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BIOLOGY

Characterization of bovine mesenchymal stromal cells from visceral adipose tissue collected *post-mortem*

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ABSTRACT: Adipose tissue can be a source of bovine mesenchymal stromal/stem cells (MSCs). However, most studies do not incorporate *post-mortem* collection or *in vitro* differentiation of these cells from bovine fat and other sources into three mesodermal lineages. This study characterized and cryopreserved bovine adipose tissue-derived MSCs (AD-MSCs) collected *post-mortem*. Cells were isolated from cattle (*Bos taurus*) and cultured under standard conditions for *ex vivo* expansion and cryopreservation. Cell growth kinetics was determined by cell doubling time after consecutive cellular passages. Clonogenic capacity was determined using the colony-forming unit fibroblast (CFU-F) assay. The trilineage differentiation assay was performed to determine its *in vitro* multipotency. The *in vitro* proliferation and clonogenic capacity of bovine AD-MSCs was maintained across successive passages (P_2–P_6). Greater clonogenic capacity was found in fresh than post-cryopreservation cells. Cells showed multipotential capacity in adipose, cartilage and bone lineages. In conclusion, no changes were observed in the growth kinetics and colony-forming capacity of the successive passages evaluated. Clonogenic capacity was higher in fresh than post-cryopreserved cells.
Key words: cattle, mesenchymal stem cells, fat, cryopreservation.

Caracterização de células tronco mesenquimais bovinas de tecido adiposo visceral coletadas *post-mortem*

RESUMO: O tecido adiposo pode ser uma fonte de células estromais/tronco mesenquimais bovinas (MSCs). No entanto, a maioria dos estudos não incorpora a coleta post-mortem ou a diferenciação in vitro dessas células a partir de gordura bovina e outras fontes em três linhagens mesodérmicas. O objetivo deste estudo foi caracterizar e criopreservar MSCs derivadas de tecido adiposo bovino (AD-MSCs) coletadas post-mortem. As células foram isoladas de bovinos (Bos taurus) e cultivadas sob condições padrão para expansão ex vivo e criopreservação. A cinética de crescimento celular foi determinada pelo tempo de duplicação celular após passagens celulares consecutivas. A capacidade clonogênica foi determinada usando o ensaio de fibroblastos de unidades formadoras de colônias (CFU-F). O ensaio de diferenciação de trilinhagem foi realizado para determinar sua multipotência in vitro. A proliferação in vitro e a capacidade clonogênica das AD-MSCs bovinas foram mantidas através de passagens sucessivas (P2-P6). Maior capacidade clonogênica foi encontrada em células frescas do que em células pós-criopreservadas. As células mostraram capacidade multipotencial nas linhagens adiposa, cartilaginosa e óssea. Concluindo, não foram observadas alterações na cinética de crescimento e na capacidade de formação de colônias das sucessivas passagens avaliadas. A capacidade clonogênica foi maior em células frescas do que em células pós-criopreservadas.
Palavras-chave: gado, células tronco mesenquimais, tecido adiposo, criopreservação.

INTRODUCTION

The possibility of *ex vivo* multipotency of bovine mesenchymal stromal/stem cells (MSCs), their immunomodulatory capacity and antimicrobial properties have increased scientific focus on these cells (HILL et al., 2019). There are relevant antecedents on the use of cell therapy in cattle diseases such as mastitis (CAHUASCANCO et al., 2019; LANGE-CONSIGLIO et al., 2019; PERALTA et al., 2020) and ovarian injuries caused by the follicular aspiration technique (MALARD et al., 2020). There is substantial interest in the development of "cultured meat" or "*in vitro* meat" from stem cells such as bovine MSCs (OKAMURA et al., 2018; RAMÍREZ-ESPINOSA et al., 2016), among other sources (SKRIVERGAARD et al., 2021).

Although, a number of studies report various aspects of bovine MSCs, few have focused on characterizing these cells. Original reports have concentrated on bone marrow (LU et al., 2014b), adipose tissue (LU et al., 2014a; SAMPAIO et al., 2015; ZHAO et al., 2012), the uterus (LARA et al., 2017) and fetal annexes, such as the placenta, umbilical cord, and amniotic fluid (RAOUFI et al., 2011; SILVA et al., 2016). However, not all characterization studies

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of bovine MSCs confirmed multipotency by *in vitro* trilineage differentiation (DOMINICI et al., 2006) of adipogenic, chondrogenic and osteogenic lines (SAMPAIO et al., 2015; ZHAO et al., 2012; ZHAO et al., 2018). In addition, only ZHAO et al. (2012) evaluated taking *post-mortem* samples to isolate bovine MSCs. Thus, the aim of this study was to characterize and cryopreserve bovine adipose tissue-derived MSCs (AD-MSCs) collected *post-mortem*. We hypothesized that collecting *post-mortem* samples is an advantageous strategy to isolate bovine AD-MSCs because they can be obtained from commercial abattoirs.

