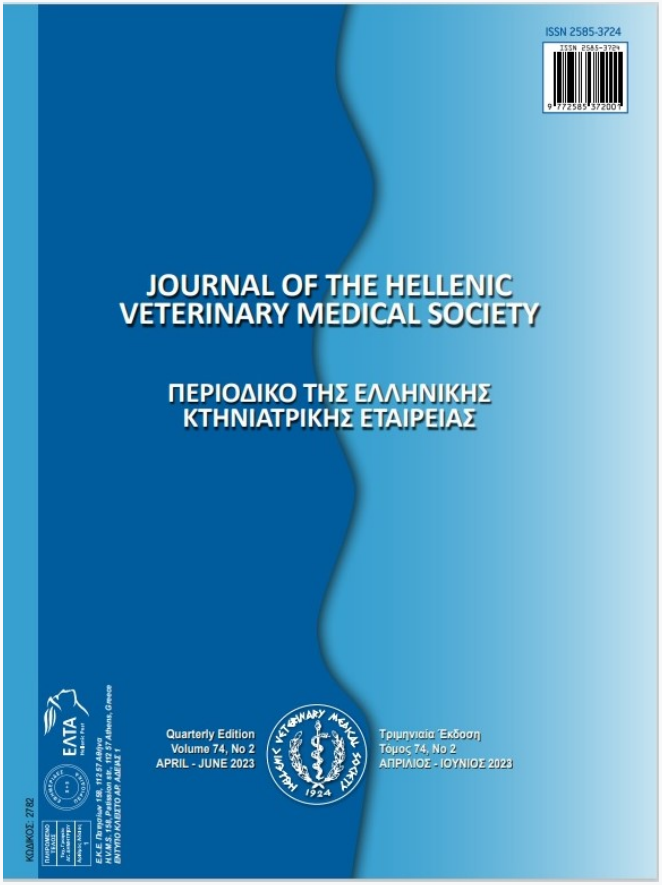
Journal of the Hellenic Veterinary Medical Society

Vol 74, No 2 (2023)



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doi: 10.12681/jhvms.28390

To cite this article:

Dikmen, T., Özden Akkaya, Ö, Nawaz, S., & Erdoğan, M. (2023). Characterization of Bovine Bone Marrow Derived Mesenchymal Stem Cells and Immunostaining of Differentiated Neurospheres . *Journal of the Hellenic Veterinary Medical Society*, *74*(2), 5599–5608. https://doi.org/10.12681/jhvms.28390 (Original work published July 4, 2023)

J HELLENIC VET MED SOC 2023, 74 (2): 5599-5608
ΠΕΚΕ 2023, 74 (2): 5599-5608

Research article
Ερευνητικό άρθρο

Characterization of Bovine Bone Marrow Derived Mesenchymal Stem Cells and Immunostaining of Differentiated Neurospheres

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ABSTRACT: Stem cell differentiation has been a key element for the therapeutical applications for various disorders. The novel anti-inflammatory and angiogenic findings and potential applications for both autologous and allogenic studies have emphasized about the usage of mesenchymal stem cells in the clinical trials. The neurosphere differentiation studies and their role for peripheral nerve injuries have gained momentum recently. Following study emphasized on the isolation of stem cells from bovine bone marrow and characterization of their mesenchymal potential by PCR followed by their differentiation into mesodermal lineages and into neurospheres under specific induction media. The generated neurospheres were further investigated for their potential expressions of neuroprogenitor markers Sox2, Nestin and neurofilament protein β-III Tubulin by immunofluorescence staining. The results of following study highlight the unique potential of bone marrow derived mesenchymal stem cells for translational research and regenerative therapy in nervous tissue injuries.

Keywords: ß-III tubulin, bone marrow derived stem cells, bovine, nestin, neurospheres, sox2.

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Date of initial submission: 21-10-2021
Date of acceptance: 31-10-2022

INTRODUCTION

The advances in the field of stem cell biology have opened new horizons for applications in the field of regenerative therapy during past decades. The breakthroughs in stem cell therapy requires the need of stem cells with broad future prospects to continue its legacy in tissue engineering and in chronic and reformative disease extenuation both in applied veterinary and human medicine. Adult tissues derivative stem cells are declared as the most promising contenders for regenerative therapies because of low to moderate immunogenicity and high plasticity **(**Gao et al., 2014**)**. Mesenchymal stem cells (MSC) signify a class of progenitor cells with specific potency that are located in the stroma of the bone marrow, facilitating the hematopoiesis and could differentiate into different mesenchymal extractions (Bosnakovski et al., 2006).

