Life Sciences 121 (2015) 174–183

Contents lists available at ScienceDirect

Life Sciences

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

Critical-size bone defect repair using amniotic fluid stem cell/collagen constructs: Effect of oral ferutinin treatment in rats

Manuela Zavatti [a,*], Laura Bertoni [a], Tullia Maraldi [a], Elisa Resca [a], Francesca Beretti [a], Marianna Guida [b], Giovanni B. La Sala [c], Anto De Pol [a]

[a] Department of Surgical, Medical, Dental and Morphological Sciences with Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy
[b] EURAC Research, Center for Biomedicine, Bolzano, Italy
[c] Unit of Obstetrics & Gynecology, IRCCS-Arcispedale Santa Maria Nuova, Reggio Emilia, Italy

ARTICLE INFO

Article history:
Received 23 June 2014
Accepted 18 October 2014
Available online 5 November 2014

Keywords:
Ferutinin
AFSCs
Bone regeneration
Collagen scaffold
Rat

ABSTRACT

Aims: This study aims to evaluate the bone regeneration in a rat calvarias critical size bone defect treated with a construct consisting of collagen type I and human amniotic fluid stem cells (AFSCs) after oral administration of phytoestrogen ferutinin.
Main methods: In 12 week old male rats ($n = 10$), we performed two symmetric full-thickness cranial defects on each parietal region, and a scaffold was implanted into each cranial defect. The rats were divided into four groups: 1) collagen scaffold, 2) collagen scaffold + ferutinin at a dose of 2 mg/kg/5 mL, 3) collagen scaffold + AFSCs, and 4) collagen scaffold + AFSCs + ferutinin. The rats were sacrificed after 4 weeks, and the calvariae were removed, fixed, embedded in paraffin and cut into 7 μm thick sections. Histomorphometric measures, immunohistochemical and immunofluorescence analyses were performed on the paraffin sections.
Key findings: The histomorphometric analysis on H&E stained sections showed a significant increase in the regenerated area of the 4th group compared with the other groups. Immunohistochemistry performed with a human anti-mitochondrial antibody showed the presence of AFSCs 4 weeks after the transplant. Immunofluorescence analysis revealed the presence of osteocalcin and estrogen receptors (ERα and GPR30) in all groups, with a greater expression of all markers in samples where the scaffold was treated with AFSCs and the rats were orally administered ferutinin.
Significance: Our results demonstrated that the oral administration of ferutinin is able to improve the bone regeneration of critical-size bone defects *in vivo* that is obtained with collagen–AFSCs constructs.

Introduction

A great challenge for regenerative medicine is the repair of bone loss due to a wide range of diseases, including osteoarthritis, osteoporosis, osteogenesis imperfecta as well as traumatic injury and orthopedic surgery. Critical-size bone defects are not capable of repairing themselves. Recently the gold standard treatments for critical-size bone defects were autologous bone grafts. However, critical-size bone defects present several limitations and complications, such as donor site pain, paresthesia, inflammation and infection [4,46,50]. Another option is the use of allografts (from humans) or xenografts (from non-humans), although these methods are associated with potential infections and immune responses [5,49].

To address these issues, many scaffolds have been investigated as potential alternatives to bone grafts for bone defect repair [34]. Scaffolds are divided in two main categories: biological (collagen type I and demineralized bone matrix) and synthetic materials (porous metals, bioactive glasses, polylactic acid, polyglycolic acid, hydroxyapatite, tricalcium phosphates) [2,21,30,52]. Biological scaffolds have significant advantages, i.e., biocompatibility, biodegradability and regenerative characteristics [25]. Among these scaffolds, collagen type I, the major component of the extracellular matrix (ECM), is the most popular biologic material used to produce tissue-engineered grafts because of its high availability, easy purification from living organisms, non-antigenicity, non-toxicity and biological plasticity [22,24,38].