MATERIALS AND METHODS

Post-mortem harvesting of adipose tissue from cow carcass

Seventeen 2-month-old Holstein crossbred (*Bos taurus*) calves weighing 82.5–103 kgs were used for *post-mortem* fat harvesting. Eight–ten-gram samples were obtained from the perirenal visceral tissue (Vs).

Isolation and cryopreservation

The tissue samples were transported in a refrigerated sterile tube with low glucose (Gibco) Dulbecco's Modified Eagle Medium (DMEM) and 2% penicillin/streptomycin (antibiotic) (Capricon). The fat was cut into small pieces, and enzymatically digested with type I collagenase (Gibco) for 40 minutes at 37 °C. The samples were centrifuged at 600g for 10 minutes, the supernatant was removed and resuspended in 10 mL of growth medium (GM) consisting of low glucose DMEM, 20% heat-inactivated fetal bovine serum (FBS) (Capricorn) and 2% antibiotic, and incubated at 37 ºC in a humidified atmosphere with 5% CO_2 (YANESELLI et al., 2018). The GM was changed every 3–4 days and 80% of confluent cells were harvested and cryopreserved in a medium composed of 95% FBS and 5% dimethyl sulfoxide (DMSO) and stored at -80 °C.

Cellular proliferation evaluation

a) Cell doubling times (DT): Post-cryopreservation bovine AD-MSCs were used to determine DT and subsequent subcultures at P_2–P_6. To that end, the cells were seeded in triplicate in a 6-well plate at a concentration of 1.7–5×10^3/cm^2 in GM with 10% FBS under standard conditions described above. The GM was changed every 3 days. After 5–7 days, the cells were trypsinized, subcultivated and quantified by trypan blue viability. The formula used was previously described (VIDAL et al., 2006).

b) Colony-forming unit fibroblast (CFU-F) assay: Cells were seeded in triplicate in 6-well culture plates, at a concentration of 1×10^3 cells/well, in GM with 10% FBS in fresh culture in P_1 and post-cryopreservation in P_2–P_6. After 7 days of culture, cells were fixed with methanol at -20°C for 15 min and stained with Giemsa for 30 min (GUERCIO et al., 2013). Colonies were macroscopically identified and counted using ImageJ software.

Trilineage differentiation assay

The cultured bovine AD-MSCs were seeded in duplicate at an initial concentration of 9.4×10^3 cells/well in 24-well culture plates, and induced with the specific medium for each lineage for 21 days in GM with 10% FBS. The medium was changed every 3–4 days. Adipogenic differentiation: 5 µM isobutyl methylxanthine (Sigma), 60 µM indomethacin (Sigma), 1 µM dexamethasone (Sigma) and 50 µg/mL insulin (Gibco). Cells were then fixed with a 4% paraformaldehyde solution for 15 minutes. The presence of lipid droplets was detected by Red O staining. Chondrogenic differentiation: 1.7 mM ascorbic acid (Sigma), 10 ng/mL of TGF-β (Sigma) and 62.5 µg/mL of insulin (Gibco). The culture was fixed and stained with Alcian Blue to detect the presence of cartilaginous matrix. Osteogenic differentiation: 60 µM dexamethasone, 10 mM β-glycerophosphate (Sigma) and 1.7 mM ascorbic acid. Alizarin Red S staining was used to determine the presence of mineralized matrix. The controls were cultured in GM with 10% FBS.

Statistical analysis

DT and CFU-F in the different passages were evaluated by parametric ANOVA with Tukey's post-hoc test. CFU-F between fresh and post-cryopreservation was compared by the t-test and the results expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant. Analyses were performed with GraphPad Prism 8 software.

RESULTS

Isolation and cryopreservation

The isolation of bovine AD-MSCs from 10 animals resulted in a success rate of 58.8 % (10/17 samples). After 24–48 h of seeding, adherent stromal cells with fibroblastic-like morphology and clonogenic capacity were observed (Figure 1). A total of 48 vials with a concentration of 1×10^6 cells/vial were stored in the biobank.