The differentiation capability of MSCs to constitute many tissues such as adipose tissue, cartilage, bone, etc. has been previously well recognized during various experimental studies (Friedenstein et al., 1974; Pittenger et al., 1999). So far, the cells featuring mesenchymal precursors have been derived from the bone marrow of various mammals, including experimental animals (Friedenstein et al., 1974), humans (Pittenger et al., 1999), felines (Martin et al., 2002), canines (Huss et al., 1995), pigs (Ringe et al., 2002) and bovines (Bosnakovski et al., 2006; Dueñas et al., 2004; Lee et al., 2015). There has been limited information available regarding the use of bovine bone marrow derived stem cells (BM-MSCs) and their use in neurological studies. Previous reports suggest that although it is cost efficient and easy to preproduce experimental animals; using large animal models for translational studies such as bovine could be beneficial since inbred laboratory animal models lack genetic variations and complexity as human populations have and large animals share comparable organ sizes and equivalent physiological properties (Harding et al., 2013; Ireland et al., 2008; Roth and Tuggle, 2015). So, it was assumed that the bovine model for stem cell isolation studies could be considered as good experimental model for long term studies and trials as compared to laboratory animals. In addition, the outcomes of such studies could also help facilitating new approaches in veterinary medicine as well.

According to minimum essential criteria which have been the characteristic feature for MSCs, the BM-MSCs have been reported to express the common mesenchymal markers like CD105, CD73 and CD90 and lack the expression of haemopoietic markers like CD45, CD34, CD14, CD11b, CD19 and HLA-DR surface markers (Cortes et al. 2013; Dominici et al. 2006; Duenas et al. 2014). Previously, it has been described that bovine BM-MSCs successfully differentiate into mesodermal cell types such as chondrocytes, adipocytes, osteoblasts (Bosnakovski et al., 2003; Bosnakovski et al., 2006; Friedenstein et al., 1966; Hill et al., 2019) as well as into endodermal (hepatocytes) and neuroectodermal (neurons) cells (Dueñas et al., 2014).

As neural tissue does not possess self-renewal properties, stem cell therapy is being considered as a great hope for neural disorders. Neurospheres are free-floating, spherical bodies which consist of neural precursor cells and can be differentiated from stem cells. Neurospheres are known to have capacity to give rise to different types of neural cells such as neurons, astrocytes, etc. (Reynolds and Weiss 1992). Therefore, a better understanding of neurospheres holds a great importance for neurodegenerative medicine. Previous studies have reported the neurosphere generation from adipose and bone marrow derived stem cells (Chung et al., 2013) and olfactory mucosa derived stem cells (Altunbaş et al., 2015) in canines yet studies in larger animal models are still rare. One of the previous studies in bovines indicated the neurogenic differentiation potential of bovine BM-MSCs using serum-based induction media however did not characterize the cells for neurosphere specific markers including Sox2 and β-III tubulin (Dueñas et al., 2014). As all *in vivo* applications require the serum-free inoculation of stem cells and their derivatives, testing a serum-free approach for generating neurospheres is crucial for road mapping the futuristic approaches in the same area. With this point of view, we previously tested the potential of bovine adipose derived stem cells (Akkaya et al., 2019) and amniotic fluid derived stem cells (Nawaz et al., 2020) to generate Sox2 and ß-III Tubulin positive neurospheres using serum-free induction medium. In this study, an another source was used as bovine bone marrow derived mesenchymal stem cells were isolated. After confirming their mesenchymal character, their ability to perform neurospheres were tested. A serum free neruosphere differentiation media with the supplementation of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) was preferred and immunostaining assays were performed to highlight the expressions of Sox2, β-III Tubulin and Nestin.

MATERIALS AND METHODS

Isolation and Culture of Bone Marrow Derived Cells in Bovines

All procedures opted during this study were performed after the consensus of the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-50-19; 30.04.2019). Briefly, bone marrow was aspirated from bone marrow cavities of sternum by using Jamshidi biopsy needle in a local abattoir of Afyonkarahisar province, Turkey soon after slaughtering the animal. The bone marrow samples were transferred into 50 ml tubes (VWR, USA) containing Modified Eagle Medium (MEM, Gibco, UK) supplemented with 1000 U heparin (Nevparin, Turkey). Bone marrow samples were centrifuged at 1000 rpm in centrifuge machine (NÜVE NF 800R) for 10 minutes. Then, the supernatant was discarded and the samples were centrifuged again at 350 g for 5 minutes each twice after adding 2 ml MEM. Later, the cells were plated in MEM complemented with 10% FBS (Biowest, South America Origin), 1% penicillin-streptomycin (10.000 U/ml) (Gibco, UK) and 0.1 % amphotericin-B (0.25 mg/ml) (Biochrom GmbH, Germany) and 2 mM/ml L-Glutamin (Gibco, UK). Cells were allowed to proliferate at 37°C in a CO_2 incubator containing 5% CO_2 in a humidified atmosphere. Following the first 2 days of initial cell seeding, the medium was changed in every 2–3 days intervals. After cells reach 80-90% confluence, cells were passaged by using 0.25% trypsin in 0.1% EDTA (Capricorn, Germany).

