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Synthesis and characterization of gold nanoparticles combined with curcumin and its effects on experimentally induced osteoarthritis

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ABSTRACT: *Osteoarthritis (OA) is the clinical term for a combination of pathological conditions that involve the progressive degeneration of articular cartilage and subchondral bone remodelling. Curcumin, a potent anti-inflammatory agent, has been extensively studied; however, it does not provide good systemic bioavailability. Gold nanoparticles (AuNPs) have potential applications in the administration of therapeutic substances in order to increase the transport efficiency of drugs. The objectives of this study were to explore the synthesis and characterization of a system combining AuNPs with curcumin and evaluate its therapeutic potential in an experimental model of OA in mice by the destabilization of the medial meniscus (DMM). The AuNPs were conjugated with curcumin and the systems were characterized by UV-VIS spectroscopy, dynamic light scattering (DLS), and zeta potential. Four groups of eight animals each were formed and labelled A, B, C, and D, which received intra-articular injections of AuNPs, curcumin, AuNP-curcumin, and physiologic solution, respectively. After seven weeks, the cartilage of the stifle joint (SJ) was rated on a scale ranging from 0 to 24. Combination of AuNP-curcumin demonstrated good stability and therapeutic applications, but it did not differ significantly ($P>0.05$) from groups A and B. However, the control group had a significantly lower score ($P<0.001$). Results of this study demonstrated the importance of developing new nanodrugs. In this case, the combination of AuNPs and curcumin yielded the nanodrug effects suggestive of a potential for application in the treatment of OA.*
Key words: *curcumin, mice, nanodrugs, nanotherapy, osteoarthritis.*

Síntese e caracterização de nanopartículas de ouro conjugadas com curcumina e seus efeitos na osteoartrite experimental induzida

RESUMO: *A Osteoartrite (OA) é uma denominação clínica para uma combinação de condições patológicas que envolvem a degeneração progressiva da cartilagem articular e remodelação de osso subcondral. A curcumina, um potente agente anti-inflamatório, têm sido extensivamente estudada, no entanto não oferece boa biodisponibilidade sistêmica. Nanopartículas de ouro (AuNPs) apresentam aplicações potenciais na administração de substâncias terapêuticas aumentando a eficiência do transporte de fármacos. O objetivo deste estudo foi realizar a síntese e caracterização de um sistema conjugando as AuNPs à curcumina e avaliar seu potencial terapêutico em um modelo experimental de OA em camundongos por desestabilização do menisco medial (DMM). As AuNPs foram conjugadas com curcumina e os sistemas foram caracterizados por espectroscopia no UV-VIS, espalhamento de luz dinâmico (DLS) e determinação do potencial zeta. Formou-se 4 grupos de oito animais cada, denominados A, B, C, D que receberam injeção intra-articular de AuNPs, curcumina, AuNP-curcumina e solução fisiológica, respectivamente. Após 7 semanas, a cartilagem da articulação-femoro- tibio-patelar (AFTP) foi avaliada em uma variação de escore de 0 a 24. A conjugação de AuNP-curcumina mostrou boa estabilidade e aplicação terapêutica, mas não diferiu significativamente ($P>0,05$) dos grupos A e B, no entanto, mostrou menor valor de escore e significância ($P<0,001$) em relação ao grupo controle. Os resultados deste trabalho mostram a importância do desenvolvimento de novos nanofármacos. Neste caso a conjugação de AuNPs com a curcumina permitiu a obtenção de um nanofármaco com sugestivo potencial para aplicação no tratamento da OA.*
Palavras-chave: *camundongos, curcumina, nanofármacos, nanoterapia, osteoartrite.*

INTRODUCTION

Osteoarthritis (OA) is the most common chronic musculoskeletal disease and a major cause of locomotor disability in dogs (INNES, 2010). Analgesics, anti-inflammatory steroids, and steroidal anti-inflammatory drugs (NSAIDs) only treat symptoms of the disease by reducing pain and inflammation. For these reasons, alternative OA treatments are desirable and, recently, nutraceuticals,

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such as curcumin, have been proposed for this purpose (INNES et al., 2010; HENROTIN et al., 2013). Curcumin is characterized as a chemopreventive agent and is a highly pleiotropic molecule capable of interacting with numerous factors and mediators involved in inflammation (AGGARWAL & HARIKUMAR, 2009). Recent studies have demonstrated that curcumin is rapidly metabolized but has limited systemic bioavailability, which has limited its use in clinical cases (IRESON et al., 2011).

