Phenotypic and functional characterization of bovine adipose-derived mesenchymal stromal cells

Jeremy G. Powell
   University of Arkansas at Fayetteville
Sriya Pokharel
   University of Arkansas at Fayetteville
Vitali V. Maldonado
   University of Arkansas at Fayetteville
Rebekah Margaret Samsonraj (✉ rmsamson@uark.edu)
   University of Arkansas at Fayetteville

Research Article

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Abstract

Objective: Mesenchymal stem cells (MSCs) are increasingly trialed in cellular therapy applications in humans to treat several degenerative and inflammatory disorders. Not only are MSCs useful as a therapeutic for human diseases, but they can also be applied to treat a range of diseases in animals. Particularly, larger animals such as cattle can benefit from MSC therapies to combat inflammatory conditions and aging-associated degenerative disorders. Given that MSCs have multilineage differentiation and immunomodulatory properties, we sought to demonstrate the feasibility of obtaining MSCs from adipose tissue and characterizing cells using established phenotypic and functional assays.

Animals: Adipose tissue from three individual healthy cows was obtained from the tail head area and processed for MSC isolation.

Procedures: MSCs were isolated using in-house optimized tissue digestion protocols and characterized by performing colony formation assay, cell growth assessments, cell-surface marker analysis by immunocytochemistry, osteogenic and adipogenic differentiation, and secretion of indoleamine 2,3-dioxygenase (IDO).

Results: MSCs were successfully isolated from bovine adipose (bv-Ad) tissue and demonstrated the ability for prolonged culture expansion, colony formation, and differentiation into osteogenic and adipogenic lineages. Bv-AdMSCs secreted significant amounts of IDO with or without interferon-gamma stimulation, indicating the ability for immunomodulation.

Clinical Relevance: Research outcomes point toward the feasibility of achieving autologous bv-AdMSC cellular preparations that can be applied in the cattle industry as a cell therapy. Our methodology can be utilized by veterinary cell therapy labs for preparing autologous or allogeneic MSCs for disease management in cattle.

Lay Summary

Our laboratory has been studying a type of stem cell called mesenchymal stem cells (MSCs) for their potential use in treating degenerative and inflammatory diseases in both humans and animals. In this study, we focused on obtaining MSCs from the fat tissue of cows and investigating their characteristics and functions. We collected fat tissue samples from healthy cows and used specific methods to isolate the MSCs. We performed various tests to assess the cells’ ability to form colonies, grow and divide, differentiate into bone and fat cells, and produce a substance called indoleamine 2,3-dioxygenase (IDO) that helps regulate the immune system. The results showed successful isolation of MSCs from the cows’ fat tissue which could be grown and expanded in culture for an extended period. The MSCs also secreted significant amounts of IDO, indicating their potential to modulate the immune system and control inflammation. This research has important implications for the cattle industry, as it suggests that autologous (from the same individual) MSCs derived from fat tissue could be used as a complementary therapy for various diseases in cows. These MSCs may provide additional benefits compared to conventional treatments by addressing inflammation and tissue scarring associated with common cattle diseases. The methodology used in this study can be adopted by veterinary cell therapy labs to prepare MSCs for managing diseases in cattle, either from the same individual or from other donors.

Teaser Text
Our research has successfully enabled obtaining and characterizing mesenchymal stem cells (MSCs) from cows’ fat tissue, offering a promising therapy for cattle diseases and providing a methodology for veterinary cell therapy labs.

INTRODUCTION

Among all domesticated species, cattle have major significance in the economics of the livestock industry, with 69.6 million tons of meat and 811 million tons of milk produced worldwide in 2017 (DairyMarketReview, 2018; FoodOutlook, June 2017). There are several medical conditions that can negatively impact meat and milk production as well as reproductive efficiency in cattle. For cattle with high economic potential, these losses pose significant costs to the livestock industry. For example, bovine respiratory diseases in cattle is economically devastating in the US cattle industry costing producers over an estimated $500 million each year, and treatment has exclusively involved antibiotics (Duff & Galyean, 2007). There is heightened immune activity (McGill & Sacco, 2020) associated with respiratory diseases in cattle (Fig. 1) that could result in lung tissue destruction. Other diseases that affect cattle include mastitis, digestive disorders, encephalopathies, white muscle disease, to name a few (Perry et al., 2013). Considering the potential of stem cells in tissue repair and regeneration, obtaining MSCs from bovine adipose tissue could be applied for addressing a variety of disorders that require anti-inflammatory interventions and pro-regenerative functions.

