MedCrave
Step into the World of Research

Journal of Stem Cell Research & Therapeutics

Research Article

Open Access

CrossMark

# Effect of different serums on culture and growth pattern on equine adipose derived mesenchymal stem cells (hrs-AT MSC)

## Abstract

Equine multipotent mesenchymal stem cells (hrs-MSC) can be isolated from various tissues including adipose tissue (AT). We have analyzed the effect of different serum sources on hrs-AT-MSC cultured and assessed proliferation, morphology, viability and immunophenotype and plasticity. The hrs-AT-MSC was cultured separately with growth media containing three different serums: 20% FCS (Gibco), 20% horse serum (Sigma) and 20% allogenic horse serum (Belgium lab) in $CO_2$ incubators. The hrs-AT-MSC growth and proliferation was better in cultural conditions where 20% FCS and 20% horse serum (Sigma) were used. Mesenchymal stem cell count was highest in the condition where horse serum (sigma) was used than both FCS and horse allogenic serum. The viability was more in where allogenic serum (Belgium lab) was used than both FCS (Gibco) and horse serum (Sigma). Like FCS (Gibco), horse serum (Sigma) and allogenic horse serum (Belgium lab) also showed promising / positive effects on equine adipose tissue derived mesenchymal stem cell (hrs-At-MSC) culture and proliferation. Horse serum was found as efficient as fetal calf serum in supporting proliferation and differentiation of equine mesenchymal stem cells in vitro. Further studies are needed to analyze these aspects of MSC in tissue regeneration.

Volume 7 Issue 1 - 2021

Swapan Kumar Maiti,[1] Guy Wouters,[2] Dimitry Spitkovsky,[3] Jurgen Hescheler[3]

[1]Principal Scientist, Surgery Division, ICAR-Indian Veterinary Research Institute, India

[2]Scientific Director and CEO, Fat Stem Laboratories, Belgium

[3]Institute of Neurophysiology, University of Köln, Robert-Koch Str. 39, Germany

**Correspondence:** Swapan Kumar Maiti, Principal Scientist, Surgery Division, ICAR-Indian Veterinary Research Institute, Izatnagar-243122 UP, India, Email swapanivr@gmail.com

**Received:** December 04, 2020 | **Published:** March 18, 2021

Stem cell biology has attracted tremendous interest recently. It is hoped that it will play a major role in the treatment of a number of incurable diseases via transplantation therapy. Several verities of stem cells have been isolated and identified in vivo and in vitro. Very broadly they comprise of two major classes: embryonic and adult mesenchymal stem cells.[1] Mesenchymal stem cells (MSCs) because of their self replication, differentiate into various types of mature cells and tissues, and regeneration capabilities are regarded as an excellent source of cells for tissue engineering and for treatment of various incurable diseases and therapeutic uses in gene therapy, drug delivery, and reconstructive surgery.[2,3] Recently, induced pluripotent stem cells (iPSC) and embryonic stem cells (ESCs) attracted researchers in organogenesis and cell-mediated therapy experiments, however, teratoma formation, ethical issues, and graft vs host rejection are the major limitations in development and therapeutic application of these cells.[4] Due to these limitations, mesenchymal stem cells (MSCs) from adult tissues are now attractive material for and tissue engineering and cell-mediated therapy.[5]