Bone tissue engineering merges scaffolds with cells and growth factors to create a tissue engineered construct to enhance bone regeneration [39]. Adipose-derived stem cells (ASCs), bone marrow mesenchymal stem cells (BM-MSCs), amniotic fluid stem cells (AFSCs) and dental

* Corresponding author at: Department of Surgical, Medical, Dental and Morphological Sciences with Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Via Del Pozzo 71, 41124 Modena, Italy. Tel.: +39 0594224853; fax: +39 0594224859.
E-mail address: manuela.zavatti@unimore.it (M. Zavatti).

http://dx.doi.org/10.1016/j.lfs.2014.10.020
0024-3205/

pulp stem cells (DPSCs) have been demonstrated as good candidates for *in vitro* and *in vivo* bone regeneration [7,26,27,37,40,42,54,57].

Recently, in our laboratories, we used a bioengineered construct of collagen scaffold-AFSCs to reconstruct a critical-size bone defect in an animal model [27]. We demonstrated that cell-seeded scaffolds have better and faster bone reconstruction capabilities than collagen alone and this improvement was due to the osteogenic potential of AFSCs [27].

Many compounds and growth factors are known to promote osteogenic differentiation; among them, 17β-estradiol plays an important role in bone metabolism. It has previously been demonstrated that 17β-estradiol enhances osteoblastic activity and bone formation [10,41]. Moreover, it is known that the lack of 17β-estradiol after menopause in women results in enhanced bone resorption, which is accompanied by impaired bone formation [43]. In this condition, the estrogen administration exerted a positive effect on bone mineral density, preventing and reducing the progress of osteoporosis [15,28]. However, estrogen treatment is associated with an increased risk of breast cancer as well as cardiovascular diseases [6,44]. In this context phytoestrogens have attracted much attention among researchers because of their estrogenic activities and lack of adverse side effects associated with estrogens [16]. Many natural compounds promote the osteogenic differentiation of mesenchymal stem cells, such as the isoflavonoids genistein and daidzein, resveratrol, kaempferol, xanthoumol, with the involvement of estrogen receptors (ER) signaling [45]. Moreover, these compounds exerted a positive effect *in vivo*, thereby preventing ovariectomy-induced bone loss [19,31,53].

Ferutinin is one such phytoestrogens that is a daucane sesquiterpene found in the roots of plants in the *Ferula* genus, particularly *Ferula hermonis* Boiss [1]. Ferutinin has a binding affinity for human ERα and ERβ that is approximately 10% of estradiol for both receptors [3]. Different from the majority of phytoestrogens, which have a higher affinity for ERβ than ERα, ferutinin affinity is higher for ERα ($IC_{50} = 33.1$ nM) than ERβ ($IC_{50} = 180.5$ nM) [20]. Moreover, the estrogenic activity of ferutinin after oral administration has been widely demonstrated [47,55,56]. We demonstrated that ferutinin was able to prevent, as well as treat, osteoporosis induced by estrogen deficiency in ovariectomized rats [13,36]. In particular, in the preventive protocol, ferutinin had the same anti-osteoporotic effects as estradiol benzoate [36] without exerting negative effects on the uterus or mammary glands [14]. Recently, we evaluated the role of ferutinin on the osteoblastic differentiation of AFSCs and DPSCs [57]. After 14 days of culture in an osteogenic medium in the presence of ferutinin, we observed a greater expression of osteoblast phenotype markers, an increased calcium deposition and osteocalcin secretion in the culture medium [57].

Accounting for the osteogenic potential of AFSCs and the enhancing properties of ferutinin in promoting the osteogenic differentiation of these stem cells, in this study we investigated the role of the oral administration of this phytoestrogen on bone regeneration *in vivo*. For this purpose, we employed a collagen scaffold seeded with AFSCs and implanted it in place of the parietal bone of rat calvaria. The aim of this study was to compare the capability of the collagen scaffold and the construct collagen scaffold + AFSCs, in the presence or absence of orally administration of ferutinin, to repair a critical-size bone defect using a well-established animal model and to evaluate the involvement of the estrogen receptors ERα and GPR30 in this regeneration process.