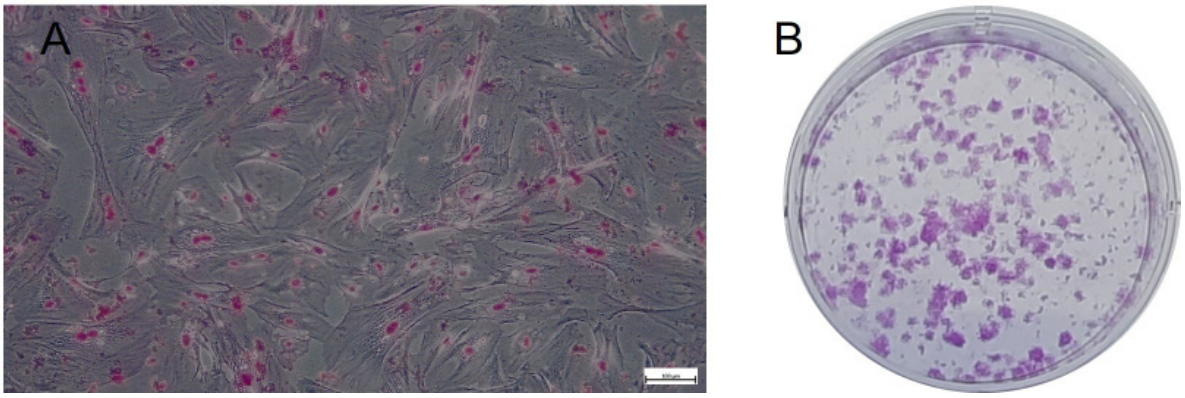

Figure 1 - Primary culture of bovine adipose tissue-derived mesenchymal stromal/stem cells (AD-MSCs) stained with Giemsa. Microscopic image of: A) primary culture showing fibroblastic-like adherence to the plastic cells and monolayer growth (10×); B) macroscopic colonies in a 6-well culture plate.

Proliferation capacity

The DT and CFU-F assays were used to determine the *in vitro* proliferation capacity of the AD-MSCs (n = 7). The DT assay showed P_2–P_6 subcultures with a range of 2.4 ± 0.9 to 4.4 ± 3 days (Figure 2A). There was an increase in time between P_4–P_5, albeit not significant. As determined by the CFU-F assay, clonogenic capacity varied from 24 ± 4 to 28 ± 1 colonies (Figure 2B). There were no post-cryopreservation differences between P_2–P_6. However, greater clonogenic capacity was reported in fresh cells (P_1) (74 ± 1 *vs*. 28 ± 1 of post-cryopreservation cells in P_2, see Figure 2C).

In vitro multipotency

To determine *in vitro* bovine AD-MSCs multipotency (n = 5), a trilineage differentiation assay was used. After induction, cells showed morphological and affinity changes to specific staining that indicated their differentiation to adipose (Figure 3A), cartilage (Figure 3C) and bone lineages (Figure 3E). The controls showed no affinity for any dye (Figures 3B, D and F).

DISCUSSION

In this study, we reported three important findings. First, we demonstrated for the first time, the

