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Development of hydroxyapatite nanoparticles loaded with folic acid to induce osteoblastic differentiation

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ABSTRACT

Recently it has been shown that folic acid can have an important role in bone regeneration. For this reason, combining a classic bone regeneration system as, hydroxyapatite, loaded with folic acid, may be an important issue to be developed. To address this issue, hydroxyapatite nanoparticles loaded with folic acid were designed as an effective bone regenerative system, to induce osteoblast differentiation and improve the bone regeneration. HapNP were prepared by a hydrothermal method that used citric acid as a tailoring agent of particles morphology and, simultaneously, had the particularly to let carboxylic pendant groups in the particle surface, which provided a platform for the immobilization of folic acid (FA), producing HapNP-FA. A comparative study among hydroxyapatite nanoparticles loaded and unloaded with folic acid in presence of human mesenchymal stem cells was performed. The results demonstrate, that nanoparticles were able to be internalized by human mesenchymal stem cells. In addition, cell proliferation and viability were not affected in a wide concentration range. Both particles induced the expression of Runx2 and the expression and activity of alkaline phosphatase. However, HapNP-FA caused a significantly higher overexpression of Runx2. The osteoblastic differentiation confirms the potential applicability of HapNP-FA in the local bone regeneration.

1. Introduction

Folic acid/folate (neutral molecule/deprotonated ion), both coexisting in aqueous medium, need to be supplied through the diet to meet daily requirements. Folic acid is used by virtually all mammalian cells to synthesize pyrimidines and purines needed for the synthesis and maintenance of nucleic acids (DNA, RNA). Also, it has a key role in the one-carbon metabolism for the methylation of essential molecules (DNA, RNA and proteins), and is a cofactor in a variety of reactions (Bailey et al., 2015; Crider et al., 2012). As such, it is required for cellular division, having an essential role in conditions demanding rapidly proliferating cells, as well as in maintaining the methylation cellular status. Cellular uptake of folic acid occurs mainly by an endocytic pathway mediated by high affinity folate receptors, cysteine-rich cell-surface glycoproteins (Chen et al., 2013).

During the last years, folic acid (vitamin B9) has gained particular attention in bone metabolism since a number of studies have reported a relationship between vitamin B9 status and bone health. There is evidence from experimental, observational and intervention studies that folic acid deficiency may affect bone structure, bone quality, bone mass and fracture risk (Bailey et al., 2015; Dai and Koh, 2015; Holstein et al., 2009; van Wijngaarden et al., 2014).

Deficient folic acid intake appears to be associated to the presence of increased plasma levels of homocysteine (Vacek et al., 2013). The mechanisms of increase in osteoclast activity, reduction in osteoblastic activity and increase in matrix metalloproteinases that degrade the bone matrix can be modulated using folic acid (Kim et al., 2006; Vacek et al., 2013; Vaes et al., 2009).

Abbreviations: HapNP, hydroxyapatite nanoparticles; HapNP-FA, hydroxyapatite nanoparticles conjugated with folic acid; FA, folic acid; Hap, hydroxyapatite.

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Hiperhomocysteinemia has been associated with decreased bone quality and increased fracture risk (Cagnacci et al., 2008; Gerdhem et al., 2007; Gjesdal et al., 2007; Morris et al., 2005; van Wijngaarden et al., 2014). Folic acid (through the activated form, 5-methyltetrahydrofolate) acts as a methyl donor, and is needed for the remethylation of homocysteine to methionine which can be converted to S-adenosylmethionine (SAM), the universal donor for the methylation of target molecules. Regarding this, protein methylation appears to be a critical mechanism for the osteogenesis and bone maintenance (Vaes et al., 2009; Wang et al., 2014). In this way, folic acid, with its role in the recycling of homocysteine to methionine, contributes to the upkeep of the cellular methylation status, including in the bone environment (Loenen, 2006). Additionally, folate appears also to affect bone by homocysteine independent pathways, namely by its role in nitric oxide synthesis in bone cells McCarty (2005).

In the last decade growing interest has been devoted to the design of bioinspired hydroxyapatite nanoparticles. They closely look like calcium phosphate particles present in the bone mineral matrix explaining their natural biocompatibility and usefulness in bone related applications (Fox et al., 2012; Rawat et al., 2016; Zhou and Lee, 2011). The ease of surface functionalization with cell-targeting agents (drugs and other bioactive molecules) greatly increases their potential application in bone regeneration strategies, treatment of bone diseases and imaging (Fox et al., 2012; Luo et al., 2016; Zhou and Lee, 2011). In previous studies we reported the preparation of hydroxyapatite nanoparticles by a hydrothermal method (Santos et al., 2012). These particles were promptly incorporated by osteoblastic cells (Santos et al., 2012) and induced osteogenic-related markers in MG63 cells (Santos et al., 2012) and human mesenchymal stem cells (Amjadian et al., 2016; Bohner et al., 2012). The same nanoparticles were able to modulate the differentiation and function of human osteoclastic cells in a dose- and time-dependent manner (Costa-Rodrigues et al., 2014). Also, a recent study showed the possibility to functionalize these hydroxyapatite particles with gold nanoparticles, using the citrate ions present on hydroxyapatite surface as a targeting agent (Ferreira dos Santos et al., 2015).

