IN VITRO EFFECTS OF CAFFEINE IN GROWTH CARTILAGE OF RATS

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ABSTRACT

Objective: To evaluate the *in vitro* effetcs of caffeine on proliferation, apoptosis and gene transcripts expression of chondrogenic differentiation in growth cartilage. Methods: The cartilaginous epiphyses of femurs of newborn rats, which were divided into two subgroups: treated with caffeine and control group, both observed over the time periods of 0, 7, 14 and 21 days. The cartilaginous epiphyses of femurs of each subgroup and each time span were subjected to histomorphometric, immunohistochemical analysis, Tunel technique and RT-PCR in real time. Results: The decrease in proliferative activity and the increase of apoptotic chondroblasts at 21 days were found regardless of the subgroup. However, the decrease in cell proliferation caused by caffeine was lower than in the control group and significantly increased the expression of gene transcripts for chondrogenic differentiation, represented by SOX-9 and RUNX-2. However, the *in vitro* culture with caffeine revealed antagonistic effects: despite the positive effect on chondroblasts proliferation and differentiation, caffeine increased apoptosis, characterized by increased expression of caspase 3 and of the number of cells undergoing apoptosis ($p<0.05$). Conclusion: Caffeine presents antagonistic effects *in vitro* on growth cartilage, increasing the proliferation, differentiation and cell apoptosis. ***Experimental Study.***

Keywords: Caffeine. Cartilage. Cell proliferation. Cell differentiation. Apoptosis.

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INTRODUCTION

Caffeine is a methylxanthine found in many foods and is widely consumed by the human population. Therefore, many of its effects and mechanisms of action have been extensively studied in various tissues. However, despite changing the postnatal bone growth, there are few studies on its effect on cartilage growth. In osteoblasts, caffeine acts negatively, reducing cell viability and increasing apoptosis of this cell, also reducing protein synthesis and gene expression of collagen, alkaline phosphatase, osteocalcin, osteopontin, histone, and CBFA1/RUNX2.[1-4] Due to these effects on bone, caffeine is considered a risk factor for osteoporosis and periodontal disease.[5] However, although there are several studies that examined the *in vitro* effects of the xanthine on bone cells, there is still lack of information about the *in vitro* effects of caffeine on cartilage cell of growing individuals. The aim of this study was to evaluate the *in vitro* effects of caffeine on the proliferation, apoptosis and expression of gene transcripts of chondrogenic differentiation in cartilage growth.

MATERIALS ANS METHODS

We used the femoral epiphyseal cartilage of 80 femurs of Wistar newborn rats, which were divided into two groups, namely growth with 2 mMol caffeine (Sigma-Aldrich, St. Louis, MO, USA) and control at 0, 7, 14 and 21 days. The femoral epiphyseal cartilage of four femurs in each group at each time interval were subjected to histomorphometric and immunohistochemistry examination for the evaluation of cell proliferation and tunel technique for assessment of apoptosis. The cartilaginous epiphyses of six femurs were subjected to RT-PCR assays in real-time for evaluation of the expression of caspase-3, Forward 5´TGGAG GAG GC - TGACCGGCAA´3 and reverse 5´ CTCTGTACCTCGGCAGGCCTGAAT´3; Runx-2, Forward 5´ GCGT CAACA- CCATCATTCTG´3 and reverse 5´ CAGACCAGCAGCACTCCATC´3; and Sox-9, Forward 5´ CCCGATCTGAAGAAGGAGAGC´3 and reverse 5´ GTTCTTCACC GA C- TTCCTCCG´3. The experimental design was factorial (2x4), i.e. two groups and four periods. For each variable it has been determined the mean and standard deviation. We performed ANOVA and comparison of means test was done by the Student-Newman-Keuls (SNK). Differences were considered significant if $p<0.05$. The experiment was approved by the UFMG Ethics Committee on Animal Experiments (protocol N° 177-2010).

All the authors declare that there is no potential conflict of interest referring to this article.

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RESULTS

Both the control group and in the group treated with caffeine, the morphology of chondroblasts was similar for most of the growth period. The percentage of empty chondroblasts lacunae in the cartilaginous epiphysis of the femur significantly increased over the growth period. However, the percentage of chondroblasts gaps with pyknotic nuclei increased on day seven, remaining so until the 21th days compared to day zero. However, at 21 growth days, the group treated with caffeine showed a number of empty lacunae of chondroblasts significantly lower compared to the control group. (Table 1 and 2, Figure 1) In the control group, the rate of cell proliferation characterized by the expression of the CDC-47, significantly decreased throughout the culture period compared to day 0. In the group treated with caffeine, the rate of cell proliferation was also significantly reduced at day seven compared to day zero, but remained steady until culture day 21, unlike the control group, which showed a progressive decrease in cell proliferation. At 21 days, despite the expression of CDC-47 being higher in the group treated with caffeine, there was no significant difference between the groups in any of the periods. (Table 3 and Figure 2) Both in the control group and in the group treated with caffeine, the number of apoptotic cells increased significantly during the entire culture period. However, at seven and 21 days of culture, apoptosis was more intense in epiphyseal cartilage cultured with caffeine. (Table 4 and Figure 3)