Mesenchymal stem/stromal cells (MSCs) are multipotent stem cells that are characterized by their ability to adhere to plastic, express cell surface markers CD105, CD73 and CD90, and lack surface markers CD45, CD34, CD14 or CD11b, as well as the ability to differentiate into osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). In addition, MSCS have been shown to secrete growth factors and other soluble molecules such as interleukins, cytokines, and chemokines that play critical roles in tissue repair and regeneration. Owing to their self-renewal, regenerative and immunomodulation properties, MSCs function as medicinal signaling cells by virtue of their secretion of soluble factors and proteins that are anti-inflammatory, anti-fibrotic, anti-microbial, anti-apoptotic, and pro-regenerative (Caplan, 2019). MSCs inhibited *Staphylococcus aureus* biofilm formation in vitro and disrupted the growth of established biofilms (Chow et al., 2020). MSCs have been trialed in treating bovine mastitis (Cahuascanco et al., 2019; Peralta et al., 2020), and bone repair in small animal models (Grayson et al., 2015). In treating respiratory infections, the risk of antibiotic resistance may remain, however it can be modulated with the adjunct use of MSCs wherein the MSCs may serve to aid in the recovery of damaged tissue. MSCs also produce antimicrobial peptides (AMPs) - short peptides commonly found in neutrophils or epithelial cells (Alcayaga-Miranda et al., 2017). AMPs kill bacteria directly by disrupting the integrity of the microbial membrane, or by inducing the release of proinflammatory cytokines and in turn the recruitment of immune cells. MSCs do not necessarily engraft or differentiate in vivo but secrete such bioactive factors that stimulate the microenvironment to fight infection and restore host tissue. In humans, MSC clinical trials have been successful in the treatment of graft-vs-host disease (Kelly & Rasko, 2021), amyotrophic lateral sclerosis (Gugliandolo et al., 2019), stroke (Li et al., 2021), rheumatoid arthritis (Lopez-Santalla et al., 2021), and respiratory disorders including COVID-19 related complications (Shi et al., 2022).

Of the several tissue sources of MSCs, adipose tissue-derived MSCs are particularly advantageous owing to their ease of isolation and higher yield in cell numbers obtained from primary tissue digests compared to bone marrow tissues (Rider et al., 2008; Samsonraj et al., 2018). In cellular therapy applications, significant numbers of cells are required during transplantation or administration, and therefore, adipose tissues are regarded as a better source of MSCs. Furthermore, acquiring adipose tissue from animals is comparatively less invasive than bone marrow aspiration.
In this research article, we demonstrate the feasibility of isolating bovine MSCs from adipose tissue and characterizing the cells *in vitro* based on established phenotypic and functional assays (Samsonraj et al., 2013; Samsonraj et al., 2015). MSCs were assessed for colony formation, self-renewing proliferative ability, presence of characteristic cell surface markers, differentiation capacities, and immunomodulatory features. Our preliminary findings in obtaining potent populations of MSCs highlight the viability of our approach which holds significant potential for the development of a new generation of ‘living’ drugs for treating a range of diseases that may require anti-inflammatory and regenerative properties.

**MATERIALS AND METHODS**

1. **Collection of adipose (fat) tissue from cows**

All procedures were performed in accordance to institutionally approved IACUC protocol #21092. After restraining cow in a squeeze chute, the tailhead area (either side of the mid-line over the pin bones) was shaved of hair, disinfected with chlorhexidine scrub solution and isopropyl alcohol, then the incision site was injected with 10 mL of 2% lidocaine solution under the skin and into the adipose tissue. After sterilizing the site, a straight-line incision (approximately 1.5 to 2 inches) through the skin was made parallel to the spine using a sterile scalpel. Adipose tissue was removed using the scalpel, tissue scissors and sterile forceps. The incision area was closed with absorbable suture and tissue glue. The incision site was then cleaned, and an insect repellent was applied. Once retrieved from the animal, adipose tissue was washed extensively with sterile cold saline supplemented with 400 µg/mL streptomycin and 400 units/mL penicillin prior to further tissue digestion.