Isolation of MSC derived from equine species has been reported in a number of different tissues, including bone marrow,[6] peripheral blood,[7] fat tissue[8] and umbilical cord blood.[9] Adipose tissue derived equine MSC (hrs-AT-MSC) exhibit the ability to differentiate into different types of cells and tissues in appropriate culture conditions using growth factors and specific hormones into osteoblast, chondroblat and adipocytes and a profound proliferative ability without hampering their own genetic firmness.[8] Serum is an integral component for MSC culture in vitro and also it is essential for osteogenic differentiation of MSC along with other factors includes β glycerophosphate, ascorbic acid, and dexamethasone as osteogenic supplements.[10] Serum is an essential component of complete growth media for MSC culture because it provides growth factors, nutrients and extracellular matrix proteins which support MSC cells in vitro.[11,12] There is also evidence that serum may act as an antioxidant for cells.[13] Despite its zoogenic content, animal serum has been used since the first isolation of MSCs and remains a prime component for their culture and differentiation. Different methods for reducing animal antigens in fetal calf serum (FCS) have been suggested but none alleviate 100% percent risks,[14] which leads to current researches for the development of substitute culture conditions, and a move towards the possible use of cheap, readily available as well as potentiate serum from other sources. The purpose of this study was to use three different types of serum for the culture and proliferation of equine adipose tissue derived mesenchymal stem cells (hrs-AT-MSC).

## Materials and Methods

Equine adipose derived mesenchymal stem cells (hrs-AT-MSC) were obtained from the Fat stem cell Laboratories, Aalst, Belgium. For culture and growth of hrs-AT-MSC three different serums have been used. The hrs-AT-MSC- was cultured in 20% FCS (Fetal Calf Serum, Gibco); 20% horse serum (Sigma) and 20% allogenic horse serum obtained from Fat Stem Laboratories, Aalst, Belgium.

We have used Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG) (Sigma) which contains 1000mg/L, L-glutamine, and sodium-bicarbonate; Anti-Anti (100X) Antibiotic-Antimycotic (Gibco); Petri dishes and different sizes culture flasks (25ml, 75ml) (Nunc) for cell isolation and cell culture; Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher) for cell washing; 0.25% Trypsin- EDTA (1X) Trypsin-EDTA (Gibco) for cell lifting and cell harvesting.

Cell viability was examined by flow cytofluorometry (FACS) method adding Propidium iodine (Pi) stain. Mesenchymal stem cell's morphological features were examined under an Axiovert 25 phase contrast microscope (Zees) attached with a digital camera. Mesenchymal stem cell's proliferative activities were evaluated by cell counting with the help of Casy I cell counter on day 0, 1, 2, 3, 4, 5, 6, 7 and d 8 cultures under normoxic condition. The Mann-Whitney test was carried out to estimate the significance of differences. Mesenchymal stem cells (MSC) isolated from equine fat tissues, passages 1-4, and were used for this study.

### MSC Isolation:

Equine adipose derived MSCs were isolated as per previously described method.[8] Mononuclear cells were obtained using a commercially available kit, as per the manufacturer's instructions, followed by Ficoll-Paque density gradient centrifugation, and plated

in non-coated tissue culture flasks in the growth medium having three types of serum i.e. 20% FCS (Gibco), 20% horse serum (Sigma) and 20% allogenic horse serum (Belgium Company/lab). Cells were allowed to adhere overnight and non-adherent cells were washed out with medium changes. Medium changes were carried twice weekly thereafter.

**Limiting dilution:**

After 7-10 days at near confluence of the cultures, the cells were detached using Trypsin/EDTA. The cells were passage at $5x10^3$cells/$cm^2$ and cultured to reach 80-90% confluence at passsage2 (P2).

**Maintenance and culture expansion:**

Once adherent cells reached approximately 80 % to 90% confluence, they were lifted from the culture medium with addition of Trypsin-EDTA, washed three times with Dulbecco's Phosphate Buffered Saline (DPBS); centrifugation @1000 rpm for 5 minutes, and repeated under the same culture conditions. Cells were incubated at $37^0$C in a humidified $CO_2$ incubator under standard conditions ($5\%CO_2$, $21\%O_2$).

