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Easy Isolation, Propagation, Characterization and Multilineage Differentiation of Equine Amniotic Fluid Derived Stem Cells

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Özet

İzolasyon ve uygulama kolaylığı açısından kök hücre tedavisi alanında uygun bir alternatif hücre kaynağı gerekmektedir. At amniyotik sıvı kaynaklı kök hücreler (AASKH) proliferasyon kabiliyetleri, yüksek farklılaşma kabiliyetleri ve izolasyonunda etik sorunların olmaması bakımından bilim insanları için ön plana çıkmaktadır. Çalışmada at amniyotik sıvı kaynaklı kök hücreler izole edilmiş, kültüre edilmiş ve karakterizasyonu, çoğalma kabiliyetleri ve çoklu farklılaşma potansiyelleri incelenmiştir. AASKH'lerin başarılı izolasyonunun ardından bu hücrelerin osteojenik, adipojenik farklılaştırma ve nörosfer oluşturma bakımından optimal özellikler göstermesi bu hücre tipini birçok amaç için yapılacak klinik uygulamalarda uygun bir aday haline getirmektedir. AASKH'lerin klinik uygulamalarda kullanılabilirliğini görebilmek adına bu hücre tipi hakkında yapılacak daha detaylı moleküler çalışmalar yol gösterici olacaktır.

Anahtar kelimeler: amniyotik sıvı, at, çoklu farklılaştırma, kök hücre

Abstract

Easy harvesting and handy application of stem cells therapy want convenient and alternative source. Equine amniotic fluid derived mesenchymal stem cells make hope for scientists as of no ethical concern and of higher proliferation and differentiation potency. Equine AFSCs were isolated, cultured and propagated for characterization of potency of growth and multilineage differentiation study. Successful harvesting and optimum differentiation of equine AFSCs into osteogenic, adipogenic and neurospheres through this study could make a ground for their clinical application for various purposes. Further extensive and comprehensive molecular studies are important to conclude the precious procedures for clinical approach of amniotic fluid derived stem cells.

Keywords: amniotic fluid, equine, multilineage differentiation, stem cells

Introduction

Application of stem cells in regenerative therapy is focusing on harvesting of healthy cells from convenient source with higher potency but less or no risk of immune rejection. Embryonic stem cells (ESCs), adult stem cells (ASCs) as well as induced pluripotent stem cells (IPS) are considering the three main sources of stem cells[1]. Though embryonic stem cells are considered to be more than pluripotent, there are reports of teratogenicity in human[2], destruction of embryos conflicting the ethical, political and religious issue [3] and difficult in isolation of cells. Newly programmed biologically engineered stem cells are IPS cells[4] have been successfully recorded for mouse[5], human[6], rat[7], monkey[8] and also from horse [9]. These findings hope for establishment of any mammalian cells as IPS[1]. But, unlimited division of IPS with pluripotency has got the risk of uncontrolled growth and teratoma formation[10]. Nevertheless, extensive in vitro manipulation necessary for reprogramming or genetic modification may lead to

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long-term negative effects that are yet to learn[1]. In this context, scientists and physicians are paying more attention to non-invasive and easy harvesting process of cells. Among the adult stem cells, amniotic fluid derived stem cells has drawn remarkable attention besides other sources like bone marrow[11], amniotic membrane[12], adipose tissue[13], umbilical cord[14] etc.

