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# Isolation and characterization of neural stem cells from caprine

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ABSTRACT

Central nervous system (CNS) has no or very little capacity to self repair. Neural tissue transplantation is impractical because it is hard to find a brain donor, and because of the low survival rate of grafted neural cells. Therefore, the need for constructing a functional brain tissue is very critical. In this study neural stem cells were isolated and expanded from caprine fetal brain in adherent culture. After 4–5 days of culture there were formation of small colonies and after about 10 days cells become confluent. Cells from passage 1 to 4 were used in study. These neural stem cells were characterized with neural stem cell specific markers nestin *(NES*), *PAX6* and *SOX2* by PCR and immunocytochemistry. The cells were expressed neural stem cell markers nestin, pax 6 and Sox 2.

**Key words**: Caprine, Neural stem cell, Neural stem cell marker

Regenerative therapy for organ dysfunction is a rapidly growing domain and involves application of multiple enabling technologies incorporating stem cells, genes and growth factors that can accelerate the recovery of a failing organ through cell and tissue regeneration within the organ. Several strategies are currently being evaluated for regeneration of damaged organs. These are aimed at 'reviving' existing malfunctioning cells, repopulating the organ by new cells from exogenous or endogenous sources, altering the extracellular matrix, or increasing blood supply by enhancing vasculogenesis. Stem cells with their unique and facile potentialities, offer building blocks for organ development and tissue repair.

The stem cell research has emerged as one of the most vibrant areas of biological research because of emergence of novel cell based therapies for the treatment of various diseases. This is particularly true for neurological disorders that currently lack effective therapies. The stem cell has the capacity of self renewal and the potential to differentiate into one or more cell types depending on the *in vivo* signals (Smith *et al.* 1992). Stem cells play a central role in the normal growth and development of animals and human. Three properties that distinguish stem cells from other cell types and make them interesting to scientists: they are unspecialized, they are able to divide and produce copies of themselves, and they have the potential to produce other cell types.

Neural stem cells (NSCs) are a subtype of progenitor cells in the nervous system that can self-renew and generate both neurons and glia. The early studies led to the isolation of stem-like cells from the embryonic mammalian central nervous system (CNS) and the peripheral nervous system (PNS). Since then, stem cells have been isolated from many regions of the embryonic nervous system, indicating their ubiquity.

In addition, NSCs have been utilized as one of the potential sources for the cell replacement therapy of CNS disorders (Ronaghi *et al*. 2010). NSCs are not a viable option for large-scale therapeutic application due to a lack of available tissue and ethical considerations (Piccini *et al*. 1999). Isolation and expansion of NSCs of human origin are crucial for successful development of cell therapy approaches in neurodegenerative diseases. The neural stem cells have been isolated from the human fetal spinal cord and brain tissue (Uchida *et al*. 2000), adult mouse periventricular region (Azari *et al*. 2010), fetal cortex (Caccia *et al*. 2007) and rat mandibular process during embryonic development (Zhang *et al*. 2006). Neural progenitor cells were isolated and characterized from postmortem human cortex (Schwartz *et al*. 2003). Neural precursors are relatively resistant to post-mortem ischemic and oxidative stress compared with neurons (Safar 1979).

Isolated and cultured stem cells not only provide an important source of cells for *in vitro* studies, but they are also an important source of CNS cells that can be used in transplantation studies (Ray *et al*. 1997, 1998). As per our knowledge there is no information regarding isolation and characterization of NSCs of domestic animals.