Molecular Characterization Studies of Undifferentiated Cells.

The BM-MSCs were cultured till the 3rd passage and onward. Around 1.5 $\times 10^6$ cells from all animal samples were centrifuged and stored at -80 °C for PCR studies after trypsinization. RNA isolation procedures were accomplished by using a commercially available kit (GeneJet RNA Purification, Thermo K0732). RNase free DNase-I enzyme (Thermo Scientific, EN0525) was used to remove DNA contamination from isolated RNA samples. Maxima First Strand cDNA synthesis kit (Thermo Scientific, K1672) was used to perform cDNA synthesis. PCR primers were designed by using FastPCR 6.0 (Kalendar et al., 2009) computer software. All primer base pairs and Tm values of used genes are given in Table 1. Specific expressions of mesenchymal (CD73) and hematopoietic and endothelial (CD34, CD45, CD74) genes were investigated by PCR analysis on undifferentiated cells.

Differentiation Studies

Osteogenic Differentiation

Differentiation protocol was conducted as previously described by Jaiswal et al. (1997). BM-MSCs were collected after 3rd passage and cells were plated into 4-well plates at a concentration of 9000 cells/cm^2 in growth culture media. After the cells reached the confluence, the osteogenic induction media which consisted of 10% FBS, 1% penicillin-streptomycin, 0.1% amphotericin-B, 50 µg/ml L-ascorbic acid (Sigma, USA), 10 mM β-glycerophosphate (Gibco, UK), 0.01 µM dexamethasone (Sigma, USA) in α-MEM (Lonza, Belgium). The media was changed in every 3 days and the osteogenic differentiation was executed till the 21st day. The osteogenic differentiation was demonstrated by Alizarin Red-S staining.

Alizarin Red-S Staining

For demonstrating calcium deposits as a result of osteogenic differentiation; Alizarin Red-S staining was performed. For staining, the culture media was aspirated carefully and cells were rinsed with Hank's Balanced Salt Solution (HBSS) (Sigma, USA). Then cells were fixed by 4% paraformaldehyde (Merck, Germany). After fixation, the paraformaldehyde was discarded and cells were washed with distilled water. Afterwards, cells were stained with 80 mM Alizarin Red S (pH 4.2) (Merck, Germany) in deionized distilled water for 30 minutes. After 30 minutes, the staining solution was washed carefully and plates were observed under inverted microscope. The calcium deposits were observed in orange-red.

Table 1. The list of genes used in the PCR analysis, with primer sequence, base pairs length and temperatures.

Gene	Forward (5' →3')	Reverse (3' →5')	cDNAbp	Tm °C
CD73	GGAAATCCCATTCTTCTCAACAGC	AATCAGATTGCCCATGTTGCA	180	60
CD74	TGGCTCTTGTTTGAAATGAGCA	CGGCATATAGTTGCCGTTCTCG	159	60
CD34	TTGCACTGGTCACCTCGGGGA	ATAGCCCTGGCCTCCACCGTTCTC	143	60
CD45	TAAACGGAGATGCAGGATCAA	TCTGCTCCCAGATCATCCTCC	135	60

Adipogenic Differentiation

After the 3rd passage, BM-MSCs were cultured into 4-well plates at a plating density of 9000 cells/cm^2. Cells were cultured in growth media till they reached confluence. After reaching confluence, cells were cultured with adipogenic induction media (AIM) and adipogenic maintenance media (AMM) for 21 days. The AMM was only used at 7th and 15th days of differentiation in order to maintain adipogenesis while the AIM was used in the rest of the study. The AIM consisted of 10% FBS, 1% pen- streptomycin, 0.1% amphotericin-B, 1μM dexamethasone, 500 μM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, Germany) 10 μg/mL insulin (Sigma) in MEM. On the other hand, AMM consisted of 10% FBS, 1% penicillin-streptomycin and 10 μg/mL insulin in MEM. After 21 days, Oil Red O staining was performed in order to demonstrate oil droplets.