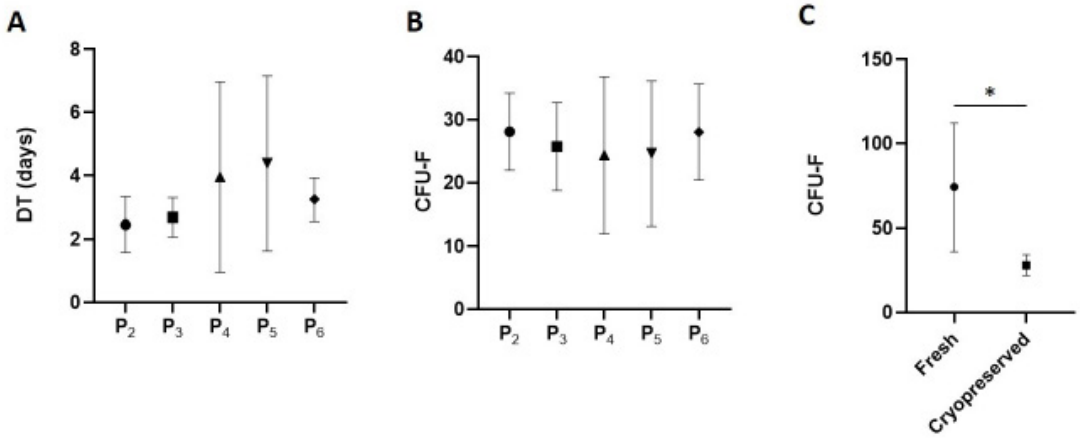

Figure 2 - Proliferation capacity of bovine adipose tissue-derived mesenchymal stromal/stem cells (AD-MSCs). A) Cell doubling times (DT) and B) colony-forming unit fibroblast (CFU-F) were evaluated across successive passages. C) Colony-forming capacity comparison between fresh cells in P_1 and post-freezing cells in P_2 (cryopreserved). The results are expressed as mean ± SD; *= $P < 0.05$.

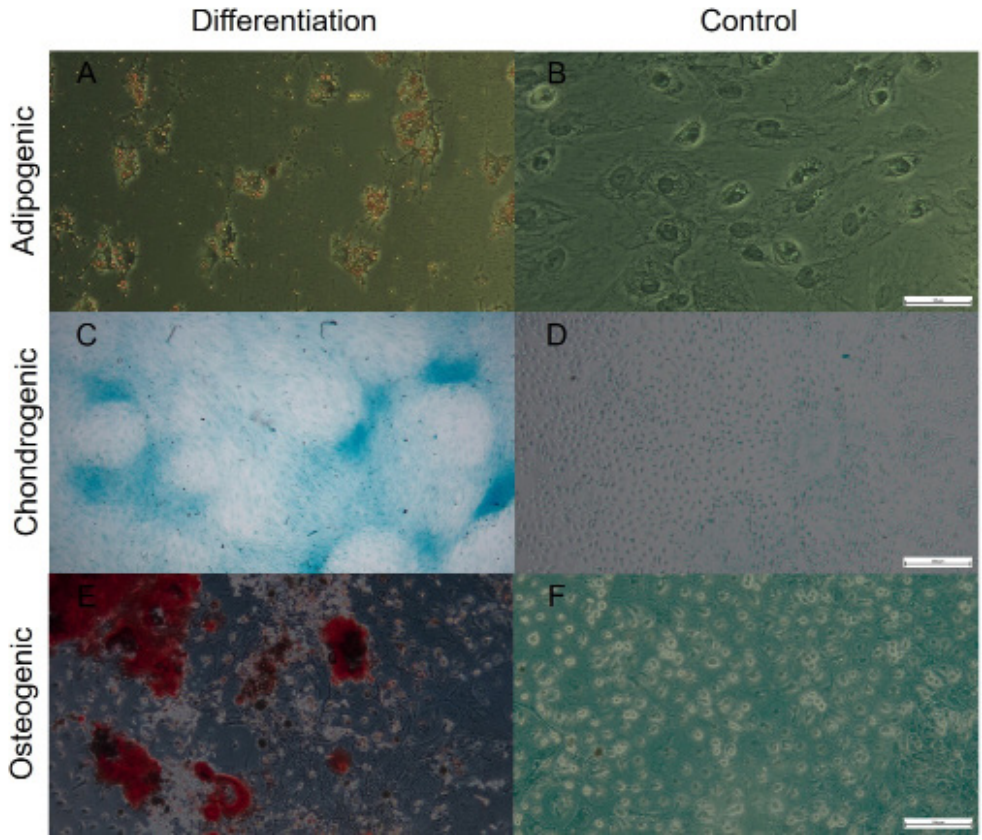

Figure 3 - Microscopic images for multilineages of bovine adipose tissue-derived mesenchymal stromal/stem cells (AD-MSCs). A) Adipogenic differentiation (40×) showing cell lipid droplet accumulation stained in red. C) Chondrogenic differentiation (4×) showing cartilaginous matrix stained in blue. D) Osteogenic differentiation (4×) showing mineralized matrix stained in red. B, D and F are negative controls.

feasibility of *post-mortem* isolation from cattle of Vs AD-MSCs with fibroblastic morphology and *in vitro* multipotency. A previous study described *post-mortem* isolation of these bovine cells but not from Vs fat (ZHAO et al., 2012). Second, growth kinetics and clonogenic post-cryopreservation capacity evaluation indicated that these cells were capable of proliferating without major variations between P_2–P_6; other authors have described the culture of bovine MSCs over a prolonged period with similar kinetics (LU et al., 2014a; SAMPAIO et al., 2015). Third, clonogenic capacity was higher in fresh than post-cryopreservation cells. There are few literature reports on the effect of cryopreservation on colonic or proliferative capacity and they do not describe important changes (DE MORAES et al., 2016).

