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Original article

The protective effects of silibinin in the treatment of streptozotocin-induced diabetic osteoporosis in rats

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ABSTRACT

Diabetic osteoporosis (DO) is a complication of diabetes mellitus. Our previous study showed that silibinin can attenuate high glucose mediated human bone marrow stem cells dysfunction through antioxidant effect. However, no study has yet investigated the effect of silibinin in diabetic rats. Therefore, we assessed the effects of silibinin on bone characteristics in streptozotocin-induced diabetic rats. The aim of our study was to determine whether providing silibinin in the different supplementation could prevent bone loss in diabetic rats or not. Rats were randomly divided into four groups: (1) control group (CG) (n = 10); (2) diabetic group (DG) (n = 10); (3) diabetic group with 50 mg $kg^{-1}day^{-1}$ of silibinin orally (DG-50) (n = 10); and (4) diabetic group with 100 mg $kg^{-1}day^{-1}$ of silibinin orally (DG-100) (n = 10). 12 weeks after streptozotocin (STZ) injection, the femora from all rats were assessed and oxidative stress was evaluated. Bone mineral density was significantly decreased in diabetic rats; these effects were prevented by treatment with silibinin (100 mg kg^{-1} day^{-1} orally). Similarly, in the DG and DG-50 groups, changes in microarchitecture of femoral metaphysis assessed by microcomputed tomography demonstrated simultaneous existence of diabetic osteoporosis; these impairments were prevented by silibinin (100 mg kg^{-1} day^{-1} orally). In conclusion, silibinin supplementation may have potential use as a possible therapy for maintaining skeletal health and these results can enhance the understanding of diabetic osteoporosis induced by diabetes.

1. Introduction

Diabetes mellitus is a devastating and life-altering disease, affecting over 20 million people in the USA [1]. Diabetes mellitus causes many complications such as nephropathy, neuropathy, and retinopathy. Diabetic osteoporosis (DO) is also a complication of diabetes mellitus [2–4], which is caused by reduced bone mineral content due to the abnormal levels of sugar, protein, fat, and microelements.

Many human and experimental studies on the complications of diabetes mellitus have demonstrated extensive alterations in bone and mineral metabolism [5,6]. The mechanisms responsible for diabetic osteoporosis have been reported partly. Increasing evidence suggested that oxidation played a role in the pathogenesis of diabetic bone disease. Studies showed the increase in oxidative stress may partly contribute to the development of diabetic osteoporosis [7,8]. And in our previous study, we found that high glucose suppressed osteogenic differentiation of human bone marrow stem cells, manifested by an increase of oxidative damage markers [9].

The optimal therapies for DO include hormone therapy, bisphosphonates, calcium and vitamin D, while none of these therapies have been found to decrease the oxidation induced by high glucose. Given the prevalence of diabetic osteoporosis and the lack of effective therapies, there is a need to develop harmless and affordable alternative therapies for preventing osteoporosis. Natural products and dietary components have positive effects on bone remodeling, particularly by inhibiting bone resorption [10,11].

Silibinin, the active component of silymarin, has been shown to have a broad spectrum of pharmacological activities such as

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hepatoprotective activity [12], anti-cancer [13], antioxidant [12,14], neuroprotective [15] and cardioprotective effects [16]. Milic et al. have summarized the effects of silibinin and suggested that the milk thistle may be a possible agent in the prevention or treatment of cancer, atherosclerosis, hepatitis, and cirrhosis [17]. Further, It has been reported that silibinin had the potential to prevent bone loss in vitro by suppressing osteoclastogenesis and enhancing osteoblastogenesis in murine MC3T3-E1 pre-osteoblastic cells [18–20]. In addition, we found that silibinin could increase osteogenic effect by stimulating osteogenic biomarkers of alkaline phosphatase, type I collagen and osteocalcin expression in bone marrow stem cells (hBMSCs) [21]. Further we found that silibinin can attenuate high glucose-mediated hBMSCs dysfunction through antioxidant effect and modulation of PI3K/Akt pathway [9]. However, the action mechanisms of silibinin for promoting bone formation process in the state of high glucose in vivo still remain unclear. Hence, we investigate whether silibinin supplementation can be protective against bone loss induced by diabetes mellitus or not.

To investigate the effects of silibinin in DO, we focused on the streptozotocin (STZ)-induced diabetic rat, which is one of the most common animal models of type 2 diabetes mellitus. In the current study, we attempted to determine whether silibinin could prevent diabetic osteoporosis, assessed the bone microarchitecture and evaluated oxidative stress in STZ-induced diabetic rats.

2. Materials and methods

2.1. Animal feeding study

All animal procedures were approved by the Animal Care and Use Committee at Wenzhou Medical University and were conducted in accordance with the policies of the Ethics Committee for Animal Research. This study employed STZ injection technique for inducing diabetes mellitus and diabetic osteoporosis. Female Wistar rats, purchased from the Shanghai Animal Experimental Center (Shanghai, China), 180 g–190 g, 7–8 weeks of age, were used for the experiment. Upon arrival in the animal care facilities, rats were individually caged and kept in rooms maintained at $22 \pm 2\,^{\circ}C$ with a 12 h light – 12 h dark cycle (lights on at 06:00, lights off at 18:00).