Identification of appropriate sources of NSCs for isolation

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Table 1. Primer sets used in study

| Genes | Primers | Product (bp) | Tm | Accession No. |
|---|---|---|---|---|
| Nestin | F: 5'-AACGCTGAGTCATTGAGAAC-3'R: 5'-CACTGCCTCCTGGTCTTC-3' | 276bp | 60°C | NM_001206591.1 |
| Sox-2 | F: 5'-CGAGTCAAGCGGCCCATGAAC-3'R: 5'-TGGCAGCCATCTTGCGTAGG-3' | 187bp | 60°C | NM_001105463.1 |
| Pax6 | F: 5'AACAGAGTTCTTCGCAACCTGGCTAG-3'R: 5'-TGGCAGCCATCTTGCG TAGG-3' | 164bp | 60°C | NM_001040645.1 |
| GAPDH | F : 5'-GGAGAAACCTGCCAAGTATG-3'R: 3'-TGAGTGTCGCTGTTGAAGTC-3' | 126bp | 60°C | DQ152956.1 |

and expansion remains a crucial step in the effort to develop cell transplantation strategies for neurological disorders. Characterization of the self-renewal capacity of these cells as well as the assessment of their ability to generate different neuronal phenotypes should first be performed *in vitro*. Culture systems also allow for testing the cellular properties of the isolated lines, and for characterizing the different factors and conditions which could affect the fate of the expanded cells. In the next step, the cells should be tested *in vivo* in animal models. After intracerebral transplantation, the cells might behave differently as compared to the *in vitro* conditions depending on implantation site and local environment.

In present study, we describe the generation of caprine somatic NSC lines, isolated from 8 to 9 weeks old fetal cortical tissue, and immortalized. One of these clonal cell lines has been maintained in culture for more than 7 passages, with an estimated population doubling time of about 48–72 h, and shown self-renewal capacity without any alteration in proliferation rate. This cell line has also the capacity to generate different types of neurons.

## MATERIALS AND METHODS

This present study was conducted for isolation and characterization of neural stem cells from caprine fetal brain. The gravid uterus (30–45 days) of goat was collected from nearby slaughterhouse and placed in a container containing normal saline. Gravid uterus was incised to collect the fetus in a beaker containing 1XPBS. Head of fetus was decapitated and incised to get brain tissue under laminar flow cabinet. The fetal brain tissue was chopped in a plate containing Dulbecco's modified eagle media (DMEM) supplemented with fetal bovine serum (5%) and gentamycin (5µl/ml) antibiotics. Cells were separated by in-out pipetting. Cell suspension was centrifuged at 500 rpm for 5 min and collected the supernatant in a separate centrifuge tube. The supernatant was centrifuged at 1000 rpm for 5 min to get cell pellet. Cell pellet was cultured in a 25 $cm^2$ flask containing Dulbecco's modified eagle media supplemented with fetal bovine serum (15%), leukemia inhibitory factor (10 ng/ml) and antibiotics. Cells were cultured in an incubator with 95% humidity, 5% $CO_2$ at 37°C. When cells reached up to 80–90% confluence, cells were trypsinized with 0.25% trypsin and some used for the experiment and remaining cells were passage up to P4 and these cells were also used for the study.

*RNA extraction and reverse transcription:* For gene expression analysis, cells were separated from monolayer culture and washed with 1X PBS. Then, the cell suspension was transferred into 2 ml centrifuge tube for RNA isolation. The total RNA was isolated using RNA kit in accordance with the manufacturer's instructions. cDNA was synthesized using iScript select cDNA synthesis kit. After preparing cDNA, concentration of cDNA was maintained at 10.00 ng/µl and used for real time PCR study.

*Real time PCR:* Relative quantification was performed using a real time polymerase chain reaction method. Briefly, a Biorad CFXManager™ Software and EvaGreen supermix, as a double stranded DNA-specific fluorescent dye were used to determine the cDNA copy number. For amplification of genes, primers were designed in beacon software. Primers for real time PCR were Nestin, Sox-2 and Pax6 (Table 1). The real time PCR thermocycling conditions were: an initial denaturation step at 95ºC for 30 sec followed by 40 cycles of 95ºC for 3 sec, annealing at 60ºC for 10 sec. Transcript level of all 4 genes were quantified using the relative quantification method ($2^{-ÄÄCT}$) based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene GAPDH. Primers designed for Nestin, *SOX*2, *PAX*6 and *GAPDH* were optimized prior to quantification experiments using polymerase chain reaction. For these genes, the expected sizes of the products were confirmed by gel electrophoresis on a 2% agarose gel with ethidium bromide stain.

