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A novel bone targeting delivery system carrying phytomolecule icaritin for prevention of steroid-associated osteonecrosis in rats

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ABSTRACT

One of the effective strategies for prevention of steroid-associated osteonecrosis (SAON) is to inhibit bone resorption and fat formation and promote bone formation at osteonecrotic sensitive skeletal sites. We identified a novel phytomolecule that showed positive effects on osteogenesis, anti-bone resorption and anti-adipogenesis *in vitro* and also developed a bone-targeting delivery system (BTDS) for *in vivo* experimental study. The study investigated if our innovative synthesized BTDS carrying this phytomolecule would be able to effectively prevent SAON in a rat model. SAON was induced by combined injections of lipopolysaccharide and methylprednisolone. SAON rats were divided into four groups, one SAON untreated control group and three SAON treatment groups with different types of delivery systems (Asp_8-liposome-icaritin, liposome-icaritin and Asp_8-liposome) for two weeks. SAON lesions were identified and osteoclasts activity, osteogenesis and adipogenesis at these sites were evaluated by immunohistochemistry. *Ex vitro* study was also designed to evaluate the osteogenic and adipogenic potential of the isolated bone marrow stromal cells (BMSCs) *via* real-time PCR and histochemical staining. Our results showed that as a bone surface-specific BTDS, Asp_8-liposome-icaritin effectively prevented steroids-treated rats from SAON with significantly decreased osteocytes apoptosis, down-regulated osteoclatsogenesis and up-regulated osteogenesis. However, both liposome-icaritin and Asp_8-liposome treatment did not show significant efficacy for SAON prevention. In summary, this proof-concept-study showed for the first time that the innovative Asp_8-liposome-icaritin BTDS was effective for prevention of SAON in terms of bone resorption prevention, adipogenesis suppression, and bone-formation enhancement.

1. Introduction

Corticosteroids are routinely prescribed for inflammatory and autoimmune diseases treatment, such as rheumatoid arthritis [1,2]. Steroid-associated osteonecrosis (SAON) is however one of the most severe complications that often results into subchondral bone collapse, especially in the hip joint [1,3]. Prevention and earlier therapy of SAON are effective strategy for prevention of joint collapse. However, pathogenesis of SAON still remains incomprehensive [4,5]. General consensus on pathogenesis of SAON is increased adipogenesis, impaired intraosseous blood supply and subsequent enhanced bone death [6,7].

Skeleton undergoes remodeling for maintaining its homeostasis. Whereas, subchondral collapse is directly attributed to excessive bone resorption without adequate bone regeneration [7–9]. Recent alternative pathogenic mechanism of SAON is bone cell apoptosis that was induced by steroids as they targeted osteoclasts by protecting these cells from apoptosis and thus enhancing bone-resorption but compromising bone formation and integrity [10–12].

In addition, the recent advances in understanding the mechanism of the inadequate repair at early stage of SAON are decreased activity of marrow stem cells (MSCs) pool and trabecular bone matrix degeneration [13,

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14]. The therapeutic strategy shall therefore inhibit adipogenic and osteoclasts differentiation and/or provide sufficient precursor cells (osteogenic progenitors) to promote osteogenesis around necrotic region [15,16]. It should be a desirable approach that the special intervention to MSCs in early stages of ON before pathological joint collapse occurs [15,17].

Our previous research reported that the flavonoid glycosides derived from herb *Epimedium* Flavonoids (EF) exerted dose-dependent effects on inhibition of both thrombosis and lipid-deposition and accordingly reduced incidence of SAON in rabbits *via* counteracting endothelium injury and excessive adipogenesis [18]. We subsequently identified an intestinal metabolite of EF in serum icaritin, a small phytomolecule [19]. Our recent *in vitro* using bone marrow stem cells (BMSCs) derived form SAON rabbits demonstrated that icaritin directly inhibited adipogenic differentiation and promoted osteogenic differentiation [20,21].

However, pharmacokinetic study showed that icaritin had relative short half-life time about 0.43 h after intravenous injection, rapidly excreted into intestinal track through bile-hepatobiliary circulation [22]. Due to insufficient blood supply in osteonecrotic bone, bioactive drug would hardly be delivered to bone lesions, which might result in limited therapeutic effects. In order to improve the efficacy of icaritin treatment, proper time window for intervention, maintain serum concentration and its targeting to bone surface are key considerations. The authors of current study developed a bone targeting delivery system (BTDS) that adopted liposome as a pharmacokinetic strategy to prolong the retention time of icaritin in blood circulation and Asp_8, eight repeating sequences of asparate to concentrate phytomolecule icaritin on bone surface undergoing active bone remodeling. BTDS might therefore target treatment already at the early stage of SAON. A recent report confirmed that BTDS, *i.e.* aspartate-serine-serine Asp_8-liposome system was able to target bone surface to facilitate anabolic therapy [23].

Accordingly, we postulated that our novel BTDS Asp_8-liposome-icaritin (ASP-LP-ICT) would effectively prevent osteonecrosis through maintaining higher concentration of icaritin on bone surface, where icaritin directly promoted osteogenesis *via* inhibiting adipogenesis of BMSCs, as well as suppressed the bone resorption, and then effectively prevented SAON lesion where undergoing bone degeneration and repair with fast bone remodeling around ON lesions.