Amniotic Fluid Stem Cells (AFSCs) are multipotent, non-teratogenic cells of mesenchymal origin[15,16] considering the easiest tool for harvesting cells in least invasive process with closer connections with embryonic stem cells. Lately, the amniotic fluid (AF) has been acknowledged as another but underutilized source of stem cells for tissue regeneration[17]. It is mentioned as the best tool for the minimal invasive access to stem cells comparable to embryonic stem cells[18]. Cells form all three germ layers has contribution to yield a large heterologous cell types[12,19]. However, some studies suggested the origin of AFSCs may from both extra-embryonic structures, embryonic and fetal tissues having combination of differentiated and undifferentiated cells from placental membrane, fetal skin, digestive, respiratory as well as urogenital tract [20, 21]. The clear-cut verdict for the origin of AFSCs is still under investigation [22, 23]. Though some reserchers suggested cells form amniotic fluid are multipotent, some other findings considered them as intermediate stage between the potency of embryonic stem cells and lineage based multipotent adult stem cells[24]. At the transcriptional lavel AFSCs express the OCT3/4 that is one of the pluripotency marker[25]. Rsearchers[26] recorded higher proliferation ability than fetal and adult cells in in vitro study. Furthermore, AFSCs were succesfully differentiated into many other cell lineage such as adipogenic, osteogenic, myogenic, nurogenic, hepatogenic and endothelial cell types[15]. Higher proliferation rate with shorter population doubling time and higher potency for differentiation AFSCs make them a better applicant in cell therapy[27] . Additionally, expression of neurogenic marker and formation of neurospheres ascend the pathway for application of AFSCs in neurodegenerative cases[27].

The insufficiency of proper harvesting of AFSCs as well as information on composition and properties of cells from equine encouraged the augmentation of this study. Several approaches have been noticed to isolate and characterize these group of stem cells. There were record of plastic adherent round shaped epitheloid cells and spindle shaped fibroblast like cells in earlier passages though epitheloid cells disappear with the passaging time and propagation of mixed primary cell cultures[28]. Therefore, the following experiment dealt with easy harvesting protocol, morphological analysis and multiliniage differerentiation of horse AFSCs for the purpose of regenerative cyto-therapies.

Materials & Methods

Collection of equine amniotic fluid and culture of stem cells

This study was conducted following the ethical guideline and protocol of ethical committee of Afyon Kocatepe University (AKUHADYEK-01-16, 28.01.2016). Accordingly, during collection of tissues for thesis work, simultaneous collection of the amniotic fluids form three horses from a well maintained horse farm nearby city (Eskişehir) but did not used previously. To harvest amniotic fluid cells in this study, a little modification of the protocol performed by earlier scientists 1 was practiced. Briefly, using a 60 ml sterile syringe (company) having 18 gauge needle AF samples were collected from three animals immediately after seen of placental membrane with fluid just before complete coming out of foal. This fluid was transferred into 50 ml falcon tube having 24µl heparin (Nevparin, Turkey) was added at the rate of 2.4 U/ml with [29] to avoid clot formation. The AF samples were brought to Histology and Embryology laboratory, XXX at 4 ºC in sterile environment. In the laboratory, samples were diluted with Phosphate Buffer Solution (PBS) containing 2% Pen-strep (Gibco, UK) and 0.2% amphotericin-B (Biochrom GmbH, Germany) and divided into 20 ml solutions in each 50 ml falcon. Using 20 ml 70% percoll (ficoll-paque plus; sigma) solution (AF samples in PBS: Percoll = 1:1) very carefully centrifugation was performed for 30 min at 1500g at 25 ºC. Cells with a little fluid was collected from interphase of solution after carefully aspiration and discarding of supernatant that was again undergone centrifugation with 15 ml PBS at 450g for 10 minutes and pellet was collected after discarding supernatant. Then the pellet was suspended in 5 ml of low glucose Dulbecco's Minimum Essentials Medium (LG-DMEM) (Sigma, USA) containing of 15% Fetal Bovine Serum (FBS) (Biowest, South American Origin), 1% penicillin-streptomycin (Gibco, UK), 200 mM L-Glutamine (Gibco, UK) and 0.1% Amphotericin-B (Biochrom GmbH, Germany). Then again centrifugation was done at 350g for 10 minutes and washing of cells done one more time using culture medium. Afterwards, re-suspended cells were seeded into cell culture flasks (25cm2) in afore mentioned culture medium and incubated with 5% CO2 at 37 ºC in a humidified atmosphere. Every 3rd day medium was changed to remove the floating cells and to supply nutrition for growing cells. Passaging of cells performed after they reached the required confluence. Cells from 3rd passages were used for differentiation studies.