2. **Isolation of bovine adipose-derived mesenchymal stem cells (BvAdMSCs)**

The adipose tissue sample was collected in PBS solution supplemented with 1% penicillin-streptomycin (P/S) (Gibco 15140-122) solution. After sterilization of the cell culture hood, the collected tissue sample was transferred to a 10 cm cell culture plate. The tissue sample was then minced carefully for about one hour using a sterile scalp blade (Fig. 2). For the digestion of minced tissue, the tissue was placed in a tube containing Alpha Minimum Essential Medium (MEM α, Gibco™ 12571-063) supplemented with 0.001% type I collagenase (Gibco™), 1% penicillin-streptomycin (P/S) and 50 µg/ml nystatin. The tissue was then incubated for digestion for 3 hours. After the completion of 3 hours digestion, the collagenase media was neutralized by adding equal volume of MEM α (Gibco™) supplemented with 15% Fetal Bovine Serum (FBS, Gibco™), 50 µg/ml nystatin, 80 µg/ml amikacin sulfate and 1% penicillin-streptomycin (P/S). After neutralization, digested tissue was centrifuged at 300g for 10 minutes. The cell pellet was then resuspended in 20 ml of Alpha Minimum Essential Medium (MEM α, Gibco™) supplemented with 15% Fetal Bovine Serum (FBS, Gibco™), 50 µg/ml nystatin, 83.4 µg/ml amikacin sulfate and 1% penicillin-streptomycin (P/S) solution and was centrifuged again for 10 minutes at 300g. After centrifugation, the supernatant was aspirated without disturbing the cell pellet and the cell pellet was resuspended in Alpha Minimum Essential Medium (MEM α, Gibco™) supplemented with 15% Fetal Bovine Serum (FBS, Gibco™), 50 µg/ml nystatin, 80 µg/ml amikacin sulfate and 1% penicillin-streptomycin (P/S) solution (hereafter referred to as ‘culture maintenance medium’). The cells were then plated in 10cm dishes with the same culture maintenance medium (Fig. 2). Plated cells were maintained in incubators at 37.5°C with 5% CO₂. The culture medium was changed 24 hours after the initial seeding to remove any non-adherent cells from the culture. All *in vitro* cell cultures were maintained in humidified incubators maintained at 37.5° C with 5% CO₂.
3. Cell culture and maintenance

The culture medium was replaced every other day after the first media change done 24 hours post seeding. The cells were monitored regularly to assess their growth and the first colonies were observed within a 10–14-day period after the initial plating. At around 70–80% confluence, the cells were trypsinized (TrypLE™ Express, Gibco) and replated in new 10 cm dishes at around 3000 cells/cm$^2$. For this process, the cell plate was washed two times with phosphate buffered saline (PBS, pH 7.4, Gibco™) and the cells were incubated with trypsin for 4 minutes. The trypsin activity was neutralized with double the volume of culture maintenance media after which the cells were scrapped carefully using a cell scrapper. The cell suspension was centrifuged at 250 g/rcf for 5 minutes and the supernatant was aspirated without disturbing the cell pellet. The cell pellet was resuspended in culture maintenance media, and the cells were plated in new 10 cm dishes.

4. Morphological characterization

The first cells were seen attached to the plastic dish at around 4–8 days post seeding. The cells formed circular colonies within 2–3 days after the first cell was seen. This represents the ability of bovine adipose derived mesenchymal stem cells to adhere to plastic as well as form colonies (Fig. 3). The cells were monitored every day to check for the formation of new cell colonies as well as growth of existing cell colonies. The cells were monitored using EVOS XL CORE imaging system at 4x magnification. The cells were split and passaged when multiple healthy colonies were observed in the flask.

5. Colony forming unit-fibroblastic assay

Colony forming unit – fibroblastic (CFU-F) ability was tested for the cells. The cells were divided into 2 groups with 150 cells per dish in one group (n = 3) and 500 cells per dish in the other group (n = 3). The cells were then maintained in culture for 2 weeks with a medium replacement at the 7th day. On 14th day, the cells were washed twice with PBS and stained using crystal violet stain. The cell colonies were counted on each plate and the CFU-F ability was analyzed by plotting average number of colonies formed against the number of cells seeded.

