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# Comparison of the chondrogenic differentiation potential of bone marrow mesenchymal stem cells between female and male Wistar rats

*[Comparação do potencial de diferenciação condrogênico de células tronco mesenquimais da medula óssea de ratos Wistar, fêmeas e machos]*

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## ABSTRACT

This study aimed to compare the chondrogenic differentiation potential between bone marrow mesenchymal stem cells from female (fBMSCs) and male (mBMSCs) Wistar rats. For this purpose, female and male BMSCs were subjected to chondrogenic differentiation for 7, 14 and 21 days for evaluation of cell morphology (14 and 21 days), formation of PAS+ chondrogenic matrix (21 days) and gene expression of aggrecan (Agg), collagen II (Col II) and Sox9 by RT-qPCR at 7, 14 and 21 days. Both fBMSCs and mBMSCs showed cell viability greater than 90%. After differentiation, the two groups showed morphologically similar cells at 14 and 21 days; PAS+ matrix formation was higher in fBMSCs when compared with mBMSCs. The Agg gene expression of mBMSCs was higher when compared with fBMSCs ($p<0.05$) at 21 days; however, the expression of Col II and Sox9 genes at 14 days was higher in fBMSCs compared to mBMSCs ($p<0.05$). Regarding the expression of genes over time, both in fBMSCs and in mBMSCs, the expression of Agg was lower at 21 days. BMSCs from female Wistar rats showed a greater chondrogenic differentiation potential compared to BMSCs from males.

Keywords: gene expression, mesenchymal stem cells, bone marrow, chondrogenesis, Wistar rats

## RESUMO

*Objetivou-se comparar o potencial de diferenciação condrogênica entre célula-tronco mesenquimais da medula óssea de ratos Wistar, fêmeas (CTM-MOf) e machos (CTM-MOm). Para isso, as CTM-MO de fêmeas e machos foram submetidas à diferenciação condrogênica por sete, 14 e 21 dias, para avaliação quanto à morfologia celular (14 e 21 dias), à formação de matriz condrogênica PAS+ (21 dias) e à expressão gênica de agrecano (Agg), colágeno II (Col II) e Sox9 por RT-qPCR aos sete, 14 e 21 dias. As CTM-MOf e as CTM-MOm apresentaram viabilidade celular superior a 90%. Após a diferenciação, os dois grupos apresentaram células morfologicamente semelhantes aos 14 e 21 dias, a formação de matriz PAS+ foi maior nas CTM-MOf quando comparada às CTM-MOm. A expressão gênica de Agg de CTM-MOm foi superior em relação às CTM-MOf ($P<0,05$) aos 21 dias; no entanto, as expressões dos genes Col II e Sox9 aos 14 dias foram maiores nas CTM-MOf em comparação às CTM-MOm ($P<0,05$). Com relação à expressão dos genes ao longo do tempo, tanto nas CTM-MOf quanto nas CTM-MOm, a expressão de Agg foi menor aos 21 dias. Conclui-se que as CTM-MO de fêmeas de ratos Wistar apresentam maior potencial de diferenciação condrogênica em comparação às CTM-MO de machos.*

*Palavras-chave: expressão gênica, células-tronco mesenquimais, medula óssea, condrogênese, ratos Wistar*

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## INTRODUCTION

Studies with stem cells have been increasing significantly in recent years and they are mainly involved in the treatment of diseases with the goal of not only relieving symptoms, but also to regenerate some types of tissues, such as bone and cartilaginous tissues (Scibetta *et al*., 2019). Cartilaginous tissue has a difficult regeneration process in case of disease or trauma due to its poor vascularization and innervation. This is the reason why stem cell therapy has been widely studied and used as treatment alternative (Matsumoto *et al*., 2008).