In the present study, *post-mortem* Vs AD-MSCs were isolated, characterized and cryopreserved. Previous studies describe bovine AD-MSCs from different anatomic sites, such as the base of the tail (SAMPAIO et al., 2015, 2009), interdigital fat pad of the hoof (ZHAO et al., 2012), and abdominal fat (LU et al., 2014a). However, currently, there are no comparative studies on how the anatomic site can affect either the *in vitro* proliferative or multipotential capacity of AD-MSCs from cattle. By contrast, studies on species such as dogs (HENDAWY et al., 2021), humans (BAGLIONI et al., 2012) and rabbits (PEPTAN et al., 2006) described differences between harvesting sites.

We agreed with the researches of other investigators that isolation of *post-mortem* bovine adipose tissue (ZHAO et al., 2012) may be a convenient strategy because samples can be obtained from commercial abattoirs. However, in the present study, samples were taken from cow carcasses rather than the interdigital fat pad of the hoof.

Our MSC isolation success rate was 58.8 %, while other studies with cattle obtained 77% from umbilical cord blood (RAOUFI et al., 2011). However, the MSC isolation success rate is seldom reported (LU et al., 2014a; SAMPAIO et al., 2015; ZHAO et al., 2012). Moreover, bovine AD-MSCs post-cryopreserved for at least 1 year were used for proliferation capacity in successive passage and multipotency assays. In another study, MSCs retained their post-freezing characteristics (DE MORAES et al., 2016), and no significant change in proliferation capacity, multi-differentiation and chromosomal stability was reported in bovine endometrial MSCs. By contrast, in our study, only the colony-forming capacity of fresh and cryopreserved cells was compared, with greater capacity found in the former.

When proliferation capacity was evaluated through DT in successive passages, there were no significant variations for the subculture analyzed. The antecedents of bovine AD-MSCs are scarce; ZHAO et al. (2012) found no differences in DT until P_5, with a range of 16 to 22 h. This differs from other authors who reported 30 ± 1.9 h between P_1–P_9 (SAMPAIO et al., 2015), and from our results, which were greater than 24 h in all passages. One of the reasons for this short period could be the GM used, with the addition of Ham's F-12 nutrient mixture. Another difference is that the authors used fresh cells (ZHAO et al., 2012) instead of their post-freezing counterparts, as used in this study. Finally, the anatomical site of cell extraction was also different, which could affect growth kinetics.

We obtained a mean of 79 h for DT between P_2–P_6, greater than the 30 h for P_1–P_9 described by SAMPAIO et al. (2015). This discrepancy could be because sampling was carried out from Sc fat, instead of Vs. In other species, differences in growth kinetics were observed, such as Sc AD-MSCs, which had a greater proliferative capacity (BAGLIONI et al., 2012; HENDAWY et al., 2021; PEPTAN et al., 2006). Culture conditions were different and their incubation temperature of 38.5 °C was higher than ours. They used α-MEM supplemented with 15 % FBS as GM, unlike our conditions. Additionally, the increase of FBS in the culture medium accelerates MSC growth kinetics (RUSSELL & KOCH, 2016).

Our DT results showed no significant changes across the passages, corroborating other authors who reported that bovine AD-MSCs could be used in this subculture range without losing their proliferative capacity (LU et al., 2014a; SAMPAIO et al., 2015; ZHAO et al., 2012). However, the heterogeneity of adipose tissue-harvesting sites, GM, and incubation conditions make it difficult to compare the DT of bovine cells. Furthermore, clonogenic capacity was evaluated by the CFU-F assay, with no significant variations between successive post-cryopreservation passages. This indicated that cells conserve their colony-forming capacity in culture, as other authors have described for this range of passages (LU et al., 2014a). Although, the literature indicates that colony-forming rates decrease as the number of passages increases, bovine MSCs retain their long-term colony-forming capacity even at > P_{20} with cells from sources such as adipose tissue (LU et al., 2014a), bone marrow (LU et al., 2014b) and umbilical cord (XIONG et al., 2014).

