Clinical safety of bovine intra-ovarian application of allogeneic mesenchymal stem cells

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Received: April 19, 2023 Accepted: May 03, 2023 Published: September 01, 2023

DOI: 10.14295/bjs.v2i9.383 URL: https://doi.org/10.14295/bjs.v2i9.383

Abstract

A basic premise of reproductive physiology is that females have a finite and non-renewable stock of germinative cells, which results in a decrease in reproductive capacity over time. For female bovines, a further factor associated with this decrease is follicular puncture (ovum pickup - OPU), a technique widely used for production of embryos *in vitro*. As such, it is necessary to seek therapeutic or preventive options for fertility problems, and one potential option is treatment with mesenchymal stem cells (MSC), which exercise a paracrine effect in combating inflammatory and degenerative processes. However, as important as evaluating the efficacy of such treatments is an evaluation of safety. In this context, the current study was carried out with the application of 2.5 x 10^6 allogenic MSC derived from adipose tissue, to the ovarian cortex of healthy nelore (n = 5) and girolando (n = 5) cows. The animals were subsequently evaluated by ultrasonography, clinical examination, number of viable oocytes collected, and rate of embryo production. None of the animals presented any clinical alteration or any alteration on ultrasonography after receiving the MSC. Furthermore, comparison between the number of viable oocytes, embryos produced, and rate of embryo production before and after MSC application did not show a difference. Based on these data, it can be concluded that intraovarian application of 2.5 x 10^6 adipose-derived MSC is safe, and this technique represents a potential for study as a therapy in cases of ovarian degeneration or lesions.

Keywords: bovine fertility, *in vitro* fertilization, ovarian lesion, mesenchymal stem cell.

Segurança clínica da aplicação intra-ovariana bovina de células-tronco mesenquimais alogênicas

Resumo

Uma premissa básica da fisiologia reprodutiva é que as fêmeas possuem um estoque finito e não renovável de células germinativas, o que resulta em uma diminuição da capacidade reprodutiva ao longo do tempo. Para as fêmeas bovinas, outro fator associado a essa diminuição é a punção folicular (ovo pickup - OPU), técnica amplamente utilizada para produção de embriões *in vitro*. Assim, é necessário buscar opções terapêuticas ou preventivas para problemas de fertilidade, sendo uma opção potencial o tratamento com células-tronco mesenquimais (CTM), que exercem efeito parácrino no combate a processos inflamatórios e degenerativos. No entanto, tão importante quanto avaliar a eficácia de tais tratamentos é a avaliação da segurança. Nesse contexto, o presente estudo foi realizado com a aplicação de 2,5 x 10^6 MSC alogênicas derivadas do tecido adiposo, no córtex ovariano de vacas nelore (n = 5) e girolando (n = 5) hígidas. Os animais foram posteriormente avaliados por ultrassonografia, exame clínico, número de oócitos viáveis coletados e taxa de produção de embriões. Nenhum dos animais apresentou qualquer alteração clínica ou ultrassonográfica após receber o MSC. Além disso,

a comparação entre o número de oócitos viáveis, embriões produzidos e taxa de produção de embriões antes e depois da aplicação de MSC não mostrou diferença. Com base nesses dados, pode-se concluir que a aplicação intraovariana de MSC de origem adiposa 2,5 x 10^6 é segura, e essa técnica representa um potencial para estudo como terapia em casos de degeneração ou lesões ovarianas.

Palavras-chave: fertilidade bovina, fertilização *in vitro*, lesão ovariana, células-tronco mesenquimais.

1. Introduction

The ovary is an organ in constant change, as in each reproductive cycle there is development of new hormone-secreting ovarian follicles. This development occurs from a set of primordial follicles that constitute the main reproductive structures of the ovary, and whose numbers determine both the reproductive potential and the reproductive life expectancy (McGee; Hsueh, 2000). In females of all vertebrate species, more than 65% of the pool of oocytes present in the primordial follicles are lost at birth. Once the number of primordial follicles is established, depletion of the majority of the remaining oocytes occurs indirectly as a result of atresic degeneration of follicles not selected for ovulation (Hirshfield, 1991).

As the oocyte pool decreases, ovarian senescence begins, guaranteeing that the reproductive lifespan is specific to each species, including bovines. For more than 150 years, it has been recognized as a basic physiological premise that female bovines have a finite and non-renewable store of germinative cells. The progressive decline in oocyte number throughout post-natal life is also one of the factors that leads to a decrease in the reproductive capacity of these animals (Byskov et al., 2005).