Mesenchymal stem cells (MSC) can differentiate into several specific cell lineages, such as bone and cartilage (Kangari *et al*., 2020), being very important for regenerative medicine and for tissue engineering (Scibetta *et al*., 2019). Studies show that bone marrow mesenchymal stem cells (BMSCs) have the potential for chondrogenic differentiation (Assis *et al*., 2018), which allows for their use in the treatment of injuries or repairs of the cartilaginous tissue (Le *et al*., 2020).

Some researchers have focused on what they call donor-to-donor MSC variation, source that include age, sex, species, and previous diseases (Kim *et al*., 2018). Other studies are more specific and address the relationship between the sex of individuals (animals or humans), effects of transcription and growth factors, and hormonal influence (Nasatzky *et al*., 1993; Lee *et al*., 2016; Kim *et al*., 2018; Scibetta *et al*., 2019).

Unraveling the mechanisms and factors that influence chondrogenic differentiation is important since diseases such as osteoarthritis are more common in women than in men. The possible hormonal factors involved in this process may be the key to possible treatments (Matsumoto *et al*., 2008; Le *et al*., 2020).

The sex or sex hormone dependent relationship in chondrogenic differentiation has been reported in more than one species and from different sources. In humans, different sources of stem cells from men and women have undergone chondrogenic differentiation, such as MSC extracted from femur (Payne *et al*., 2010) and MSC derived from skeletal muscle (Scibetta *et al*., 2019). Furthermore, a study conducted with mini-pig BMSCs found that E2 (17β-estradiol) increased the proliferation of these cells in both males and females in a dose-dependent manner, improved osteogenic potential only in females, and induced similar chondrogenic differentiation both in males and in females (Lee *et al*., 2016).

Despite the number of studies in the stem cells area and their chondrogenic potential, no research reports compare the chondrogenic differentiation potential of BMSCs between female and male Wistar rats. The results of this study could make an important contribution to explaining if there is a difference between male and female BMSCs regarding chondrogenic differentiation potential, and it would also influence the use of these cells in cell therapy. Therefore, this study aims to compare the chondrogenic differentiation potential of bone marrow mesenchymal stem cells between female and male Wistar rats.

## MATERIAL AND METHODS

Animals used in this study were kept in accordance with the Brazilian Law for Scientific Use of Animals (#11794/2008). The experimental procedures were reviewed and approved by the Animal Ethics Committee of Universidade Federal do Espírito Santo, according to protocol 062/2013.

*Rattus norvegicus* of the Wistar lineage, 30 days old, three females and three males, were euthanized with an overdose of sodium pentobarbital (100mg/kg, intraperitoneal). In an aseptic environment, trichotomy and antisepsis were performed on the skin of the abdominal region and hindlimbs of the animals. Subsequently, the limbs were dissected, and the right and left femurs and tibias were disarticulated, and the bones placed in DMEM medium (Dulbecco's Modified Eagle Medium, Gibco, USA). In laminar flow, bone marrow was washed with DMEM enriched with gentamicin (60µg/L), penicillin (100U/mL), streptomycin (100µg/mL) and amphotericin (25µg/L), followed by centrifugation for 10 minutes at 1400g.

Cells ($100\times10^5$) were grown for approximately four replicates in T75 bottles (Sarstedt, Germany) containing DMEM enriched with antibiotics and antimycotics and 10% fetal bovine serum (LGC Biotecnologia, Brazil) in an

incubator at 37ºC and 5% $CO_2$. The culture medium was changed twice a week. All solutions and culture media were prepared with pure microorganism-free water.

Before chondrogenic differentiation, the cells were assessed for viability by trypan blue. To perform the test, the cells were washed with 0.15 M phosphate buffered saline (PBS) and trypsinized, then harvested and centrifuged at 1400 rpm for 10 minutes, resuspended, and then stained. Thus, the non-viable cells could be observed in blue and the viable ones transparent, which were analyzed and quantified in the Neubauer chamber.

