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FULL PAPER

Theriogenology

Isolation and characterization of buffalo (*bubalus bubalis*) amniotic mesenchymal stem cells derived from amnion from the first trimester pregnancy

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ABSTRACT. Amniotic mesenchymal stem cells (AMSCs) from livestock are valuable resources for animal reproduction and veterinary therapeutic. The purpose of this study is to explore a suitable way to isolate and culture the buffalo AMSCs (bAMSCs), and to identify their biological characteristics. Digestion with a combination of trypsin-EDTA and collagenase type I could obtain pure bAMSCs more effectively than trypsin-EDTA or collagenase type I alone. bAMSCs could proliferate steadily *in vitro* culture and exhibited fibroblastic-like morphology in vortex-shaped colony. bAMSCs were positive for MSC-specific markers *CD44*, *CD90, CD105*, *CD73*, *β-integrin* (*CD29*) and *CD166*, and pluripotent markers *OCT4*, *SOX2*, *NANOG*, *REX-1*, *SSEA-1*, *SSEA-4* and *TRA-1-81*, but negative for hematopoietic markers *CD34*, *CD45* and epithelial cells specific marker Cytokeratin 18. In addition, bAMSCs were capable of differentiating into adipogenic, osteogenic, chondrogenic and neural lineages, with expression of *FABP4*, *Ost*, *ACAN*, *COL2A1*, *Nestin* and *β III-tubulin*. Glycogen synthase kinase 3 inhibitor: kenpaullone promoted bAMSCs to differentiate into neural lineage. This study provides an effective protocol to obtain and characterize bAMSCs, which have proven useful as a cell resource for buffalo cell reprogramming studies and transgenic animal production.

KEY WORDS: amniotic mesenchymal stem cell, buffalo, differentiation, pluripotency

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Mesenchymal stem cells (MSCs) were first found in bone marrow [10]. Because of their self-renewal ability, low immunogenicity and potential for multiple differentiation, MSCs have a promising future in regenerative medicine. For some application purposes, it is of great importance to get a large quantity of high quality MSCs through a noninvasive method [1]. Up till now, MSCs from different sources have been reported, including adipose [39], bone marrow [10], umbilical cord blood [12] and so on. In't Anker *et al*. even isolated MSCs from human amnion membrane, and confirmed that the expansion potency of amnion membrane–derived MSCs were higher than adult bone marrow–derived MSCs [16].

Amnion MSCs (AMSCs) are ideal candidates for regenerative medicine and future clinical treatments. In most cases, the amnion is abandoned at birth, which makes it easily accessible and avoid invasive ethical issues [35]. Amnion is a semi-transparent membrane, which is free of neural tissues, blood vessels, lymph and muscle on the surface, and mainly made of amniotic epithelial cells and amnion mesenchymal cells, so the AMSCs obtained are less likely to be contaminated by other cells [2]. Owing to its biological characteristic, a large number of cells can be obtained with a small amount of tissue, which is feeder-free during culture [5]. Moreover, the mainly histocompatibility complex (MHC) class I and MHC class II antigens are weakly detected on the surface of human amnion membrance. Therefore, AMSCs appear to be low immunogenicity and are desired seed cells in regenerative medicine and allograft [18].

The non-embryonic pluripotent stem cells in livestock are valuable cell resources for the models of human cell therapies and regenerative veterinary medicine. Many reports revealed the research on stem cells in livestock also assisted reproductive biotechnological applications. Evidence indicates that using bone marrow MSCs as nuclear donors in somatic cell nuclear transfer (SCNT) increases the developmental competence of porcine and bovine cloned embryos [3, 22]. So far, AMSCs from different

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species have been reported, including human [16], horse [21], bovine [4], sheep [25], canine [9], chicken [23], porcine [19] and cat [37]. In buffalo, owing to its characteristics of heat resistance, high humidity resistance and strong disease resistance, it has tremendous potential development capabilities in agriculture across the globe. Only a few researches have reported the bAMSCs [7, 14, 15, 24, 31]. There are many controversies about these cells, such as the derivation of the AMSCs from different gestational stages, the different isolation methods used, the expression of specific markers to determine cell types and the ability of the cells to differentiate. So, this study is aimed to explore the isolation method and culture conditions of AMSCs derived from buffalo amnion from the first trimester. In addition, the biological characteristics of bAMSCs were identified comprehensively, including the ability of proliferation, colony formation, the expression of the specific markers and the ability of differentiation, especially for neurogenic differentiation.

MATERIALS AND METHODS

Reagents and medium

All cell culture medium with supplements were obtained from Gibco (Carlsbad, CA, U.S.A.). The culture plastic dishes and tubes were obtained from Corning (Steuben County, NY, U.S.A.). The primary antibodies: Oct4, Sox2 and the secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.), and the other antibodies were obtained from Abcam (Cambridge, U.K.). The RT-PCR related reagents were obtained from Takara bio, Inc. (Kusatsu, Japan). The other reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), unless otherwise indicated.

Cell isolation and passage

This study was conducted in accordance with the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bio-resources guide for the care and use of laboratory animals. Buffalo gravid uterus from the first trimester were harvested from an abattoir, cleaned, removed the placenta, collected the amniotic membranes and put into centrifuge tubes with PBS (phosphate buffer saline) at room temperature, and brought to laboratory within 1 hr of slaughtering. Amniotic membranes were rinsed in 70% alcohol for 20 sec, and washed with PBS more than 5 times. Amniotic membranes were cut into small pieces with sterilized nippers. In order to compare three different isolation methods, tissue was divided into three similar volume groups and put into centrifuge tubes. The first group was digested with a combination of trypsin-EDTA and collagenase type I (T+C): 0.25% (w/v) trypsin-EDTA digested at 37°C for 30 min, and the digestion was terminated with complete medium (high glucose-Dulbecco's Modified Eagle Medium, DMEM+10% FBS+10,000 U/m*l* penicillin +10,000 μg/m*l* streptomycin). The dispersed samples were filtered through 70 μm nylon cell strainer, collected and moved to centrifuge tubes. The digestion was repeated with 0.25% (w/v) trypsin-EDTA for another 30 min and the undigested tissue was collected using a 70 μm nylon cell strainer. 0.1% (w/v) collagenase type I was added into the remaining tissue and this was digested at 37°C for 60 min. Complete medium was used to terminate the digestion. The remaining tissue was filtered through a 70 μm nylon cell strainer, the liquid collected and centrifuged for 5 min at 1,500 rpm. Cells were re-suspended in complete medium supplemented with 20% FBS and live cells were counted after trypan blue staining.