Another factor associated with decreased reproductive capacity in female bovines is follicular puncture occurring as part of the technique of ovum pick-up (OPU). This procedure is widely used for *in vitro* production of embryos, an important biotechnological tool in the productive chain (Cavalieri et al., 2018), which, however, can be associated with the appearance of often irreversible ovarian lesions. As such, even with many advances, OPU still presents challenges that compromise the efficiency of the technique in the long term (Galli et al., 2014), thus necessitating the search for therapeutic alternatives to attempt to attenuate the creation of these ovarian lesions.

Cell therapy is considered one of the most promising areas in regenerative medicine, including in the reproductive field. A large part of the therapeutic effect of mesenchymal stem cell (MSC) treatment is attributed to their capacity to produce growth factors and other chemokines (Murphy et al., 2013). Contrary to initial beliefs, MSC are not involved only in processes of homeostasis and repair of the tissues from which they are isolated, but also possess diverse indirect effects that contribute to the recuperation and regeneration of other cell types and tissues in the organism.

This is related to the production and secretion of a wide variety of cytokines, chemokines and growth factors that have paracrine effects on in-vivo tissue regeneration (Prockop, 2007). MSC have the capacity to produce a range of growth factors and interleukins, that play an important role in immunomodulation (Caplan, 2009; Caplan, 2017). In this context, due to the possibility of using stem cell therapy for treatment of infertility both in animals and in humans, it is important to first evaluate the safety of this method.

As such, a study was carried out incorporating intra-ovarian application of allogenic MSC derived from adipose tissue of healthy cows. Through analysis of ultrasound images, clinical evaluation, counting of viable oocytes, and embryo production, it was possible to confirm the safety of this technique in bovines.

2. Material and Methods

2.1 Animal model

This work was carried out between June and September with cows from the Nelore (*Bos indicus*; n = 5 and Girolando (*Bos indicus x Bos taurus*; n = 5) breeds. All animals were four years old, weighed beteen 390 – 420 kg^{-1}, and were from a farm in Flores de Goiás – Goiás State, Brazil. The selected animals had unremarkable fertility histories, and each had a two-month-old calf from a second pregnancy.

2.2 Evaluation of oocytes, in vitro fertilization, and evaluation of embryos

Each follicular aspiration was carried out using a guide coupled to a 5 MHz sectoral transducer (AlokaSSD-500, Japan), and a vacuum pump. At the moment of aspiration, the transducer was positioned in front of the ovaries, such that the follicles were aligned with the line of the biopsy, as shown on the ultrasound. With the pump

adjusted to a pressure of 80-100 mm Hg, the follicles were perforated, and the oocytes aspirated. The oocytes were transferred to a tube containing 2 mL of Dulbecco´s modified phosphate-buffered saline (DMPBS), supplemented with sodium heparin (500 UI/L, Hemofol – Cristália, São Paulo State, Brazil).

The oocytes were washed, counted, and classified according to their morphology, number of cell layers in the cumulus oophorus (COCs), and appearance of the cytoplasm. The classifications were: viable, with cell layers in the cumulus and uniform cytoplasm, or, non-viable, without cumulus cells, and/or cytoplasm too clear or dark.

Only viable oocytes were selected and acclimatized in cryogenic tubes (Corning, 1.2 mL – New York, United States, USA), containing maturation media, consisting of tissue culture medium (Earle Salts, Gibco, New York, United States, USA), with 0.05 UI/mL of Plused® FSH/LH (Hertape-Calier, Barcelona, Spain), 1 mg/mL estradiol, and 10% fetal bovine serum (FBS). Maturation continued for 24 h at 38.5 °C, and the oocytes were then washed and transferred to a 75 μL drop if FIV media, supplemented with 10 μg/mL heparin, 20 μM D-penicillamine, 10 μM hypotaurine, and 1 μM epinephrine.

For the *in vitro* fertilization procedure, a pre-validated sperm suspension was added to the oocytes in FIV media, which were then incubated at 38.5 °C in a humidified incubator with 5% CO_2- The bull used to collect semen for all experiments was of the Aberdeen Angus breed and the sample was the same for all. After the fertilization period, the possible zygotes were transferred to drops of synthetic oviduct fluid (SOFaa), supplemented with essential and nonessential amino acids, 0.34 mM sodium tricitrate, 2.77 mM myo-inositol, and 10% (*v/v*). FBS. The quality of embryos was evaluated according to rate of development, which was determined according to the stage achieved on the seventh day (d7) post-fertilization.