Viable bone marrow mesenchymal stem cells with 80% to 90% confluence were cultured in chondrogenic medium for 21 days (Stem Pro® Chondrogenesis Differentiation Kit; Gibco, USA) in an incubator at 37ºC and 5% $CO_2$. Thereafter, the cells were cultured in the pellet system in a previously standardized quantity ($5\times10^5$ cells) in three repetitions (for analysis of cell morphology and PAS+ chondrogenic matrix formation) and in sextuplicate (for gene expression analysis), in 15 mL Falcon tubes for 7, 14 and 21 days in a volume of 500 µL of chondrogenic medium per tube; chondrogenic matrix formation was assessed after 21 days, and aggrecan, collagen II and Sox9 gene expression was performed after 7, 14 and 21 days of differentiation.

Cell morphology was assessed after 14 and 21 days of differentiation. Samples were washed with 0.15 M PBS and fixed in 10% buffered formaldehyde for one hour, processed by the routine paraffin embedding method (Assis *et al*., 2018; Solchaga *et al*., 2011). Subsequently, the samples were subjected to microtomy to obtain 4 µm thick sections and stained with Hematoxylin and Eosin for morphology analysis and stained with Periodic Schiff Acid (PAS) for the analysis of chondrogenic matrix formation.

Total RNA extraction from cells grown in chondrogenic differentiation medium was performed using Trizol (Trizol® Reagent, Invitrogen, CA, USA), according to the manufacturer's recommendations. Reverse transcription reactions were performed using a commercial SuperScript® III First-Strand Synthesis SuperMix for RT-qPCR kit (Invitrogen, CA, USA). The RT-qPCR reactions were conducted with 1 µg of cDNA, 500nM of each primer and 5µL of SYBR Green reagent in a final volume of 10 µL of reaction in the 7500 Real-Time PCR apparatus (Applied Biosystems Inc., CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the $2^{-\Delta\Delta Ct}$ method was used as a normalizer of the expressions. Primers were delineated based on the *Rattus norvegicus* mRNA sequence: forward CACACGCTACACACTGGACT and reverse TCACACTGGTGGAAGCCATC for aggrecan; forward CTGAAGGGCTACGACTGGAC and reverse TACTGGTCTGCCAGCTTCCT for Sox9; forward GTTCACGTACACTGCCCTGA and reverse AAGGCGTGAGGTCTTCTGTG for collagen II and forward TTCTTGTGCAGTGCCAGCC and reverse GTAACCAGGCGTCCGATACG for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). The relative expression of each gene was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method.

For statistical analysis, the design was entirely randomized. For the analysis of cell viability results, data were subjected to analysis of variance (Anova) and means were compared by Student's *t*-test (Instat, version 3.00, 32 Win 95/NT; GraphPad Software San Diego, CA, USA). Differences were considered significant if $p<0.05$. Cell morphology data were arranged descriptively.

The evaluation and comparison of PAS+ chondrogenic matrix formation was performed using the following parameters: distribution [negative=0, focal=1, multifocal=2, diffuse=3] and intensity [negative=0, discreet=1, moderate=2, strong=3]. The parameters were multiplied, and the resulting score used (Trivilin *et al*., 2017). For assessing the PAS+ chondrogenic matrix formation, the slides were analyzed in an optical microscope by two evaluators, who received the same slides for analysis independently, without previous discussion of the findings. After the evaluations, the result was computed, and the scores were multiplied and the figures were generated.

For the analysis of gene expression results, the data were subjected to analysis of variance (Anova) and the means were compared by

Student's *t*-test (Instat, version 3.00, 32 Win 95/NT; GraphPad Software San Diego, CA, USA) when comparing the gene expression between the groups. Moreover, means were compared by the Student-Newman-Keuls (SNK) test when comparing gene expression over the evaluated times. Differences were considered significant if $p<0.05$.

## RESULTS

The viability of bone marrow mesenchymal stem cells obtained from female (fBMSCs) and male (mBMSCs) *Rattus norvegicus* Wistar was at least 90% without significant difference for this parameter ($p<0.05$) (Figure 1).