The second group was digested with collagenase type I alone (C): the first digestion was carried out using 0.1% (w/v) collagenase type I at 37°C for 60 min, the digestion terminated and filtered through 70 μm nylon cell strainer, collected and moved to centrifuge tubes. Digestion was repeated for another 60 min under the same conditions, terminated and the cells collected, counted and cultured in a similar way to the first group. The third group was digested with trypsin-EDTA alone (T): the processes were the same as the second group with 0.25% (w/v) trypsin-EDTA instead of collagenase.

All collected cells were supplied with complete medium and cultured at 37°C, 5% CO_2, in the incubator (Thermo). When cells reached 80% confluence, 0.25% (w/v) Trypsin-EDTA were used to dissociate cells from the plates.

Proliferation assays

Passage 3, 6, 9 and 20 of bAMSCs were collected respectively, and seeded in 24-well plates at a density of 10^4 cells/well. 3 wells were randomly harvested each day to count for 7 successive days. According to the every-day mean values, growth curves were plotted. Dependent on the growth curve, population double time (PDT) was calculated.

PDT=(t−t0) lg2/(lgNt−lgN0) where t0=beginning time of culture, t=ending time of culture, N0=seeded cell number of culture and Nt=final cell number of culture.

Colony formation

Passage 3, 6, 9 and 20 of bAMSCs were collected respectively, and seeded in 100 mm plates at a low density of 15 cells/cm^2. The colonies were stained by Giemsa after 14 days of culture. Colonies larger than 2 mm in diameter in each dish were counted.

Immunofluorescence

Cells at passage 3 to passage 10 were seeded at a density of 5,000 cells/well in 4-well plates and cultured for 3 days. Cells were washed with PBS 3 times, fixed with 4% (w/v, dissolved in PBS) paraformaldehyde for 20 min, and washed again with PBS 3 times. Cells were incubated with 0.1% (v/v, dissolved in PBS) Triton™ X-100 for 15 min in order for cells to be permeabilized, and further washed 3 times with PBS. Then the cells were incubated with 5% (w/v, dissolved in PBS) BSA for 1 hr, and washed 3 times with PBS. Next, the cells were incubated with primary antibodies (dissolved in PBS at a concentration of 1:250) overnight

Table 1. Sequence of primers used for RT-PCR analysis

Gene	Primer sequence	Amplicon (bp)	TM (°C)	NCBI accession number
18S	F:5′-GATGGGCGGCGGAAAATTG-3′ R:5′-TCCTCAACACCACATGAGCA-3′	79	60	NM_001033614
OCT4	F:5′-GTTCTCTTTGGAAAGGTGTTC-3′ R:5′ –ACACTCGGACCACGTCTTTC-3′	306	60	JN991003
SOX2	F: 5′-CGTGGTTACCTCTTCTTCC-3′ R: 5′- CTGGTAGTGCTGGGACAT-3′	139	60	JN986576
NANOG	F:5′-CACCCATGCCTGAAGAAAGTT-3′ R:5′-TGGAAAGTTCTTGCATTTGCTG-3′	306	55	JN991004
REX-1	F:5′-GTCCTTCGATTACAACCCCA-3′ R:5′-CACGTACTTGCTGCTGGAGA-3′	226	60	XM_015472188
CD44	F:5′-CGGAACATAGGGTTTGAGA-3′ R:5′-GGTTGATGTCTTCTGGGTTA-3′	301	60	XM_015474843
CD73	F:5′-CAATGGCACGATTACCTG-3′ R:5′-GACCTTCAACTGCTGGATA-3′	428	56	NM_174129
CD166	F: 5′-TATCAGGATGCTGGAAAC-3′ R: 5′-TAGCCAATAGACGACACC-3′	498	56	XM_005201256
β-integrin	F:5′-GAAACTTGGTGGCATCGT-3′ R:5′-CTCAGTGAAGCCCAGAGG-3′	493	55	NM_174368 XM_006063210.1
CD34	F:5′-CCTCATCAGCTTTGCGACTT-3′ R:5′-CCAGGAGCAAGGAGCACA-3′	314	56	NM_174009
CD45	F:5′-CTACCCAACCTTCTACTCAA-3 R: 5′-TTCACATCCAGGAGGTTC-3′	221	56	XM_015475267
FABP4	F:5′-CTGGCATGGCCAAACCCA R:5′-GTACTTGTACCAGAGCACC	182	56	NM_174314
Ost	F:5′-AGCGAGGTGGTGAAGAGA R:5′-CCTGGAAGCCGATGTGGT	145	56	NM_174249
COL2A1	F:5′-CGCGGATTTGTTGCTGCTTGC-3′ R:5′-AGGTCCCATCAGCCCCATTGGT-3′	268	56	NM_174520
ACAN	F:5′-CGCTGTCTCGCCAAGTGTATGG-3′ R:5′-CGGTTCAGGGATGCTGACACTC-3′	175	60	NM_173981
Nestin	F: 5′-TGAAACACCTGTGCCAACCT-3′ R: 5′-GCTTCAGCCCACATGACTTC-3′	204	60	NM_001206591

at 4°C. The next day, cells were washed 3 times with PBS, and incubated with secondary antibodies (dissolved in PBS at a concentration of 1:500) for 1 hr at room temperature in the dark. The blank controls were incubated with secondary antibodies only nuclei were counterstained with Hoechst 33342 (dissolved in PBS at a final concentration of 5 μM). Finally, immunostaining was assessed using a fluorescent microscope (Nikon, Tokyo, Japan).

Molecular characterization

The expression of MSC-specific genes *CD44*, *CD73*, *CD166*, *β-integrin* (*CD29*), hematopoietic stem cell specific genes *CD34*, *CD45*, and pluripotency-related genes *OCT4*, *SOX2*, *NANOG*, *REX-1* in bAMSCs were detected by RT-PCR. Total RNA were extracted from passage 3 to passage 10 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. Retrotranscription was carried out according to the PrimeScript RT reagent Kit with genomic DNA Eraser in a total volume of 20 μl. After DNA removal and reverse transcription, PCR was performed in a 20 μl final volume with Taq DNA polymerase under the following conditions: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 sec, annealing temperature (TM) for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 10 min. Buffalo specific oligonucleotide primers were designed by Primer Premier 6 and was dependent on availability of NCBI bovine and buffalo gene sequences. PCR products were visualized after electrophoresis on a 2% agarose gel. RT-PCR was also used to detect the expression of specific genes in induced differentiated cells, referring to the above protocols. The primers (intron-spanning primer) and PCR conditions are listed in Table 1.