2.3 Collection and characterization of mesenchymal stem cells

Mesenchymal stem cells (MSC) were isolated and cultured from adipose tissue extracted from a healthybovine, recently killed at an abattoir, according to previously described protocols (Lindroos et al., 2011; Romagnoli; Brandi, 2014; De Francesco et al., 2015). The adipose tissue was washed in saline solution, exposed to hyaluronidase, and filtered. The isolated cells were placed in culture flasks with Dulbecco´s modified Eagle´s media (DMEM), and incubated at 37.5 °C, with 5% CO_2. After 24 h, the media was discarded along with non-adherent cells, andfresh media was added to the flasks. At 80% confluence, the cells were trypsinized, counted in a *Neubauer* chamber, and placed in freezing straws with DMSO and FBS in liquid N_2, as described previously (De Rosa et al., 2009; Cui; Pu, 2010).

Cell characterization was carried out according to the guidelines of the International Society for Cellular Therapy (Dominici et al., 2016). The positive surface markers were analyzed with CD29 (rat anti-hum n), CD44 (rat anti-equine), and CD90 (goat anti-canine). The negative surface marker was CD34 (rat anti-human). MSC function was also evaluated through the presence of transcription factors SOX2 and OCT3/4. All markers were evaluated by immunophenotyping with a flow cytometer and Amnis image quantifier.

The cells were evaluated for their ability to differentiate into osteoblasts, chondrocytes and adipocytes, as described previously (De Francesco et al., 2015; De Rosa et al., 2009; Cui; Pu, 2010; Dominici et al., 2016; Marx et al., 2015). Contaminants (bacteria, fungi and mycoplasma) were ruled out using PCR. Finally, cell viability after thawing was evaluated by flow cytometry using annexin-Alexa Fluor 488 and propidium iodide (Thermo-Fisher Scientific, USA). Clinical evaluation and treatment with MSC. The initial evaluation was carried out by rectal palpation and trans-rectal ultrasonography using a 7.5 MHz probe, followed by OPU in each animal. After 15 days, a second OPU was carried out, followed by immediate intra-ovarian application of MSC, which were injected into the ovarian cortex at three different points of the ovary. Access to the ovarian cortex was guided by transvaginal ultrasound, and injection of 2.5×10^6 MSC in each ovary was performed with an inverted follicular aspiration system. A maximum volume of 0.4 ml was administered to each ovary (Figure 1).

The animals were clinically evaluated at 24, 48 and 72 h after MSC application. Parameters were ingestion of water and food, as well as gauging the temperature and coloration of the mucosa. At 15, 30 and 45 days post-application of MSC (d30, d45 and d60 of the study), the cows were subjected to fresh aspirations, followed by evaluation of the number of oocytes and rate of *in vitro* production of embryos, for comparison with the data from before the treatment. The ovaries and uteri were also evaluated, by ultrasonography, for signs of pathology, hemorrhage, or adhesions that may have developed after the OPU and cell application.