Similar to the number of apoptotic bodies, the expression of caspase 3 also significantly increased in both groups at 21 days of culture compared to day 0. However, at 21 days, the expression of caspase 3 gene transcript was significantly higher in the group treated with caffeine compared to the control group during the same period. (Table 5) Only in the group treated with caffeine, the expression of SOX-9 increased significantly at 21 days of culture compared to day zero, being also higher compared to the control group at 21 days of culture. (Table 5) The expression of RUNX-2 was significantly reduced in the control group at 21 days of culture, compared to day zero. However, in the group treated with caffeine, the expression of RUNX-2 at 21 days did not differ with respect to day zero, but was significantly higher than in the control group. (Table 5)

Table 1. Mean, standard deviation and results of statistical analysis of the percentage of empty lacunae of chondroblasts in cartilaginous epiphysis of femurs of neonatal rats cultured in medium with or without caffeine (control).

Group	Day 0	7 Days	14 Days	21 Days
Control	6.26±6.61C a	13.37±11.0C a	36.8±11.7 B a	50.7±15.56 A a
Caffeine	6.26±6.61C a	28.5±9.57 B b	45.4±8.81A a	24.2±7.56B b

*Means with the same uppercase letters in the line or the same lowercase letters in columns do not differ to each other to Student-Newman-Keuls test (SNK) (P≥0.05).

Table 2. Mean, standard deviation and results of statistical analysis of the percentage of gaps chondroblasts with picnotic core of the cartilaginous epiphysis of femurs of neonatal rats cultured in medium with or without caffeine (control).

Group	Day 0	7 Days	14 Days	21 Days
Control	9.40±4.036 B a	23.37±10.18 A a	19.25±5.47 A a	30.57±12.16 A a
Caffeine	9.40±4.036B a	17.4±10.32A a	24.71±7.95A a	28.7±13.12A a

*Means with the same uppercase letters in the line or the same lowercase letters in columns do not differ to each other to Student-Newman-Keuls test (SNK) (P≥0.05).

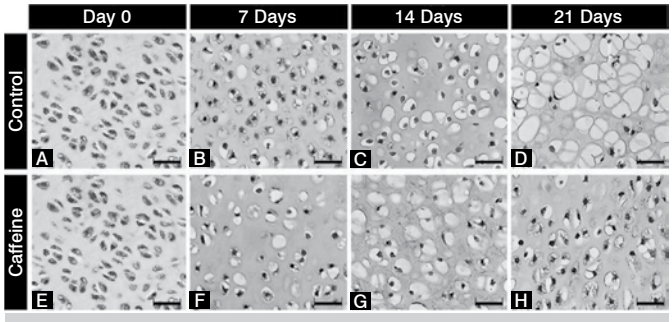

Figure 1. Epiphyseal cartilage of newborn rats cultured in medium with or without caffeine (control). HE, Bar = 23, 64 μm. A, B, C, and D) Control group at zero, 7, 14, and 21 days of culture, respectively. E, F, G, and H). Caffeine treated group at zero, 7, 14, and 21 days of culture, respectively.

Table 3. Mean, standard deviation and results of statistical analysis of the percentage of chondroblasts with CDC-47 expression in cartilaginous epiphysis of the femur neonatal rat cultured in medium with or without caffeine (control).

Group	Day 0	7 Days	14 Days	21 Days
Control	384.62±28.15 A a	245.4±82.4B a	267.87±46.7 B a	162±37.86 C a
Caffeine	384.62±28.1A a	283.7±98.8B a	248.5±57.64B a	228.6±65.16B a

*Means with the same uppercase letters in the line or the same lowercase letters in columns do not differ to each other to Student-Newman-Keuls test (SNK) (P≥0.05).

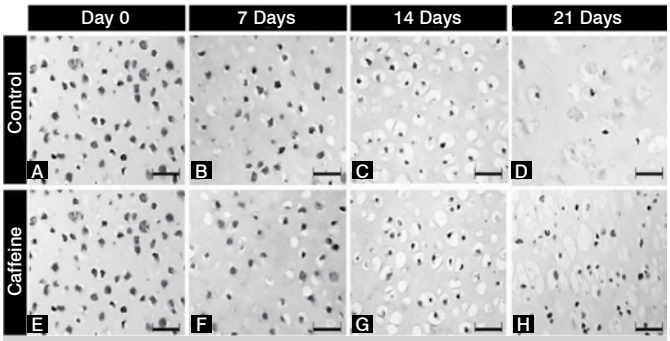

Figure 2. Epiphyseal cartilage of newborn rats cultured in medium with or without caffeine (control). Streptavidine-biotin-peroxidase, counterstaining by Methil green. Nuclear expression of CDC-47. Bar = 23, 64 μm. A, B, C, and D) Control group at zero, 7, 14, and 21 days of culture, respectively. E, F, G, and H). Caffeine treated group at zero, 7, 14, and 21 days of culture, respectively.