In vitro multiple differentiations

Adipogenesis, osteogenesis and chondrogenesis differentiation: bAMSCs at passage 3 to passage 10 were seeded in 6-well plates at a density of 200 cells/cm^2. Inducing medium was added when cells reached 80% confluence respectively. The medium was changed every 3 days. Control group was filled with culture medium, and changed at the same frequency. Both groups were cultured under the same culture conditions (37°C, 5% CO_2). After 1 week (chondrogenesis differentiation for 2 weeks), induction was terminated. The following staining and RT-PCR were carried out for testing the differentiation potential: for adipogenesis differentiation, Oil Red O (0.3% in 60% isopropanol) staining and specific expression of *FABP4*; for osteogenesis differentiation, alizarin red (1.37% in Tris-HCl) staining and specific expression of *Ost*; for chondrogenesis differentiation, alcian blue (1% in

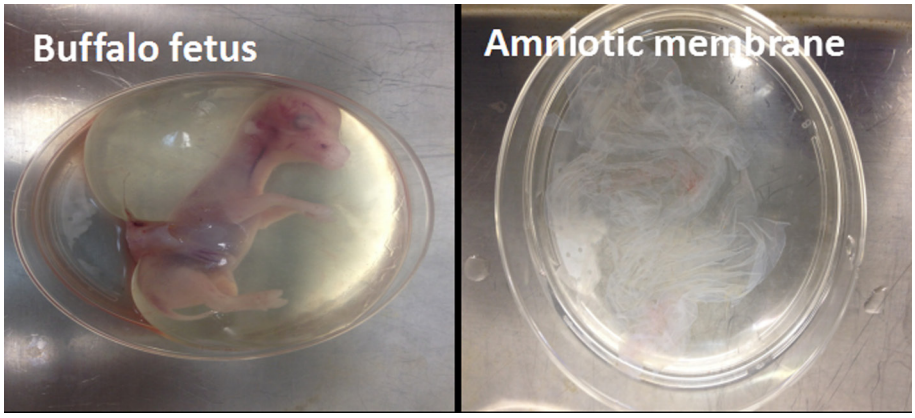

Fig. 1. Three-month-old buffalo fetus and amniotic membrane.

0.1 M HCl, pH=1) staining and specific expression of *COL2A1* and *ACAN*.

For neural differentiation, bAMSCs were exposed to a different differentiation medium. The basic medium consisted of DMEM plus FBS (8%), and this was used as control. The differentiation medium contained basic medium that was supplemented with bFGF (10 *n*g/m*l*), forskolin (an activator of adenylate cyclase, 10 μM), kenpaullone (the glycogen synthase kinase 3 inhibitor, 10 μM), bFGF+forskolin, forskolin+kenpaullone, bFGF+kenpaullone and bFGF+forskolin+kenpaullone, respectively. During the differentiation process, cells were cultured under the same culture conditions (37°C, 5% CO_2). Induction was terminated after 2 days, immunofluorescence of *β III-tubulin* and RT-PCR was performed to detect the expression of the specific neural cell gene, *nestin*.

Statistical analysis

A Student's paired *t*-test was used for statistical analysis and *P*-values <0.05 were considered statistically significant.

RESULTS

Comparison of different isolation methods

Buffalo amniotic membrane from the first trimester were collected and isolated with T+C, C and T (Fig. 1). Five hours later, most of the primary cells had adhered to plates. The adhered mixture of cells presented fibroblast-like and epithelial-like morphology (Fig. 2A). In terms of the trypan blue staining, the living cell numbers were $4.04 \pm 0.04 \times 10^5$, $5.16 \pm 0.13 \times 10^5$ and $3.6 \pm 0.29 \times 10^5$, respectively (Fig. 2B). Living cells derived from only collagenase type I digestion was in greater numbers than the other two isolation methods. Immunofluorescence of the epithelial cell specific marker, Cytokeratin 18, was taken to detect epithelial cells, which constituted a proportion of the mixture of from the three isolation methods. Ten fields were randomly selected to count for percentages of positive Cytokeratin 18 in each group. The results revealed that the collagenase type I group had the highest percentage of positive Cytokeratin 18 (CK18+), which was 39.5% ± 3.28. While the trypsin-EDTA+collagenase type I group and the trypsin-EDTA group showed almost no positive staining for Cytokeratin 18 (CK18-, Fig. 3). The trypsin-EDTA+collagenase type I isolation method could obtained more pure CK18- cells and the biological characteristics of these cells will be identified in the following study.

Cell proliferation and colony formation

Trypsin-EDTA was used to dissociate the adhered cells when the cells reached 80% confluence. After serial subculture, cells arranged closely and displayed vortex-like shapes. The cultured cells could proliferate steadily *in vitro* during the early passages. However, cells grew noticeably slower and showed a tendency of exhibiting apoptosis after 20 passages. Cells showed increased vacuolization and tended to detach easily from the surface (data not shown). Cell growth curve were drawn according to the cell counts at passage 3, 6, 9 and 20 (Fig. 4A). The cultured cells grew slowly in the first two days of the latent phase, and showed obvious fast growth in the following 3 days of logarithmic growth phase and almost no growth at the day 6–7 of the plateau phase. Accordingly, the PDT of cells from passage 3, 6, 9 and 20 were 43.9 ± 2, 45.9 ± 3, 46.7 ± 3 and 60 ± 5 hr, respectively (Fig. 4B). PDT was prolonged as passage number increased and showed a significant difference after passage 20 ($P<0.05$).

Colony formation capacity is one of the prominent characteristics of mesenchymal stem cells. Colonies of buffalo amnion derived cells were stained by Giemsa (Fig. 5A). Two mm or greater in diameter colonies were counted. The colony numbers were 68 ± 4, 72 ± 8, 56 ± 4 and 31 ± 6 for cells from passage 3, 6, 9 and 20, respectively (Fig. 5B). The colony number reduced significantly after passage 9 ($P<0.05$). This is consistent with the growth curves seen and statistical analysis of the PDT. These results indicate the proliferation and self-renewal ability of buffalo amnion derived cells.