The conjugation of drugs and molecules to metals, especially nanoparticles in aqueous media, can provide enhanced activity, increased half-life and greater resistance to specific metabolic processes (SINDHU et al., 2014). Advantages in AuNP conjugation may include controlled and/or prolonged release of the substance therein encapsulated, which reduces adverse effects associated with the substance, compound protection from inactivation before reaching the site of action, increased intracellular penetration, and increased pharmacological activity (MARANGONI et al., 2013). The aim of this study was to explore the synthesis and characterization of AuNPs with curcumin and thus evaluate their therapeutic action in an experimental osteoarthritis model in mice.

MATERIALS AND METHODS

Synthesis and characterization of AuNP

10 mL of an aqueous solution of Chloroauric acid ($HAuCl_4$) at 0.001 mol L^{-1} were added with magnetic stirring to 10mL of an aqueous solution of polyallylamine hydrochloride (PAH). Then, 1mL of sodium borohydride at 0.01mol L^{-1} at approximately 0°C was added to this solution. The system was stirred for approximately 30min on a magnetic stirrer, then allowed to stand at room temperature and was protected from light for 24 hours. Finally, the particles were washed by centrifugation (12,000rpm 10min^{-1}) to remove excess PAH. A curcumin solution of 500µL at 1.0mg mL^{-1} in ethanol were added to 5mL of AuNP-PAH suspension. The system was stirred for four hours at room temperature and then washed twice by centrifugation (12,000rpm 10min^{-1}) to remove excess curcumin.

An UV-VIS spectroscopy was used to identify and quantify AuNP-PAH and curcumin as well as to observe their interactions. Measurements were performed on a Hitachi U-2900 using quartz cuvettes with an optical path of 1cm, as proposed by MARANGONI et al. (2013)

Particle size distribution was obtained by DLS measurements at 25°C in triplicate using a ZS® Nano spectrometer (Malvern Instruments, UK). Zeta potential of suspensions was measured at 25°C in triplicate using a ZS® Nano spectrometer. All samples were measured in 10mmol L^{-1} phosphate buffer.

Animals and surgical induction

Thirty two male C57BL6 strain mice at 8 weeks of age were used. After trichotomy and antisepsis of the surgical area, the destabilization technique was performed on the medial meniscus (DMM), as proposed by GLASSON et al. (2007).

Four groups were formed, each with eight animals, as follows: A (360mg of AUNP-PAH), B (20mg of curcumin), C (360mg of AUNP-PAH-curcumin (20mg)) and D (physiologic solution); they were distributed in accordance with the administered therapy. All animals were treated by intra-articular injection every 15 days, with the first treatment given on day 14 after the induction of OA. Seven weeks after the induction of OA, animals were euthanized.

Histopathological evaluation

All knee joints (KJs) were fixed in 10% buffered formalin for 24 hours and decalcified in EDTA solution monosodium 15% for two weeks. They were then processed according to routine procedures and the slides were stained with hematoxylin and eosin (H&E) and Safranin O fast green to evaluate the concentration of proteoglycans. They were assessed by 2 independent observers in a blind study using the criteria established by MANKIN et al. (1971). The score for each joint was obtained by calculating the formula, standardized by the evaluation system of Osteoarthritis Research Society International (OARSI), and established by BAO et al. (2009) and RUTGERS et al. (2010) thus, each KJ received a score ranging from 0 to 24.