6. Proliferation assay

The growth of bovine adipose derived mesenchymal stem cells was assessed over a 6-day period. On day 0, cells were seeded into eighteen 6cm cell culture dishes at 3000 cells/cm$^2$ in culture maintenance medium. Starting from day 1 until day 6, cell count was obtained from 3 dishes each day (n = 3). To get the cell count, the cells were trypsinized for 4 minutes after which the trypsin activity was neutralized by adding double the amount of culture maintenance medium. The cells were gently scrapped, and a cell suspension was obtained. The cells were then counted using hemacytometer and trypan blue staining. For a comparative understanding, human bone marrow derived mesenchymal stem cells (hBM-MSCs) were also plated in identical manner as the BvAdMSCs and cell count was obtained for the human cells.

7. In-vitro osteogenic and adipogenic differentiation

The potential for bovine adipose derived mesenchymal stem cells (BvAdMSC) to differentiate into osteogenic and adipogenic lineages were tested in vitro. For osteogenic differentiation, BvAdMSC cells were plated in a 6 well plate at 3000 cells/cm$^2$. Twenty-four hours after the initial plating, the culture medium for experimental group was
replaced with alpha minimum media containing 15% FBS, 0.1 µM dexamethasone (Sigma D4902), 50 µM ascorbic acid-2 phosphate (Sigma A4544), 10 mM β-glycerophosphate (Sigma G9422), 83.4 µg/ml amikacin sulfate and 1% penicillin-streptomycin solution. For the control group, media change was done using the regular culture maintenance medium. The cells were cultured in this medium for 28 days with a culture medium replacement after every 2 days. For adipogenic differentiation, the cells were plated in a 6-well plate at 5000 cells/cm². Cells were maintained in culture for 2 days at above mentioned culture conditions after which media for the experimental group was changed with high glucose DMEM (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10% FBS, with or without 1 µM dexamethasone, 10µM insulin (SAFC- 91077C), 100 µM indomethacin (Sigma-Aldrich I7378), 11.5µg/ml 3-isobutyl-1-methylxanthine (Sigma-Aldrich I5879), 83.4 µg/ml amikacin sulfate and 1% penicillin-streptomycin solution. The cells were maintained in culture for 35 days with a medium replacement after every 2 days.

To confirm whether the cells have differentiated into osteogenic and adipogenic lineages, the cells were stained using Alizarin Red staining and Oil Red O staining respectively. For visualization of osteogenic differentiation, the cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes. The cells were washed again twice with PBS before adding 1% Alizarin Red Solution (pH 4.2). The cells were incubated at room temperature for one hour before washing twice with PBS. To visualize the adipogenic differentiation, the cells were washed with PBS before fixing with 4% PFA for one hour at room temperature. After one hour, the cells were washed once with diH₂O before adding the 0.36% oil red O solution. The cells were incubated at room temperature for one hour after which the cells were washed with 60% isopropanol twice. The cells were then washed multiple times with water (H₂O) to replace any background/precipitates. The dishes were left to dry inside a hood and the stained cells were visualized using a bright field microscope.

### 8. Surface marker characterization

The surface marker characterization was performed for the Bovine Adipose Tissue derived Mesenchymal Stem Cells (BvAdMSCs) using immunocytochemistry techniques. For the characterization of surface markers, cells were seeded at a density of 3000 cells/cm² in 35 mm glass bottom dishes (MATTEK P35G-1.5-14-C). The cells were grown until 80% confluency after which they were fixed using 4% paraformaldehyde (PFA) solution. CD90 antibody conjugated with Alexa Fluor 488 (Novus Biologicals NBP2-47755AF488) was used. For CD45 staining, purified CD45 antibody and Goat anti-Mouse IgG (H+L) secondary antibody [rhodamine] (Pre-adsorbed) (Novus Biologicals NBP1-73133-1mg) were used. Cells fixed in 4% PFA were permeabilized using 0.5% Triton-X 100, after which they were blocked in 5% Horse Serum. The cells were washed 3 times with PBS and incubated overnight in 15 µg/ml antibody prepared in 1% horse serum. After overnight incubation, the cells were washed in PBS and double stained for nucleus visualization using 5 µg/ml DAPI stain. The cells were then imaged using a fluorescence microscope to look for the markers.