Population Doubling Time

After primary cultures, cells were trypsinized with 0.05% trypsin-EDTA (Sigma, USA) counted and re-seeded at 25

cm2 flasks at a density of 20×103 cells/ cm2 under 5% CO2 in humidified incubator at 37 °C. Every three days interval complete renewal of medium was done till cells become 80-90% confluence. Thus the AFSCs were cultured for population doubling potential from passage 1 (P1) to passage (P10) using trypsin at afore mentioned density. For each passage, three flasks were cultured and counted to find out the mean of population doubling time (PDT) that was detrmined with the help of following formula [30].

PDT(h)=Duration of culture(h)*log(2)/(log(Final concentration (M)of cell)-log(Initial concentration (M)of cells))

Growth Curve

Using four-well culture plate (Thermo Scientific, Denmark) cells were seeded at the density of 8×103 cells/ cm2 to find out the behavior of growth of AF-MSCs. Cellular proliferation was observed for consecutive 14 days and in every 2 days interval cells from two wells were trypsinized and counted by Neubauer chamber and Trypan blue dye. To guarantee the optimum growth of cells, the cultured medium was changed at every 3rd day completely.

In-vitro Multilineage Differentiation

Cells from Passages 3 and passage 5 having 80-90% confluence were considered and cultured at density of 15×103cells/cm2 in specific differentiation culture medium for induction of osteogenic and adipogenic cell differentiation. There was a control group cultured under normal cell culture medium for each type of differentiation.

Osteogenic Differentiation

Osteogenic differentiation medium containing α-MEM (Lonza, Belgium) with 10% FBS (Biowest, South America Original), 1% Pen-strep (Gibco, UK), 0.1% Amphotericin-B (Biochrom GmbH, Germany), 2 mM L-gmutamine (gibco, UK), 0.1 µM dexamethasone (sigma, Belgium) 0.05 mM ascorbic acid (Dr.ehrenstorfer GmbH) and 10 mM β-glycerophosphate (gibco, UK) were used. AFSCs were cultured into 24-well cell culture plate at 29×103 cells/ cm2. Conditions of cells were checked regularly under inverted microscope and medium was changed at regular interval of 72 h. Cultured cells were fixed with 4% neutral buffered formalin after discarding of medium at 21 day. Intercellular calcium deposition as a result of osteogenic differentiation was confirmed by Alizarin Red S (Merck, Germany) staining of formalin fixed cells [31]. Photographs were taken using DP-25 software under inverted microscope.

Adipogenic Differentiation

AFSCs were cultured at the density of 29×103 cells/cm2 for execution of adipogenic differentiation into 24-well cell culture plate using adipogenic induction medium having LG-MEM (Sigma-Aldrich, USA) along with 10% FBS (biowest, South America Original), 1% Pen-Strep (Gibco, UK) 01% Amphotericin-B (Biochrom GmbH, Germany), LG-DMEM (Sigma-Aldrich, USA) containing 10% FBS (Biowest, South America Original), 0.5 mM 3-isobutyl-1-methylxanthine; IBMX (Sigma, Germany), 1 µM dexamethasone (Sigma, Belgium), 10 µg/mL Insulin (Sigma-Aldrich, Germany) and 100 µM indomethacin (Cayman Chemical Company, USA) were considered. The cultured continued for 21 days long and the adipogenic culture medium was changed at every 3rd day. At the day 7 and 15, the adipogenic induction medium was replaced with adipogenic maintenance medium having LG-DMEM (Sigma-Aldrich, USA), 10% FBS (Biowest, South America Original), 1% Pen-strep (Gibco, UK), 0.1% Amphotericin-B (Biochrom GmbH, Germany) and 10 µg/mL insulin (Sigma-Aldrich, Germany) according to the instruction of the cell culture manual (Cat# SCR020, Chemicon® International). After fixation with neutral buffered formalin, the cells at day 21 stained with (0.1% in 60% isopropanol) 5 mM Oil Red O (Sigma-Aldrich, USA) to recognize the intracellular cytoplasmic fat droplets[32]. Under inverted microscope photographs were taken using DP-25 software.

