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Original article

Ethanolic extract of *Dalbergia sissoo* promotes rapid regeneration of cortical bone in drill-hole defect model of rat

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ABSTRACT

Leaves of *Dalbergia sissoo* is known to have protective actions against postmenopausal bone loss in rat. In this study, we have evaluated the fracture healing properties of ethanolic extract (EE) of *Dalbergia sissoo* leaves. To observe the fracture healing property in the drill-hole injury model, we randomly divided total 32 adult female Sprague Dawley rats (180 ± 200 g) into 4 groups: (i) Control operated group; (ii) EE (250 mg/kg/day); (iii) EE (500 mg/kg/day) and (iv) EE (1000 mg/kg/day). The right femora were fractured at the mid-diaphysis region and each group of rats received their respective treatment for 15 days. Ethanol extract dose dependently induced bone regeneration at the fracture site assessed by fluorochrome labeling. All of three doses, 250 mg/kg/day dose significantly increased bone volume fraction, trabecular thickness, trabecular number, and connectivity density and decreased trabecular separation in bone. Furthermore, the extract induced the expression of osteogenic genes including BMP-2, BMP-4, RunX-2 and COL-1 compared to the control group. The EE improved fracture healing much earlier (day 15) than the normal healing process, as assessed by the increased callus volumes and mineralized nodule formation. This extract is found beneficial in fracture healing of rat.

1. Introduction

The main function of the musculoskeletal system is to transmit forces from one part of the body to another under controlled strain and to protect vital organs (e.g. lungs, brain) [1]. Bone adaptability allows for efficient repair, which in turn assists to prevent fractures. However, fractures are still quite common, usually caused by the sudden appearance of a load that surpasses bone strength, or the cyclic activity of loads [2]. Predicting and preventing bone fractures is an important issue in orthopaedics due to their high frequency, surgical problems and socio-economic impact [3]. For example, world-wide hip fracture numbers were predicted to be 1.66 million in 1990 and expected to increase to 6.26 million by 2050 [4]. Once a fracture takes place, the basic healing process is auto-activated naturally to heal the site that includes the differentiation of numerous tissues (cartilage, bone, granulation, etc.) with different patterns that are directly affected by the mechanical environment, which is in turn governed by the load applied and the stability of the fracture site [5,6]. In fact, not all fractures are completely healed. From time to time, there are non-unions or delayed fractures relying on particular geometric, mechanical and biological elements, mitigating the many different kinds of fixations used to increase fracture stabilization [7]. Till now these are no proven and established studies to show drug potential for fracture healing. Medicinal plants are the source of health care management of a large section of the world's population and continue to play a vital role in the health delivery systems. Many reports from our group have confirmed strong osteogenic actions of several compounds isolated from medicinal plants [8–10]. In this study, we have investigated *Dalbergia sissoo* plant leaves extract for its effects on fracture healing in rats using a drill hole injury model of bone. Fracture healing ability was studied using (a) Dynamic histomorphometry to evaluate new bone formation rate at the site of drill hole, (b) Static histomorphometry to assess the micro-architectural characteristics of the newly formed callus and (c) Osteogenic efficacy of genes at the fracture site.

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Dalbergia sissoo commonly known as Shisham or Indian rosewood in India. It is a deciduous tree, widely distributed throughout the Indian subcontinent [4,11,12]. There are many reports in which various pharmacological properties of Dalbergia sissoo have been shown which include stimulation of new bone cells and tissue regeneration. Other study suggests that, Dalbergia sissoo leaves juice used for eye ailments. Our study shows that Dalbergia sissoo leaves extract rich in several phytoestrogens, particularly methoxyisoflavones, exhibit in vitro bone-forming (osteoblast mineralization) activity. Various compounds isolated from butanol and ethanol soluble fraction of Dalbergia sissoo leaves and pods show osteogenic property [8,10,14]. Purified compound like biochanin A, genstein, pratensein, Quercetin, Kaempferol, Caviunin and their analogues from Dalbergia sissoo extract show enhanced osteoblast differentiation as well as mineralization. In-vivo studies show that isolated purified compounds like Kaempferol, Caviunin 7-O-[β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside and genstein show bone formation as well as anti-resorptive activities [8,10,14]. Caviunin 7-O- [β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside show osteogenic activity by regulating β-catenin/Wnt pathway [15]. Our phytopharmacological evaluation program aimed to find effective therapy for fracture healing [15]. We have reported that a standardized phytopreparation from an Indian medicinal plant (*Dalbergia sissoo*) has antiresorptive and bone-forming effects on a postmenopausal osteoporosis model of rat [14]. We hypothesized that cumulative presence of several flavonoids in *Dalbergia sissoo* leaves extract could have fracture healing effects in drill hole model of rats.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals, cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA) and Sigma Aldrich (USA).