### 9. Indoleamine 2,3-dioxygenase (IDO) activity assay

The bovine adipose derived MSCs were tested for secretion of IDO to assess immunosuppressive capabilities. The IDO assay was performed using the IDO Elisa kit (MyBioSource MBS028821). BvAdMSCs were seeded in a 12-well plate at 5000 cells/cm². Cells were plated as control and IFN-γ treatment groups. The treatment group cells were further divided into different IFN-γ concentrations of 50 ng/ml and 100 ng/ml. The cells were grown to 75–80% confluency after which the IFN-γ groups were treated with 50 ng/ml and 100 ng/ml concentrations. After 24 hours, the conditioned media of cells treated with IFN-γ was collected for IDO assay and the cells were lysed using RIPA buffer to obtain a cell lysate. The conditioned media and cell lysate were centrifuged at 6,000 g at 4°C for 10
minutes to obtain debris-free supernatant samples for the IDO assay. IDO assay was performed following manufacturer’s instructions using the IDO Elisa kit (MyBioSource MBS028821).

10. Statistical analyses

To compare the IDO secretion between stimulated and non-stimulated cells, and proliferative potential of MSCs from human bone marrow and bovine adipose tissues, t-test analyses were performed at each time point. A \( p \) value < 0.05 was considered to be significant.

RESULTS

1. Bovine adipose tissue yields self-renewing and proliferative stromal cells

The tissue sample obtained from bovine adipose tissue was successfully cultured to isolate mesenchymal stem cells. Further characterization of isolated cells using morphological characteristics, differentiation abilities and surface marker characterization confirmed the cells were mesenchymal stem cells. Bovine MSCs show morphological characteristics of MSCs: The cultured bovine adipose derived mesenchymal stem cells adhered to the plastic dishes after seeding and formed colonies at around 10–14 days after plating. The cells formed circular cell colonies with cells showing fibroblastic phenotype (Fig. 3).

The bovine adipose derived mesenchymal stem cells also exhibit colony forming units-fibroblastic (CFU-F) abilities. The group with higher density of cells seeded (i.e. 500 cells per dish) showed a higher numbers of colonies formed compared to the group with lower density of cells seeded (i.e. 300 cells per dish) (Fig. 4A-B). The colonies formed by the bovine adipose derived mesenchymal stem cells were of variable sizes (Fig. 4A).

Bovine adipose derived mesenchymal stem cells (BvAdMSCs) and human bone marrow derived mesenchymal stem cells (hBM-MSCs) were plated as mentioned above to analyze their growth over a 6-day period. The BvAdMSCs showed growth over the 6-day period with a peak growth observed at day 5 post seeding. Comparatively, hBM-MSCs also showed growth over the 6 days period with peak cell growth observed at day 4 post seeding (Fig. 4C). This shows that BvAdMSCs have self-renewal ability without any inducing agents or exogenous supplementation of growth factors, and it exhibits proliferation rate similar to human MSCs.

2. Bovine adipose MSCs express characteristic cell surface markers

The bovine adipose derived mesenchymal stem cells were tested for the positive MSC surface marker CD90 and negative MSC surface marker CD45. CD90 (Thy-1) is a 25-30kDa glycoprotein which is routinely used as a positive MSC marker. High expression of CD90 surface protein has been reported in undifferentiated MSCs(Moraes et al., 2016; Yeola et al., 2021). We found that the bovine adipose derived MSCs were positive for CD90 surface marker. The CD90 surface marker was conjugated with Alexa 488 fluorophore represented by a distinct green color on the stained cells (Fig. 5A, left panel). The CD45 surface marker is a membrane glycoprotein with a molecular mass of about 200kDa, that is a leukocyte common antigen and is expressed on most hematopoietic cells(Kumar et al., 2005; Nakano et al., 1990). The cells were negative for the CD45 surface marker, which is characteristic to MSC populations (Fig. 5A, right panel).
3. Bovine adipose MSCs differentiate into osteo and adipogenic lineages