*Immunofluorescence*

To stain cell membrane, the living cells were washed with 1X PBS and fixed with 4% paraformaldehyde (PFA) for 20 min. For intracellular epitopes, cells were incubated for 5 min with 0.1% Triton X-100 to permeabilize the cell membrane, non specific sites were blocked with 10% goat serum at room temperature for 2 h, and then incubated with primary antibody (1: 300) for 3 h at room temperature and subsequently incubated with secondary antibodies (1:600) labeled with FITC for 2 h. Stained cells were covered with pro-Long gold antifade agent and observed under fluorescent microscope immediately.

## RESULT AND DISCUSSION

Cultured cells were attached to plate after 4–5 days of

initial culture and, by the end of 9–10 days cell reached 70–80% confluency. Primary colonies expressed neural stem cell markers Nestin, *PAX* 6, *SOX* 2 after passaging cells. After 4 passages, the cells also maintained the expression of neural stem cell markers (Figs 1, 2). Survival, proliferation, and differentiation of individual stem cell colonies can be monitored in the presence of growth factors in culture, allowed us to observe effects of growth factor (leukemia inhibitory factor-LIF).

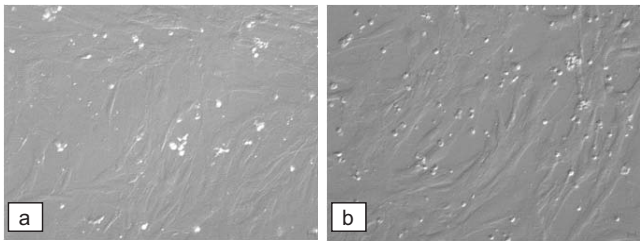


Fig. 1. Neural stem cell monolayer. (a) Passage I (P1), (b) Passage 2 (P2) (Scale bar-50μM).

The fact that human precursors were still viable after such long postmortem intervals suggests that neural precursors are relatively resistant to post-mortem ischemic and oxidative stress compared with neurons (Safar 1979). Numerous investigators have isolated and expanded neural stem/progenitor (precursor) cells *in vitro* using a variety of mitogens and culture conditions (Gage *et al*. 1995). Although stem/progenitor cells from different species respond to growth factors, alone or in combination, their growth properties differ depending on conditions (Hitoshi *et al*. 2002). Activation of the leukemia inhibitory factor (LIF) receptor reportedly promoted gliogenesis and also supported neural stem cell (NSC) renewal. LIF was soon identified to promote the self-renewal of embryonic stem cells (Smith *et al*. 1988), and it exerts numerous effects within the nervous system, including upon NSCs (Turnley and Bartlett 2000). The LIF-related cytokines have 2 seemingly contradictory effects on NSCs. Several groups have reported that LIF, or CNTF (ciliary neurotrophic factor) acts directly on NSCs, causing them to become astrocytes (Bonni *et al*. 1997). Although LIF-related cytokines increase astrocyte production at the expense of neuronal generation (Bonnie *et al*. 2007). Li *et al*. (2005) used the DMEM/F12 media supplemented with EGF, FGF and LIF for isolation of neural stem cells from human fetal striatum.