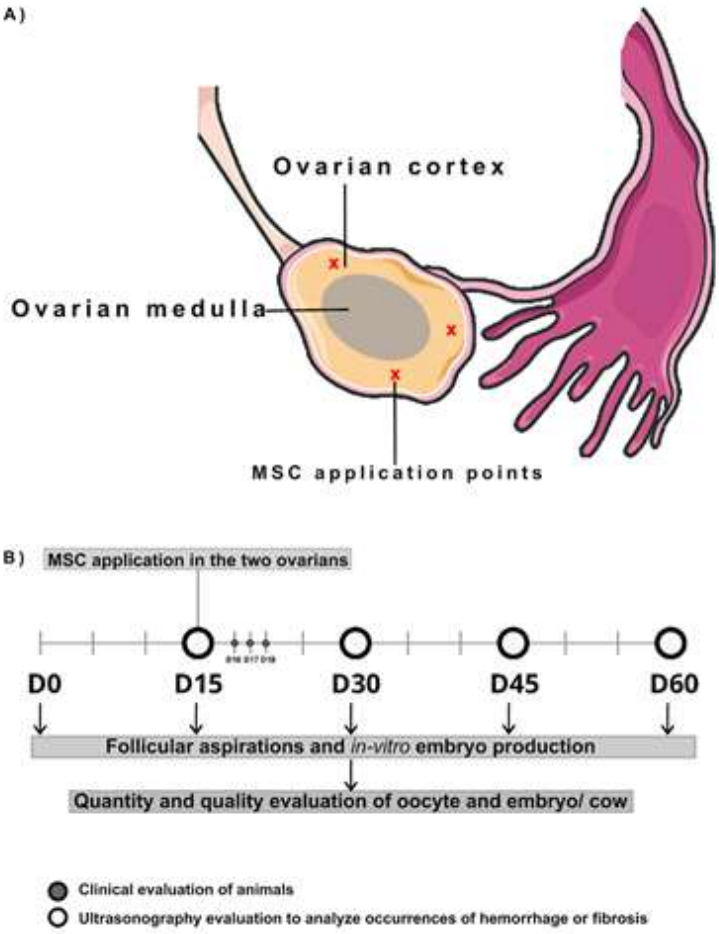

Figure 1. A: Schematic representation of the MSC application points in the ovaries. **B**: Experimental delineation for the MSC safety test. At day 0 (d0), day 15 (d15), day 30 (d30), day 45 (d45) and day 60 (d60), follicular aspirations were carried out in healthy cows. The collected oocytes were analyzed qualitatively and quantitatively and used for in vitro fertilization. At d15, d30, d45 and d60, the ovaries were evaluated by ultrasonography, and occurrences of hemorrhage or fibrosis were recorded. The rate and quality of embryo production were measured. At d15, intra-ovarian MSC application was performed. The animals were clinically evaluated one day (d16), two days (d17) and three days (d18) after application of the MSC. Source: Authors, 2023.

2.4 Ethical statement

This study was carried out according to the rules defined by the National Council for Control of Animal Experimentation (CONCEA – Brazil), and the National Institutes of Health guide for the care and use of Laboratory animals and was approved by the Animal Ethics committee of the Catholic University of Brasília, with protocol number 003/18.

2.5 Statistical analysis

The variables ´oocytes collected´ and ´embryos produced´ and ´rate of embryos´ were tested for normaldistribution (assessed by the Lilliefors test). For the Nelore cows, the variables ´oocytes collected´ and ´embryos produced´ showed normal distribution and were evaluated by ANOVA, taking into consideration the variables ´cow´, ´OPU session´ and ´treatment´. For the Girolando cows, the variables ´collected oocytes´ and ´produced embryos´ were analyzed with a nonparametric Wilcoxon test, taking into consideration the effect of ´treatment´. The variables ´rate of embryo production´ for the two breeds were analyzed by the Chi-squared method.

3. Results

On initial evaluation of the animals by rectal palpation and ultra-sonography, no animal presented alterations to the genital tract, and all animals presented various ovarian follicles at different stages of development, and corpus luteum, thus demonstrating that the animals were in cycle. The MSCs were obtained from adipose tissue from a recently killed donor and were isolated and characterized before use. After 24 h, small colonies of adherent cells were found throughout the surface of the culture vessel. After two days, the cells began to grow and elongate, adopting a spindle shape similar to fibroblasts, in large numbers. When the cells reached 80% confluence, they showed phenotypic characteristics typical of MSC, adhering to the plastic with a fusiform shape.

The flow cytometry results (Figure 2) demonstrated that the MSC expressed high levels of CD29, CD44 and CD105 (90.23% triple positive cells), and did not express the hematopoietic marker CD34. They also highly expressed SOX2 (93.73%), and OCT3/4 (99.05%) (Table 1).