Statistical analysis

Data were analysed for normal distribution using the Kolmogorov-Smirnov test. Therefore, normal data were submitted to analysis of variance, and, in cases of significance, means were compared using Tukey's test. For all statistical analysis, the 5% significance level was adopted. Values were analysed using GraphPad Prism 4.

RESULTS

The AuNP-PAH-curcumin complex and its precursors were characterized by UV-VIS spectroscopy. The formation of AuNPs was detected by UV-VIS measurements by the plasmonic features

of nano-sized particles. In the spectrum corresponding to AuNP-PAH, a strong absorption band was observed at approximately 530nm. In the spectrum of the AuNP-PAH-curcumin complex, a shift of approximately 15nm was identified (Figure 1A).

Zeta potential values for both systems presented a highly positive surface charge, +55mV and +35mV in the AuNP-PAH and AuNP-PAH-curcumin suspension, respectively, which indicated electrostatic stability. Decrease of surface charge of AuNP-PAH after conjugation with curcumin suggested that the modification of the surface with curcumin was efficient. The size distributions for the solutions obtained by DLS measurements are shown in figure 1B. The average diameter of AuNP-HAP was reported to be approximately 20nm while the AuNP-PAH-curcumin complex had a diameter of approximately 60nm.

Histological analyses showed a clear distinction in the joint cartilage lesion between the different groups (Figure 2). Focal discontinuity of the cartilage surface area and erosion with loss were the most superficial lesions reported in groups A and B, as well as the mild loss of proteoglycans as evidenced by decreased Safranin O/Fast Green staining. In group C, there were milder SJ lesions, of which edema and fibrillation of the cartilage surface were the most common changes, emphasized by the greater degree of proteoglycan staining by Safranin O/Fast Green. In the control group (group D), more severe lesions were observed such as microfractures with the presence of fibrocartilage, remodelling, and bone repair, while the severity and stage of the lesions increased compared to the other groups.

A higher value was reported for group D's SJ at a mean score of 18.25. In addition, a significant difference ($P<0.001$) was reported when group D was compared to all other groups. In contrast, joints evaluated in groups A and B reached average values of 7.87 and 9.50, respectively, with no statistically significant difference ($P>0.05$) between these groups. Animals that received the AuNP-PAH-curcumin complex (group C) showed a significant decrease ($P<0.001$) in their mean score values compared to the control group; however, no significant difference was found compared with the other groups, as shown in figure 3.

DISCUSSION

Nanoparticles are thermodynamically unstable and have a natural tendency to aggregate and grow. Thus, the great challenge today consists precisely in preparing stable nanomaterials that may be