Table 4. Mean, standard deviation and results of statistical analysis of the number of apoptotic chondroblasts/field marked by tunneling technique in the cartilaginous epiphysis of the femurs of neonatal rats cultured in medium with or without caffeine (control).

Group	Day 0	7 Days	14 Days	21 Days
Control	20.6±8.12 D a	95.18±21.32 C b	198.33±61.34B a	262.95±54.28 A b
Caffeine	20.6±8.12 D a	171.71±59.39 C a	224.51±36.623 B a	360.55±54.49A a

*Means with the same uppercase letters in the line or the same lowercase letters in columns do not differ to each other to Student-Newman-Keuls test (SNK) (P≥0.05).

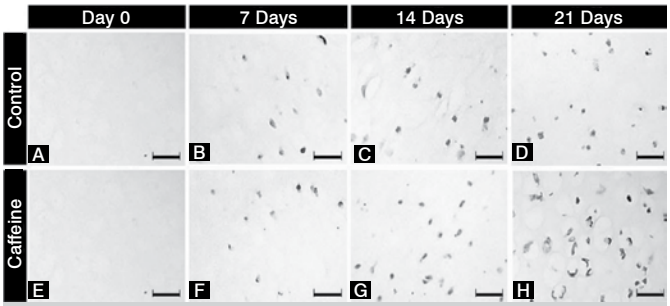

Figure 3. Epiphyseal cartilage of newborn rats cultured in medium with or without caffeine (control). Tunneling technique, counterstaining with Methyl green. Bar = 23, 64 μm. A, B, C, and D) Control group at zero, 7, 14, and 21 days of culture, respectively. E, F, G, and H). Caffeine treated group at zero, 7, 14, and 21 days of culture, respectively.

Table 5. Mean, standard deviation and results of statistical analysis of the expression of gene transcripts for caspase-3, Runx-2 and Sox-9 by RT-PCR in real time cartilaginous epiphysis of the femur of neonatal rat cultured in medium with or without caffeine (control) on day zero and after 21 days of culture.

Gene Transcripts	Control Day 0	Control 21 Days	Caffeine 21 Days
Caspase	0.804±0.09 B	2.04±1.34 B	4.22±2.09 A
Sox-9	1.45±1.58 B	2.72±1.22 B	8.35±3.36 A
Runx-2	0.87±0.29A	0.35±0.11B	0.73±0.22 A

*Means with the same uppercase letters in the line or the same lowercase letters in columns do not differ to each other to Student-Newman-Keuls test (SNK) (P≥0.05).

DISCUSSION

The present study aimed to further verify the *in vitro* mechanisms of action of caffeine on the cartilage tissue. However, the results demonstrated in part a different effect to that observed *in vivo*, since the epiphyseal cartilage treated with caffeine exhibited greater cell viability and greater expression of chondrogenic differentiation factors. On the other hand, caffeine also resulted in increased apoptosis with higher number of apoptotic chondroblasts and higher expression of caspase 3 gene transcript. This apoptotic effect of caffeine on cartilage cells *in vitro* might be the reason why we observe cell death in *in vivo* cartilage growth both in pups and neonates after nursing.[6] However, an antagonistic effect was observed *in vitro* where caffeine increased the number of apoptotic cells and expression of the transcript for caspase - 3, but also the proliferating cells remained viable for a longer time with increased gene transcripts of cell differentiation represented by SOX-9 and RUNX-2. These results show that the *in vitro* effects of caffeine may be different than the effects *in vivo*. However, antagonistic effects of caffeine seem to occur also in hair follicles. While caffeine stimulates *in vitro* the growth of hair human follicles[7] and it has been used in local treatment of alopecia,[8] offspring of rats treated with caffeine presented alopecia due to the lower proliferative activity of the cells of the hair follicle.[9] This difference may be explained, since the *in vitro* experimental model is simplified, and it is not possible to reproduce an environment with all the factors and signaling molecules that act *in vivo*. Although there are several studies that examined the *in vitro* effects of caffeine on bone cells,[4,5] the effect of caffeine on this model seems to have been the first attempt to study the *in vitro* effects of the xanthine in the cartilage cells of growing individuals.

CONCLUSION

We concluded that caffeine has *in vitro* antagonistic effects on cartilage tissue, increasing apoptosis, but at the same time, increasing cell viability by preventing the fall of proliferative index and increasing expression of gene transcripts of cell differentiation represented by SOX-9 and RUNX-2.

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