BMSCs from males and females were morphologically similar at both 14 and 21 days of chondrogenic differentiation, showing a predominantly oval shape, oval and sometimes pyknotic nuclei.

The chondrogenic matrix of fBMSCs at 21 days of differentiation presented a moderate diffuse form, scoring 6. While in mBMSCs at 21 days of differentiation, the matrix formation presented a mild diffuse form, scoring 3. The comparison between the scores showed that the chondrogenic matrix of fBMSCs at 21 days of differentiation was significantly greater than that of mBMSCs (Figures 2 and 3).

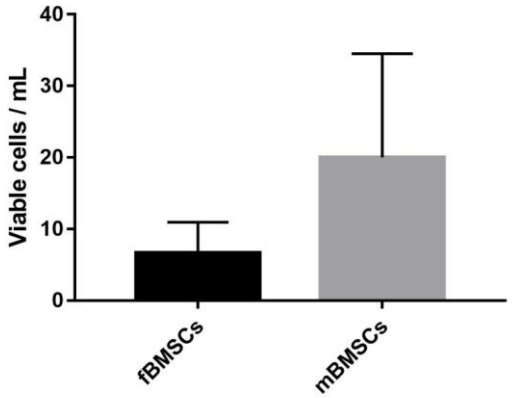



Figure 1. Comparison of cell viability (mean and standard deviation) between bone marrow mesenchymal stem cells from females (fBMSCs) and males (mBMSCs) using Student's *t*-test ($p<0.05$).

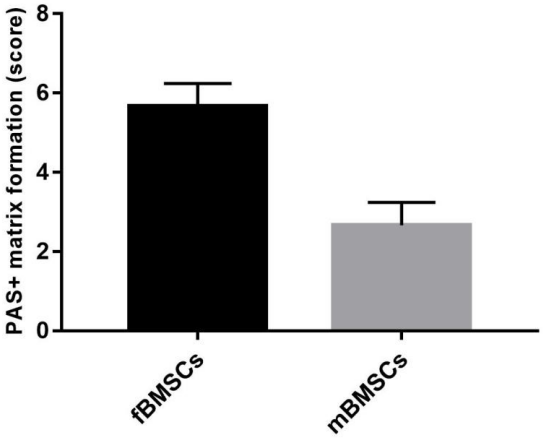
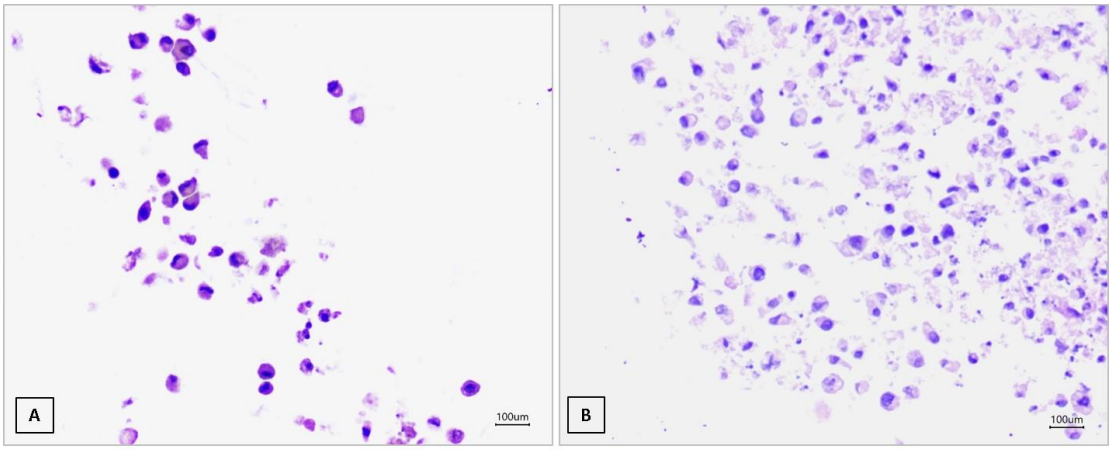



Figure 2. Comparison of PAS+ chondrogenic matrix formation (mean and standard deviation) between bone marrow mesenchymal stem cells from female (fBMSCs) and male (mBMSCs) *Rattus norvegicus* Wistar rats at 21 days of chondrogenic differentiation using Student's *t*-test ($p<0.05$).