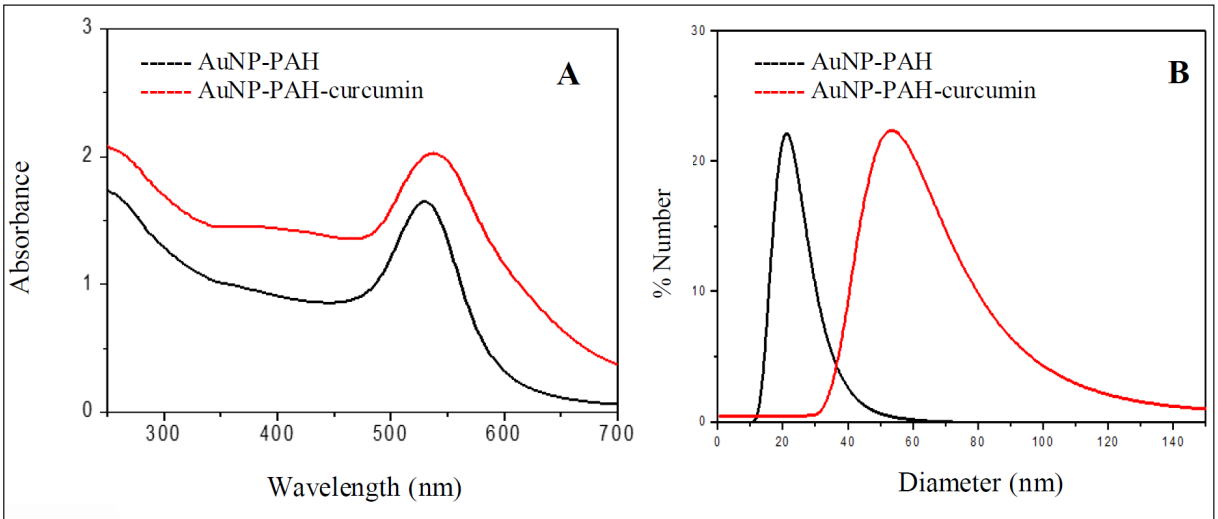

Figure 1 - Schematic representation of the characterizations of complex AuNP-PAH and AuNP-PAH-curcumin. A) UV-VIS absorption spectra showing the spectrum corresponding to AuNP-PAH a strong absorption band was observed at approximately 530nm. In the spectrum of the AuNP-PAH-curcumin complex, a shift of approximately 15nm can be identified. B) DLS measurements showed that the average diameter of AuNP-PAH was approximately 20nm, while the AuNP-PAH-curcumin complex had a diameter of approximately 60nm. AuNP: gold nanoparticles; PAH: polyallylamine hydrochloride; UV-VIS: Ultraviolet visible spectroscopy; DLS: dynamic light scattering.