The multilineage differentiation ability of bv-AdMSCs was assessed in vitro to verify their identity as MSCs as part of the minimal criteria to define a mesenchymal stem cell. The cells treated for osteogenic differentiation showed morphological changes within 2 weeks of treatment indicated by mineralization accumulated between cells and increased cell size. The cells were stained positively with Alizarin Red staining that could be visualized both macroscopically as well as under a bright field microscope (Fig. 5B) at 10X magnification. Under adipogenic induction, bv-AdMSCs exhibited a rounded morphology compared to the control group cell’s spindle like morphology, early in the culture. After 3–4 weeks of adipogenic induction, lipid droplets began to form. Upon staining with Oil Red O, the lipid droplets were noted to be stained bright red with visible clusters of droplets under examination the light microscope (Fig. 5C).

4. Bovine adipose MSCs have inducible IDO activity and immunosuppressive potential

Indoleamine 2,3-dioxygenase is an immunosuppressant enzyme secreted by MSCs. IDO is a cytoplasmic protein responsible for converting tryptophan into N-formyl-kynurenine which suppresses the proliferation of T-cells (Zheng et al., 2017). MSCs are routinely investigated for their functional features as a measure of their efficacy and potency. IDO secretion is indicative of MSC ability for immunomodulation. We assessed bv-AdMSCs for secretion of IDO to evaluate their immunosuppressive abilities. At 70–80% confluence, bv-AdMSCs were treated with IFN-γ. We observed that bv-AdMSCs, even without stimulation, secreted significant amounts of IDO without any significant differences upon stimulation with IFN-γ (Fig. 6). This implies that naïve adipose MSCs are capable of immunomodulation even without external stimulus. Together, our results suggest the functional potency of bv-AdMSCs in mediating immunomodulatory functions.

DISCUSSION

Cell-based therapies for bovine diseases are valuable therapeutic tools for a wide range of applications that require regenerative and anti-inflammatory treatment regimens (Alzheimer’s Association: 2016 Alzheimer’s disease facts and figures, 2016). In this preliminary research, we demonstrate the feasibility of successful isolation and characterization of bovine adipose tissue-derived MSCs in vitro. We report our in-house optimized protocol to acquire and generate functionally potent MSCs from adipose tissue through a minimally invasive tissue acquisition approach. One of the major limitations in cellular therapy is the generation of sufficient cell numbers required to promote regenerative or immunomodulatory effects upon transplantation. While bone marrow is a viable tissue source of MSCs, the cellular yield per gram of tissue is comparatively lesser than adipose MSCs. According to a study by Nuvo Pain Management Center in 2019, an adipose tissue sample of the same size as a bone marrow sample had a higher volume of mesenchymal stem cells. This suggests that as well as not being as invasive, adipose tissue removal requires a smaller sample size compared to bone marrow. In addition, obtaining bone marrow aspirates from animals is rather invasive and painful, requiring the use of high dose sedatives or anesthetics. These bottlenecks prompted us to investigate the feasibility of obtaining alternate tissue sources of MSCs, such as the adipose tissue. In our study, the tailhead region of the animal was deemed a safe site for adipose tissue collection, eliminating the needs for both general anesthetics and costly, invasive procedures.
Post-tissue harvest, rigorously established protocols developed from our laboratory permitted effective isolation of adipose MSCs from digested tissues. In cellular manufacturing, efficient isolation involves obtaining single-cell colonies of MSCs in culture within 7–10 days of plating. In our work, visible colonies were observed to develop as early as 3 days post-plating, demonstrating the efficacy of our collagenase-mediated digestion protocols. Cellular yield of 0.8 million primary cells per gram of adipose tissue at 14 days post-plating indicated the robust self-renewal and proliferative capacities of adherent MSC colonies. Both proliferation rates and cell numbers acquired were indicative of efficient culture expansion without the need for exogenous addition of growth factors. Adipose MSCs, in general, are documented to have higher proliferative capacities than their bone marrow counterparts (Burrow et al., 2017; Mohamed-Ahmed et al., 2018). This self-renewing ability of MSCs documented in vitro is a critical determinant in successful treatment outcomes in translational and veterinary MSC therapy trials. We have previously demonstrated that the growth capacity of MSCs is an important attribute in determining the in vivo potency of MSCs (Samsonraj et al., 2013; Samsonraj et al., 2015; Sathiyanathan et al., 2020). In an earlier subcutaneous transplantation study led by our team, human MSCs with higher growth capacity were shown to promote increased new bone formation compared to low growth capacity MSCs (Samsonraj et al., 2015). Therefore, the proliferation ability demonstrated by all three bv-AdMSC cultures in our study holds significance in translational and cellular therapy applications.