Neurospheres generation

Firstly, four-well cell culture plate was coated with 0.01% poly-L-Lysine (Sigma, USA) and dried. AFSCs from passages 3 and passage 5 were cultured at a density of 5×104cells/cm2 using DMEM/F-12 (Gibco, UK), 1% Pen-strep (Gibco, UK), 0.1% Amphotericin-B (Biochrom GmbH, Germany), 1% Insulin-Transferrin-Selenium (ITS) (Gibco, USA), 20 ng/mL EGF (Sigma, USA), and 20 ng/mL FGF (R&D system, USA) as culture medium in 95% humidity and 5% CO2 at 38.5 °C. In every 48 h half of the culture medium was renewed with care to avoid aspiration of formed neurospheres. The neurospheres differentiation was monitored at 24h, 50h, 70h, 90h, 100h, 120h, and 180h and photograph was taken under inverted microscope using DP-25 software. There were 3 replicates for each.

Statistical Analysis

Data were recorded and all analysis was accomplished by using SPSS v4.1 software. One way ANOVA followed by bonferroni was performed for population doubling time analysis.

Results

Harvesting of AFSCs and cellular proliferation

At P0, the AFSCs showed dissimilar shape and sizes of cell types coming from different sources. However, cells were

recorded with rapid growth in LG-MEM with higher of foetal bovine serum. Usually cultured cells took 7-8 hours for plastic adherence to culture flasks after seeding. Though many cells remain unattached at first seeding, the floating cells normally get removed upon changing of culture medium. It took slight extended time for the formation of the niche at P0 but in P1 and subsequent passages it is shorter. During P0, cellular morphology may be epitheloid (Figure1B) or spindle shaped (Figure1A) and fibroblast like cells (Figure1A, C, D, E, F)) demonstrating separate origin of cells. Cellular morphologies progressively became stable from P2 onward having fibroblasts like appearance (Figure- 1C, D, E, and F).

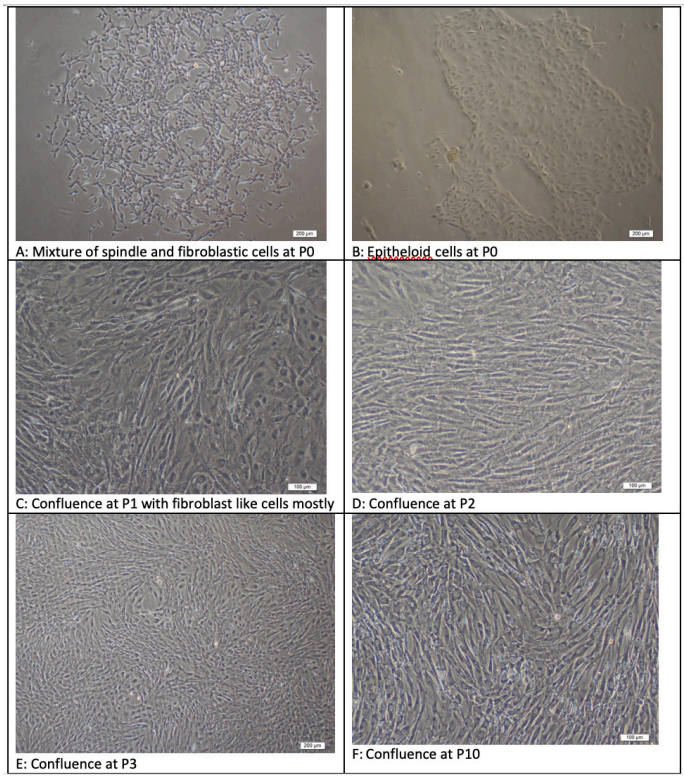

Figure 1: Morphology of equine AFSCs at primary and subsequent cell culture. Figures showed nearly cells of similar morphology except a few dissimilar cell types at earlier stages of passages

Proliferation pattern

The growth curve diagram was produced by plotting the number of cells in mL against time over a period of 14 days. Here the initial phase was noticed for 2-4 days following the subsequent log phase for 4th-8th days in with an inclined plateau of 8-12th and then declined (Figure 2). The symmetrical uprising skew from 4-8th day of culture expresses unimodal morphology.