**Flow cytometry/ Immunophenotyping:**

About $2\times10^5$ hrs-AT-MSCs was concentrated by centrifugation at 500g for 5 min, suspended in 50 µl DPBS containing 1% (w/v) bovine serum albumin, and labeled with the primary antibodies (Integrin alfa 5). The samples were incubated for 30 min at room temperature and, then for 20 min at room temperature with the secondary antibody. The samples were then analyzed by flow cytometry (FC; FACS caliber, BD Biosciences, San Jose, Calif., USA) with Cell Quest software. To assay cells by the forward and side scatter of light, the instrument was standardized with microbeads (Dynosphere Uniform Micro spheres, Bangs Laboratories, Fisher Scientific, USA).The experiments were repeated with MSCs from three separate preparations.

## Results and discussion

Primary culture of hrs-AT-MSC cultured on complete growth media containing three different serums revealed same type of cells and they were on active proliferative stage. The population of MSC, however, became heterogeneous in subsequent time lapse and revealed different morphological types of cells but they were mostly under actively proliferative stage. The hrs-AT- MSC culture in three different serums revealed the following cells: triangular or spindle-shaped cells with homogenous cytoplasm-actively proliferating stage; fibroblast-like cells with large nucleus-also in active proliferative stage; and polygonal, oval, or irregular shape highly spread cells with heterogeneous cytoplasm-in slowly proliferative stage. This proliferative activity of cells in three different types of serum cultures decreases by 4th passage and then the spread cells were chief/main cells in the culture medium. This "proliferative inhibition" was abolished during further sub-cultures and proliferative activity was increased rapidly which lead to developed morphologically similar cells predominantly in the three different serum containing cell culture.

Morphological features of mesenchymal stem cells were examined on three different serums on different culture days (Figures 1-4). On day 8th post seeding, 80- 90% confluence with typical spindle shaped MSC was observed in 20% FCS; 70-80% confluence with spindle but more elongated MSC was observed in 20% HS-Sigma, whereas, 50-60% confluence with varied shape (spindle to cuboidal), round nuclei, more number of nucleolus and stunted cytoplasmic process with signs of mineral deposition was observed in 20% allogenic horse serum (Belgium lab).