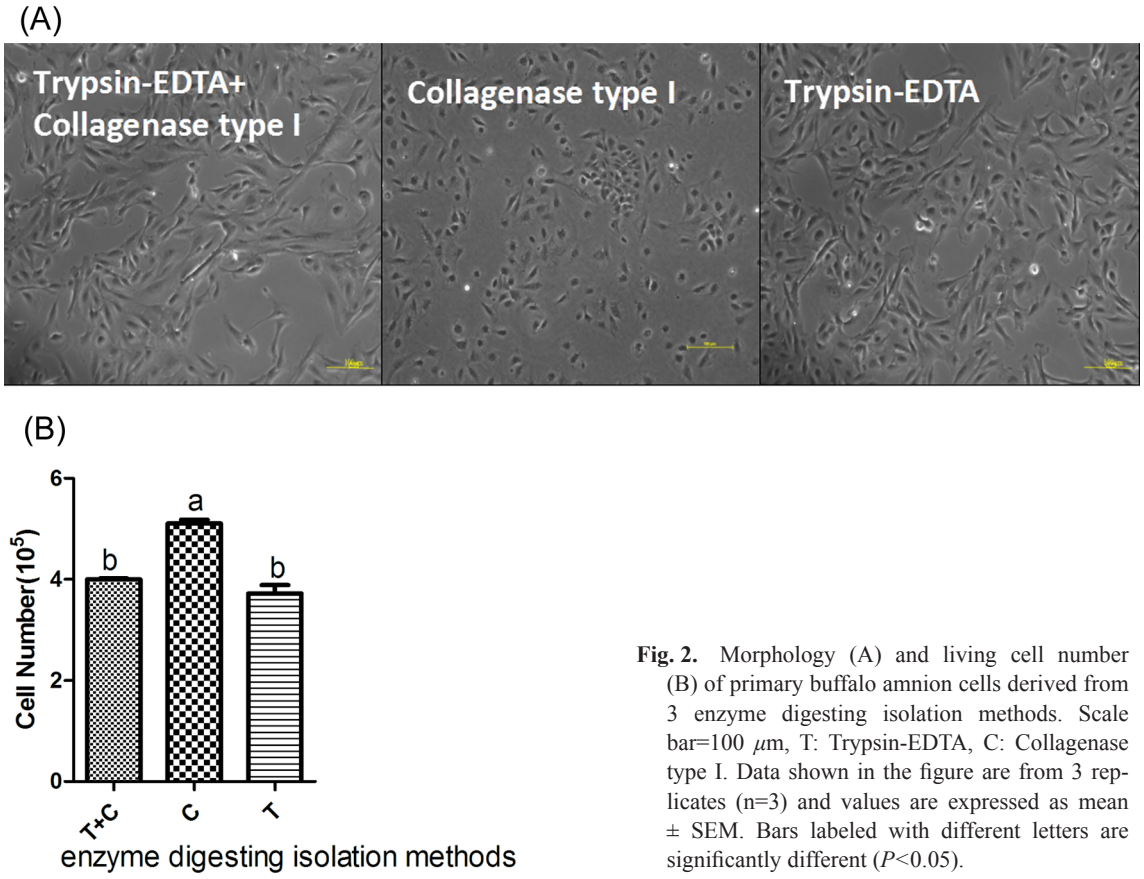

Fig. 2. Morphology (A) and living cell number (B) of primary buffalo amnion cells derived from 3 enzyme digesting isolation methods. Scale bar=100 μm, T: Trypsin-EDTA, C: Collagenase type I. Data shown in the figure are from 3 replicates (n=3) and values are expressed as mean ± SEM. Bars labeled with different letters are significantly different (P<0.05).

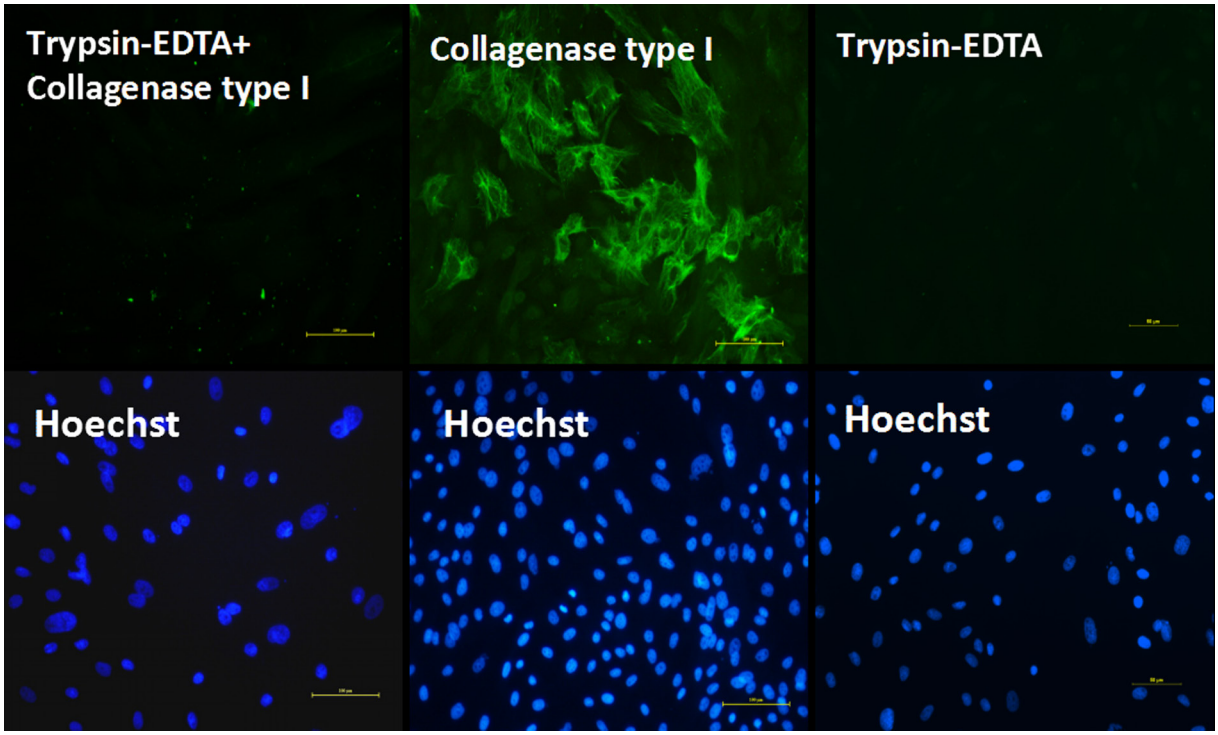

Fig. 3. Cytokeratin 18 immunofluorescence of primary amnion cells derived from 3 enzyme digesting isolation methods. Scale bar=100 μm.