In assessing the quality of the in-house isolated adipose MSCs in vitro, an important parameter that ensures multipotency is the ability to differentiate into mesodermal lineages. Bv-AdMSCs used in our study successfully differentiated into osteogenic, adipogenic and chondrogenic lineages (Fig. 4). Confirming successful differentiation ability in vitro is critical to ensure that the cells have passed the necessary minimum criteria for MSC definition and would be suitable candidates for in vivo tissue repair (post-transplantation) as well as for cell-based therapies, for example, in respiratory disease management in cattle which would require engraftment and differentiation to repair damaged lung tissue (Samsonraj et al., 2017; Samsonraj et al., 2015).

Given the trophic milieu of MSCs are largely mediated by secreted proteins, MSCs may act as living drugs exerting beneficial paracrine effects that are immunomodulatory and tissue-reparative, together providing an overall improvement in fighting infection as well as encouraging faster tissue healing. MSCs secrete soluble molecules such as prostaglandin E2 (PGE2), indoleamine 2, 3-dioxygenase (IDO), interleukin IL-10, and transforming growth factor-beta 1 (TGFβ1) that suppress proliferation and/or activity of T cells, B cells, dendritic cells, and activate regulatory T-cells (Tregs) (Samsonraj et al., 2017). One of the principal immunosuppression mechanisms of human MSCs is the production of IDO. IDO is involved in the L-tryptophan catabolism leading to its depletion in the surrounding microenvironment and accumulation of kynurenin, which then inhibits T cell activation, proliferation, and overall functional activity of T cells, dendritic cells, and natural killer cells, among other important effects (Noronha et al., 2019; Samsonraj et al., 2017; Wang et al., 2018). Our work confirms the secretion of IDO by bv-AdMSCs even in the absence of IFNγ, strongly suggesting that the baseline immunomodulation levels of our culture-expanded cells are significant and potent to mediate immunosuppressive effects. In the vision for our ongoing extensions to apply the current methodology in animal health research, our innovative MSC intervention involves use of living cells that display immunomodulatory capacities, while also having the potential towards enhancing tissue recovery.

**CONCLUSION**

Through cell-to-cell interaction and secretion of multiple bioactive molecules, MSCs have functional capacity to create a reparative environment and promote regeneration. Together, our data suggests that MSCs can be
successfully isolated from bovine adipose tissue and cultured in the laboratory to generate sufficient cells with self-renewal and multi-lineage differentiation capacities. Creativity lies in harnessing the soluble factors and proteins secreted by bv-AdMSCs which, as we have thus demonstrated here, are easily obtainable in large quantities from the animal’s own body fat (autologous) and can also be applied as allogeneic therapies by following appropriate biobanking strategies. Clinical trials in human subjects are increasing with promising results for treating musculoskeletal disorders such as osteoarthritis and bone fractures; nervous system conditions such as Alzheimer’s and spinal cord injuries; cardiovascular disorders such as myocardial infarction and cardiomyopathy; and respiratory disorders such as pulmonary hypertension, acute respiratory distress syndrome, COVID-19, and chronic obstructive pulmonary disease, to name a few (clinicaltrials.gov). Given these successes, it is no doubt that MSCs hold potential to ameliorate bovine diseases, and our study has now confirmed the feasibility of producing cellular preparations of potent stem cells in the laboratory, as well as validated stem cell phenotypic characteristics for use in farms and veterinary trials.