**fBMSCs** **mBMSCs**

Figure 3. Comparison of PAS+ staining (basophilic) between bone marrow mesenchymal stem cells from female (fBMSCs) (A) and male (mBMSCs) (B) *Rattus norvegicus* Wistar rats at 21 days of chondrogenic differentiation. PAS staining. Bar = 100 µm. Purple staining indicates that the fBMSCs are more stained by PAS and therefore have more glycosaminoglycans in compared to mBMSCs.

Aggrecan (Agg) gene expression at 7 and 14 days of chondrogenic differentiation showed no significant difference between the groups fBMSCs and mBMSCs ($p>0.05$) (Figure 4A,B). However, at 21 days, group mBMSCs showed significantly higher expression compared to fBMSCs ($p<0.05$) (Figure 4C).

As for the expression of Col II and Sox9 genes at 7 and 21 days, the expression was similar between the groups studied ($p>0.05$) (Figure 4D,F,G,I). However, at 14 days, the expression of these genes was higher in fBMSCs compared to the mBMSCs ($p<0.05$) (Figure 4E,H).

When comparing the expression of genes over time, both in fBMSCs and in mBMSCs, there was no difference in the expression of Col II and Sox9 genes, and only the Agg gene showed a change in expression. fBMSCs showed a higher expression of Agg at 14 days of differentiation and even statistically higher at 21 days ($p>0.05$) (Figure 5A,B,C). mBMSCs, in turn, showed a the highest Agg expression at 7 days of differentiation, which decreased over time and was statistically higher at 21 days ($p>0.05$) (Figure 5D,E,F).

## DISCUSSION

This study aimed to compare the chondrogenic differentiation potential between BMSCs of *Rattus norvegicus* of female and male Wistar lineage. In general, fBMSCs present a better potential than mBMSCs, if they have greater formation of chondrogenic matrix at 21 days of differentiation and greater expression of Col II and Sox9 genes at 14 days of differentiation.

Before the BMSCs of this study were subjected to chondrogenic differentiation, cell viability was evaluated to ensure the continuity of the experiment and the differentiation process as performed by other previous studies (Alves *et al*., 2016; Assis *et al*., 2018). In this regard, both studied groups showed viability greater than 90% without a significant difference between these groups. Thus, both were viable for the performance of chondrogenic differentiation, and it was not a limiting factor for the results obtained following the experimentation. Furthermore, these data proved that BMSCs from female and male *Rattus norvegicus* Wistar are good sources of mesenchymal stem cells and can be used experimentally, just like other previously proven sources, such as BMSCs extracted from the human femur of men and women of different ages (Payne *et al*., 2010; Contentin *et al*., 2020).

In our study, no morphological difference was observed between fBMSCs and mBMSCs at 14 and 21 days of chondrogenic differentiation. This result corroborates what was observed in a previous study, in which the analysis of human BMSCs morphology at 8 and 16 days of

chondrogenic differentiation showed no morphological difference between male and female cells (Lee *et al*., 2019). Thus, both sources can differentiate *in vitro* and form morphologically similar cells.