Oil Red O Staining

After 21 days of differentiation, the differentiation media was discarded and cells were rinsed first with PBS and then with 70% ethanol. Afterwards, the cells were fixed with 10% buffered neutral formalin for 20 minutes. Post fixation, cells were rinsed with PBS and with 70% ethanol again. After washing the cells, Oil Red O stain was applied for 25 minutes. The stock solution for Oil Red O (12 mM Oil Red O) (Sigma-Aldrich, USA) in isopropyl alcohol was diluted with deionized distilled water in 2/3 ratio to be used in staining procedure. After 25 minutes, staining solution was discarded and cells were washed first with 70% ethanol and then with deionized distilled water. Counter staining was done by Harris' Haematoxylin for nuclear staining. Similar studies and protocols have been performed already (Lillie and Ashburn, 1943; Pittenger et al., 1999; Strutt et al., 1996).

Neurospheres generation

The neurospheres culture protocol was adapted from Bez et al. (2013) with some modifications. Briefly, the BM-MSCs after trypsinization from the 3rd passage were collected and cultured in DMEM/F-12 (Gibco, UK), containing %1 insulin-transferrin-selenium (ITS) (Gibco, USA), 50 ng/mL EGF (Sigma, USA), and 50 ng/mL bFGF (R&D Systems, USA) at 5×10^4 cells/cm^2 density on 0.01% poly-L-Lysine (Sigma, USA) coated 4-well plates. Cells were allowed to differentiate in neurospheres under optimal conditions. To prevent the nutrient depletion, the half of the medium was replenished every 2nd day.

Neurospheres Collection and Immunofluorescence

Neurospheres were stained for particular markers by immunofluorescence studies as previously described by Sasaki et al. (2010). Neurospheres were collected at 72h post differentiation and were allowed to pool down. After pooling, cells were washed with PBST (Phosphate buffer saline containing 0.1% Tween 20 (BioRad, USA) three times. Later the neurospheres were fixed by applying 4% paraformaldehyde into the wells for 10 min. After fixation, the neurospheres were again washed with ice-cold PBS for three times and later treated with 0.1% Triton X-100 (MP Biomedicals-France) in PBS. After washing step with PBST again, the blocking was performed using 10% serum from the species in which the secondary antibodies were raised. In this case, the blockings were made by applying 10% goat serum (Abcam-UK, ab7581) for 30 minutes at room temperature. Anti-Nestin (1:250, Mouse Monoclonal; Millipore-mab5326), anti-β-III tubulin (1:300 Mouse monoclonal; Abcam-UK, ab78078) and anti-Sox2 (1:50 Rabbit polyclonal; Abcam-UK, ab5603) primary antibodies were used after diluting in PBST with 1% bovine serum albumin. The secondary antibodies used were, Goat anti Mouse Texas Red conjugated (1:100; Abcam-UK, ab6787) for Nestin and ß-III tubulin and Goat anti Rabbit Alexa fluor 488 conjugated (1:200; Abcam-UK, ab150077) for Sox2. The secondary antibody solutions were diluted in PBS with 1% BSA and all the manipulations were performed in a dark room. The nuclear staining was performed by applying DAPI fluoroshield mounting medium (Abcam, UK, ab104139) on neurospheres for 1 minute. The immunofluorescence expressions were checked immediately after the staining by Zeiss Axio Observer Z1 inverted microscope.

RESULTS

The bovine BM-MSCs represented a plastic adherent population of cells which proliferate easily under suitable culture conditions. At the initial passage, cells exhibited fibroblast-like morphology after the elimination of lymphocyte-like cells within first 1 or 2 medium changes and the fibroblast-like morphology stayed persistent in further passages (Figure 1; A, B). Cells were cultured till the 3rd passage for further analysis.

To prove the mesenchymal and non-immunogenic character of the cells, samples were collected at the 3rd passage and PCR analysis were performed to investigate mesenchymal marker CD73, hematopoietic and

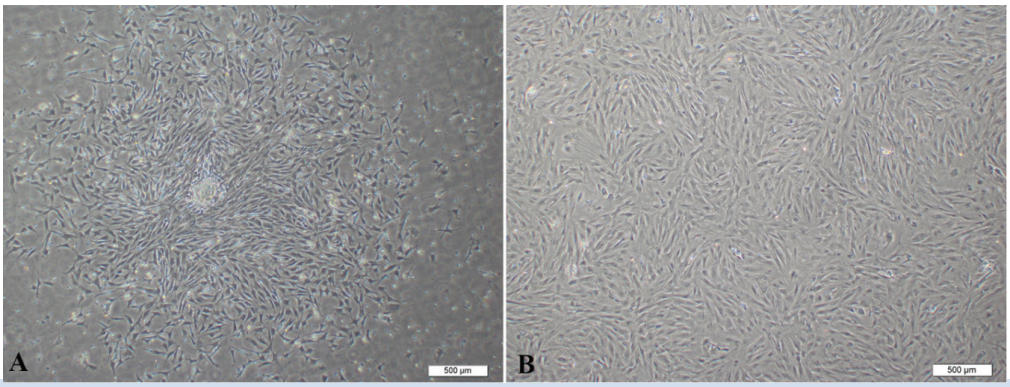

Figure 1. Morphological structure of BM-MSCs at different passages. (A): Fibroblast-like cells at P0, (B): Fibroblast-like morphology at P3. Bar = 500 µm