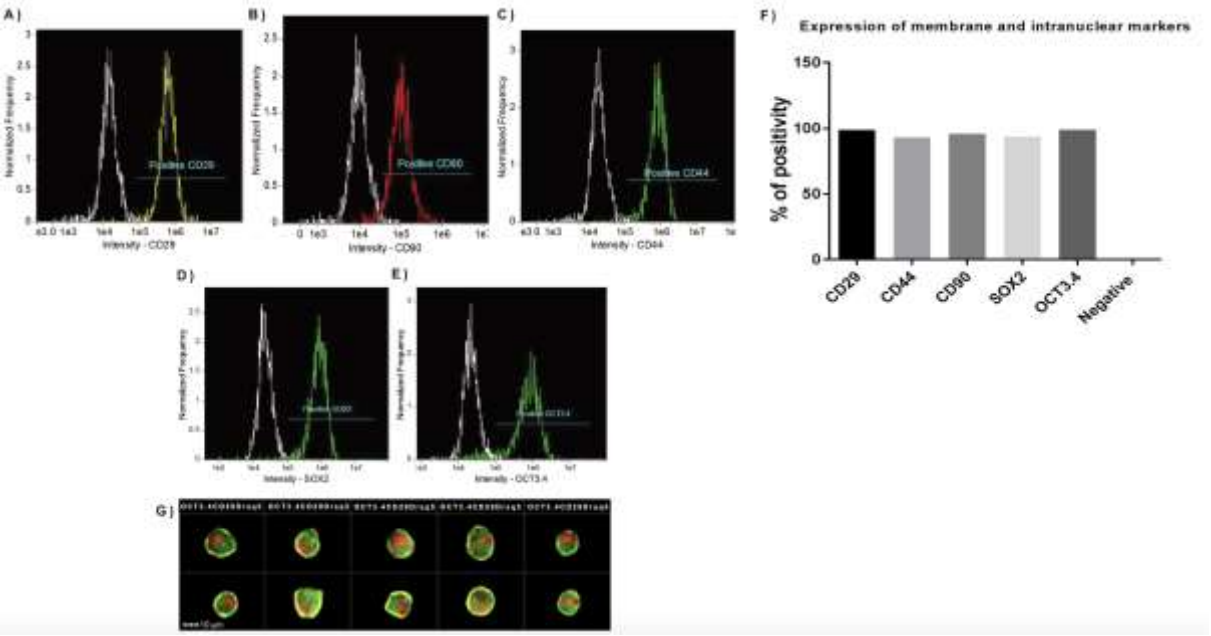

Figure 2. Expression of stem cell molecular markers. Frequency of expression of: CD29 (A), CD90 (B), CD44 (C), SOX-2 (D) and OCT3-4 (E), by flow cytometry. The graphs represent the frequency of labeled cells (Y-axis), and the signal intensity (X-axis) produced by the fluorochrome conjugated to the primary antibody (anti-CD29-PE (amarelo), anti-CD90-DyeLight642 (vermelho), anti-CD44-FITC (verde), anti-SOX2-DyeLight488, anti-OCT3-4-DyeLight488 (verde), SOX2-DyeLight488 (verde). F: Graphical representation of percentage positive cells for each marker. G: representative images of stem cells labeled with Anti-CD29-PE (yellow), Anti-SOX-2-DyeLight488 (green) specific DNA dye Draq7 (red). Source: Authors, 2023.

Table 1. Expression levels of membrane markers CD29, CD44, CD90, and of the intranuclear markers SOX2 and OCT3/4 as determined by immunophenotyping.

Triple Positive	Double Positive			Single Positive			Triple Negative	SOX2	OCT3.4
CD29/CD44/CD90	CD29/CD44	CD29/CD90	CD44/CD90	CD29	CD44	CD90			
90.23	2.32	5.39	0.41	1.04	0.41	0	0.20	93.73	99.05

Source: Authors, 2023.

Analysis of cell viability by flow cytometry demonstrated 90.2% live cells, two hours after thawing (Figure 3). These results show the robustness of the isolation and expansion process. Results of adipogenic, chondrogenic, and osteogenic differentiation assays were similar to previously described data (Marx et al., 2015).

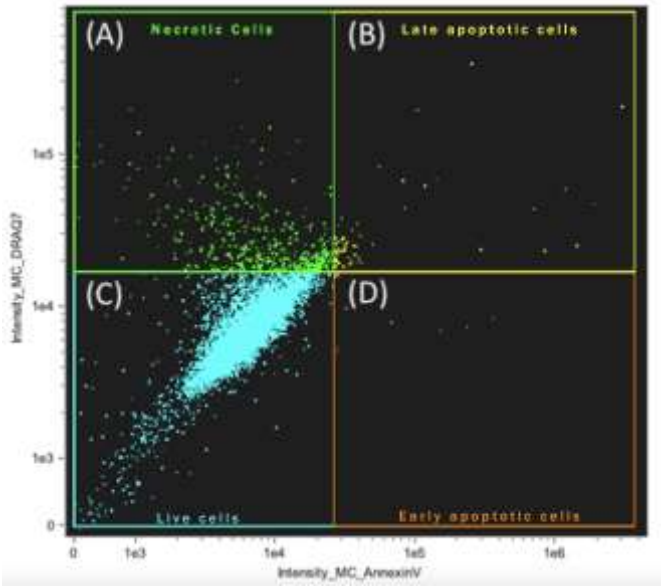

Figure 3. Flow cytometry analysis of MSC after thawing shows necrotic cells (A), late apoptotic cells (B), viable cells (C), and early apoptotic cells (D). Source: Authors, 2023.