Our results showed that the cells exhibited *in vitro* trilineage differentiation capacity (adipogenic, chondrogenic and osteogenic cell lineages), known lineages for MSC characterization (DOMINICI et al., 2006). We agreed with other authors who have achieved complete trilineage differentiation in bovine MSCs (SAMPAIO et al., 2015; ZHAO et al., 2018; ZHAO et al., 2012). Conversely, other studies characterized bovine MSCs but do not indicate the three mesodermal lineages (DE MORAES et al., 2016; LARA et al., 2017; LU et al., 2014a; LU et al., 2014b). However, some of these studies describe *in vitro* differentiation to other cell lineages such as hepatocytes, pancreatic islet cells and neurocytes (LU et al., 2014b; XIONG et al., 2014). Thus, it seems important to characterize bovine MSCs by demonstrating trilineage differentiation capacity, since the use of specific surface markers for bovine MSCs has yet to be established, as it has for humans (BOURIN et al., 2013; DOMINICI et al., 2006). Despite this situation for domestic animal MSCs (RANERA et al., 2011; SCREVEN et al., 2014), some studies have immunophenotyped bovine AD-MSCs (LU et al., 2014a; SAMPAIO et al., 2015).

CONCLUSION

The isolation, characterization, and cryopreservation of bovine AD-MSCs from *post-mortem* Vs fat was feasible, confirmed by fibroblastic morphology, adherence to plastic and *in vitro* trilineage differentiation capacity. Furthermore, these cells were cultured under standard conditions and successive cell passages up to P_6 showed proliferative capacity. Clonogenic capacity was higher in fresh than post-cryopreserved cells. Thus, *post-mortem* sampling of cattle carcasses for MSC isolation

appears to be a promising source and future studies should be conducted to explore its applicability in cell therapy and the food industry.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was carried out in strict accordance with the recommendations of the Honorary Commission for Animal Experimentation (CHEA) of Uruguay under approval identification CEUA-FVET no. 685.

REFERENCES

BAGLIONI, S. et al. Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. **PLoS ONE**, v.7, n.5, 2012. Available from: <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0036569>. Accessed: Aug. 15, 2023. doi: 10.1371/journal.pone.0036569.

BOURIN, P. et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: A joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). **Cytotherapy**, v.15, n.6, p.641–648, 2013. Available from: <https://www.isct-cytotherapy.org/article/S1465-3249(13)00387-3/abstract>. Accessed: Aug. 15, 2023. doi: 10.1016/j.jcyt.2013.02.006.

CAHUASCANCO, B. et al. Bovine fetal mesenchymal stem cells exert antiproliferative effect against mastitis causing pathogen Staphylococcus aureus. **Veterinary Research**, v.50, n.1, p.1–10, 2019. Available from: <https://veterinaryresearch.biomedcentral.com/articles/10.1186/s13567-019-0643-1>. Accessed: Aug. 15, 2023. doi: 10.1186/s13567-019-0643-1.

DE MORAES, C. N. et al. Bovine endometrial cells: a source of mesenchymal stem/progenitor cells. **Cell biology international**, v.40, n.12, p.1332–1339, 2016. Available from: <https://onlinelibrary.wiley.com/doi/abs/10.1002/cbin.10688>. Accessed: Aug. 15, 2023. doi: 10.1002/cbin.10688.

DOMINICI, M. et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. **Cytotherapy**, v.8, n.4, p.315–317, 2006. Available from: <https://www.isct-cytotherapy.org/article/S1465-3249(06)70881-7/abstract>. Accessed: Aug. 15, 2023. doi: 10.1080/14653240600855905.

GUERCIO, A. et al. Canine mesenchymal stemcells (mscs): Characterization in relation to donor age and adipose tissue-harvesting site. **Cell Biology International**, v.37, n.8, p.789–798, 2013. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/cbin.10090>. Accessed: Aug. 15, 2023. doi: 10.1002/cbin.10090.

HENDAWY, H. et al. Tissue harvesting site effect on the canine adipose stromal vascular fraction quantity and quality. **Animals**, v.11, n.2, p.1–14, 2021. Available from: <https://www.mdpi.com/2076-2615/11/2/460>. Accessed: Aug. 15, 2023. doi: 10.3390/ani11020460.

HILL, A. B. T. et al. Applications of mesenchymal stem cell technology in bovine species. **Stem Cell Research and Therapy**, v.10, n.1, p.1–13, 2019. Available from: <https://stemcellres.biomedcentral.com/articles/10.1186/s13287-019-1145-9>. Accessed: Aug. 15, 2023. doi: 10.1186/s13287-019-1145-9.