endothelial markers CD34, CD45 along with HLA-DR receptor marker CD74. While the mesenchymal marker CD73 gave positive results; CD34, CD45 and CD74 markers were negative (Figure 2).

Figure 2. BM-MSCs exhibiting positive expression of mesenchymal marker CD73 and negative expression for CD34, CD45 and HLA-DR surface marker CD74

The bovine BM-MSCs were also conducted to multi-lineage differentiation studies. For this purpose, cells were successfully differentiated into adipogenic and osteogenic lineages. Cells were differentiated with specific induction mediums for 21 days for both studies. After the process, lineage specific staining was performed to prove differentiations. Both differential staining demonstrated positive results and confirmed osteogenic and adipogenic differentiation capacity of BM-MSCs into desired cell types (Figure 3). In addition to that, BM-MSCs were also differentiated

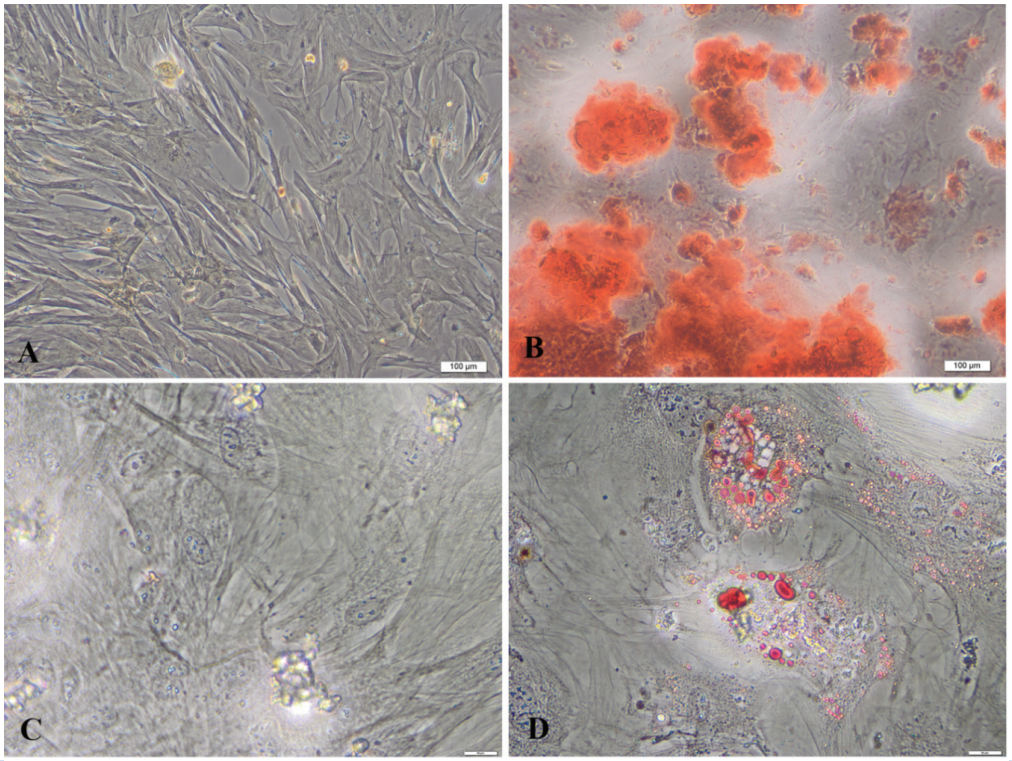

Figure 3. Staining of BM-MSCs for osteogenic and adipogenic differentiation at Day 21 (A): Negative control group for osteogenic differentiation after Alizarin Red-S staining. (B): Osteogenic differentiation in BM-MSCs; stained for calcium deposits in orange-red colour with Alizarin Red-S; Bar = 100 µm. (C): Negative control group for adipogenic differentiation after Oil Red-O staining. (D): Adipogenic differentiation in BM-MSCs; oil droplets stained in bright red colour with Oil Red-O staining; Bar = 50 µm.