The investigation of adverse and collateral effects, including any events during and after therapy with MSC showed that no animal presented adverse clinical signs at 24, 48, and 72 h after MSC application. On follicular aspiration at 15, 30, 45, and 60 days after MSC application, it was observed that the stem cells did not cause any alterations in ovarian imaging, such as hemorrhage or fibrosis. This was true for all animals (Figure 4).

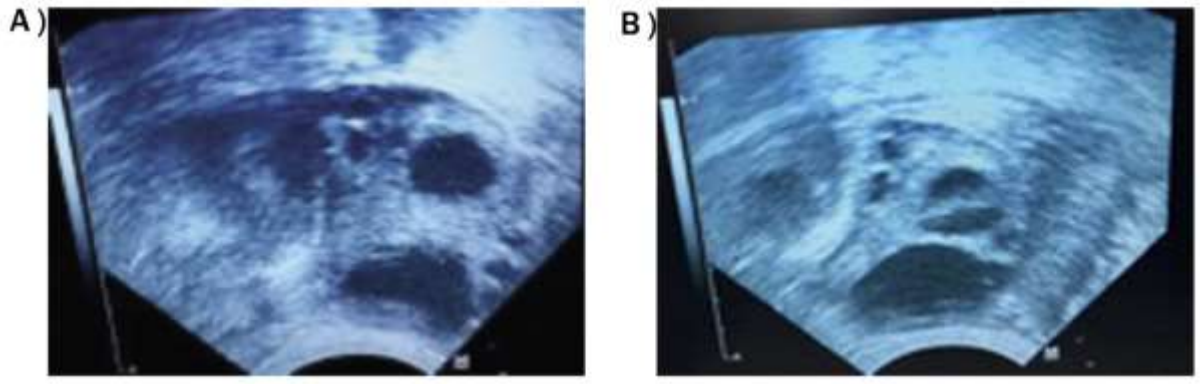

Figure 4. Ultrasonographic examination showing healthy ovaries before (**A**) and after (**B**) application of MSC. Source: Authors, 2023.