Materials and methods

Cell culture

Human amniotic fluid stem cells (AFSCs) were obtained from supernumerary amniocentesis provided by the Laboratorio di Genetica, Ospedale Santa Maria Nuova (Reggio Emilia, Italy). All samples were collected with the informed consent of patients according to Italian law and the Ethical Committee guidelines of Modena and Reggio Emilia University.

AFSCs were isolated as previously described [26]. Human amniocentesis cultures were harvested by trypsinization and submitted to cKit immunoselection using MACS® technology (Miltenyi Biotec, Cologne, Germany) [26]. cKit positive AFSCs were subcultured at a 1:6 dilution and were not allowed to reach 80% confluence.

AFSCs were cultured in minimum essential medium (αMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents, EuroClone, Milan, Italy) at 37 °C and 5% CO_2.

In vitro osteogenic differentiation

Collagen disks that had 13 mm diameters and 1.5 mm heights (horse-derived collagen—Condress, Istituto Gentilini, Pisa, Italy) were used as the 3D scaffold and were placed in 12-well culture plates. The scaffolds were washed twice with culture medium (1 hour for each rinse). The cells were seeded on each scaffold at density of 1×10^6 cells per disk and cultured for 24 hours with 2 mL culture medium. Then, the culture medium was changed to osteogenic medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate and 100 μM ascorbic acid-2-phosphate (all reagents, Sigma-Aldrich, St Louis, MO, USA). The cell-scaffold constructs were maintained in the osteogenic medium for 1 week before *in vivo* implantation, according to a previous protocol [27].

Selected samples were stained with 6-carboxyfluorescein diacetate (CFDA, Sigma-Aldrich, St Louis, MO, USA) to detect viable seeded cells. The cells were observed for green fluorescent staining with a Nikon A1 confocal laser scanning microscope (Nikon Instruments S.p.A., Firenze, Italy).

Surgery, implantation procedure and treatments

CD® IG5 male rats that were 12 weeks old were purchased from Charles River Laboratories (Lecco, Italy). They were housed one per cage and maintained in standard conditions with a 12:12 light/dark cycle, at temperature of 22 ± 1 °C and 55%–60% relative humidity. Commercial rat pellets (Global Diet 2018, Mucedola Srl, Milan, Italy) and drinking water were available *ad libitum*. For the implantation procedure, the animals were anesthetized with an intraperitoneal injection (0.2 mL/100 g body weight) of ketamine hydrochloride (Ketavet 100®, Farmaceutici Gellini SpA, Aprilia, Italy). We performed two symmetric full-thickness cranial defects (5 mm × 8 mm) on each parietal region of 10 animals. A midline skin incision was performed from the nose-frontal area to the external occipital protuberance. The skin and underlying tissues, including the periosteum, were reflected laterally to expose the full extent of the calvaria. The cranial areas to be removed were marked at the parietal bones using stereotaxic coordinates using the cranial structures as references. The cranial defect was created with a micromotor drill under constant sterile saline solution irrigation to prevent bone overheating [27]. One scaffold (5 × 8 × 1.5 mm size) was implanted into each cranial defect and adapted to fill the entire defect area. Each animal received two constructs.

To evaluate the bone regeneration in different conditions, the animals were divided into 4 groups:

- Group 1: collagen type I scaffold
- Group 2: collagen type I scaffold + ferutinin oral administration
- Group 3: collagen type I scaffold seeded with AFSCs
- Group 4: collagen type I scaffold seeded with AFSCs + ferutinin oral administration.

After scaffold implantation, the incisions were sutured with prolene 4-0 sutures (Ethicon, Roma, Italy). The animals were immunocompromised using cyclosporine A (Sandimmun, Novartis SpA, Origgio, Varese, Italy) at a dose of 15 mg/kg body weight administered 4 hours before