into neurospheres in serum free media with the influence of bFGF and EGF. Upon application of induction media, the bovine BM-MSCs differentiated into free floating, spherical and three dimensional neurospheres. There was no differentiation as early as 24h post induction (Figure 4A). After 48h post induction free floating, three-dimensional spherical bodies were visible (Figure 4B). The generated neurospheres were round to oval in shape and few carried minute outward projections as well. Neurospheres appeared to have variations in size which varied with duration of induction, however size of the neurospheres were not recorded. The neurospheres attained the maximum size after 72h where only minute difference in the size were observed apparently. Besides, it was found there were populations of BM-MSCs which remain un-induced even at 48 hours of study. All the neurospheres analyzed through immunofluorescence studies were found to be positive for their characteristic markers including Sox2, Nestin and β-III Tubulin protein expressions after 72h of differentiation (Figure 5).

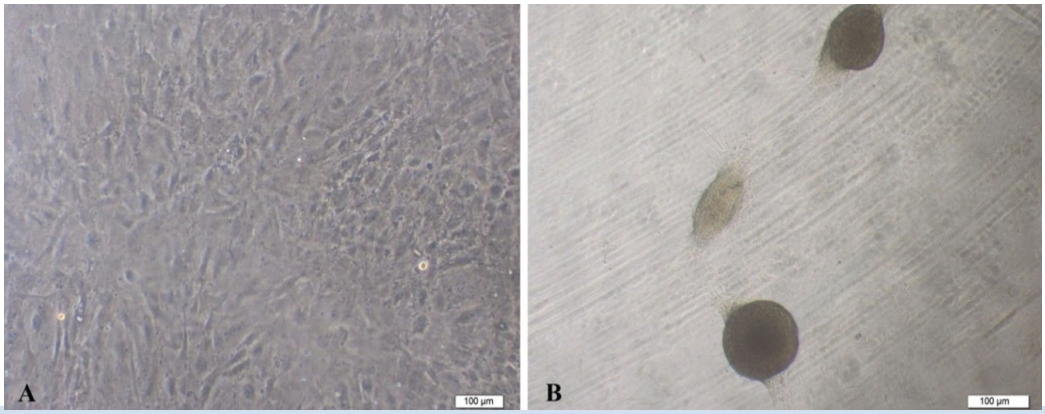

Figure 4. Generation of neurospheres at different time intervals. (A) BM-MSCs at 24h post induction; (B) Generated neurospheres at 48h post induction. Bar =100 µm.

