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Nonlinear effects of caffeine on the viability, synthesis and gene expression of chondrocytes from the offspring of rats treated during pregnancy

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ABSTRACT

Objective: Evaluate the effects of doses of caffeine administered to pregnant rats on the articular cartilage chondrocytes of their offspring.

Methods: Twenty-four adult Wistar rats were randomly assigned to four groups, with one control group and three groups being treated with caffeine at doses of 25, 50 and 100 mg/kg throughout pregnancy. At birth, three offspring/females were euthanized so that the chondrocytes could be extracted. At 7, 14 and 21 days of culture, the chondrocytes were subjected to the MTT cell viability assay and an evaluation of their alkaline phosphatase activity and collagen synthesis. Chondrocytes were also stained by Hematoxylin-eosin, PAS, Safranin-O and Alcian Blue. The Sox-9, Runx-2, aggrecan, collagen-II and alkaline phosphatase gene transcript levels were also evaluated. Mean comparisons were performed by the Student–Newman–Keuls test.

Results: Chondrocyte cultures from the 25 mg/kg group had the lowest results, as chondrocytes from this group had reduced viability, percentage of cells, alkaline phosphatase activity and collagen and chondrogenic matrix synthesis. A reduced expression of Sox-9, alkaline phosphatase and collagen-II was also detected in the 25 mg/kg group. Chondrocyte cultures of the group treated with 50 mg/kg caffeine showed reduced collagen synthesis and Sox-9 expression. The caffeine dose of 100 mg/kg also reduced collagen and Sox-9 and alkaline phosphatase expression.

Conclusion: Caffeine administered to pregnant rats negatively alters the articular cartilage chondrocytes of their offspring, reducing the synthesis of collagen and Sox-9 expression regardless of the dose. This study also concluded that the effects of caffeine are not linear or dose-dependent.

1. Introduction

Cartilaginous tissue consists of chondroblasts (young, newly differentiated cells) and chondrocytes (mature cells), which are highly specialized cells of mesenchymal origin responsible for the synthesis, maintenance and degradation of the extracellular chondrogenic matrix. This matrix is mainly composed of type II collagen associated with the macromolecules of proteoglycans, hyaluronic acid and adhesive glycoproteins (Gosset et al., 2008; Studer et al., 2012). During chondrogenesis, endochondral bone formation involves multiple stages; chondroprogenitor cells first undergo condensation and then differentiate into extracellular matrix-producing chondroblasts, which differentiate into chondrocytes that become entrapped by the produced matrix. Among the factors involved in regulating the molecular mechanisms of this differentiation, we highlight the Sox-9 protein, which acts on aggregated mesenchymal cells to control the genes of extracellular matrix proteins and align the parallel cell columns in the growth plate (Crombrugghe et al., 2001; Akiyama et al., 2002; Ikeda et al., 2005; Oh et al., 2010). Later, chondrocytes become hypertrophic and replete with rough endoplasmic reticulum, Golgi complexes and secretory vesicles, thus expressing Runx-2. This, in turn, induces the expression of type X collagen, alkaline phosphatase, vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs) and inhibitors of tissue-specific metalloproteinases (TIMPs) (Gerstenfeld and Landis, 1991; Kergosien et al., 1998). These factors facilitate the invasion of cartilage by blood vessels, resulting in the formation of long bone ossification centers (Vortkamp, 1998; Crombrugghe et al., 2001; Komori, 2003; Horton and Degnin, 2009; Ding et al., 2012).

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There are many agents that can affect the consecutive stages of chondroblastic differentiation and the critical regulators of this process (Yan et al., 2012), including caffeine, which is a pharmacologically active alkaloid consumed in copious amounts by the present population (Heckman et al., 2010). Caffeine is also fed to production animals in the form of pulp or coffee husk (Cabezas et al., 1987; Mazzafera, 2002). Caffeine and its metabolites can act directly or indirectly on various tissues of the body, including cartilage and bone tissues, and the effects of caffeine on tissue and bone cells have been well investigated (Tassinari et al., 1991; Kamagata-Kiyoura et al., 1999; Tsuang et al., 2006; Rapuri et al., 2007; Lu et al., 2008; Zhou et al., 2010; Reis et al., 2014; Choi et al., 2017). Thus, it is well known that this alkaloid is considered a risk factor for osteoporosis (Macedo et al., 2012), periodontal disease (Kamagata-kiyoura et al., 1999) and fetal malformation (Bergman et al., 1990; Narod et al., 1991). Caffeine can indirectly influence bone metabolism by increasing urinary calcium excretion (Bergman et al., 1990), and it acts directly or indirectly on the osteogenic differentiation of mesenchymal stem cells (MSCs) (Liu et al., 2011). In addition, caffeine can also influence the viability and synthesis of osteoblasts (Zhou et al., 2010), thus reducing the expression of genes, enzymes and proteins that are naturally expressed during osteogenesis, such as Runx-2 and Sox-9 (Tassinari et al., 1991; Zhou et al., 2010). In neonatal and infant rats, caffeine consumption by the mother during gestation and lactation affects cartilage growth (Wink et al., 1996; Tan et al., 2012; Reis et al., 2014) causing less differentiation of the chondroblasts. Consequences of this include disorganized and indistinguishable epiphyseal plaques on the long bones and vertebrae, as well as glycosaminoglycan deficiency in the chondrogenic matrix (Reis et al., 2014). In addition, changes have been observed in the long bones and vertebrae, such as reduced length and osteopenia (Huang et al., 2002; Reis et al., 2014; Shin et al., 2015). However, to elucidate the genesis of changes caused by caffeine on chondrocytes, more studies on cellular and molecular mechanisms are needed. This appears to be the first study that evaluates the effects of different doses of caffeine administered to pregnant rats on the viability and activity of chondrocyte synthesis of their offspring.