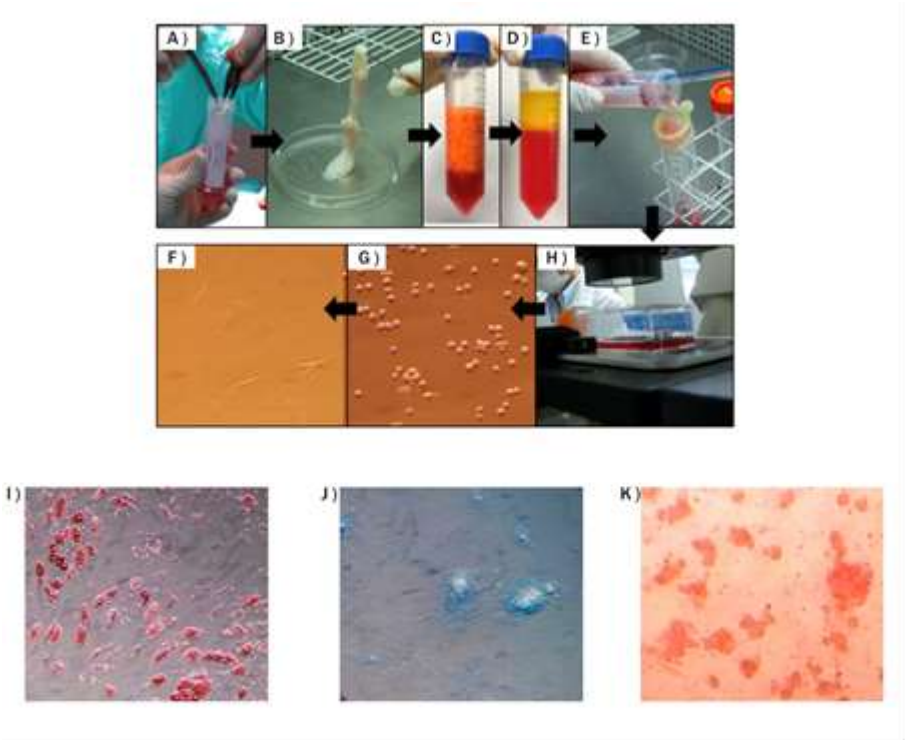

Figure 4. "Supplementary Figure". MSC isolation process and cellular differentiation. Adipose tissue collection (**A**), tissue fragmentation (**B**), enzymatic digestion (**C**), tissue after digestion (**D**), filtration after enzymatic digestion (**E**), microscopic examination of cells (**F**), image of MSC after isolation: magnification 400x (**G**), and image of MSC at 24 hours after isolation: magnification 400x (H). Differentiation: MSC induced to differentiate into adipogenic (**I**), chondrogenic (**J**) and osteogenic (**K**) lineages. Source: Authors, 2023.

Furthermore, the clinical evaluations did not show any alterations in temperature, feeding, or ingestion of water after application of the MSCs. For Nelore cows, there was effect according to ´cow´ for ´oocytes collected´ ($p < 0.001$) and according to ´cow´ and ´OPU session´ for ´embryos produced´ ($p < 0.0001$ and $p < 0.05$ respectively). For the Girolando cows, none of the variables met normality through the Lilliefors test. There was no effect for ´treatment´ ($p > 0.05$) in Nelore nor in Girolando cows. There was no alteration in average numbers of viable oocytes or embryos produced before and after application of MSC ($p > 0.05$), for any animal from either of the breeds involved. There was also no difference in the rate of embryo production ($p > 0.05$) (Table 2).

Table 2. Average number of viable oocytes obtained, embryos produced, and rate of embryos before and after MSC therapy in cows of the Nelore and Girolando breeds.

Nelore cows		
Parameter	Before MSC	After MSC
Viable oocytes	17.0±10.9 [a]	13.2±7.9 [a]
Embryos produced	8.7±9.9 [a]	7.0±6.4 [a]
Embryo rate	51.2 [a]	53.0 [a]
Girolando cows		
Parameter	Control	MSC
Viable oocytes	19.2±8.8 [a]	22.5±9.1 [a]
Embryos produced	9.6±6.1 [a]	11.9±6.9 [a]
Embryo rate	50.0 [a]	52.8 [a]

[a]Values followed by the same by the same letter in each line do not differ significantly ($p > 0.05$). Source: Authors, 2023.

4. Discussion

In the current study, allogeneic MSC derived from adipose tissue were transplanted into ovaries of cows with normal reproductive histories, and with clinical and ultrasonographic evaluations consistent with healthy animals. The goal was to evaluate the safety of intra-ovarian application of MSC. Recent studies have described the efficiency of MSC derived from bone marrow (Fu, He, Xie and Liu, 2008) and amniotic fluid (Chang et al., 2018) transplanted into lesioned or degenerated ovaries. As such we hypothesized that MSC derived from adipose tissue may also improve ovarian function; however, an initial evaluation of the safety of this type of therapy in this species is very relevant.

For this study, bovines were selected as the model, as this species has been the basis for discovery of several factors related to ovarian dynamics in humans. In women, the occurrence of follicular waves, the number of waves during the menstrual cycle, the selection of the dominant follicle and the ovulation of a single follicle are events that are highly similar to those in cows (Adams, 1995; Baerwald, Adams, Pierson, 2003). Ageing in bovines is associated with elevated levels of gonadotrophin and reduced concentrations of steroid hormones, and these changes are consistent with those described during precocious reproductive ageing in women. The changes in follicular dynamics and endocrine control in cows of 13-14 years old are similar to those previously described for women approaching menopause. As such, bovines are a viable model for studying reproductive ageing in women (Hori et al., 2019).

Adipose tissue is an important source of MSC, as aspiration of subcutaneous abdominal adipose tissue is commonly used for esthetic applications in humans (Takehara et al., 2013), and for bovines, this tissue is also easily obtained, for example from the base of the tail, an accessible region without large blood vessels (Sampaio et al., 2015). Furthermore, it was recently reported that not only subcutaneous, but also visceral adipose tissue possesses MSC with regenerative properties (Baglioni et al., 2019). These data are relevant to studies investigating MSC therapies, such as in the present work. In this context, thorough studies regarding possible adverse effects will facilitate the safe therapeutic clinical application of MSC.