Bone is an active organ undergoing modeling and remodeling throughout life. These phenomena involve highly regulated and coupled bone formation and bone resorption events, respectively accomplished by the concerted activities of osteoblasts and osteoclasts. Due to this high metabolic dynamics, upon injury, bone has a remarkable ability to regenerate. Mesenchymal stem cells are recruited to the local and committed to an osteogenic differentiation pathway needed to the formation of new bone. In such a dynamic environment, the local availability of folic acid is anticipated to play a positive role in the cellular activities involved in the bone regenerative process. In this context, this study reports the synthesis and characterization of hydroxyapatite nanoparticles loaded with folic acid aiming a potential usefulness in local strategies of bone regeneration. Such a system will take advantage of the bone biocompatibility of hydroxyapatite, the surface properties of the nanoparticles, the prompt particles cellular uptake and the localized delivery of folic acid. This approach has not been explored either in normal conditions or in those of compromised bone metabolic activities.

A convenient chemical method has been developed to couple folic acid to the hydroxyapatite nanoparticle surface. At first, the hydroxyapatite nanoparticles (HapNP) were synthesized by a hydrothermal method using citric acid to get HapNP with pending carboxylic groups on the surface (Santos et al., 2012). Subsequently, the carboxyl groups on HapNP were coupled with folic acid (FA). This hydroxyapatite loaded with folic acid (HapNP-FA) was characterized for the physic-chemical profile. Also, human mesenchymal stem cells were exposed to HapNP-FA and evaluated for proliferation, metabolic activity and osteoblastic parameters, as well as particle internalization, to explore the potential of these particles in bone regenerative applications.

2. Materials and methods

2.1. Synthesis of hydroxyapatite nanoparticles

Hydroxyapatite nanoparticles (HapNP) were synthesized by a hydrothermal method (HS). Ammonia solution (NH_4OH, 25%, Riedel-deHaën) was added to a citric acid solution (0.6 M; $C_6H_8O_7{\cdot}H_2O$, 99.5%, Riedel-deHaën) until pH = 8.1 was reached, as previously reported (Santos et al., 2012).

2.2. Hydroxyapatite nanoparticles loaded with folic acid

The HapNPs prepared by HS were selected for loading folic acid (FA) onto the nanoparticles surface. Folic acid (FA, $C_{19}H_{19}N_7O_6$, 99%, ABCR), 0.05 g, was added to 100 ml phosphate buffer solution (PBS) with a pH of 7.5. After that, 0.1 g of the HapNPs were suspended in the PBS added with FA for 48 h, in the dark, at room temperature. Finishing this process, the nanoparticles were then repeatedly washed with Millipore water followed by filtration (0.22 μm millipore). The particles conjugated with folic acid were labeled as HapNP-FA.

2.3. Physic-chemical characterization of HapNP and HapNP-FA

2.3.1. Structure and morphology

The identification of the crystalline phase of the loaded folic-acid onto hydroxyapatite nanoparticles (HapNP-FA) as well as the prepared hydroxyapatite nanoparticles (HapNP) was performed on an X-ray diffractometer, model Rigaku PMG-VH with a Cu-Kα incident radiation (1.5405 Å). High-resolution transmission electron microscopy (JEOL-TEM 2200 FS, operated at 200 kV) was used to characterize the microstructure of the particles. For TEM study, the nanoparticles were thoroughly dispersed in 2-propanol by ultrasonication. A drop of the suspension was placed on a carbon coated copper grid, air-dried and then the images were obtained.

2.3.2. Zeta potential measurements

The zeta potentials of the HapNP and HapNP-FA were measured using a Malvern equipment with zetasizer nano series 6.00 software. A small amount of the particles was suspended in a 2 mM KCl solution in order to ensure a constant electrical double-layer thickness and then allowed to equilibrate for 2 h before measurement. HCl (0.1 M) and NaOH (0.1 M) were used for pH adjustment.

2.3.3. Surface chemistry

The surface composition of the hydroxyapatite nanoparticles (HapNP) as prepared and modified with folic acid (HapNP-FA) was determined from Fourier transform infrared (FTIR) spectra. The FTIR spectra were recorded at room temperature with a FT-IR spectrometer (Mattson galaxy 3020) using the KBr pellet technique in the range 400–4000 cm^{-1}.

The existence of folic acid on the HapNP was also confirmed by X-ray photoelectron spectroscopy, using a Microlab 310 (VG Scientific) equipped with an Mg (non-monochromated) anode and a concentric hemispherical analyzer. The spectra were taken at constant analyzer mode (CAE = 30 eV).

2.3.4. Identification of folic acid on HapNP by UV–vis spectroscopy

The binding of folic acid on HapNP was revealed by UV–vis spectroscopy analysis. A destructive analysis of HapNP and HapNP-FA was conducted by UV–vis spectroscopy. For this, the nanoparticles (0.01 g) were dissolved in 0.1 M HCl (3 ml), and the