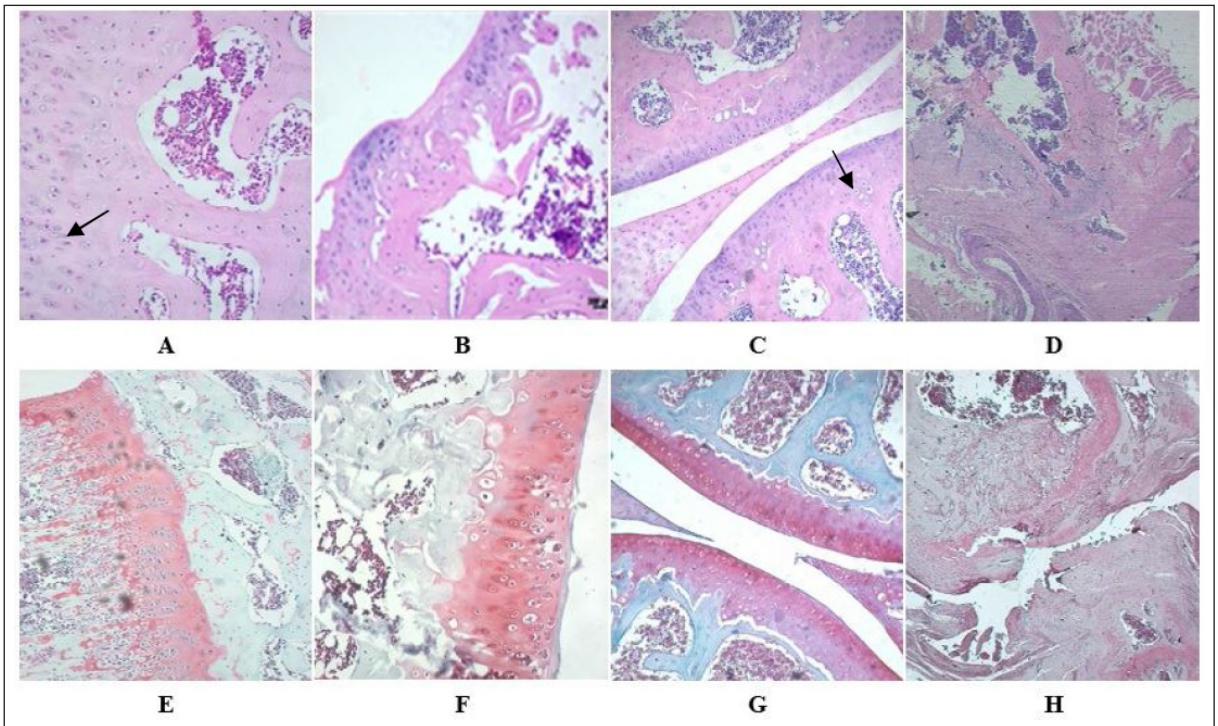

Figure 2 - Photomicrographs of the joint cartilage of mice after the experimental induction of OA in DMM: A, E), group treated with AuNP-PAH; A: slight unevenness of the joint surface (arrow) with disorganization of the chondrocyte columns; B, F) group treated with curcumin; C, G) group treated with AuNP-PAH-curcumin; C) Integrity of the joint surface can be seen despite the slight disorganization of chondrocyte columns and cluster formation (arrow); D, H) control group treated with saline solution; D) Intense fibrocartilage formation, disorganization, and bone remodelling; H) intense decrease in proteoglycans shown by the decrease in Safranin O/Fast Green staining. A, B, C, D: H&E staining; E, F, G, H: Safranin O/Fast Green staining. 20X. AuNP-PAH: gold nanoparticles; PAH: polyallylamine hydrochloride; H&E: haematoxylin and eosin; DMM: destabilization of the medial meniscus.

handled without losing their characteristics so that they can be efficiently used in technological and biomedical applications (MARANGONI et al., 2013).

An UV-VIS spectroscopy has been widely used in characterizing the optical properties of metallic nanoparticles. The red shift observed in this study for the spectrum of AuNP-PAH-curcumin was related to the changes in their surface due to modification with curcumin. This is relevant because large shifts to longer wavelengths may indicate a system aggregation process (YEN et al., 2009). The strong band of AuNP-PAH complex absorption observed at approximately 530nm was attributed to the collective oscillations of electrons on the surface of gold nanoparticles called plasmon resonance. According to BARTH (2007), the absorption wavelength is directly related to the average diameter of nanoparticles.

Furthermore, the small band at approximately 400nm in the spectrum of AuNP-PAH-curcumin corresponded to the characteristic absorption band of curcumin, indicating conjugation. The increase in diameter reported by DLS for the AuNP-PAH-curcumin complex was also related to the formation of a curcumin layer around the nanoparticle, as observed by SINDHU et al. (2014), who compared gold nanoparticles of different sizes in aqueous solution to experimental data. The measured value of the zeta potential provides an indication of the stability of the suspension. This study observed that the suspensions are highly stable because, according to BARTH (2007), suspensions with zeta potential values above +30mV and below -30mV are stable; whereas, those with values above -30mV and below +30mV are unstable and can flocculate. The decrease of the zeta potential after conjugation with the curcumin molecules proves that the functionalization was efficient. As shown, these properties of the suspensions enable the objective assessment of whether or not they have