LANGE-CONSIGLIO, A. et al. Antimicrobial Effects of Conditioned Medium From Amniotic Progenitor Cells in vitro and in vivo: Toward Tissue Regenerative Therapies for Bovine Mastitis. **Frontiers in Veterinary Science**, v.6, n.December, p.1–13, 2019. Available from: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00443/full>. Accessed: Aug. 15, 2023. doi: 10.3389/fvets.2019.00443.

LARA, E. et al. Characterization of mesenchymal stem cells in bovine endometrium during follicular phase of oestrous cycle. **Reproduction in Domestic Animals**, v.52, n.5, p.707–714, 2017. Available from: <https://onlinelibrary.wiley.com/doi/10.1111/rda.12969>. Accessed: Aug. 15, 2023. doi: 10.1111/rda.12969.

LU, T. et al. Isolation and characterization of adipose-derived mesenchymal stem cells (ADSCs) from cattle. **Applied Biochemistry and Biotechnology**, v.174, n.2, p.719–728, 2014a. Available from: <https://link.springer.com/article/10.1007/s12010-014-1128-3>. Accessed: Aug. 15, 2023. doi: 10.1007/s12010-014-1128-3.

LU, T. et al. Multi-lineage potential research of bone marrow-derived stromal cells (BMSCs) from cattle. **Applied Biochemistry and Biotechnology**, v.172, n.1, p.21–35, 2014b. Available from: <https://link.springer.com/article/10.1007/s12010-013-0458-x>. Accessed: Aug. 15, 2023. doi: 10.1007/s12010-013-0458-x.

MALARD, P. F. et al. Intraovarian injection of mesenchymal stem cells improves oocyte yield and in vitro embryo production in a bovine model of fertility loss. **Scientific Reports**, v.10, n.1, p.1–12, 2020. Available from: <https://www.nature.com/articles/s41598-020-64810-x>. Accessed: Aug. 15, 2023. doi: 10.1038/s41598-020-64810-x.

OKAMURA, L. H. et al. Myogenic differentiation potential of mesenchymal stem cells derived fromfetal bovine bone marrow. **Animal Biotechnology**, v.29, n.1, p.1–11, 2018. Available from: <https://www.tandfonline.com/doi/full/10.1080/10495398.2016.1276926>. Accessed: Aug. 15, 2023. doi: 10.1080/10495398.2016.1276926.

PEPTAN, I. A. et al. Comparison of osteogenic potentials of visceral and subcutaneous adipose-derived cells of rabbits.

Plastic and reconstructive surgery, v.117, n.5, p.1462–1470, 2006. Available from: <https://journals.lww.com/plasreconsurg/abstract/2006/04150/comparison_of_osteogenic_potentials_of_visceral.13.aspx>. Accessed: Aug. 15, 2023. doi: 10.1097/01.prs.0000206319.80719.74.

PERALTA, O. A. et al. Safety and efficacy of a mesenchymal stem cell intramammary therapy in dairy cows with experimentally induced Staphylococcus aureus clinical mastitis. **Scientific Reports**, v.10, n.1, p.1–12, 2020. Available from: <https://www.nature.com/articles/s41598-020-59724-7>. Accessed: Aug. 15, 2023. doi: 10.1038/s41598-020-59724-7.

RAMÍREZ-ESPINOSA, J. J. et al. Bovine *(Bos taurus)* Bone Marrow Mesenchymal Cell Differentiation to Adipogenic and Myogenic Lineages. **Cells Tissues Organs**, v.201, n.1, p.51–64, 2016. Available from: <https://karger.com/cto/article-abstract/201/1/51/91527/Bovine-Bos-taurus-Bone-Marrow-Mesenchymal-Cell?redirectedFrom=fulltext>. Accessed: Aug. 15, 2023. doi: 10.1159/000440878.

RANERA, B. et al. Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. **Veterinary immunology and immunopathology**, v.144, n.1–2, p.147–154, 2011. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0165242711002315?via%3Dihub>. Accessed: Aug. 15, 2023. doi: 10.1016/j.vetimm.2011.06.033.

RAOUFI, M. F. et al. Isolation and differentiation of mesenchymal stem cells from bovine umbilical cord blood. **Reproduction in Domestic Animals**, v.46, n.1, p.95–99, 2011. Available from: <https://onlinelibrary.wiley.com/doi/full/10.1111/j.1439-0531.2010.01594.x>. Accessed: Aug. 15, 2023. doi: 10.1111/j.1439-0531.2010.01594.x.

