and applying them in neurobiological studies have been so limited and their application was more in virology

studies such as investigation of infectious pancreatic necrosis (IPN) virus, isolation of alpha virus-1 and so on (Servilia *et al.,* 2008). Only a few neural cell lines have been reported up to now (Lai *et al.,* 2003; Servilia *et al.,* 2008; Wen *et al.,* 2008; Ku *et al.,* 2009).

In general, producing long term cell cultures from the brain of different fish species is important for basic studies and applied research. Unfortunately, despite the importance of these cultures and the application in various fields of study, few studies have been conducted on preparing neural stem cell culture from the fish brain and determining optimal condition for *in vitro* cultivation all of which were limited to teleost fish such as *Dicentrarchus labrax* (Servilia *et al.,* 2008) or *Sparus aurata* (Centoducati *et al.,* 2013) and some other teleost fish. In this regard, there have been no reports on developing cell culture from the brain of chondrostei fish. Therefore this present study aims to develop an *in vitro* culture of Persian sturgeon (*A. persicus*) brain cells.

Materials and methods

Persian sturgeon

Sturgeon fish (*Acipenser persicus*; Acipenseriformes; Chondrostei) (15 specimens) were obtained from Shahid Marjani hatchery in 2012 and transported to the laboratory of aquaculture at the fisheries faculty, Gorgan University of Agricultural Sciences and Natural Resources, Iran. Fish were kept in 400-L tanks filled with water (pH at about 8±0.2, oxygen at 5.6±0.3 mg L^{-1}, temperature at 23±1 ºC, hardness at 256±5.8 mg L^{-1}) and fed daily on Biomar food. The weight of fish used for cell cultures was 250±40 g.

Isolation and preparation of brain tissues

For collecting brain tissue, Persian sturgeon were anaesthetized in a tank filled with water plus cloves powder (0.5 g L^{-1}) and then decapitated. After isolating the brain from the skull, the anterior, middle and posterior parts of the brain were characterized and isolated. Samples were washed twice in tubes containing Hank's balanced salt solution (HBSS) (Bio idea) (Servilia *et al.,* 2008).

Cell culture

Tissue samples were homogenized mechanically by pipetting in tubes containing HBSS, penicillin-streptomycin (P-S) (Gibco, USA) and amphotericin (AP) (Gibco, USA) (2×P-/AP). After adding trypsin-EDTA (Gibco, USA) for 5 min for enzymatic digestion, pieces of tissues and cells were placed in DMEM/F12 medium (Bio idea, Iran) supplemented with fetal bovine serum (%15FBS), penicillin-streptomycin and amphotericin (2×P-S/AP). Then, the samples were centrifuged for 5 min at 3000 rpm. The pelleted cells were resuspended by adding DMEM/F12 and pipetted using sterile Pasteur pipette. The cells from each part of the brain were separately cultivated in 12.5 cm^2 flasks containing DMEM/F12 medium supplemented with 15% FBS, 2X P-S/AP and then

placed in incubator (temperature at 23 °C with 5% CO_2). The medium was refreshed with the medium for the first time after 2 days and later on every 3 days. The cultivated cells were investigated using an inverted microscope, every 3 days.

The confluent cells were subcultured at the ratio of 1:2. Briefly, after detaching the cells using trypsin-EDTA and adding DMEM/F12 medium, samples were centrifuged for 5 min in 3000 rpm. The pelleted cells were resuspended in DMEM/F12 medium by pipetting and transferred into the new flasks containing fresh medium (Servilia *et al.,* 2008). Finally the flasks were placed into the incubator.

Determination of optimal temperature

To determine optimal temperature for in vitro culture of Persian sturgeon brain cells, the cells from posterior part of the brain (CPS) were seeded into the 12-well tissue plates in 2 mL of the normal growth medium (5.0×10^4 cells per well). To adhere to the culture plates, the cells were incubated overnight at their initiation temperature of 23°C. After adhering, one duplicate of cells was detached via trypsinization and counted using a hemocytometer (day 0). The rest of the plates were incubated at temperatures of 20, 22, 25 and 28°C. Cells from the duplicate of wells from each tested temperature were trypsinized and counted every three days (days of 3, 6, and 9). The cell numbers were expressed as a percentage of that on the initial day 0 used as the reference count (Grunow *et al.,* 2011).