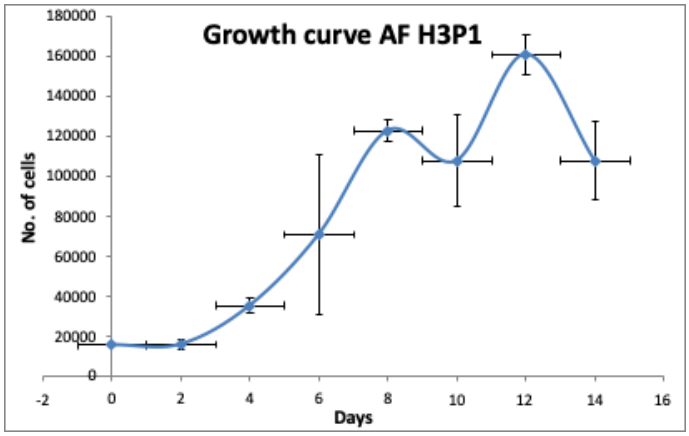

Figure 2: Growth curve of horse AFSCs at P3. After day 12 there was decrease in proliferation potency of cells.

Population Doubling Time

The results of one way ANOVA with bonferroni presented a variation from P1 to P10 for equine AFSCs population doubling time (PDT) ((Figure 3). The PDT graph revealed that P3 yielded maximum number of cells in the shortest time period where as P6 took the longest time period. Here P3 expressed significantly difference ($p< 0.05$) value from P5, P6, P7 and P10 where there was absence of noteworthy and significant difference ($p> 0.05$) from P2 and p4.

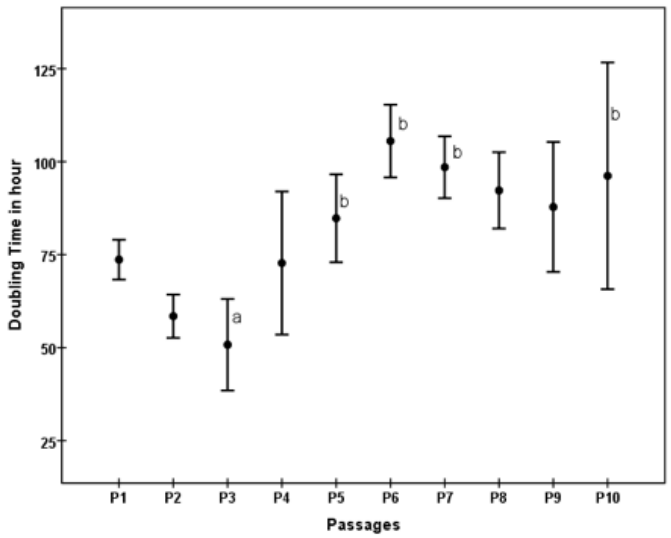

Figure 3: Population doubling time of horse amniotic fluid. P3 exhibited the shortest time for PDT. Difference in the superscript significantly difference ($p<0.05$, Bonferroni)

Osteogenic differentiation

As, the AFSCs are multipotent in nature they were effectively differentiated into osteogenic lineage. During differentiation study, changing of morphology was noticed from 5/6th day onward and became larger and polyhedral. The extracellular deposition of calcium was confirmed by Alizarin Red S at day 21. The bright red color confirmed the extracellular calcium deposition (Figure 4B). However, the negative control cells remained confluent and undifferentiated under the influence of complete growth medium

(Figure 4A).