Regarding the analysis of chondrogenic matrix formation, our study showed that fBMSCs obtained a greater matrix formation compared to mBMSCs. In this line, Scibetta *et al*. (2019) compared the chondrogenic differentiation of muscle-derived mesenchymal stem cells (MDSCs) from men and women and its relationship with donor age and concluded that, unlike the results of this study, MDSCs from young and older men show greater chondrogenic matrix formation when compared with MDSCs from young and older women (Scibetta *et al*., 2019). Moreover, BMSCs from humans were evaluated for chondrogenic matrix formation, noting that the chondrogenic matrix of BMSCs from men showed that the higher the donor age, the lower the amount of staining, what which did not occur in the group where in which the source of cells came from women (Payne *et al*., 2010). Based on this result, it is necessary to point out that several factors can influence the formation of chondrogenic matrix *in vitro*, such as MSC source, species, age, and sex of donors. However, in mice, the sex factor favored BMSCs from young females.

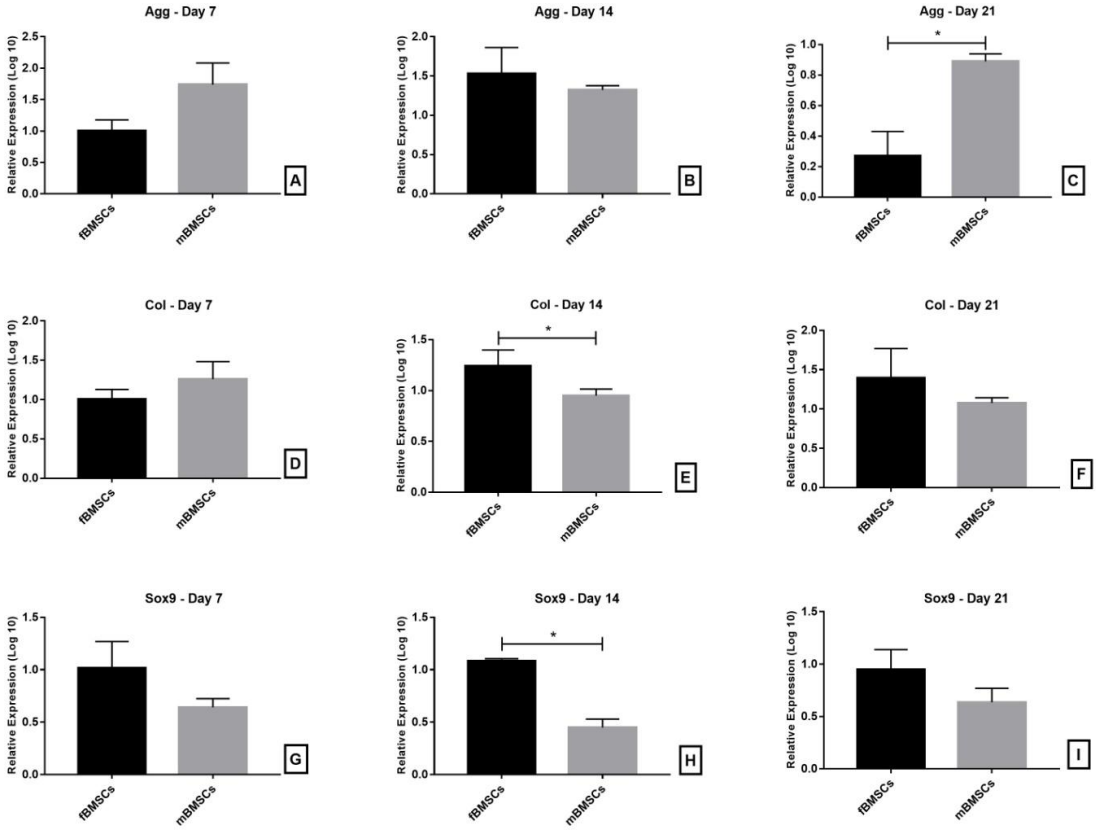



Figure 4. Gene expressions of bone marrow mesenchymal stem cells from female (fBMSCs) and male (mBMSCs) *Rattus norvegicus* Wistar rats, subjected to chondrogenic differentiation. Comparison of Aggregan (Agg) gene expression between fBMSCs and mBMSCs at 7 (A), 14 (B) and 21 (C) days of chondrogenic differentiation. Comparison of collagen II (Col II) gene expression between fBMSCs and mBMSCs at 7 (D), 14 (E) and 21 (F) days of chondrogenic differentiation. Comparison of Sox9 gene expression between fBMSCs and mBMSCs at 7 (G), 14 (H) and 21 (I) days of chondrogenic differentiation. Student's *t*-test. *Indicates significant difference between groups ($p<0.05$).