Immunocytochemistry

For immunocytochemistry, anti-nesting antibody from rabbit (Sigma) was used as the neural stem cell marker. The CPS culture was grown after the 5th passage on poly-L-Lysine coated coverslips for 72 hrs. Fixation was done using paraformaldehyde (4%). The cells were washed 5× with PBS containing triton X-100 (0.3%). The antibody was diluted (1:500) in normal serum (10%) in triton X-100 (0.3%) PBS. The goat anti-rabbit IgG (coupled to FITC, dilution; 1:300) was used as a secondary antibody. After incubation of cells with the secondary antibody they were washed in PBS. Controls were run by replacing the primary or secondary antibodies with the corresponding normal serum. Finally, the cells were observed under a florescence microscope (Servilia *et al.,* 2008).

Cryopreservation

For cryopreservation of cell cultures from the posterior part of the Persian sturgeon brain, the cells were trypsinized and then centrifuged at 3000 rpm for 5 min after adding FBS. The pelleted cells were resuspended in the medium containing 90%FBS and 10% dimethylsulfoxide-DMSO and transferred into sterile vials. The vials were stored at -20°C for 3 hrs and -80°C for 24 hrs. Finally, the vials were transferred into a tank containing liquid nitrogen (Servilia *et al.,* 2008).

For reseeding, the frozen cells were thawed in regular culture medium and centrifuged at 3000 rpm for 5 min. The pelleted cells were resuspended by adding 2 mL of the normal medium and

pipetted up and down. The obtained cell suspension was transferred into the 12.5 cm^2 flasks containing the fresh culture medium. Trypan blue dye (Bio idea) was used to evaluate the viability of thawed cells (Servilia *et al.*, 2008).

Statistical analysis

Differences among the mean values of cell proliferation in different temperatures were assessed by one-way analysis of variance (ANOVA). Also the differences were considered significant at $p \leq 0.05$.

Results

A suspension of cells was obtained directly after seeding in which most of the cells were small and round. The round cells observed after plating changed morphologically so that different shapes from spindle-like to mesenchymal- and neuron-like types were observed all the time from the beginning of the seeding (Fig. 1). The cells continued to grow and proliferate; they reached confluence and filled the bottom of dish in about 3 weeks (Fig. 2). Most of the confluent cells changed into approximately spindle-forms because of contact inhibition. These cells were able to do all the above stages after sub-culture.

Among all the prepared cultures, the cells from the posterior part of the Persian sturgeon brain (CPS: cerebellum of Persian sturgeon) showed continues growth so that these have survived and proliferated successfully during more than 11 months and 16 passages and their stock is now available in our laboratory.

To maintain the obtained cell stocks, the cells of CPS were cryopreserved (rapid freezing) in liquid nitrogen using 10% dimethyl-sulfoxide supplemented with 90% FBS. The cryopreserved CPS cells were thawed successfully with a survival rate of about 70%.

To determine optimal temperature for *in vitro* culture of Persian sturgeon neural stem cells, four temperatures, 20, 22, 25 and 28°C, were evaluated (Figs. 3 and 4). Results showed that although the cultivated cells were able to grow at the lowest tested temperature, they grew more slowly than the cells at temperatures of 22 and 25°C ($p \leq 0.05$). Also, it seems that 28°C is not good for the growth of Persian sturgeon brain cells and temperatures above 28°C are higher than the tolerance limit of the cells that can result in brain cell death. However, there was no significant differences ($p > 0.05$) in proliferation level among the cultivated cells at temperatures of 22 and 25 °C and the best temperatures for Persian sturgeon brain cells were 22-25°C in that the cells showed the highest proliferation at 25°C.