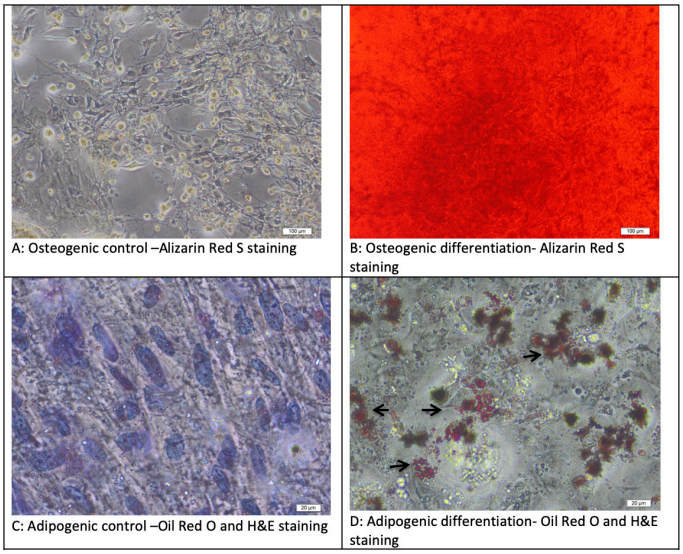

Figure 4: Osteogenic and adipogenic differentiation potential of equine AFSCs. (A) Control: AFSCs not showing any extracellular calcium deposition after Alizarin Red S staining in cells from P3 (Bar=100µm) (B) Experiment: AFSCs demonstrating extracellular calcium deposition after Alizarin Red S staining at P3 (Bar = 100µm). The strong red stained confirmed the secreted calcium by differentiation of osteocytes from AFSCs. (C) Control: AFSCs after Oil Red O staining derived from P3; No fat droplets seen in negative control (Bar = 20 µm) (D) Experiment: AFSCs after Oil Red O staining at P3; Red colored fat droplets observed in cytoplasm pointed by arrow heads (Bar= 20 µm).

Adipogenic differentiation

In adipogenic differentiation study, cell morphology was changed after one week of induction medium in more apparent. Cytoplasmic vacuoles were noticed as intracellular lipid droplets became developed. To confer the adipogenic differentiation, the cells were stained with Oil Red O at Day 21. The neutral formalin fixed Oil Red O stained cells exhibit clear presence of red colored lipid droplets in the cytoplasm in experimental group (Figure 4D) whereas the control group remained unaffected after staining that was cultured in complete growth medium (Figure 4C).

Neurospheres characterization

There were record of a mix population of neurospheres from AFSCs using specific neurosphere induction medium on lysine coated culture plate after 24h of culture from P3 (Figure 5A). The neurospheres were spheroid to oval in shape, three-dimensional structures of variable sizes. Gradual increase in the size of neurospheres was observed approximately from 24 to 70h post generation (Figure 4B, C) and gets fully matured. Near about 90h neurospheres (Figure 4D) became free floating spheroids during culture. Later on, neurospheres were started to disintegrate (Figure 4E). After 180h neurospheres (Figure 4F) were almost disappear leaving few cells under the influence of neurospheres medium.

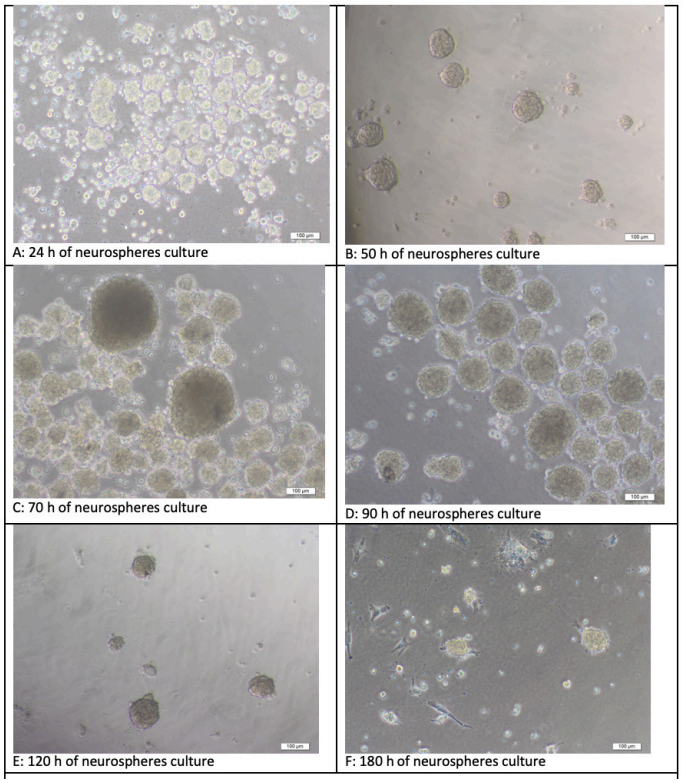

Figure 5: Morphology of neurospheres generated from equine amniotic fluid derived stem cells. A, B, C, D, E, F: AFSCs yielded neurospheres from P5 at 24h, 50h, 70h, 90h, 120 h and 180h respectively (Bar = 100µm).