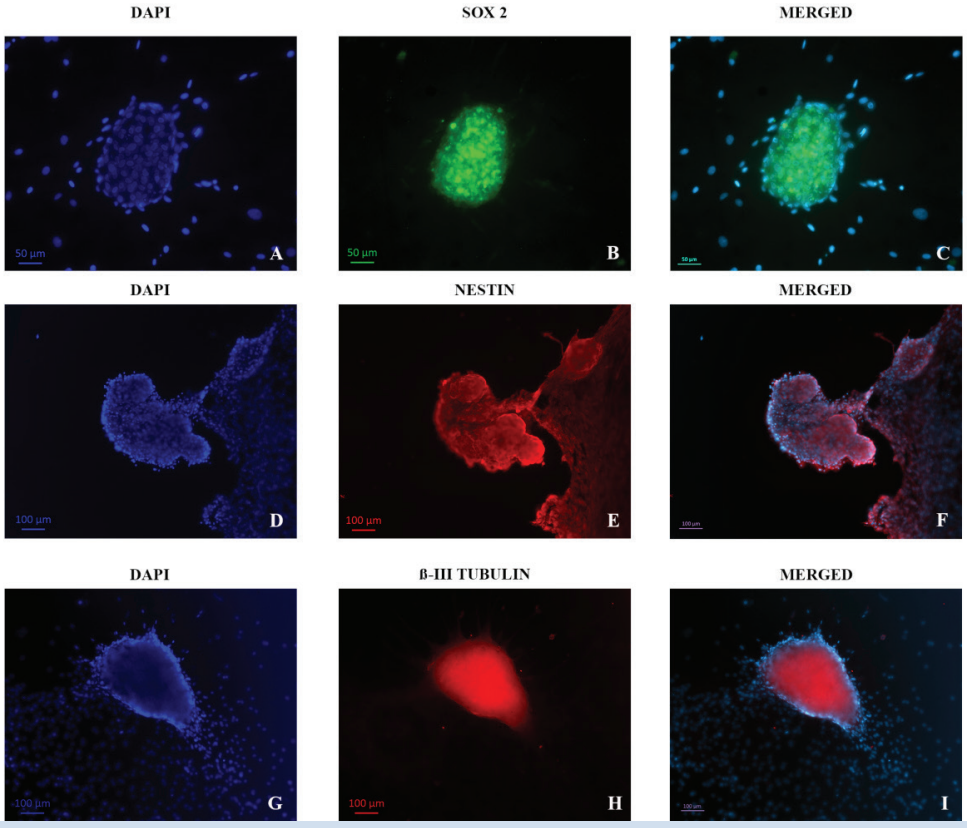

Figure 5. Immunofluorescence staining of BM-MSCs derived neurospheres. (A), (D), (G): Nuclear staining with DAPI. (B) anti-Sox2 (E) anti-Nestin (H) anti-ß-III Tubulin (C), (F), (I) Merged images. Bar =100 µm.

DISCUSSION

The current study focused on generation of neurospheres from bovine BM-MSCs and confirmation of their neurogenic potentials at early stages. For that, BM-MSCs were isolated and their mesenchymal character was confirmed. Then a neurosphere differentiation study was performed under serum-free conditions and the neural character of the collected neurospheres were affirmed by IF.

The bovine BM-MSCs represent an adherent population of cells which propagate easily upon suitable culture conditions. Similar to our study, Dueñas et al. (2014) also reported the adherence, plasticity and mesenchymal nature of bovine BM-MSCs. The BM-MSCs upon proliferation demonstrate fibroblastic morphology (Figure 1A, B). In a similar manner, Hill et al. (2019) reported that BM-MSCs exhibit fibroblastic morphology and express pluripotent markers like Oct3/4, Sox2 and Nanog in addition to MSCs markers.

MSCs are expected to express MSCs specific markers such as CD73, CD105, etc. while a negative expression is required for haemopoietic and immunogenic markers (Dominici et al., 2006). In the study, the PCR analysis showed that BM-MSCs expressed mesenchymal stem cell marker CD73, but cells were found to be negative for hematopoietic and endothelial markers CD 34, CD45 and HLA-DR receptor gene CD74 (Figure 1). Our findings were similar to previous studies where BM-MSCs have been reported to express the common mesenchymal markers like CD105, CD73 and CD90 and be short of the expression of haemopoietic markers like CD45, CD34, CD14, CD11b, CD19 and HLA-DR surface markers (Cortes et al., 2013; Dueñas et al., 2014).

In order to meet the requirements for the minimal criteria for mesenchymal stem cells, the cells have to be capable to undergo multilineage differentiation (Dominici et al., 2006). BM-MSCs were formerly announced to have the capacity to differentiate into osteocytes and adipocytes. Bosnakovski et al. (2005) narrated that BM-MSCs show multipotent properties as they were able to differentiate into both osteogenic and adipogenic lineages. Parallel to previous studies, a successful differentiation of BM-MSCs into adipogenic and osteogenic lineages was performed (Friedenstein et al., 1966; Hill et al., 2019). The adipogenic differentiation was confirmed by Oil Red O staining by demonstrating the oil droplets within the cytoplasm of differentiated cells. After the staining, oil droplets were visible in red-pink color under the microscope (Figure 3D). The osteogenic differentiation, on the other hand, was demonstrated by Alizarin Red S staining. The red-orange-stained calcium deposits had confirmed the osteogenic differentiation after the Alizarin Red S staining (Figure 3B). The characteristic staining results for both osteogenic and adipogenic differentiation prove the mesodermal linkage of BM-MSCs cells.

Upon application of induction media, the bovine BM-MSCs were successfully differentiated into spherical and three dimensional neurospheres in a short notice. There was no visible formation of neurospheres at 24th hour post induction (Figure 4A). The neurospheres were allowed to grow and expand by 48h (Figure 4B). Although neurospheres were visible in culture; it was found there were populations of BM-MSCs which remain un-induced to neurogenic differentiation even at 72nd hour of study. All the neurospheres, collected for immunofluorescence studies at 72nd hour. The immunofluorescence studies showed that all the neurospheres express Sox2, Nestin and β-III Tubulin (Figure 5).