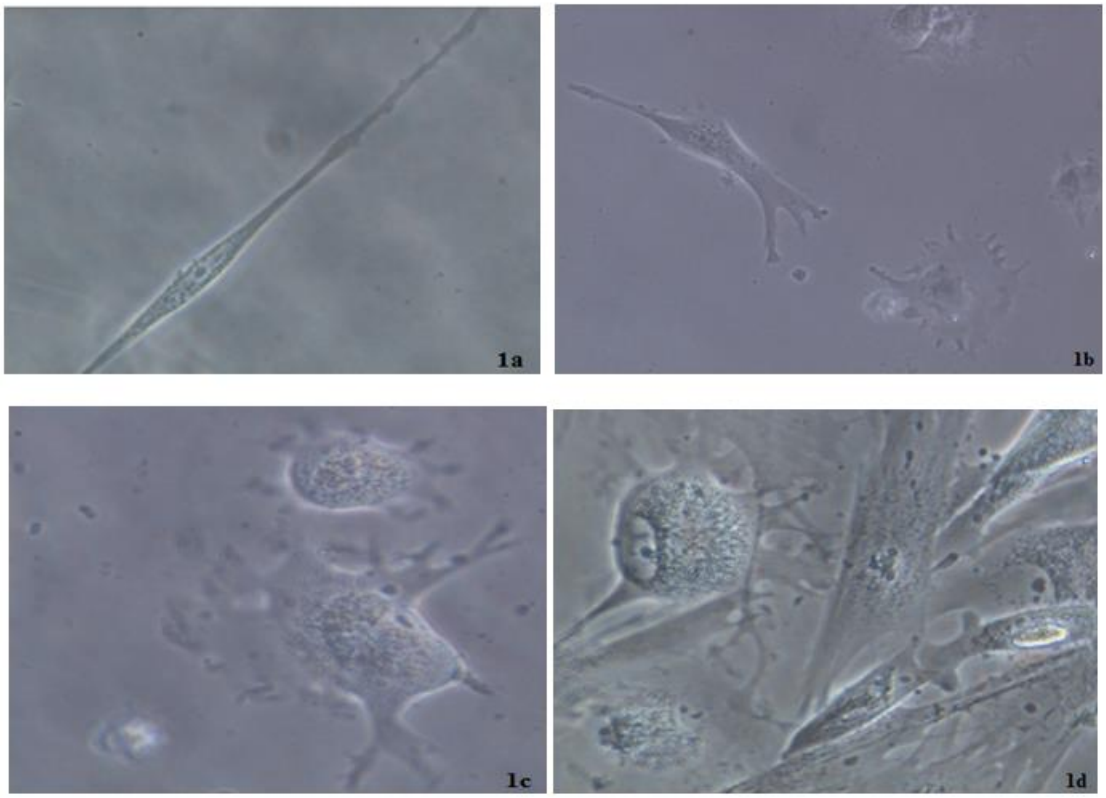

Figure 1: Cell cultures from *Acipenser persicus* brain. Different shapes from spindle-like (a) to mesenchymal- and neuron-like types (b-d) were observed over the time from the beginning of the seeding.

