European Journal of Pharmaceutics and Biopharmaceutics 82 (2012) 508–517

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

In vivo efficacy studies of layer-by-layer nano-matrix bearing kaempferol for the conditions of osteoporosis: A study in ovariectomized rat model

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ARTICLE INFO

Article history:
Received 28 March 2012
Accepted in revised form 4 August 2012
Available online 24 August 2012

Keywords:
Kaempferol
LbL nano-matrix
Bioavailability
Bone marrow cells
Osteoblast mineralization
Bone-micro-architecture

ABSTRACT

A prototype formulation based on layer-by-layer (LbL) nano-matrix was developed to increase bioavailability of kaempferol with improved retention in bone marrow to achieve enhanced bone formation. The layer-by-layer nano-matrix was prepared by sequential adsorption of biocompatible polyelectrolytes over the preformed kaempferol-loaded $CaCO_3$ template. The system was pharmaceutically characterized and evaluated for osteogenic activity in ovariectomized (OVx) rats. Data have been compared to the standard osteogenic agent parathyroid hormone (PTH). Single oral dose of kaempferol loaded LbL nano-matrix formulation increased bioavailability significantly compared to unformulated kaempferol. Three months of Formulated kaempferol administration to osteopenic rats increased plasma and bone marrow Kaempferol levels by 2.8- and 1.75-fold, respectively, compared to free Kaempferol. Formulated Kaempferol increased bone marrow osteoprogenitor cells, osteogenic genes in femur, bone formation rate, and improved trabecular micro-architecture. Withdrawal of Formulated kaempferol-in OVx rats resulted in the maintenance of bone micro-architecture up to 30 days, whereas micro-architectural deterioration was readily observed in OVx rats treated with unformulated kaempferol-within 15 days of withdrawal. The developed novel formulation has enhanced anabolic effect in osteopenic rats through increased stimulatory effect in osteoblasts. Treatment post-withdrawal sustenance of formulated kaempferol could become a strategy to enhance bioavailability of flavanoids.

1. Background

In the past few decades, the layer-by-layer (LbL) polyelectrolyte assembly has been designed to encapsulate various biomolecules [1]. The robustness of the system has been proven due to flexibility in choosing the template and tailoring the surface by using different types of biocompatible polyelectrolytes [2]. It is evident from the past work that the main emphasis has been given only in tuning of pore sizes of the core template to achieve high drug payload, modulate release profile and to immobilize various therapeutic molecules such as enzymes, nucleic acids, and DNA. There are very few reports with regard to its preclinical applications. It has been established that therapeutic molecule can be stabilized after encapsulating in nanopores of inorganic template and subsequent coating with polyelectrolytes in layer-by-layer fashion [3]. Intake of diets rich in flavonols (a subclass of flavonoids including kaempferol and quercetin] has been positively associated with better skeletal health in humans [4,5]. In animal models, dietary supplements and nutraceuticals rich in flavonols have been reported to counteract the bone deleterious effects of estrogen deficiency [4]. Furthermore, treating osteopenic rats with kaempferol mitigates bone loss without having a uterine hyperplastic effect [6]. A number of studies have demonstrated anti-osteoclastogenic effect of kaempferol *in vitro* [6,7]. In addition, kaempferol has been shown to promote osteoblast differentiation [8]. Bone sparing

Abbreviations: LbL, kaempferol – layer-by-layer; SDC, sodium deoxycholate; MP, $CaCO_3$ microparticles; $CaCO_3$-kaempferol, kaempferol-loaded $CaCO_3$ MP; x, zeta potential; PE, polyelectrolyte; OVx, ovariectomized rats; BMC's, bone marrow cells; PTH, parathyroid hormone; iPTH, intermittent parathyroid hormone; MAR, mineral apposition rate; BFR, bone formation rate; μCT, microcomputed tomography; BV/TV, bone volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; SMI, structure model index; DA, degree of anisotropy; CTx, C – terminal teleopeptide of type I Collagen; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; AUC, area under curve.

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http://dx.doi.org/10.1016/j.ejpb.2012.08.001