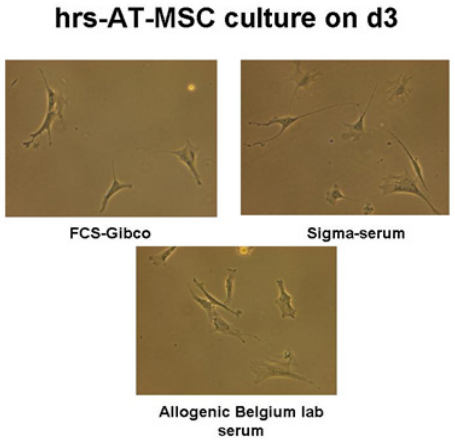


**Figure 1** Morphological characterization of hrs-AT-MSC in three different serum cultures on day 3.

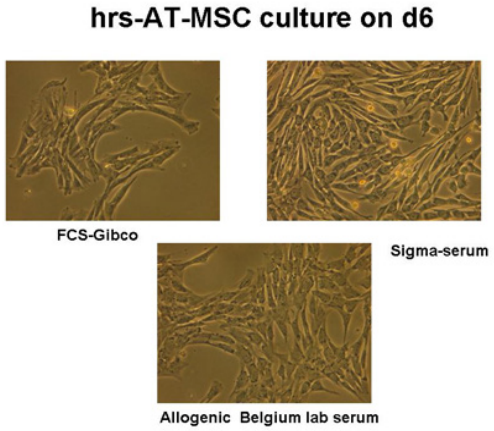


**Figure 2** Morphological characterization of hrs-AT-MSC in three different serum cultures on day 6.

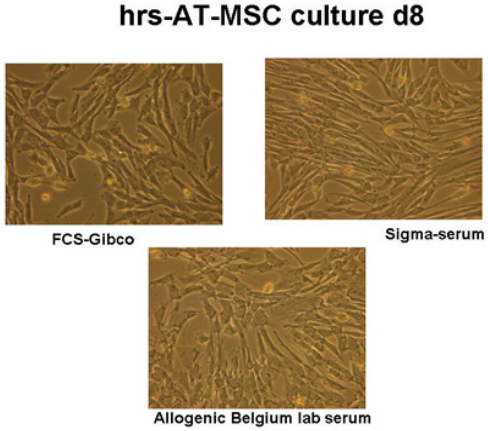


**Figure 3** Morphological characterization of hrs-AT-MSC in three different serum cultures on day 8.

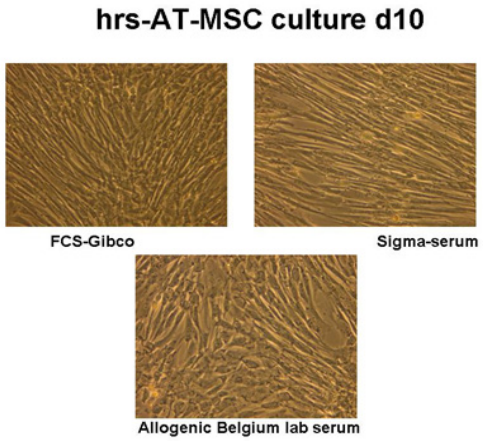


**Figure 4** Morphological characterization of hrs-AT-MSC in three different serum cultures on day 10.

Cell count and cell viability test was performed on day 10 of primary culture. Cell population, cell size, cell viability and cell mortality in three different types of serum culture is depicted in the table-1. Highest cell proliferation was recorded in 20% horse serum (Sigma), followed by 20% FCS (Gibco) and then 20% allogenic horse serum (Belgium lab) (Figure 5). Cell diameter was largest in 20% allogenic horse serum (Belgium lab) followed by 20% FCS (Gibco) and then 20% horse serum (Sigma). Cell viability was more in 20% allogenic horse serum (Belgium lab) followed by FCS (Gibco) and then 20% horse serum (Sigma) (Figure 6). Cell mortality was highest in 20% horse serum (Sigma) and lowest in 20% allogenic horse serum (Belgium lab).

**Table 1** Cell count, cell diameter and cell viability

| Parameters | 20% FCS (Gibco) | 20% Horse serum (Sigma) | 20% Allogenic Horse Serum (Belgium lab) |
|---|---|---|---|
| Cell population | CC- 8.359 x $10^5$ /ml | 1.079 x $10^6$ /ml | 4.908 x $10^5$ /ml |
| Cell Diameter | 15.34µm | 14.68µm | 16.07µm |
| Cell viability | 89.62% | 88.93% | 93.43% |
| Cell mortality | 10.38% | 11.07% | 6.57% |