Discussion

As an ideal stem cell source, the equine amniotic fluid is of great importance due to opportunity of non-invasive and non-surgical procedures for their collection. This experiment mainly intended for morphological characterization of amniotic fluid derived stem cells, their stemness via multilineage differentiation approach. The harvested AF derived stem cells were recorded for pretty good propagation and displayed better adherence properties cultured in 15% foetal bovine serum using predefined culture environments; however there are record of floating cells in some extent that did not adhere with plastic surfaces at all. Iacono at al.1 and Gao et al. [34] have recorded similar results of fibroblast like cellular morphology from amniotic fluid source using similar type's culture medium. The variance of cellular morphology along with fibroblast appearance such as spindle-shaped cells, circular flat cells even neuron like ling cells also reported by aforementioned researchers. Some researchers [35] reported three types of cells such as epithelial cells, amniotic cells and fibroblastic cells in amniotic fluid. Scientists [36] mentioned that all three layers of amniotic membrane have contribution to form amniotic

fluid and its derivatives thus make differences in morphologies of cells. The morphology of cells became fibroblastic shape mostly after initial 2-3 passages along with their proliferation. Inversely relation of cellular proliferation with advancement of passage number has noticed in this study after P4 which suggest the application of AFSCs in equine may be suitable in earlier passages. Previous findings of many workers1 revealed that after P3 equine AF derived stem cells a little bit longer time for their proliferation. Interestingly, this study got P3 with the shortest population doubling time that may differ from Iacono et. al (2012). However, other researchers [37, 27] also mentioned similar findings regarding their experiment of bovine amniotic fluid.

AFSCs reserve the potency of multilineage differentiation that was also revealed after osteogenic and adipogenic differentiations in this study. Intense red stained after Alizarin Red S and glittering red intracellular oil droplets confer their differentiation into osteogenic and adipogenic cell. Many researchers reported similar results with their experiments. Earlier works 1 expressed similar records with equine amniotic fluids. The previous reports [37, 34] also showed that culture of bovine AFSCs in respective differentiation medium was successfully differentiated into osteogenic and adipogenic lineage, analogous to this study. Furthermore, potent differentiation of horse amniotic fluid to neurospheres could stand for a promising source for application of AFSCs in neurodegenerative disorders. Under well-defined environment and medium effective differentiation to neurospheres in this study correlated with other findings. Gao et al. [34] described after plating of bovine amniotic fluid cells at specific clonal densities yielded neurospheres of varying sizes in 20 hours of post induction. Scientists [38, 27] mentioned successful differentiation of neurospheres from bovine adipose and bovine amniotic fluid using specified medium after 72 hours and 24 hours, respectively. Heterogeneous population of neural stem cells and their progenitors could be generated from developing neurospheres having potency for rising several neuronal cells, astrocytes and oligodendrocytes in appropriate differentiation induction [39]. Also, the active and quiescent cells in neurospheres can reestablish to stem cell state. Nevertheless, dissimilarity in the size of neurospheres developed after differentiation can be thought an important clue to their future lineage differentiation [27].

Conclusion

Harvesting of amniotic fluid stem cells by minimizing obstacles of inherent uric acid crystals in fluid in this study rendered the non-invasive and easy source of stem cells. The multilineage differentiations along with neurospheres generation with their proliferation potential revealed them as a prospective candidate for regenerative and replenish medicine. However, the exact application mode and procedures along with improved knowledge on cellular and molecular scenario is utmost important for application.

Conflict of Interest: There is no conflict of interest.

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