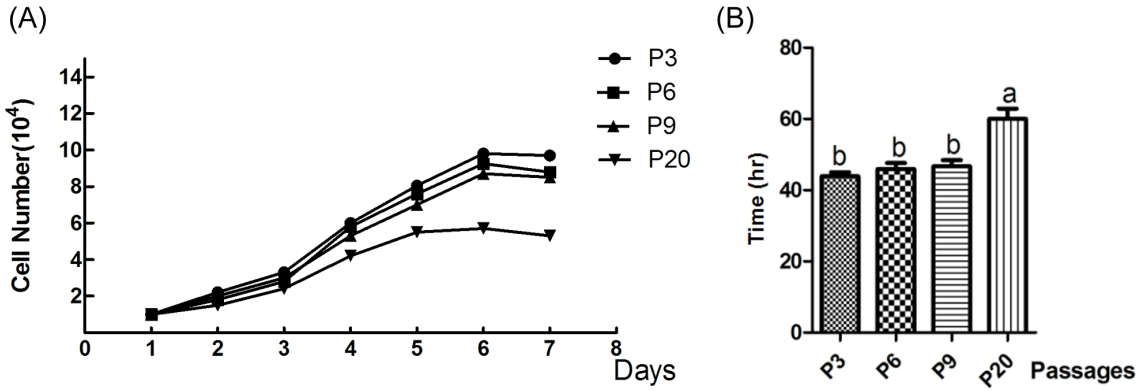

Fig. 4. Cell growth curve (A) and PDT (B) of buffalo amnion derived cells. Data shown in the right figure are from 3 replicates (n=3) and values are expressed as mean ± SEM. Bars labeled with different letters are significantly different (P<0.05). P: passage.

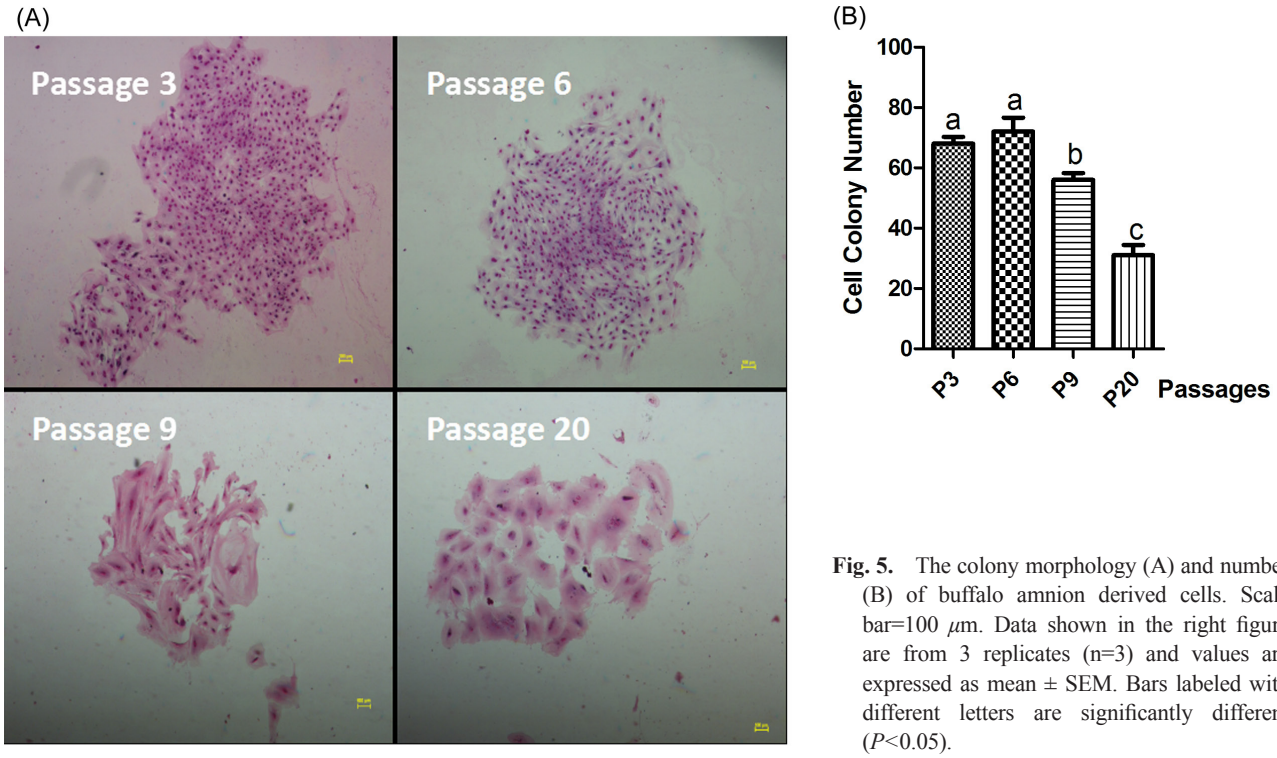

Fig. 5. The colony morphology (A) and number (B) of buffalo amnion derived cells. Scale bar=100 μm. Data shown in the right figure are from 3 replicates (n=3) and values are expressed as mean ± SEM. Bars labeled with different letters are significantly different (P<0.05).

Characterization of buffalo amnion derived cells

Expression of pluripotency markers and mesenchymal markers are characteristics of the mesenchymal stem cells. The immunofluorescence analysis revealed that the cultured cells were positive for pluripotency-related genes *OCT4*, *SOX2*, *NANOG*, *SSEA-1*, *SSEA-4* and *TRA-1-81* and mesenchymal stem cell surface markers *CD44*, *CD90* and *CD105*. However, the specific marker for hemopoietic stem cells, *CD45*, was not expressed (Fig. 6). Consistently, RT-PCR results confirmed the expression of pluripotency marker genes *OCT4*, *SOX2*, *NANOG* and *REX-1*, and mesenchymal stem cell surface markers *β-integrin* (*CD29*), *CD44*, *CD73* and *CD166*, while no expression of hemopoietic stem cell surface markers *CD34* and *CD45* was observed (Fig. 7). These results indicate the similarity of buffalo amnion derived cells to mesenchymal stem cells.