Based on the observed absence of systemic manifestations, as well as the anatomic and physiological preservation of the ovaries, it was possible to affirm that the application of 2.5×10^6 MSC to the ovarian cortex of cows is safe and does not cause reproductive harm to Nelore and Girolando cows. These data are important for cellular therapy, and are already consolidated for certain application routes, such as intra-thecal (Harris, Vyshkina, Sadiq, 2016), endovenous (Liang et al., 2018) and intra-articular (Freitag et al., 2016), all in humans. The ovarian response to MSC has previously been evaluated by others (Mohamed et al., 2018), without any report of collateral effects, similar to the result reported in the current bovine study. Despite the selection of animals according to similar reproductive histories, normalizing as far as possible reproductive characteristics, field-based research brings some limitations. This can be observed in the oocyte aspiration data, which did not present differences between collections, however it appeared to show a tendency towards lower recovery of viable oocytes after application of MSC.

This decrease may be due to the time of year in which the OPU was performed after MSC application, corresponding to the dry season. At this time of year pasture is more scarce, and the animals have overall less available food, a factor that may interfere directly in reproductive parameters of female bovines (D'Occhio et al., 2018). However, there was no observed alteration in the rate of embryo production between periods, in other words, although the number or oocytes collected was lower, the production of embryos was stable, thus showing no negative impact on the study.

Some studies carried out with mice (Takehara et al., 2013) and humans (Woods; Tilly, 2013; Tilly; Sinclair, 2013) have shown that transplanting adipose-derived MSC can increase ovarian function. The focus of the present work was to evaluate the safety of this therapy in cows, however, through the study design it was possible to see some indications of efficacy of this therapy in lesioned or failing ovaries. Despite embryo production being an important indicator of a return to fertility, no studies were found that made this evaluation after MSC treatment in bovines. Studies carried out with MSC application for reproductive applications in females have focused on a return to ovarian activity with observations of histological alterations, or of gene expression, or through measurement of growth factors, with the most commonly used animal model for these studies being rats (Fu et al., 2008; Lai et al., 2016; Takahera et al., 2013). Reports have shown that MSC from bone marrow play an important role in follicle survival and reduction of apoptosis in ovarian cells (Lai et al., 2016; Abd-Allah et al., 2013; Mohamed et al., 2018).

In a work with ovarian failure in rats, the results from MSC injection indicated that these cells improved ovarian function and folliculogenesis through influence on the ovarian epithelium or through their effects on the

microenvironment (Elfayomy et al., 2016). Lai & collaborators (2014) applied MSC in female rats treated with chemotherapy, and the results indicated that migration of these cells to the ovaries could play a role in the regulation of pro-inflammatory cytokines, as well as marker genes of oogenesis, as such restoring the function of damaged ovaries. This observation is extremely relevant to humans, especially when coupled with information regarding the safety of MSC application, as observed in the present study.

5. Conclusion

Based on the results presented here, it was concluded that the application of 2.5 x 10^6 MSC to the bovine ovarian cortex is safe in that it did not cause alterations on imaging of ovaries, or a significant variation in the number of collected oocytes, nor in the rate of embryo production for female bovines of the Nelore and Girolando breeds. Furthermore, no animal presented any clinical manifestations, corroborating previous data. As such, the application of adipose-derived MSC presents an option for study in therapy for cases of degeneration or lesions in ovaries, due to the safety of their intra-ovarian application.

6. Acknowledgements

This work was funded in part by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant number: 424074/2016-8). MP, PM and TA are funded, in part, by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors thank Ms. Rose Gonçalves and Mr. José Luis Gonçalves for supplying the animals and infrastructure for execution of this research.

7. Authors contribution

Maurício Antônio Peixer, *Patrícia Malard*, *Juliana Carvalho* & *Robert Pogue*: worked in the study protocol and written of the paper. *Joao Viana*: made the statistics. *Hilana dos Santos Brunel* and *Thuany Alencar-Silva*: worked in the written of the paper.

8. Conflict of interest

The authors declare that there is no conflict of interest.

9. Ethical Approval

Yes.

10. References

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