2.2. Plant material and extraction

Leaves of *Dalbergia sissoo* were collected from CDRI, Lucknow India. The powdered leaves of plant material were extracted with ethanol following previously described method [8]. The mixture was filtered; the filtrate obtained was concentrated under reduced pressure. The yield (10gm/700 mg), calculated from the weight of initial powder and the final powder, obtained after evaporation of solvent was 7%.

2.3. Animal study

All animal care and experimental procedures were approved by the Institutional Animal Ethics Committee. Female SD rats (180 ± 20 g) were obtained from the National Laboratory Animal Centre. Animals were kept in a 12 h light–dark cycle, with controlled temperature (22–24 °C) and humidity (50–60%) and free access to standard rodent food and water [8,13].

2.4. Drill-hole defect at the mid-diaphysis of the femur

32 Adult Sprague-Dawley rats (180 ± 20 g each) were taken and randomly divided into four groups for the drill-hole injury study. For this, the front skin of the mid-femur in rats was incised straight and longitudinally at 1 cm in length under anesthesia. After splitting the muscle, we stripped the periosteum to expose the femoral bone surface. A drill-hole injury was made by inserting a drill bit with a diameter of 0.8 mm in the anterior portion of the diaphysis of the bilateral femurs 2 cm above the knee joint. Drill-hole injury in the femur was created in all the groups as follows: control operated ±vehicle (gum acacia in distilled water), EE (250 mg/kg/day), EE (500 mg/kg/day), EE (1000 mg/kg/day). Treatments started from the next day of injury and continued for 2 weeks. After 2 weeks of the various treatments described earlier, all rats were euthanized and autopsied to collect their femurs for the measurement of bone micro-architectural parameters and dynamic histomorphometric study at the drill-hole site [14].

2.5. Dynamic histomorphometric study

Each animal received intraperitoneal administration of fluorochrome calcein (20 mg/kg) 24 h before autopsy. After 15 days of treatment as described above, all rats were killed and autopsied to collect their femurs for the measurement of bone micro-architectural parameters at the drill-hole site. Bones were embedded in acrylic material and 50 μm sections were made using an Isomet Bone Cutter (Buehler, Lake Bluff, IL, USA) and photographs taken under fluroscence microscopy aided with appropriate filters. The intensity of calcein binding, which is an indicator of the amount of new mineral deposition, was calculated using Image J software [14].

2.6. Microcomputed tomography (μCT)

Assessment of internal microstructures (both 2D and 3D) of the mineralized tissue in the drill-hole was analyzed by μCT, using Sky Scan 1076 CT scanner (Aartselaar, Belgium). Bones were cleaned of soft tissues and scanned using X-ray source of 70KV, 100 mA with a pixel size of 18 μm. The images were reconstructed using Sky Scan Nrecon software, which facilitates network distributed reconstruction carried out on four personal computers running simultaneously. Callus bone was captured by drawing ellipsoid contour with CT analyzer software. Micro-architectural parameters including bone volume fraction (BV/TV), trabecular thickness (Tb.

Table 1
Primer sequences for real-time PCR analysis of gene expression.

Gene name	Primer Sequence	Accession Number
Bone morphogenetic protein-2 (BMP-2)	F- CCCCTATATGCTCGACCTGT R- AAAGTTCCTCGATGGCTTCTT	NM_017178.1
Collagen 1 (COL-1)	F- CCCGACTGTGACTTAACATCC R-GTCCTCTTCTTCTTTGTGTAATTGG	XM_001072520.2
Runt related transcription factor2 (RunX-2)	F- CCGTGTCAGCAAAACTTCTTT R- CTCACGTCGCTCATCTTGC	NM_053470.2
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F- TGGGAAGCTGGTCATCAAC R- GCATCACCCCATTTGATGTT	DQ403054.1
Bone morphogenetic protein-2 (BMP-4)	F- CCCCTATATGCTCGACCTGT R- AAAGTTCCTCGATGGCTTCTT	NM_017178.1