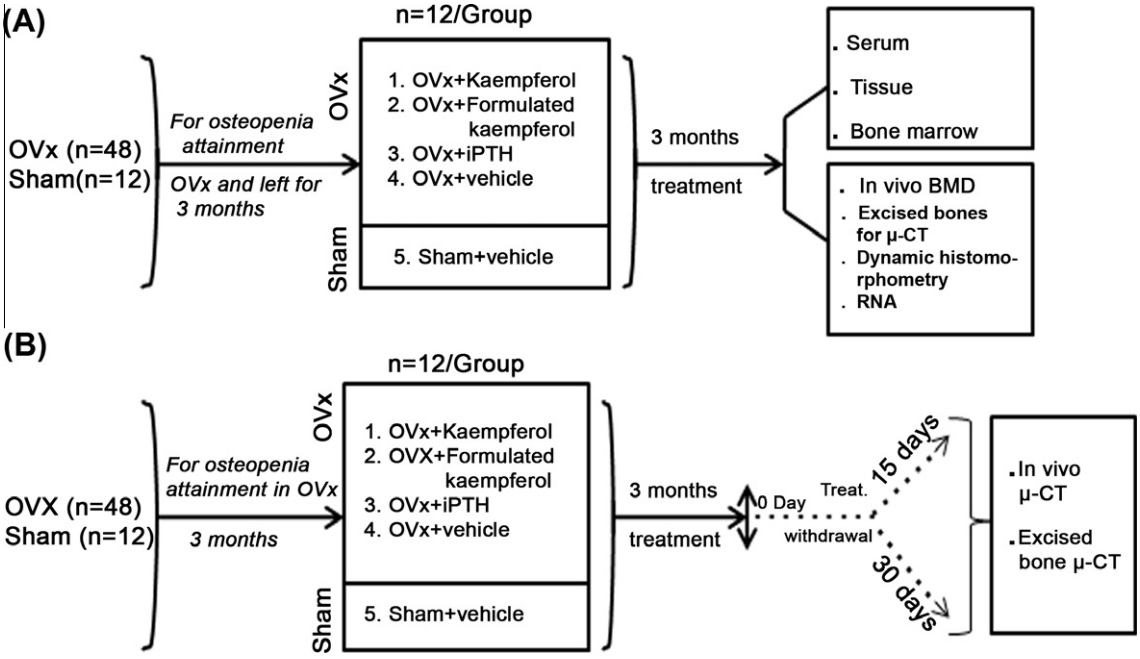

Fig. 1. (A) Schematic diagram of the experimental design and (B) the withdrawal protocol depicting time points.

action of kaempferol is thought to be mediated by anti-oxidant properties that attenuate the production of oxidation-derived free radicals from the bone resorbing osteoclasts and their precursors [7,9]. Kaempferol could also act through estrogen receptors, as phytoestrogens do [10]. Despite so many positive attributes, kaempferol is poorly bioavailable, which impedes the application of this promising and relatively non-toxic agent to develop as a pharmacological agent for postmenopausal osteoporosis.

The biological effects of kaempferol, as in case of any other flavonoids appear to be critically dependent on free hydroxyl groups that are rapidly conjugated by glucuronosyltransferases and sulfotransferases [11]. These two enzymes are abundantly present in the small intestine and liver, through which all of the oral dose must pass. Thus, a very tiny fraction of kaemferol when orally administered would reach other organs beyond sites directly along the gastrointestinal tract. Consistent with this metabolic processing of kaempferol, the bioavailability of kaempferol in rats is approximately 2%. In addition to the extensive first-pass metabolism by glucuronidation and sulfonylation, kaempferol was found to be absorbed at low to moderate levels. We hypothesize that harnessing the benefit of kaempferol as an osteoprotective agent would require enhanced systemic bioavailability and tissue delivery (in this case bone marrow) of kaempferol.

In this study, we report novel application of layer-by-layer polyelectrolyte nano-matrix designed on kaempferol loaded inorganic $CaCO_3$ template (formulated Kaempferol), which bypasses the bioavailability pitfall of this compound and improves osteogenic properties of kaempferol at molecular level on long term treatment. $CaCO_3$ as a core particle has intrinsic advantage of having application in the condition of osteoporosis as supplement. The unique advantage of this system lies in the fact that it can be delivered as such without removing the core in contrast to previous reports. Moreover, auto-digestion of the core particle in the acidic environment of the stomach and the nano-layered assembly facilitates absorption of kaempferol in a unique way to enhance osteogenic effects in ovariectomized (OVx) rats. We have compared the bone restorative effect of formulated kaempferol with its unformulated form in osteopenic rats and the associated cellular mechanisms

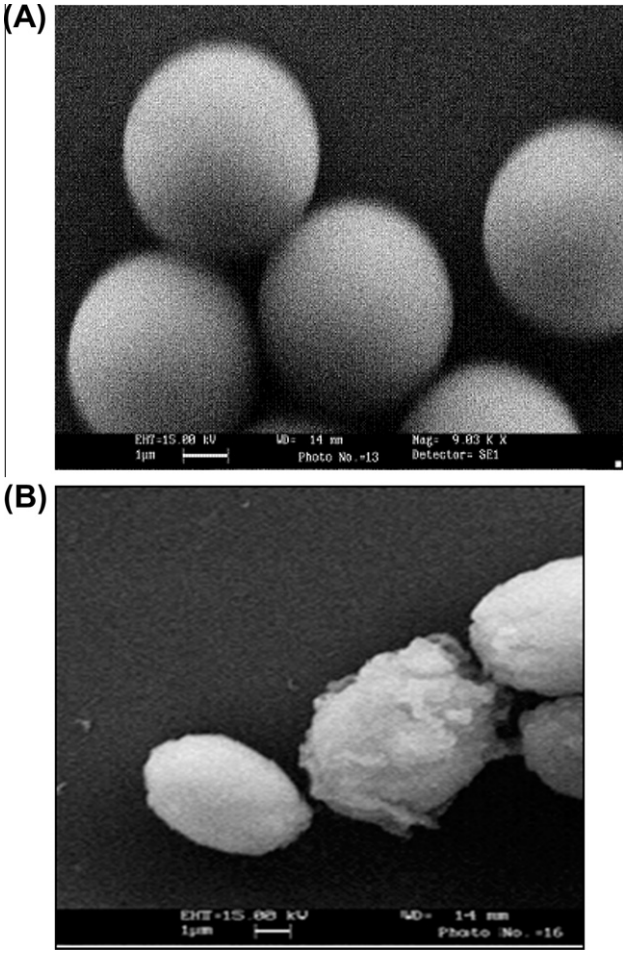

Fig. 2. (A) The morphology of the fabricated microcapsules as examined by scanning electron microscopy (SEM). The developed core particles were perfectly spherical, non-aggregated and mono-disperse in population. (B) Scanning electron microphotograph of kaempferol loaded LBL fabricated system. Scale bar: 1 µm.