Chung et al. (2013) had successfully generated neurospheres from both bone marrow derived and adipose derived stem cells of canines and made a comparison in the context of their neurogenic capacities and reported that neurospheres differentiated from adipose tissue stem cells had significantly higher number of cells. Yet, they also reported that neuron-like cells differentiated from BM-MSCs derived neurospheres had much greater percentage of ß-III Tubulin positive neuronal cells. Therefore, investigating as much stem cell source as we can could point out the best source and approach for the intended use of neurospheres. Previous attempts on neurospheres generation from bovine stem cells were performed using bovine adipose tissue derived stem cells and bovine amniotic fluid derived stem cells under serum-free induction conditions by our team, and in both studies similar to the current research, all the neurospheres were positive against Sox2, Nestin and ß-III Tubulin protein expressions (Akkaya et al., 2019; Nawaz et al., 2020). Yet, a previous neurogenic attempt made by Dueñas et al. (2014) using β-mercaptoethanol treatments on bovine BM-MSCs could not lead to successful expression of neural stem cell markers like Nestin and MAP2. However, in the same study using EGF and FGF in FBS containing medium helped in differentiation of pro-neuronal/neuronal cells that expressed

Nestin and MAP2. In contrary, an FBS free media was rather preferred in an attempt to generate neuroprogenitor cells using as high concentrations of bFGF and EGF as 50 ng/ml resulted in neurospheres which expressed Sox2, Nestin and β-III Tubulin upon immunofluorescence studies. Main role of Sox2 in neural stem cells of adult brain is regulating neurogenesis and acting as a miRNA regulator in the oligodendrogenesis process According to the studies as described by Reiprich and Wegner (2014), Sox2 is found to be expressed in totipotent inner cell mass and is a component of neural epithelium in growing central nervous system. Graham et al. (2003) and Bylund et al. (2003) stressed that the cells which express high levels of Sox2, tends to produce larger neurospheres and have amplified capability to create secondary neurospheres. Some morphological variations in the size and shape of neurospheres was observed during the study. According to studies, the variation in size and shape of neurospheres could be related to individual Sox2 expression by BM-MSCs. The Sox2 expression on all collected neurospheres in our study suggest that BM-MSCs derived neurospheres could be considered as multipotent, neuroprogenitor neurospheres. Yet, in order to justify this statement further quantitative studies are necessary for the Sox2 expression of bovine BM-MSCs derived neurospheres.

Moreover, the neurospheres isolated from bovine BM-MSCs also expressed neuronal markers such as β-III Tubulin and Nestin upon immunofluorescence analysis. In consistent to our study, Brazel et al. (2006) described that all Sox2-expressing neuro-epithelial cells also express Nestin protein. Another study conducted by D'Amour and Gage (2003) suggests that neurospheres which express Sox2, express high levels of Nestin which is a potent neural stem cell transcription factor too. It was found that nearly all constituent cells of originated neurospheres were immunoreactive for neural intermediate filament Nestin, pro-neuronal neurofilament marker β-III Tubulin and transcription factor Sox2 similar to previous reports of many researchers (Reynolds and Weiss 1992; Bez et al., 2003) described.

The outcomes of the current study suggests that bovine BM-MSCs had potential to be utilized as a source for neurospheres to be used in further research. Yet, the initial expressions of markers like Sox2 and Nestin of MSCs should be determined before the induction and any correlation between these markers and neurogenic potential of neurospheres should be noted. In the current study, neurospheres were collected as early as the 72nd hour post induction. A longer induction period might be beneficial to further evaluate other important aspects such as differentiation percentage of the MSCs; self-renewal capacity, average diameter, apoptosis rates, of neurospheres. And quantitative protein or gene expressions of neural markers should be determined before choosing the most appropriate stem cell source for *in vivo* and/or clinical experiments.

CONCLUSION

The bovine model owing the lager life span compared to traditional laboratory animal models could be well suited for clinical trials carried for humans in addition to its severe importance for veterinary medicine. It is also advantageous, because of its abundance in biological materials and similar size, anatomy and physiology compared to rest of lab animals. In this study, it was demonstrated that bovine BM-MSCs are capable to differentiate into neurospheres under serum-free conditions. After just 3 days of inductions, the neurospheres were positive for Nestin, Sox2 and ß-III Tubulin, indicating the neural character of the neurospheres. Neurosphere culture system could be employed as a valuable source to study *in vitro* neurogenesis and neural development in bovines. It was assumed after monitoring the proper immunogenic parameters and evaluating further characteristics of neurospheres that, bovine BM-MSCs derived neurospheres can be applicable for *in vitro* experiments and maybe even to treat the peripheral nerve injuries for allogenic studies.

ACKNOWLEDGEMENTS

This study has been conducted under the available resources of Histology and Embryology laboratory, Afyon Kocatepe University, Turkey. Authors would like to acknowledge the support provided by abattoir veterinarian Mr. Barış BOSTANCI during sample collection from slaughtered animals.

CONFLICT OF INTEREST

Authors declare no conflict of interest and authors solely are responsible for the content and writing of this study. Authors also likes to disclose that no part of this research has been previously presented or published elsewhere.

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