2. Materials and methods

2.1. Preparation and characterization of BTDS

Based on our established biotechnology [24], we further synthesized an Asp_8-liposome-icaritin BTDS by thin film evaporation method. Briefly, 8 mg icaritin and 250 mg lipids were dissolved in the mixed organic solvent of methanol/chloroform. Subsequently the lipid film was dried completely and then hydrated. The liposome suspension was then extruded in a Lipex™ Extruder (Northern Lipids INC., Canada) driven by pressurized nitrogen to obtain small unilamellar vesicles. Then 15 mg of Asp_8 was added to further modify the surface of this liposome and agitated. Subsequently, this liposome suspension was purified to remove the un-conjugated Asp_8. The quantification of cholesterol was conducted with Infinity® Cholesterol Lipid Stable Reagent (Thermo Electron; Melbourne, Australia) to assess the lipids concentration [24]. The liposome without Asp_8 served as control and was prepared using DSPE-mPEG2000 instead of DSPE-PEG2000-MAL.

Cryogenic transmission electron microscopy (Cryo-TEM) was used to examine the delicate structures. The particle size distribution and Zeta potential of icaritin-liposome and Asp_8-liposome-icaritin were determined by a Nicomp 380/ZLS zeta potential analyzer (Beckman Coulter, USA). The amount of icaritin encapsulation in liposome was measured using ultra performance liquid chromatography (UPLC, Waters Inc., USA) to quantify connection and encapsulation efficiency. Encapsulation efficiency was calculated by the following formula [25]: Encapsulation efficiency (%) = (Amount of drug in liposome / Initial amount of drug added) × 100; Connection efficiency (%) = [(Amount of supplemented Asp8 − Amount of dissociative Asp8) / Theoretical connective Asp8] × 100.

2.2. Icaritin release and tissue distribution of BTDS

Icaritin encapsulated in liposome and distributed in rats was imaged using Xenogen IVIS imaging system (Xenogen Imaging Technologies, CA, USA). The release profiles within 72 h were evaluated using the dialysis membrane method. Concentration of icaritin was determined using UPLC system stated above.

2.3. Animal model establishment

Thirty 16-week old male Sprague Dawley (SD) rats with body weight of 300 ± 20 g were used for SAON model according to our established protocol [26]. Briefly, the rats were intravenously injected with 100 μg/kg body weight of lipopolysaccharide (LPS, *Escherichia coli*, Sigma-Aldrich, Inc. USA). Three intramuscular injections with 40 mg/kg body weight of methylprednisolone (MPS, Pharmacia & Upjohn, USA) were given at a time interval of 24 h. After MPS injections, the rats were intravenously injected with BTDS of 16 mg/kg body weight once per week for two weeks. Asp_8-liposome-icaritin (ASP-LP-ICT) and Liposome-icaritin (LP-ICT) injection served as treatment groups, and Asp_8-liposome (ASP-LP) was named as treatment control group. Osteonecrosis control (ONC) group was injected with phosphate buffer saline (PBS) after LPS and MPS injection, and normal rats only with PBS injection were served as normal control (NC). Two weeks post injections, sequential fluorescence labeling with calcein green and xylenol orange (Sigma-Aldrich GmbH, Buchs, Switzerland) in a time sequence of 10 and 3 days before euthanasia. Serum and BMSCs was collected. Femoral heads were dissected for histology. During the treatment, the rats were kept in cage and received a standard laboratory diet and had free access to food and water *ad libitum*. All animal experiment procedures described below were approved by the animal ethics committee of the Chinese University of Hong Kong (Ref No. 13/038/MIS).

2.4. Undecalcified histology

Following our established protocol [27,28], samples were embedded in methyl methacrylate (MMA, Mecck-Schuchardt, Germany). Mid-coronal sections for trabecular bone of femoral head were prepared at a thickness of 5 μm (LEICA SM2500E, Nussloch, Germany). Fluorescence images were taken using fluorescence microscope (Leica DM5500, Germany) and evaluated using bone histomorphometry software (Osteometrics, Atlanta, GA) [29]. The region of interest (ROI) in trabecular bone below growth plate was defined for quantification. The parameters were expressed by the ratio of osteoblasts surface and bone surface (Ob.S/BS), osteoid surface (Os.Pm), the ratio of osteoid surface and bone surface (Os.S/BS), osteoid area (Os.Ar), mineralizing surface (MS), mineral apposition rate (MAR) and bone formation rate per bone surface (BRF/BS) according to the published guideline for bone histomorphometry [16,29].

2.5. Hematoxylin and eosin (H&E) staining

Coronal paraffin sections of 5 μm thickness were prepared using a microtome (LEICA RM2165, Germany). H&E stained sections were digitalized for descriptive histological examinations and quantitative histomorphometry. The parameters were calculated and expressed by thrombus area (Thr.Ar), fat cells area (Fc.Ar) and fat cells number (N.Fc) [16,29,30].