RUSSELL, K. A.; KOCH, T. G. Equine platelet lysate as an alternative to fetal bovine serum in equine mesenchymal stromal cell culture - too much of a good thing?. **Equine Veterinary Journal**, v.48, n.2, p.261–264, 2016. Available from: <https://beva.onlinelibrary.wiley.com/doi/10.1111/evj.12440>. Accessed: Aug. 15, 2023. doi: 10.1111/evj.12440.

SAMPAIO, R. V et al. Isolation and characterization of bovine mesenchymal stem cells derived from adipose tissue. **Reproduction, Fertility and Development**, v.22, n.1, p.356, 2009. Available from: <https://www.publish.csiro.au/rd/rdv22n1ab399>. Accessed: Aug. 15, 2023. doi: 10.1071/RDv22n1Ab399.

SAMPAIO, R. V. et al. Generation of bovine *(Bos indicus*) and buffalo (*Bubalus bubalis*) adipose tissue derived stem cells: Isolation, characterization, and multipotentiality. **Genetics and Molecular Research**, v.14, n.1, p.53–62, 2015. Available from: <https://www.geneticsmr.com/articles/3881>. Accessed: Aug. 15, 2023. doi: 10.4238/2015.January.15.7.

SCREVEN, R. et al. Immunophenotype and gene expression profile of mesenchymal stem cells derived from canine adipose tissue and bone marrow. **Veterinary Immunology and Immunopathology**, v.161, n.1–2, p.21–31, 2014. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0165242714001421?via%3Dihub>. Accessed: Aug. 15, 2023. doi: 10.1016/j.vetimm.2014.06.002.

SILVA, C. G. da et al. Isolation and characterization of mesenchymal stem cells derived from bovine Wharton's jelly and their potential for use in cloning by nuclear transfer. **Ciência Rural**, v.46, n.10, p.1830–1837, 2016. Available from: <https://www.scielo.br/j/cr/a/Hxxn3ygCPnMDXVr9kgvpRPn/?lang=en>. Accessed: Aug. 15, 2023. doi: 10.1590/0103-8478cr20151074.

SKRIVERGAARD, S. et al. Bovine satellite cells isolated after 2 and 5 days of tissue storage maintain the proliferative and myogenic capacity needed for cultured meat production. **International journal of molecular sciences**, v.22, n.16, p.8376, 2021. Available from: <https://www.mdpi.com/1422-0067/22/16/8376>. Accessed: Aug. 15, 2023. doi: 10.3390/ijms22168376.

VIDAL, M. A. et al. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: Adipogenic and osteogenic capacity. **Veterinary Surgery**, v.35, n.7, p.601–610, 2006. Available from: <https://onlinelibrary.wiley.com/doi/10.1111/j.1532-950X.2006.00197.x>. Accessed: Aug. 15, 2023. doi: 10.1111/j.1532-950X.2006.00197.x.

XIONG, H. et al. Biological characterization of mesenchymal stem cells from bovine umbilical cord. **Animal Cells and Systems**, v.18, n.1, p.59–67, 2014. Available from: <https://www.tandfonline.com/doi/full/10.1080/19768354.2014.880370>. Accessed: Aug. 15, 2023. doi: 10.1080/19768354.2014.880370.

YANESELLI, K. et al. Aislamiento y caracterización de células madre mesenquimales de caninos, equinos y felinos en Uruguay. **Veterinaria (Montevideo)**, v.54, n.209, p.14–19, 2018. Available from: <http://www.scielo.edu.uy/scielo.php?pid=S1688-48092018000100018&script=sci_arttext>. Accessed: Aug. 15, 2023. doi: 10.29155/vet.54.209.3.

ZHAO, X. X. et al. Inhibiting transforming growth factor-β signaling regulates in vitro maintenance and differentiation of bovine bone marrow mesenchymal stem cells. **Journal of Experimental Zoology Part B: Molecular and Developmental Evolution**, v.330, n.8, p.406–416, 2018. Available from: <https://onlinelibrary.wiley.com/doi/10.1002/jez.b.22836>. Accessed: Aug. 15, 2023. doi: 10.1002/jez.b.22836.

ZHAO, Y. et al. The effect of serial passaging on the proliferation and differentiation of bovine adipose-derived stem cells. **Cells Tissues Organs**, v.195, n.5, p.414–427, 2012. Available from: <https://karger.com/cto/article-abstract/195/5/414/91391/The-Effect-of-Serial-Passaging-on-the?redirectedFrom=fulltext>. Accessed: Aug. 15, 2023. doi: 10.1159/000329254.