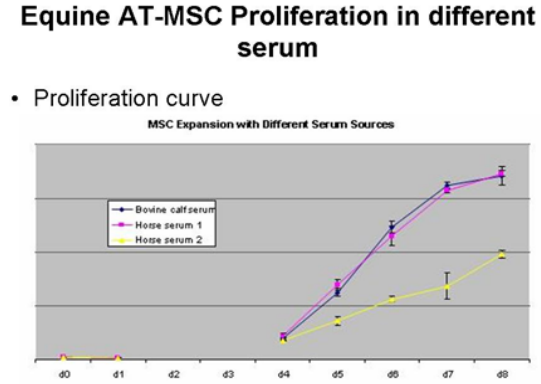


**Figure 5** Proliferation curve of hrs-AT-MSC in three different serum cultures.

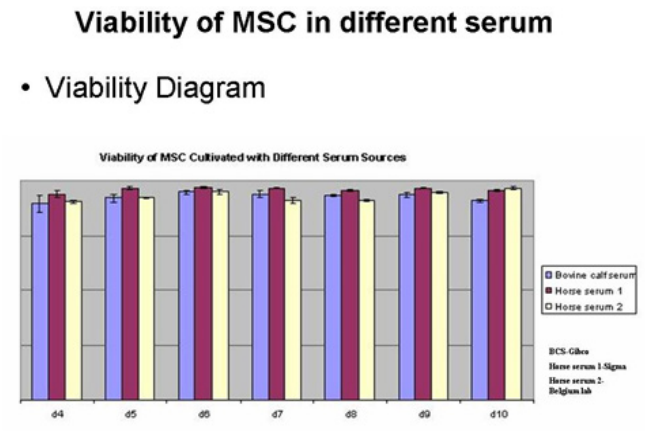


**Figure 6** Viability of hrs-AT-MSC in three different serum cultures in different days.

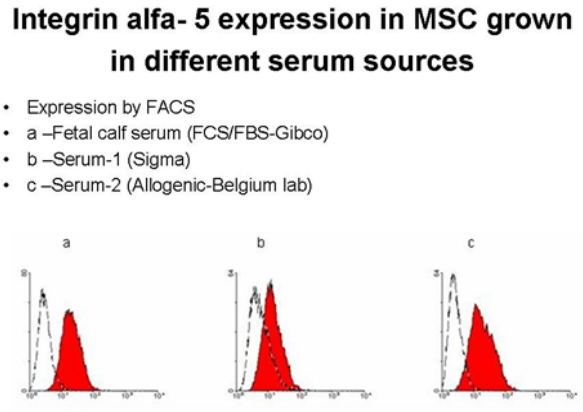


**Figure 7** Integrin alpha5 expression of hrs-AT-MSC in three different serum cultures.

The immunophenotype with Integrin alpha 5 expressions was higher in 20% FCS (Gibco) followed by 20% horse serum (Sigma) and then 20% allogenic horse serum (Belgium lab) (Figure 7). The Integrin family of cell surface receptors appears to play a major role in the mediation of the cell-extracellular matrix (ECM) interacts associated with structural and functional changes in surrounding tissues. The Integrin are heterodimeric glycoprotein that are composed of an α and a β subunit. Integrin-mediated signaling is involved in a variety of cellular process such as differentiation, adhesion and migration. Integrin-mediated signaling seems to play an important role in the generation and maintenance of the osteoblast and chondrocyte differentiation in stem cells derived from bone marrow and adipose tissue.[15,16] In our study, expression of Integrin α was found more when 20% FCS was used in equine adipose derived mesenchymal stem cell culture than 20% horse serum (sigma) and 20% allogenic horse serum (Belgium lab) which in accordance to our findings that growth and proliferation of mesenchymal stem cells were better where 20% FCS was used in culture than 20% horse serum (Sigma) and 20% allogenic horse serum (Belgium lab) was used. Integrin α may be considered as a good immunophototyping marker for adipose tissue derived mesenchymal stem cell culture and proliferation.

In general, hrs-AT-MSC growth and proliferation was better in cultural conditions where 20% FCS and 20% horse serum (Sigma) were used. Mesenchymal stem cell count was highest in the condition where horse serum (sigma) was used than both FCS and allogenic horse serum (Belgium lab). The viability was more in where allogenic horse serum (Belgium lab) was used than both FCS (Gibco) and horse serum (Sigma). Like FCS (Gibco), horse serum (Sigma) and allogenic horse serum (Belgium lab) also showed promising /positive effects on equine adipose derived mesenchymal stem cell (hrs-At-MSC) culture and proliferation.

Equine Mesenchymal stem cells (hrs-MSCs) exhibit very encouraging results in bone repair and other tissue regeneration applications.[17] Serum derived from animal sources (mainly bovine) now used profoundly for mesenchymal stem cell culture as it's a important source of extracellular matrix proteins, nutrients and growth factors or cytokines. Due to animal antigens and ill-defined composition of FCS/FBS and regular uses of bovine calf serum, leads to the development of alternatives protocols. The present study was, therefore, undertaken to reduce exposure to FCS/FBS by the use of three different source of equine serum. In this study, different equine serums were used to support the growth, proliferation and expansion of equine adipose tissue mesenchymal stem cell, and also retaining their surface marker expression during culture and proliferation. After serum exposure to the first 12-14 days of treatment; hrs-AT-MSCs was recorded higher proliferation with horse serum (Sigma) than with FCS/FBS (Gibco).