In vitro multiple differentiation of bAMSCs

In the adipogenic medium, bAMSCs grew slowly during adipogenesis differentiation, and gradually displayed a more polygonal shape. After 4 days of induced differentiation, some lipid droplets were observed in the cells. With a progression of adipogenesis differentiation, the induced cells became more rounded in shape, the number of lipid droplets increased and aggregated these

to form larger ones. The lipid droplets were observed by Oil Red O staining. The negative control of cultured bAMSCs during this process of adipogenesis differentiation showed no changes in cell shape and were negative for Oil Red O staining (Fig. 8A). RT-PCR results showed that the adipogenesis specific gene, *FABP4*, was positively expressed in the induced cells, but was not expressed in the control bAMSCs (Fig. 8C).

During osteogenic differentiation, cells gradually gathered and formed calcium nodes. After 7 days of induced differentiation, the calcium nodes increased in number and size, and were positive to alizarin red staining. Control cells cultured in complete medium kept the shuttle shape for one week after and were negative to alizarin red staining (Fig. 8A). RT-PCR demonstrated that the osteogenesis specific gene, *Ost*, was positively expressed in the induced cells, but was not expressed in control bAMSCs (Fig. 8C).

Cells morphology appeared to change markedly after 3 days of chondrogenic induced differentiation. bAMSCs changed from a shuttle shape to a round shape and tended to aggregated during the process. Scattered cartilaginous nodes appeared after one week of culture. Cartilaginous nodes increased in number and became larger as induction continued. After two weeks, the cartilaginous nodes were observed by Alcian blue staining. Cells cultured in complete medium showed no changes in cell shape and were negative for Alcian blue staining (Fig. 8A). RT-PCR detection revealed that chondrogenic specific genes, *COL2A1* and *ACAN*, were both positively expressed in the induced cells, but were not expressed in control bAMSCs (Fig. 8C).

During neurogenic differentiation, bAMSCs were cultured in the differentiation medium supplemented with both bFGF, forskolin and kenpaullone. Some bAMSCs changed from a spindle-like shape to radial, tapered, polygon and irregular shapes after the first day of induction. Some stellate cells appeared, and most cells exhibited dendritic and axon-like structures after 2 days of induction (Fig. 8B). Immunofluorescence results indicated that the induced cells were positive for *β III-tubulin*. RT-PCR results showed that the neurogenic specific gene, *nestin*, was expressed in the induced cells. However, bAMSCs cultured in the control medium showed no change in cell shape and were negative for *β III-tubulin* and *nestin* expression (Fig. 8B and 8C). bAMSCs cultured in the other differentiation medium combinations showed no significant neurite formation, and were weak for *β III-tubulin* expression (supplementary Fig. S1). These results indicate that the combination of bFGF, forskolin and kenpaullone can efficiently induce bAMSCs to differentiate into neurons.

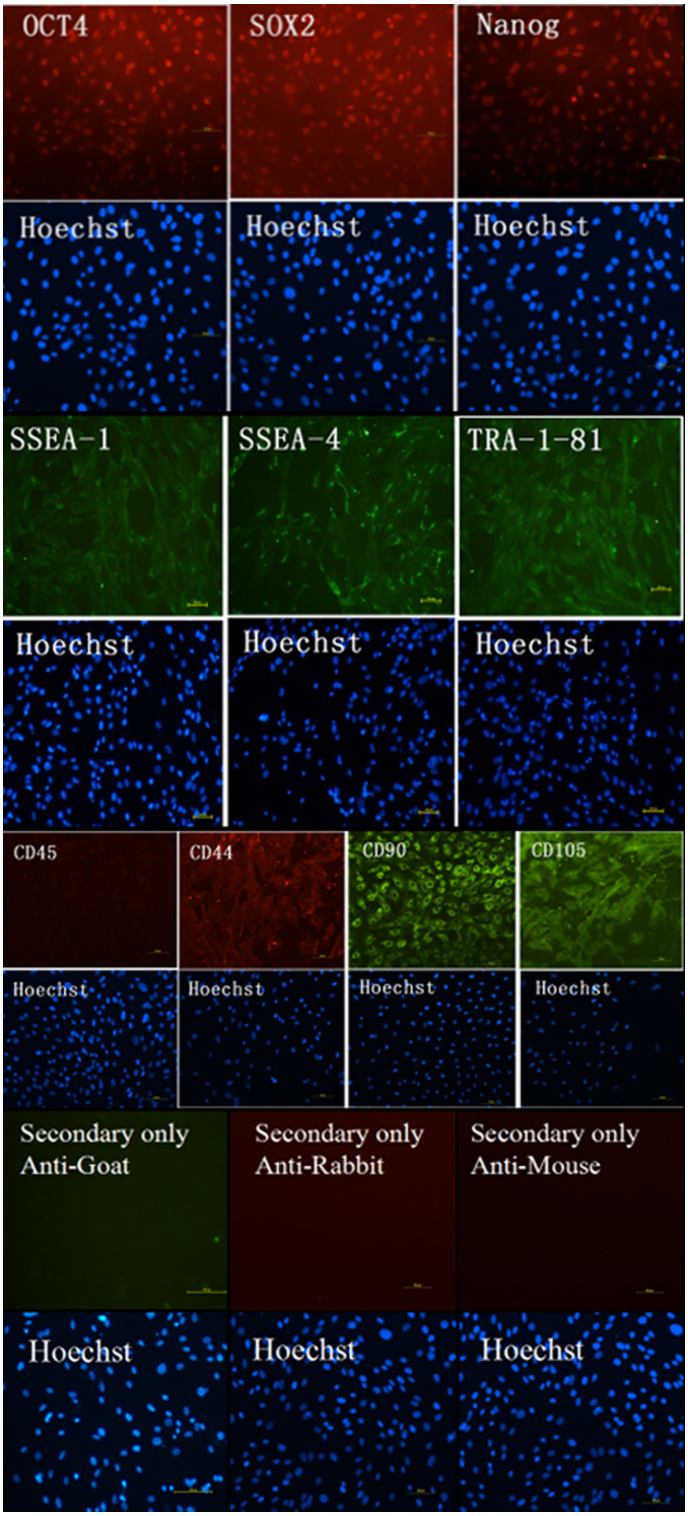

Fig. 6. Immunofluorescence analysis of pluripotent, mesenchymal and hematopoietic specific genes expression in buffalo amnion derived cells of passage 10. Scale bar=50 μm.