Therefore, the objective of this study was to evaluate cell viability by the MTT assay and the activity of chondrocyte synthesis extracted from the offspring articular cartilages of rats treated with different doses of caffeine by analyzing the percentage of cells and matrix. Additionally, the gene expression of Sox-9, Runx-2, aggrecan, type II collagen and alkaline phosphatase was analyzed using the qRT-PCR technique to elucidate some mechanisms by which caffeine reduces endochondral bone growth.

2. Materials and methods

All experimental procedures were approved by the Ethics Committee on Animal Experimentation of UFMG (protocol No. 3/2013).

2.1. Animals and study design

Twenty-four 2-month-old Wistar rats were randomly assigned to four groups, three of which were treated with caffeine, and one was a control group. The animals were housed in plastic boxes (6 animals/carton) where they received commercial feed (1.4% calcium, 0.60% phosphorus and 22% protein) and water ad libitum. They were maintained in a 12 h light - 12 h dark cycle.

After an adaptation period of 30 days, all female rats were subjected to daily vaginal cytology analysis to monitor their estrous cycle. Those that presented as being in the proestrus phase were housed in plastic boxes with adult male rats for 12 h. After this period, vaginal smears were performed daily to detect spermatozoa.

Copulation was confirmed by the presence of spermatozoa during the vaginal cytology analysis, and this day was considered day zero of gestation. After copulation, the rats were housed in separate boxes. The animals in the treated and control groups received daily caffeine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 5 mL of distilled water by a gastric-gas probe at doses of 25, 50 and 100 mg/kg administered throughout the gestational period. The animals in the control group received only distilled water as a placebo, also administered by a gastric-gas probe.

Three days after birth, three pups from each female of each group were euthanized to extract the chondrocytes from the articular cartilages, and cells from each group were pooled to perform the in vitro tests in sextuplicate or quadruplicate, as detailed below.

2.2. Harvest and culture of chondrocytes from cartilage of newborn pups

Chondrocyte extractions were performed according to previously established protocols (Gosset et al., 2008; Chen and Liu, 2009).

The three-day-old pups were euthanized with an overdose of anesthetic (2,5% Tionembutal; Abbott, São Paulo, Brazil). Next, antisepsis was performed using 70% alcohol and povidone-iodine (PVPI) on the hind leg skin of the rat. The femurs and tibias from all animals were then dissected from adjacent muscle and connective tissues with sterile instruments under laminar flow and washed in sterile 0.15 M standard phosphate buffer (PBS) (Synth, São Paulo, SP, Brazil). Cartilage fragments were immediately collected from the distal and proximal ends of these bones under a stereomicroscope.

Cartilage fragments were washed under laminar flow with DMEM (Gibco, Grand Island, NY) containing gentamicin (60 mg/L), penicillin (100 U/mL), streptomycin (10 mg/mL) and amphotericin B (250 mg)/L) (Merck, Germany). Cartilage was then digested by overnight incubation with 1.5 mg/mL collagenase II (Sigma-Aldrich, St. Louis, MO, USA) in DMEM containing 5% fetal bovine serum (FBS) (Sorali, Brazil) (Chen and Liu, 2009).

The cell suspensions were centrifuged for 10 min at 1400 *g* and washed with PBS. After further centrifugation, the cells were resuspended and cultured in T75 bottles (TPP-Tech Plastic Products in Trasadingen) containing chondrogenic medium in an oven at 37 °C and 5% CO2. This medium was composed of DMEM supplemented with 1% fetal bovine serum, ascorbic acid (50 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA), 10-7 M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 625 µg/mL insulin (Biobras, Brazil), 625 µg/ml transferrin (Sigma-Aldrich, St. Louis, MO, USA), 1 mM pyruvate (Sigma-Aldrich, St. Louis, MO, USA), and 1.25 µg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, the bottles were washed with 0.15 M PBS to remove unbound cells. The culture medium was changed twice a week.

2.3. MTT reduction assay

At the end of days 7, 14 and 21, the cells from all the experimental groups were cultured at a density of 1×10^4 cells in 24-well plates and subjected to the MTT reduction test [3-(4,5-Dimethylthiazol-2 yl)-2,5-diphenyltetrazolium] (Invitrogen, Eugene, Oregon, USA), in which MTT was reduced to formazan crystals. Briefly, the cells were incubated with MTT for 2 h at 37 °C. After incubation, the cells were treated for 12 h with a solubilizing solution (sodium dodecyl sulfate in 10% HCl) (Synth, São Paulo, SP, Brazil). Subsequently, 100 µL of cells were transferred to 96-well plates for spectrophotometer readings at a wavelength of 595 nm. The mean absorbance and standard deviation were determined six times for each experimental group.

2.4. Alkaline phosphatase activity

At the end of the 7, 14 and 21 days of culture, cells from all experimental groups were cultured at a density of 1×10^4 cells in 24-well plates to evaluate the alkaline phosphatase activity.

Cells were incubated with BCIP/NBT solution (5-bromo, 4-chloro, 3-indolylphosphate-nitroblue tetrazolium) (Gibco, Grand Island, NY) for