The animals were allowed to acclimatize for a week before beginning experiments. Following acclimation, rats were randomly assigned as follows: (1) control group (CG) (n = 10); (2) diabetic group (DG) (n = 10); (3) diabetic group with 50 mg $kg^{-1}day^{-1}$ of silibinin (sigma) orally (DG-50) (n = 10); and (4) diabetic group with 100 mg $kg^{-1}day^{-1}$ of silibinin orally (DG-100) (n = 10).

Rats in diabetic and silibinin-treated diabetic groups were fed with high fat diet (Diet #MD45%fat; Mediscience Ltd, China) for a period of 4 weeks to induce insulin resistance. After the 4 weeks of dietary manipulation, the animals were intraperitoneally injected with STZ (Sigma-Aldrich, St Louis, MO, USA) (35 mg/kg body weight in 100 μL of sterile citrate buffer, pH 4.5) on two consecutive days to induce the type II diabetes mellitus [22,23]. Rats in the CG group were fed with standard chow and water ad libitum and injected with citrate vehicle alone. After 72 h of STZ injection, animals with venous blood glucose levels of over 16.7 mmol/L were considered diabetic and selected for further studies. Venous blood glucose was measured every week to ensure the blood glucose level more than 16.7 mmol/L.

All rats had free access to standard chow and tap water throughout the experiment. All rats were provided a vehicle control or silibinin for 12 weeks after STZ injection. Due to its low solubility in water, silibinin was suspended in 0.5% sodium carboxymethyl cellulose. Silibinin was administered at a daily dose of 50 mg $kg^{-1}day^{-1}$ in the DG-50 group and 100 mg $kg^{-1}day^{-1}$ in the DG-100 group by oral gavage while the controls were administered 1 mL of deionized distill water (ddH_2O) in 0.5% CMC-Na by oral gavage for 12weeks. The dose of silibinin was according to previous published study [24].

At 12 weeks after oral gavage administration of silibinin, rats were anesthetized by administration of 2% (w/v) pentobarbital sodium (35 mg/kg, Solarbio Science & Technology, Beijing, China) via intraperitoneal injection. The bones were collected from each animal and dissected with care being taken to protect the periosteum. Each bone was individually wrapped in ddH_2O-soaked gauze and stored at $-20\,^{\circ}C$ until analyzed.

2.2. Bone morphometry and microarchitecture

Bone morphometry measurements of length, width and height were determined using a vernier caliper. Bones were dried at $110\,^{\circ}C$ for 48 h and then weighed. The femoral length was measured from the medial condyle to greater trochanter. The femoral width was measured from the lateral condyle to medial condyle and the femoral height was measured from the top to the bottom of the lateral condyle in this study. Morphometry measurements were averaged after no bilateral differences were determined using a paired *t*-test with significance level set at $P < 0.05$.

Bone radiographs of excised femur were taken with Dual-energy X-ray absorptiometry (DXA) scans (Kubetic) on the right femora. For microarchitecture, trabecular bone architecture was determined on the left femora using microcomputed tomography (μCT) (MicroCTμ100, SCANCO Medical, Switzerland). The trabecular bone within the distal femur metaphysis was scanned and 100 images (14.8 μm/slice or 1.48 mm) were analyzed with semi-automatically drawn contours beginning 50 slices (740 μm) away from the growth plate within the volume of interest (VOI). The femora were scanned at 70KV and 200 μA and 300 ms exposure time to obtain image resolution of 14.8 μm. Next, the reconstructed images were analyzed using the software (Ray V4.0-4, Switzerland). The VOI was assessed for structural parameters including trabecular bone volume fraction (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp) and structure model index.

2.3. Histological examination

For Harris hematoxylin and Shandon Instant eosin (H&E) staining, the right femur were excised, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) for 3 days at room temperature, decalcified with decalcifying solution (Sigma-Aldrich, St Louis, MO, USA) for 4 weeks. The tissue samples were then dehydrated in a graded series of ethanol solutions for 18 h, embedded in paraffin and cut into 5 μm sections in thickness. The specimens were subjected to histomorphometric analysis under a light microscope with a micrometer, using an image analyzer (Olympus, Japan).

2.4. Serum and urine biochemical analyses

After 12 weeks of silibinin treatment, rats were anesthetized by administration of 2% (w/v) pentobarbital sodium (35 mg/kg) via intraperitoneal injection, blood was obtained using cardiac puncture, and serum samples obtained by centrifugation (3000 rpm, 10 min) were stored at $-80\,^{\circ}C$ until analysis. Plasma osteocalcin, a marker of bone formation, was measured by a commercially available rat-specific enzyme immunoassay (EIA) (Shanghai Haling biological technology, PR China). Serum concentrations of parathyroid hormone (PTH) were determined using a rat PTH ELISA kit (Immutopics Inc., San Clemente, CA, USA). Serum samples were analyzed for their calcium and phosphorus contents by the arsenazo-3 dye and ammonium molybdate colorimetric methods respectively. Plasma C-reactive protein