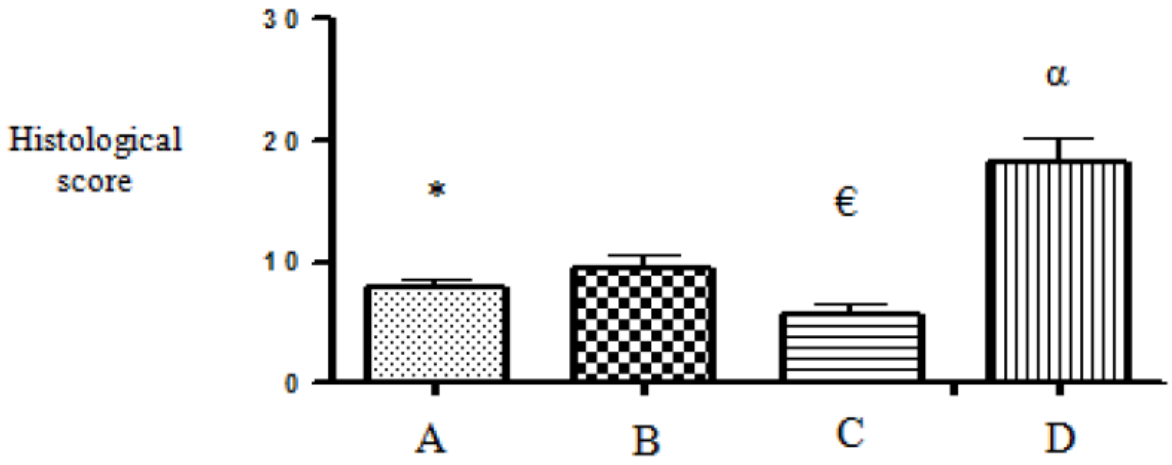

Figure 3 - Schematic representation of the mean scores in the histological assessment of joint cartilage in mice with experimental OA: A) The group treated with AuNP-PAH with score of 7.87; B) The group treated with curcumin with score of 9.50; C) The group treated with AuNP-PAH-curcumin with score of 5.75; and D) The control group treated with saline solution with score of 18.25. *: P>0.05 vs. B and C; €: P>0.05 vs. B; *α: P<0.001 vs. A, B, and C. Means were compared using Tukey's test. AuNP-PAH: gold nanoparticles; PAH: polyallylamine hydrochloride.

stable behaviours. A similar result was observed by SILVA (2015), conjugating AuNPs to a flavonoid, which exhibited concentrated suspensions measuring approximately 15nm, consistent with the findings in this study for the AuNP-PAH-curcumin complex. The synthesis of AuNPs poses challenges due to the poor reproducibility and stability of the colloidal system. LIAN et al. (2016) reported the conjugation of curcumin on the surface of AuNPs, which were synthesized by direct reduction using $HAuCl_4$ in the aqueous phase without the use of any other reducing agents. They observed a good functionalization of the system while there was a slight aggregation of curcumin particles, making it less available. In the present research, we developed a one step, facile procedure for the synthesis of AuNP-PAH combined with curcumin by using PAH as reducing agente, which led to the production of larger nanoparticles stabilized by various molecules.

As for the histological evaluation of the lesions, it was observed that the articular cartilage submitted to the DMM procedure showed typical morphological changes of OA, as described in the literature by GLASSON et al. (2007). These demonstrated cartilage and bone lesions, which probably resulted from changes in articular cartilage and secondary bone involvement (PRITZKER et al., 2006).

Although not significantly different among them, a decrease in the severity of histological lesions in groups A, B, and C was observed. In the SJ of group A, this was evidenced by the anti-inflammatory and anti-angiogenic properties of AuNPs, as reported by HUANG et al. (2012) who reported a reduction in disease progression by the inhibition of the vascular endothelial growth factor (VEGF) activity with administration of AuNPs in the early stages of OA in mice. Curcumin's anti-inflammatory properties have been documented in recent studies by JURENKA (2009) and HENROTIN et al. (2013), and this study confirms its effect on reducing injuries because it inhibits inflammatory mediators such as NF-KB, IL-1β pro-inflammatory genes, COX-2, and VEGF (CSAKI et al., 2009).

The morphological characteristics of the articular cartilage in group C were less typical of OA than those seen in the other groups. Degenerative lesions, such as edema, fibrillation, and hypercellularity, were also reported; however, they were more discreet and less extensive. These findings suggested better partial bioavailability of curcumin when conjugated with AuNP-PAH because they can provide an increase in curcumin activity, a longer half-life, greater stability, and possible resistance to metabolic processes (INNES et al., 2010).

CONCLUSION

The results of this study demonstrated the importance of the study and development of new

nanodrugs since the combination of nanoparticles can impart additional properties to such systems. Animals that received an intra-articular application of AuNP-PAH-curcumin showed lower severity of histological lesions. However, the AuNP-PAH-curcumin complex showed no significant difference compared with groups A and B, differing only compared to the control group. New studies on the functionalization and characterization of biomolecules may be necessary to continue to develop new approaches and modalities for nanodrugs. In this case, the application of AuNP conjugated with curcumin and added to a nanodrug in animals with OA symptoms demonstrated the potential for the treatment of OA in humans.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the Ethics Committee on Animal Use (CEUA-UFMT) under protocol no. 23108.043951/12-1. 32.

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