DISCUSSION

Non-embryonic derived mesenchymal stem cells are useful in human regenerative medicine and animal science studies. These cells have been isolated and characterized from many tissues and animal species. Buffalo non-embryonic derived mesenchymal stem cells have been derived from amniotic fluid [7], bone marrow [11], umbilical cord matrix [34], adipose tissue [33] and amniotic membrane [14, 15, 24, 31]. In the previous reports, there were many ambiguous results when defining the buffalo amniotic membrane derived cells, including the gestational stages, isolation methods, the identification of marker genes, the purity of the amniotic mesenchymal stem cells and

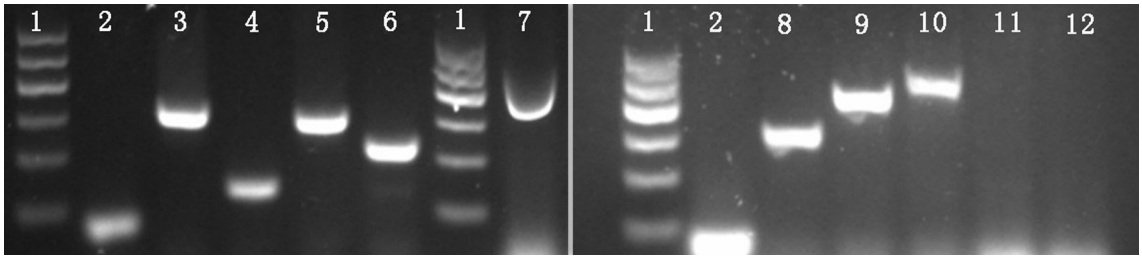

Fig. 7. RT-PCR analysis of pluripotent, mesenchymal and hematopoietic specific genes expression in buffalo amnion derived cells of passage 10. 1: DNA Marker I, 2: 18s (79 bp), 3: OCT4 (306 bp), 4: SOX2 (139 bp), 5: NANOG (306 bp), 6: REX-1 (206 bp), 7: β-integrin (493 bp), 8: CD44 (301 bp), 9: CD73 (428 bp), 10: CD166 (498 bp), 11: CD34 (314 bp), 12: CD45 (221 bp).

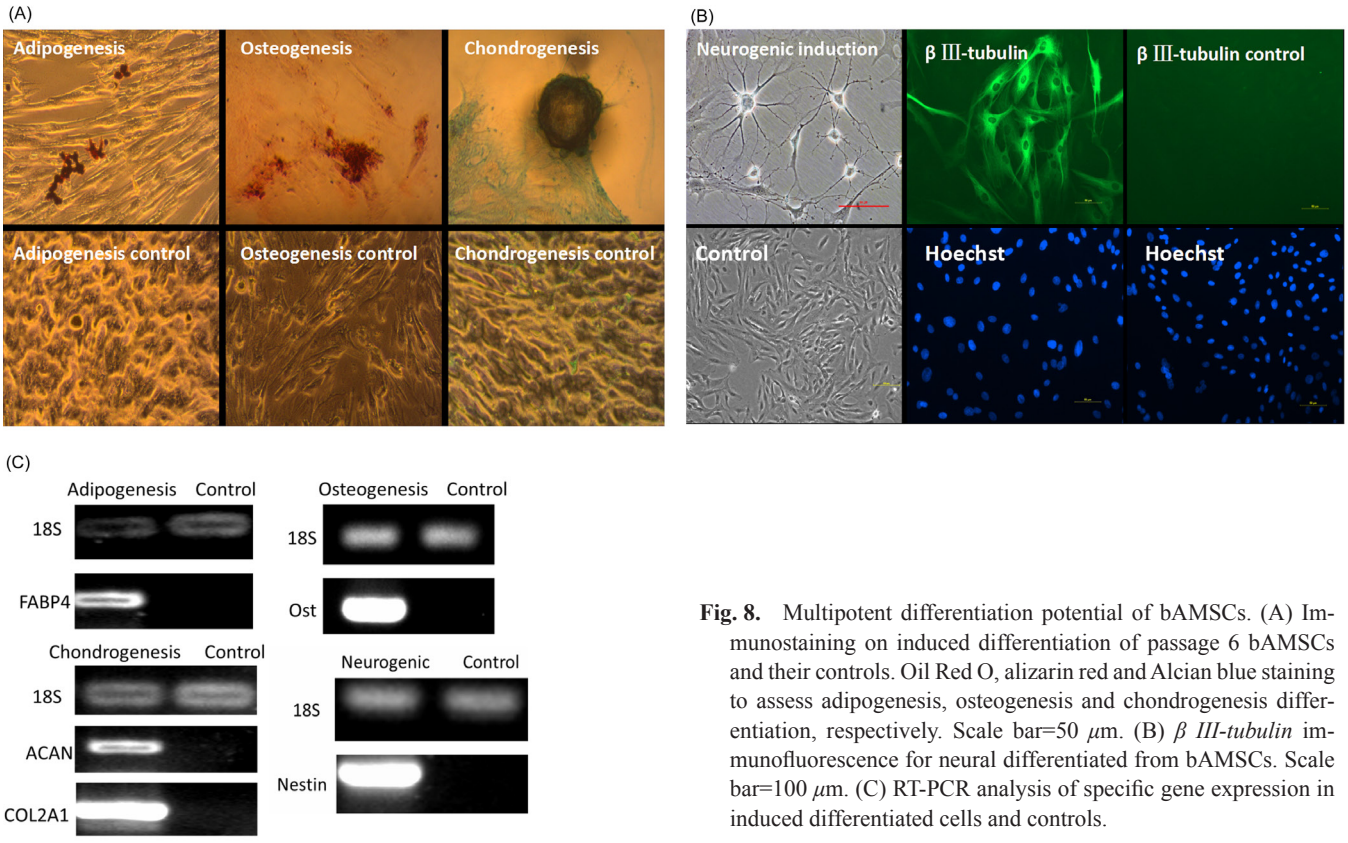

Fig. 8. Multipotent differentiation potential of bAMSCs. (A) Immunostaining on induced differentiation of passage 6 bAMSCs and their controls. Oil Red O, alizarin red and Alcian blue staining to assess adipogenesis, osteogenesis and chondrogenesis differentiation, respectively. Scale bar=50 μm. (B) *β III-tubulin* immunofluorescence for neural differentiated from bAMSCs. Scale bar=100 μm. (C) RT-PCR analysis of specific gene expression in induced differentiated cells and controls.

the differentiation potential. The first reported the presence of stem cell-like cells from buffalo amnion from the first trimester pregnancy which only expressed *OCT4*, *NANOG* and *SOX2*, and these cells could be directed to differentiate into osteocytes [24]. In this study, we established a relatively comprehensive experimental platform for the isolation, purification and identification of mesenchymal stem cells and their differentiation capacity *in vitro*.