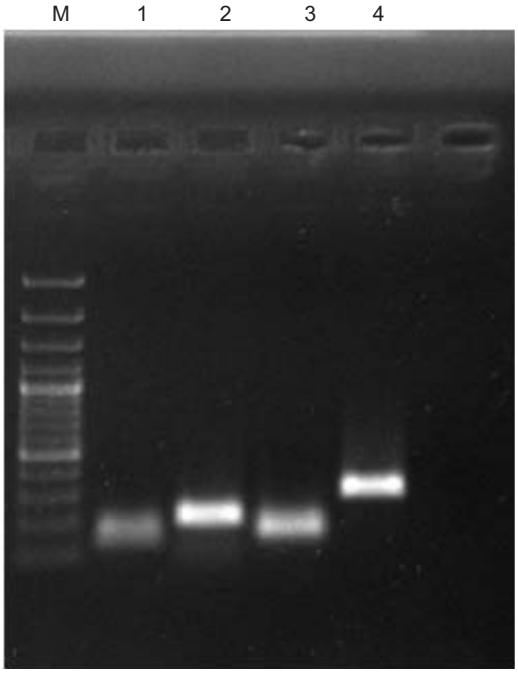


Fig. 2. Gel electrophoresis of PCR amplified product of caprine neural stem cells marker. Lane M: Marker (100bp); lane 1: GAPDH (126bp); lane 2: *SOX*2 (187bp); lane 3: *PAX*6 (164bp); lane 4: Nestin (276bp).

Under proliferation conditions, q-PCR showed expression of neuro developmental markers (Nestin, *PAX*6 and *SOX*2) and confirming their identity as NSCs. The relative expression of Nestin, *PAX*6 and *SOX*2 were 39.37, 31.49 and 11.59, respectively, compared to control media. The relative expression of Nestin and *PAX*6 were higher compared to *SOX*2 in control media. Electrophoretic analysis of final RT-PCR product on agarose gel (2%) yielded a product of 276 bp, 164 bp and 187 bp (Fig.3). Previously these markers have been described in several mammalian species as associated with neural stem cell (Tohyama *et al.* 1992, Cai *et al*. 2002, Tsai and McKay 2002), whereas there was no evidence for expression of the eye-associated gene products PAX6 (Bernier *et al*. 2001). Our results indicated that undifferentiated NSC gene have been proposed to be involved in the regionalization of the developing neocortex, and in the control of different properties of cortial progenitors such as their neuronal commitment, phenotype specification and migration (Bishop *et al*. 2002).

Immunofluorescent staining results showed that the NSC

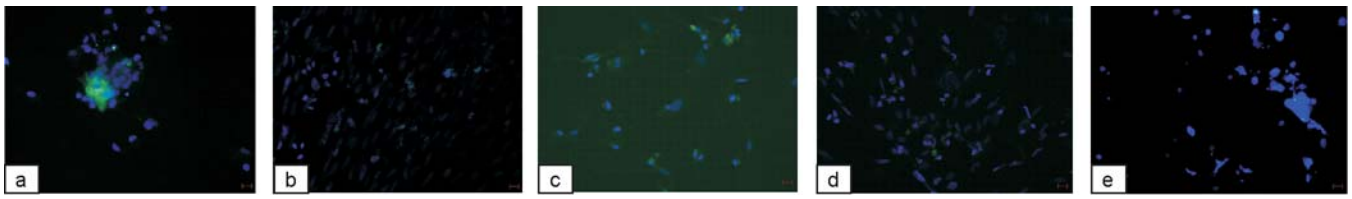


Fig. 3. (a) nestin P1, (b) nestin P4, (c) Pax6 P1, (d) Pax6 P4, (e) control.

cultures demonstrated extensive positive for sox2 and pax6 in P0 and P4 culture and no fluorescence was detected in negative controls (without primary antibody). NSC appears small, rounded epithelial like and morphologically homogeneous under phase contrast. The HMG-box transcription factors of the SOX family play roles in the maintenance of multipotency of stem cells from different tissues with a high degree of conservation across the species (Pevny and Lovell Badge 1997). Confirmation as neural precursors, cells of the cultured population expressed a number of primitive neurodevelopment markers (Tsai and McKay 2002)

The neural stem cell could be isolated from caprine striatum and expanded in adherent culture as pure population over a long time. These cells may be an ideal cell sources as alternative candidate for both research and cell replacement therapy of nervous system related disorder. These cells may be source of donor material for further therapies directed as development, degenerative, traumatic, infectious or neoplastic disease related to nervous system.

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