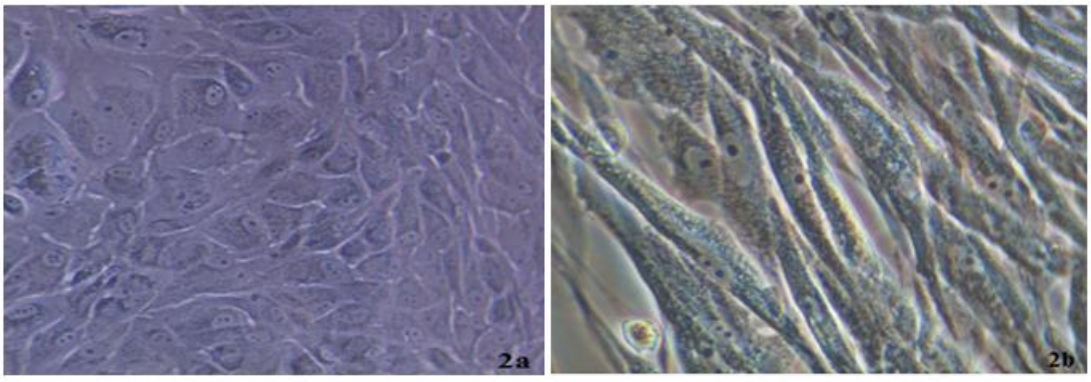

Figure 2: Cell cultures from *Acipenser persicus* brain. The cells reached confluence about three weeks after seeding time (a: 10X, b: 40X).

Since the cells of CPS culture showed high potential of proliferation without any clear sign of senescence, immunocytochemistry was done using anti-nesting (anti-N) antibody to characterize Persian sturgeon neural stem cells in CPS culture. Results from immunocytochemistry showed that some cells in CPS culture expressed anti-N as a neural stem cell marker (Fig. 5).

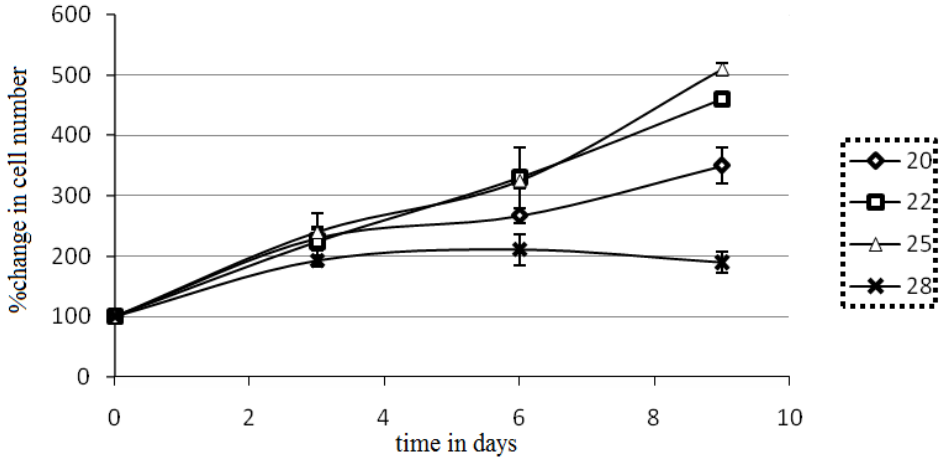

Figure 3: Neural stem cells from *Acipenser persicus* brain at different temperatures of 20, 22, 25 and 28°C. The highest proliferation of the cells was observed at 25°C ($p \leq 0.05$).