Ghosh *et al*. used tissue explant adherent methods and obtained buffalo amnion derived primary cells from post-partum placentae [14]. Initially, we attempted to isolate the buffalo amnion derived cells from the first trimester of pregnancy using the tissue explant adherent method. However, the smoothness of the amniotic membrane makes it difficult for tissues to adhere to the plate, and a relatively small number of cells were obtained making it difficult to meet the requirement of these experiments. In addition, the tissue explant adherent method is more likely to suffer from contamination problems [36]. Therefore, the enzymatic digestion method was used as the preferred method for bAMSCs isolation.

Collagenase type I [20, 37] and trypsin-EDTA [4, 21] were usually applied to obtain amniotic mesenchymal stem cells. In this study, three different combinations of enzymes digesting methods were compared, including trypsin-EDTA, collagenase type I alone and trypsin-EDTA combined with collagenase type I. Collagenase type I alone obtained more living cells than the other two methods, but higher number of cobblestone-like shaped cells were observed. The primary cells were mainly composed of

amniotic epithelial cells and mesenchymal cells [26]. The stem cell characteristics of amniotic epithelial cells can confuse the identification and clinical applications of the AMSCs [15]. So, an immunofluorescent epithelial cell specific marker Cytokeratin 18 was subsequently used to purify the bAMSCs. The results showed that almost no CK18+ cells appeared in the digestion with a combination of trypsin-EDTA and collagenase, and obtained more pure bAMSCs than the other two methods.

The important characteristics to identify mesenchymal stem cells are expression of pluripotency markers and mesenchymal markers. However, there is no uniform standard to determine these in buffalo and bovine AMSCs preparations. According to the literature, the pluripotency markers, *OCT4*, *SSEA-4*, *SOX2* and *NANOG*, and the mesenchymal markers, *CD29*, *CD44*, *CD166*, *CD73* and *CD105*, were expressed in bovine AMSCs [4, 13]. bAMSCs expressed the pluripotency markers, *OCT4*, *SOX2*, *NANOG* [24, 33], *TERT* [14], *SSEA-1*, *SSEA-4*, *TRA-1-60* and *TRA-1-81* [15], and the mesenchymal markers, *CD29*, *CD44* [31] and *CD105* [14, 15], but not expressed *CD34* [15, 31]. Immunofluorescence and RT-PCR exhibited bAMSCs (CK18-) expressed pluripotency and mesenchymal markers, but negative for hemopoietic stem cell surface markers. These results are in accordance with Sadeesh and Ghosh's reports [15, 31]. These results suggested that the bAMSCs derived from the first trimester pregnancy in this study, had the characteristics of mesenchymal stem cells. The negative expression of hematopoietic stem cells surface markers may imply the low immunogenicity of AMSCs, which may ensure that they can act as a suitable candidate for veterinary therapeutic purposes [29].

Another important characteristic of mesenchymal stem cells was the differentiation potential. MSCs had been successfully induced and differentiated into adipogenic, chondrogenic, osteogenic and neurogenic lineages in cattle [13, 30]. The differentiation potential of bAMSCs derived from the first trimester of pregnancy had not been reported previouly [24]. In this study, we successfully differentiated AMSCs derived from the first trimester of pregnancy of buffaloes, into adipogenic, osteogenic chondrogenic and neurogenic lineages. Adipogenic specific genes such as fatty acid binding protein (*FABP4*) [4], osteogenic specific gene *OST* (*osteopontin*, also known as *SPP1*) [30], chondrogenic specific genes *ACAN* (*aggrecan*) and *COL2A1* (*collagen type II alpha 1 chain*), and neurogenic specific genes such as *nestin* [13] and *β III-tubulin* [15] were often used to identify the cell lineages differentiated from human and bovine MSCs. These differentiated cells originated from bAMSCs also expressed the lineage-specific marker genes separately.

For the neurogenic differentiation of MSCs, several different induction media were used by different laboratories. Different combinations of epidermal growth factor (EGF), bFGF, valproic acid, butylated hydroxyanisole, insulin, hydrocortisone and sonic hedgehog (Shh) and a series of neural supplements were widely used in neurogenic differentiation of human MSCs [6, 17, 27, 28, 32]. Forskolin and valproic acid were used in neurogenic differentiation of bovine MSCs [8]. Ghosh *et al*. differentiated buffalo amnion derived cells into neurogenic lineage by supplementing retinoic acid in the differentiation medium for 3 weeks [15]. The neurogenic medium used here was supplemented with bFGF, forskolin and kenpaullone, and the neurons appeared only 2 days after induction. When only bFGF and forskolin was used, this delayed the formation of neurons (5–6 days), accompanied by a weak expression of *β III-tubulin*. This may be due to the role of kenpaullone, which have been reported to strongly improve the survival of human motor neurons [38].

It is worth mentioning that, the early passages of animal mesenchymal stem cells were enough to be used in the study of animal reproduction [22, 38]. The biological characteristics of bAMSCs were identified for passage 3 to 10. After passage 10, bAMSCs proliferation slowed down and colony formation capacity was reduced. This was especially evident after passage 20 when the expression of pluripotency markers and mesenchymal markers in bAMSCs became more heterogeneous (data not shown). Therefore, we would suggest that the early passages (passage 3 to 10) of bAMSCs are better to use in the study of animal reproduction and veterinary medicine.

In conclusion, bAMSCs derived from amnion from the first trimester pregnancy had the main characteristics of mesenchymal stem cells. Kenpaullone could promote the differentiation of bAMSCs into the neural lineage. Further studies will focus on optimizing the culture system of bAMSCs and expanding their application in animal breeding and genetics.

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