Human serum (HS) was used initially for the culture of human mesenchymal stem cells (h-MSC) showed an efficient replacement for routine FCS uses and revealed osteogenic differentiation of human mesenchymal stem cell in vitro culture. It was concluded that human serum (HS) well supported MSC growth; differentiation and expansion during in vitro culture and proposes a suitable replacement to FCS for clinical applications. Osteogenic differentiation rate was higher when used HS compared to FCS culture.[18] Human serum found similarly effective as fetal bovine serum in supporting growth, proliferation and differentiation of human mesenchymal stem cells in vitro and in vivo.[19]

FBS/FCS provides growth factors, cell attachment factors and different cell nutrients. Amongst suppliers or companies or amongst different batches of same company; concentrations or percentage of these growth factors and nutrients in FBS/FCS are varied. So utilizing FBS consisting uncharacterized components ultimately leads to the heterogeneity of MSC culture, MSC number and MSC quality when culture with FBS of different supplier or different batches of same supplier. It's also reported that FBS can also be a source of harmful pathogens and contains some serum proteins which potentiate elicit immune response in host. To overcome these deficiencies associated with the addition of FBS in MSC culture, use of autogenous or allogenic serum, plasma or platelet lysate are suggested for human MSCs culture and propagation.[20] The uses of blood products for the cultivation/culture of canine MSCs is also reported.[21] The concept of serum-free medium predominantly devoid of animal components to eliminate variability associated with FBS in MSC culture is not novel for culture and propagation of human and rodent MSCs, because, efficiency of MSC growth varies depending on the media formulation.[22] Similarly, uses of serum-free media developed for isolation, culture and expansion of human, rodent or canine MSCs is often met with mixed results.[22,23] More research work is needed to conclude and compare the approach to bovine serum-deprived MSC complete growth media models including the use of platelet lysate[14] or serum-deprived growth medium with supplementation of prime components present in serum[24] for hrs-AT-MSC differentiation and expansion for their ultimate uses in cell-based therapy and tissue regeneration.

## Conclusion

FCS (Gibco) was found better for equine adipose derived mesenchymal stem cells (hrs-AT-MSC) culture and proliferation; however horse serum (Sigma) and allogenic horse serum (Belgium lab) can be used as alternate to fetal calf serum. Horse serum (Sigma) and allogenic horse serum (Belgium lab) are less costly than FCS (Gibco) in equine stem cell culture and research.

## Acknowledgements

None.

## References

1. Maiti S K, Shiva Kumar, Srivastava L, et al. Isolation, proliferation and morphological characteristics of bone marrow derived mesenchymal stem cells from different animal species. *Trends Biomater Artif Organs.* 2013;27(1):29–35.
2. Shtrichman R, Germanguz I, Eldor JI. Induced pluripotent stem cells (iPSCs) derived from different cell sources and their potential for regenerative and personalized medicine. *Current Molecular Medicine.* 2013;13(5):792–805.
3. Toh WS, Lee EH, Cao T. Potential of human embryonic stem cells in cartilage tissue engineering and regenerative medicine. *Stem Cell Rev Rep.* 2011;7(3):544–559.
4. Amiri F, Jahanian–Najafabadi A, Roudkenar M H. In vitro augmentation of mesenchymal stem cells viability in stressful microenvironments. *Cell Stress Chaperones.* 2015;20(2):237–251.
5. Law S, Chaudhuri S. Mesenchymal stem cell and regenerative medicine: regeneration versus immunomodulatory challenges. *American journal of stem cells.* 2013;2(1):22–38.
6. Smith RK, Korda M, Blunn GW, et al. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Vet J.* 2013;35(1):99–102.
7. Koerner J, Nesic D, Romero JD, et al. Equine peripheral blood–derived progenitors in comparison to bone marrow–derived mesenchymal stem cells. *Stem Cells.* 2006;24(6):1613–1619.
8. Vidal MA, Kilroy GE, Lopez MJ, et al. Characterization of uine adipose tissue–derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow–derived mesenchymal stromal cells. *Vet Surg.* 2007;36(7):613–622.
9. Koch TG, Heerkens T, Thomsen PD, et al. Isolation of mesenchymal stem cells from equine umbilical cord blood. *BMC Biotechnol.* 2007;7(1):26.
10. Kuznetsov SA, Mankani MH, Robey PG. Effect of serum on human bone marrow stromal cells: Ex vivo expansion and in vivo bone formation. *Transplantation.* 2000;70:1780–1787.
11. Bieback K, Hecker A, Kocaoemer A, et al. Human Alternatives to Fetal Bovine Serum for the Expansion of Mesenchymal Stromal Cells from Bone Marrow. *Stem Cells.* 2009;27(9):2331–2341.
12. Liu CH, Wu ML, Hwang SM. Optimization of serum free medium for cord blood mesenchymal stem cells. *Biochem Eng J.* 2007;33: 1–9.