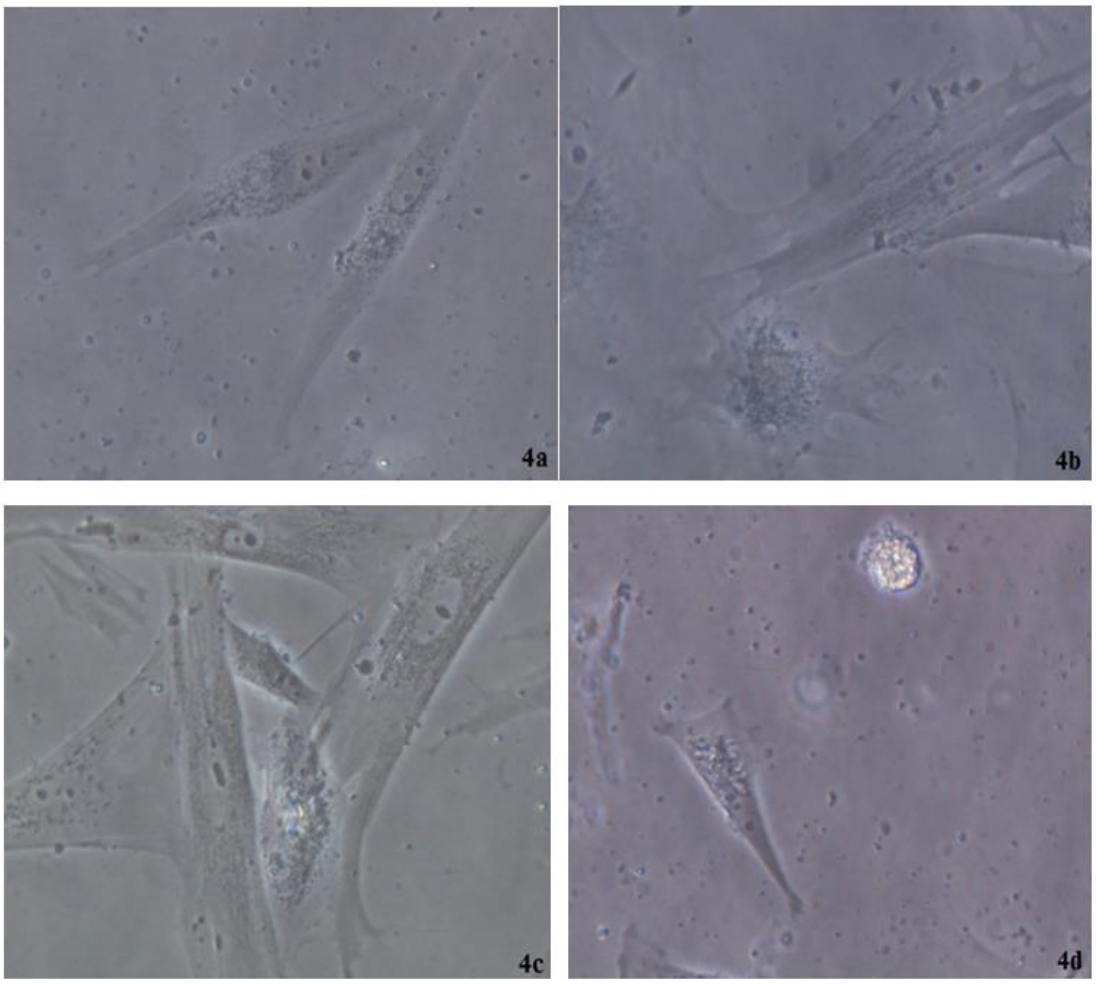

Figure 4: Neural stem cells culture from *Acipenser persicus* brain at different temperatures of 20 (a), 22 (b), 25 (c) and 28°C (d) (day 9).

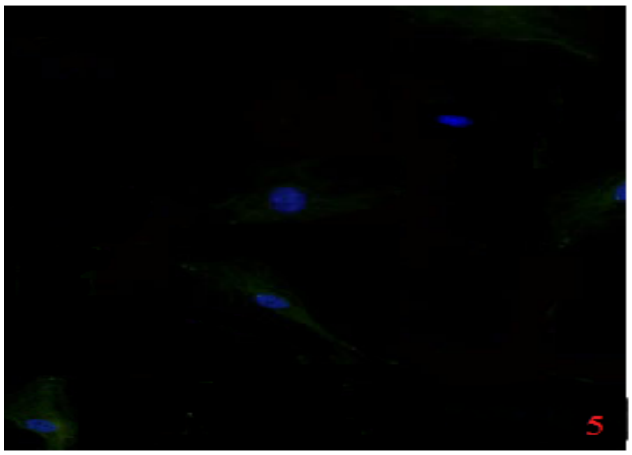

Figure 5: Immunocytochemical analysis of CPS culture using anti-nestin antibody. These cells expressed nestin.

Discussion

In vitro cell cultures of fish are important in basic and applied studies and the need for cell lines with proliferative potential is increasing (Ciba *et al.,* 2008). This is especially so important for fish with high commercial value such as sturgeon fish. Although, some cell cultures have been developed from some tissues and organs of sturgeon fish such as cell cultures from pectoral fin of *Acipenser mikadoi* (Vishnyakova *et al.,* 2009), head kidney of *Acipenser baerii* (Ciba *et al.,* 2008) and cardiac tissue of *Acipenser oxyrhinchus* (Li *et al.,* 2011), an *in vitro* long term culture from the sturgeon fish brain has never been established.