**Abbreviations**

- AMPs: antimicrobial peptides
- BvAdMSCs: Bovine Adipose derived Mesenchymal Stem Cells
- CD45: Cluster of Differentiation 45
- CD90: Cluster of Differentiation 90
- CD73: Cluster of Differentiation 90
- CFU: F–Colony Forming Unit–Fibroblastic
- DMEM: Dulbecco's Modified Eagle Medium
- EVOS: EVOS XL CORE imaging system
- FBS: Fetal Bovine Serum
- hBM: MSCs–Human Bone Marrow derived Mesenchymal Stem Cells
- IACUC: Institutional Animal Care and Use Committee
- IDO: Indoleamine 2,3 dioxygenase
- IFN: γ–Interferon gamma
- IgG: Immunoglobulin G
- IL:
Declarations

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

R.M.S. conceived the idea and designed the study; J.G.P. led the process for IACUC approvals and collected bovine adipose tissue; R.M.S., S.P., and V.V.M. conducted experiments; R.M.S. and S.P. drafted the manuscript. R.M.S. and J.G.P. generated financial support, provided input, and edited the manuscript. All authors reviewed and approved the final manuscript.

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References


Figures

![Immune activity in BRD](image)

**Figure 1**

**Key factors involved in immunopathology of bovine respiratory diseases.** Immune activity is elevated in respiratory disorders. Key cytokines and interleukins involved are interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8), interleukin 17 (IL-17), interferons and Toll-like receptors.
Figure 2

**Schematic of bovine adipose tissue processing protocol.** Stepwise illustration of processing bovine adipose tissue. Following mincing on petri dishes with sterile scalpel (Steps 1-3), minced tissue is subjected to collagenase digestion (Steps 4-5) and centrifugation (Step 6). Cell pellet obtained post-centrifugation (Step 7) is resuspended in fresh complete media and seeded for culture expansion (Steps 8-9) and incubated for one week prior to formation of colonies of MSCs.

Figure 3
**Bovine adipose tissue-derived MSC morphology.** Phase-contrast images of bovine adipose tissue-derived MSCs from three individual animals (BvAdMSC 01, BvAdMSC 02, BvAdMSC 03) isolated successfully by plastic adherence. All bovine adipose tissue derived MSCs adhered to plastic and were spindle-shaped in morphology. Colony formation was observed within 2 weeks of plating digested tissue fractions. Scale bar: 100 μm.

![Image](image_url)

**Figure 4**

**Phenotypic characterization: Colony formation and proliferative potential of bovine adipose tissue-derived MSCs.** Adherent MSCs formed colonies when seeded at low densities, indicating their self-renewal ability and colony forming potential. **A.** Scanned images of plates showing colonies of MSCs stained with crystal violet dye. Two different seeding numbers (150 cells per dish and 500 cells/dish) were tested. Scale bar: 1 cm. **B.** Bar graph showing
average number of colonies formed with two different seeding densities. The efficiency of colony formation (%) was similar between the two groups, together indicating that seeding densities did not affect overall CFU-F efficiency. **C.** Line graph showing growth of Bv-AdMSCs in comparison with human bone marrow-derived MSCs. BvAdMSCs proliferated well in vitro over a period of 6 days generating cell numbers similar to human MSCs.

![Figure 5](image)

**Figure 5**

**Phenotypic characterization: Surface marker expression and multilineage differentiation potential of bovine adipose-derived MSCs.** **A.** Immunocytochemistry performed on Bv-AdMSCs showed positive surface marker expression of CD90 (left panel) conjugated with Alexa Flour 488 (green) and nuclear staining with DAPI (blue). Right panel showing negative expression of CD45 probed with corresponding secondary antibody conjugated with rhodamine (red). Lack of CD45 on Bv-AdMSCs is indicated by the absence of signal with immunocytochemical analysis. **B.** Bv-AdMSCs subjected to osteogenic differentiation for 21 days and stained with Alizarin red. Successful osteogenic differentiation is indicated by mineralization as seen by the red deposits when stained with Alizarin Red. **C.** Bv-AdMSCs subjected to adipogenic differentiation for 21 days and stained with Oil Red O. Successful adipogenic differentiation is indicated by formation of lipid droplets as seen by the red deposits when stained with Oil Red O.
**Figure 6**

**Functional characterization: Immunomodulation ability assessment by IDO secretion.** Bar graph showing ELISA-based assessment of the amounts of secretion of indoleamine dioxygenase by Bv-AdMSCs when stimulated with or without IFN-g, indicating the ability of Bv-AdMSCs to act as immunosuppressive agents.