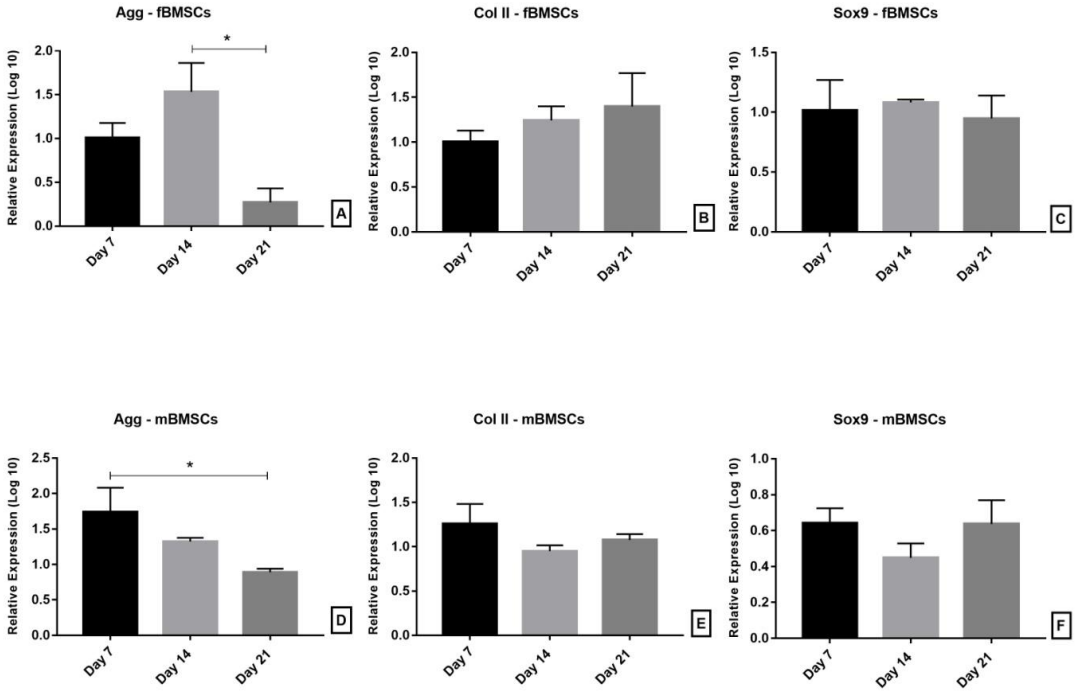



Figure 5. Gene expression in mesenchymal stem cells from female (fBMSCs) and male (mBMSCs) *Rattus norvegicus* Wistar rats over 7, 14 and 21 days of differentiation using SNK (Student-Newman-Keuls) test. Expression of aggrecan (Agg) (A,D), collagen II (Col II) (B,E) and Sox9 (C,F). *Indicates significant difference between groups ($p<0.05$).

As for the expression of Col II gene, in our study, fBMSCs showed a greater expression when compared with mBMSCs at 14 days of chondrogenic differentiation, and with a tendency for fBMSCs to have greater expression at 21 days. On the other hand, in MDSCs from males and females undergoing chondrogenic differentiation, analysis of Col II protein expression by immunohistochemistry showed a stronger labeling in the male group than in the female group (Scibetta *et al*., 2019). While in mini-pig, Col II expression was found to be similar in BMSCs from males and females subjected to E2 *in vitro* (Lee *et al*., 2016). Thus, it is important to understand that differences in Col II expression may occur depending on donor species, sex, and *in vitro* culture conditions.