Whereas viable population of cells could be obtained from the three tested regions of Persian sturgeon brain, the best results were obtained from the posterior part of the brain in that cell cultures from this region (CPS) have survived for a long time and their stock is still available in our laboratory.

The growth of Persian sturgeon brain cells were influenced by temperature like most other cells in culture therefore the optimal temperature for in vitro culture of Persian sturgeon neuronal stem cells was determined. Results indicated that temperature of 28°C, higher than the optimal temperature of Persian sturgeon in nature, is not appropriate for the growth and proliferation of brain cells. The lowest tested temperature (20°C) is still appropriate for brain cell growth, but as compared to 25°C this cannot be considered as optimal temperature. It seems that the Persian sturgeon brain cells are more sensitive to higher temperatures than the lower ones. Previously, several studies on fish cell culture represented that the best growth of cells occurs at temperatures slightly higher than the temperature of the donor (Peters, 2009). According to our results, the temperatures of 22 and 25°C, especially 25°C, appeared to support the growth of Persian sturgeon brain cells while the optimum temperature for sturgeon growth is close to 23°C in water (Iranshahi *et al.,* 2011). So, in our study the cells were

first incubated at 23°C and later at 25°C as the optimal temperature. In a study by Grunow *et al.* (2011), the best temperature for cell culture from *A. oxyrinchus* larvae was reported at 25°C while the optimal one of the intact fish, Atlantic sturgeon, is between 18 and 23°C. However, in another study by Servilia *et al.* (2008), the best growth of cells from *Dicentrarchus labrax* occurred from 16 to 22°C, especially 22°C, within the range of temperatures in which this species can be found in the wild.

Since the brain cells showed high stability, it could be said that Persian sturgeon brain has high proliferative capacity. The high stability might be attributed to the high constitutive telomerase activity in fish organs. Bols *et al.* (2005) described this phenomenon and expressed that it is an interesting subject for further studies to understand spontaneous immortalization in fish cell lines. Previously, it was found that the cerebellum of teleost fish has high potential of proliferation capacity (Zupanc and Ott, 1999). In a research on sole and sea bass, the presence of an important number of proliferative cells in the cerebellum was revealed (Servilia *et al.*, 2008). The cerebellum is thought to retain a population of radial glial cells that are thought to be neural stem cells (Zupanc and Clint, 2003) responsible for neurogenesis in adult fish and these brain cells continue their growth during the adult period (Hinsch and Zupanc, 2006). In this regard, Servilia *et al.* (2008) introduced SBB-W1 culture from cerebellar-tegmental region of sea bass brain as a permanent cell line and represented that these cells have high potential of proliferation. The CPS cells from the posterior part of the Persian sturgeon brain including cerebellum showed high capability to proliferate so the cells with proliferative potential might be present. To confirm this assumption, immunocytochemistry using anti-nesting antibody was applied for characterization of neural stem cells. Unfortunately, because there is no specific antibody against proteins from these commercially important fish, mammalian antibodies had to be used. In this regard, developing specific antibodies seems necessary since the cross-reactivity of antibodies against mammalian proteins seems to be low (Grunow *et al.*, 2011). In our study, CPS cells showed positive reactivity to anti-N, suggesting their potential role as neural stem cells (Wen *et al.*, 2008) as Yang *et al.* (1993) represented that expressing anti-N antibody by cells signify the potential of these cells as neural stem cells.

In the present study, cell cultures from the posterior part of the Persian sturgeon brain showed high proliferative potential without any clear sign of senescence during more than 11 months and 16 passages. So, CPS culture could be useful in many research areas but before considering this valuable cell culture as an international cell line, further work including studies on the differentiation potential of CPS cells and factors influencing them as well as molecular aspects, is needed.

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