13. Meuleman N, Tondreau T, Delforge A, et al. Human marrow mesenchymal stem cell culture: Serum–free medium allows better expansion than classical alpha–MEM medium. *Eur J Haematol.* 2006;76:309–316.

14. Perez–Ilzarbe M, Diez–Campelo M, Aranda P, et al. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. *Transfusion.* 2009;49(9):1901–1910.

15. Goessler UR, Bugert P, Bieback K, et al. Integrin expression in stem cells from bone marrow and adipose tissue during chondrogenic differentiation. *Int J Mol Med.* 2008;21(3):271–279.

16. Navarrete R O, Rodil SE, Hyzy SL, et al. Role of Integrin subunits in mesenxhymal stem cell differentiation and osteoblast maturation on graphitic carbon–coated microstructures surfaces. *Biomaterials.* 2015.51:69–79.

17. Mocchi M, Dotti S, Bue M D, et al. Veterinary regenerative medicine for musculoskeletal disorders: can mesenchyal stem/ Stromal cells and their secretome be the new frontier? *Cells.* 2020;11(9):143.

18. Popov A, Scotchford C, Grant D, et al. Impact of serum source on human mesenchymal stem cell osteogenic differentiation in culture. *International Journal of Molecular Sciences.* 2009;20(20):5051.

19. Aldahmash A, Haack–Sorensen M, Al–Nbaheen M, et al. Human serum is as efficient as fetal bovine serum in supporting proliferation and differentiation of human multipotent stromal (mesenchymal) stem cells in vitro and in vivo. *Stem Cell Rev Rep.* 2011;7(4):860–868.

20. Panchalingam KM, Jung S, Rosenberg L, et al. Bioprocessing strategies for the large–scale production of human mesenchymal stem cells: a review. *Stem Cell Res Therapy.* 2015;6:225.

21. Russell KA, Gibson TWG, Chong A, et al. Canine platelet lysate is inferior to fetal bovine serum for the isolation and propagation of canine adipose tissue– and bone marrow–derived mesenchymal stromal cells. *PLoS One.* 2015.

22. Clark KC, Kol A, Shahbenderian S, et al. Canine and Equine mesenchymal stem cells grown in serum free media have altered immunophenotype. *Stem Cell Rev Rep.* 2016;12(2):245–256.

23. Schwarz C, Leicht U, Rothe C, et al. Effects of different media on proliferation and differentiation capacity of canine, equine and porcine adipose derived stem cells. *Res Vet Sci.* 2012;93(1):457–462.

24. Lange C, Cakiroglu F, Spiess AN, et al. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum–free medium for transplantation and regenerative medicine. *J Cell Physiol.* 2007;213(1):18–26.