The gene expression of Sox9 and Col II were similar in both fBMSCs and mBMSCs, with a difference only in fBMSCs, in which a higher expression of these genes was observed compared with mBMSCs at 14 days of chondrogenic differentiation. The Sox9 gene is known to be one of the main regulators of the entire chondrogenesis process, controlling the expression of other genes, such as activating the expression of Col II gene, the main component of the chondrogenic matrix (Akiyama, 2008), which occurred in this study at least at 14 days of differentiation. Thus, the findings of our study corroborate previously described results (Akiyama, 2008; Augello and Bari, 2010; Wang *et al*., 2014).

When comparing Sox9 gene expression at 16 days of chondrogenic differentiation between MDSCs from men and women, no major difference was observed (Scibetta *et al*., 2019). However, in the same study, results of chondrogenic matrix formation analysis using Alcian blue staining and immunohistochemistry assays showed a higher chondrogenic potential of cells extracted from men. This result differs from those of our study, which showed a higher expression of Sox9 in fBMSCs compared to mBMSCs at 14 days of differentiation and the analysis of chondrogenic matrix formation showed that fBMSCs had a higher score. Therefore, one can attribute this difference to

factors such as heterogeneity of the cells, varied sources of mesenchymal stem cells (skeletal muscle and bone marrow) and different donors (humans and mice) (Zhou *et al*., 2020).

The genes Sox9 and collagen II maintained their gene expression constant during the three weeks of chondrogenic differentiation. Therefore, fBMSCs and mBMSCs tend to maintain their chondrogenic differentiation capacity over time (7, 14 and 21 days of chondrogenic differentiation) at least regarding the expression of these genes that are considered essential for chondrogenic differentiation.

One study demonstrated that BMSCs from female rats withstand better environments under hypoxic conditions when compared with BMSCs from male rats (Crisostomo *et al*., 2006). Furthermore, the influence of the sex hormone E2 on BMSCs extracted from dog femurs showed improved cell proliferation and decreased apoptosis in a dose dependent manner (Zhou *et al*., 2020). Thus, this information may partially elucidate the results observed in our study, in which fBMSCs presented a higher chondrogenic differentiation potential compared to males, since the environment during chondrogenic differentiation is hypoxic and fBMSCs may have withstood this condition longer, besides normally suffering more influence from E2 compared to male BMSCs, even though E2 was not added to the culture medium in our study.

Aggrecan, as well as collagen II, is one of the main components of the cartilaginous matrix (Dudhia, 2005). When Agg expression was analyzed over 7, 14 and 21 days of differentiation, fBMSCs showed higher expression at 14 days and even higher at 21 days, while mBMSCs showed a peak of Agg expression at 7 days of differentiation and decreased over time, with the lowest expression at 21 days. This result may also explain the fact that fBMSCs presented a better differentiation potential compared to mBMSCs, since Agg expression decreased in mBMSCs and remained constant until 14 days in fBMSCs, and only decreased at 21 days of differentiation. Moreover, MSCs derived from adipose tissue and treated with E2 ($10^{-8}$ M) had a decrease in Agg gene expression when compared with the untreated group (Sadeghi *et al*., 2015). Therefore, although E2 was not placed in the culture medium of our study, this information suggests that the Agg gene suffers greater influence of the E2 hormone and may also contribute to explain the result observed in our study.

## CONCLUSION

We conclude that bone marrow mesenchymal stem cells from female Wistar rats have a greater chondrogenic differentiation potential compared to bone marrow mesenchymal stem cells from male individuals of the same species. The cells from female individuals show a greater formation of chondrogenic matrix at 21 days of differentiation and a greater expression of Col II and Sox9 at 14 days of differentiation.

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