Journal of Molecular Structure 1074 (2014) 315–320

Contents lists available at ScienceDirect

Journal of Molecular Structure

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

Spectroscopic characterization of bone tissue of experimental animals after glucocorticoid treatment and recovery period

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HIGHLIGHTS

- Osteoporosis is induced by glucocorticoids in *Wistar* rats during a twelve week period of time.
- FTIR results demonstrated that the composition of mandibular bone tissue was changed.
- In recovery and treatment phase the increase of mineral content was statistically significant.

ARTICLE INFO

Article history:
Received 13 March 2014
Received in revised form 30 May 2014
Accepted 2 June 2014
Available online 12 June 2014

Keywords:
FTIR
AAS
Mandible
Glucocorticoids
Minerals

ABSTRACT

The influence of glucocorticoids on the composition and mineral/organic content of the mandible in tested animals after recovery and healing phase was investigated in this work. The results of FTIR analysis demonstrated that bone tissue composition was changed after glucocorticoid treatment. The increase of calcium, magnesium, phosphorus content and mineral part of bones was statistically significant in recovery phase and in treatment phase that included calcitonin and thymus extract. Some changes also happened in the organic part of the matrix, as indicated by intensity changes for already present IR bands and the appearance of new IR bands in the region 3500–1300 cm^{-1}.

Introduction

Bones, as well as dentin, belong to hard or mineralized tissues [1]. They are composed of inorganic, insoluble mineral component, and of organic component that determine tissue characteristics, cells and water [2]. Bone tissue is well differentiated connective tissue whose collagen-fibrous basis within extracellular substance is saturated with calcium salts [2,3]. Collagens are insoluble, extracellular glycoproteins found in almost all animals and the most abundant proteins in the human body. They are essential structural components of all connective tissues, including bone, cartilage, ligaments and skin [2,4]. Inorganic component of bones and dentin is made of tiny crystals, biological apatite and amorphous calcium-phosphate [5]. Biological apatite is made of calcium-phosphate salts, such as calcium-hydroxyapatite [6]. Apatites have general formula $Ca_{10}(PO_4)_6X_2$, where in hydroxyapatites (HAp) X stands for OH group [7]. The stoichiometric Ca/P ratio of HAp is 1.67, and usual Ca/P ratio is within the interval 1.5–2.0 and should not be lower, because the quality of crystals decreases [8]. Biological apatites also contain a small quantity of other ions: citrates, CO_3^{2-}, Cl^-, F^-, Na^+, Mg^{2+}, K^+ and Zn^{2+} [9]. The surface of apatite crystal is partially negatively charged, so it chemisorbs H_2O molecules easily [10–12].

There are three significant factors that regulate calcium metabolism in human body. In this regulatory mechanism three are three key hormones: parathyroid hormone, calcitriol ($1,25\text{-}(OH)_2\text{-}D_3$) and calcitonin [4]. Parathyroid hormone and calcitriol, but not

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http://dx.doi.org/10.1016/j.molstruc.2014.06.006
0022-2860/

calcitonin, affect three target organs: bones, kidney and small intestine [13]. Polypeptide hormone calcitonin has significant biological effect in suppression of osteoclast activities. A dynamic balance between deposited and soluble form of calcium exist in the body, so calcium metabolic imbalances lead to disarrangement in mineralization of bone tissue [14,15]. Osteoporosis is a systemic disease that seizes all parts of the skeleton and is characterized by reduction of bone mass per volume unit. Problems of bone tissue mineralization decreases due to osteoporosis are significant in pathology, medical and stomatological sciences [16,17].

IR spectroscopy can provide molecular structure information about mineralized and non-mineralized connective tissues [1–3] and different composite biomaterials were used as bone substitutes [18–21]. FTIR spectroscopy with FTIR microspectroscopic imaging has been extensively applied to the analyses of tissues in health and disease. Spatially resolved mid-FTIR data has provided insights into molecular changes that occur in diseases of connective or collagen-based tissues, including, osteoporosis, osteogenesis imperfecta, osteopetrosis and pathologic calcifications [1–3].

The goal of this study was to examine the effects of applied glucocorticoids on the composition of mandibular alveolar bones in experimental animals (*Wistar* rats) during intensive growth and mineralization of bones, as well as to assess the effects of Salmon calcitonin and Thymus extract therapies in the recovery treatment of glucocorticoids induced osteoporosis, as evidenced by the changes in Ca, Mg and P quantitative content in bone tissue by atomic absorption spectrometry (AAS) and inductively coupled plasma - atomic emission spectrometry (ICP-AES) techniques and the qualitative composition changes in bone tissue by FTIR spectroscopy.

Materials and methods

Experimental animals

Healthy female *Wistar* rats, weighing about 165 g in average, 6 to 8 weeks old, were taken for the experiment. This age is the period of intensive sexual maturation and growth, as well as development and mineralization of bone tissue. The animals were maintained under controlled conditions: 12-h light–dark cycle and a constant temperature of 22 ± 1 °C. The animals were given a standard pelleted chow and tap water. They were allowed to eat and drink *ad libitum*. The animals were allowed to acclimatize for one week before beginning of experiment. Experiment procedure was performed in accordance with the guidelines and decision of the Ethical Committee of the Faculty of Medicine, University of Niš, Serbia.

Osteoporosis induction

Glucocorticoid (GC) treatment was used as an experimental model of induction of osteoporosis and was explained in details previously [16,17].

Experimental design

A total of 40 rats were randomly divided into five groups (8 per group) as follows: four groups were consisted of GCs treated rats (labeled as I–IV), and one group was untreated (labeled as 0). Three groups among GC treated ones (II–IV) were left for recovery of bone loss for 3 weeks after finishing of GC treatments.

Animals of group **I** were sacrificed immediately after 12 weeks of GC treatment. In the group **II** after 12 weeks of GC treatment rats were treated as follows: Salmon calcitonin (Calcitonin hubber, Galenika, Serbia) was applied *i.m.* at a dose of 1 μL/g of body mass daily and Calcium gluconate (Calcium Sandoz, Novartis), at a dose of 0.05 mL per animal daily, as well as Alphacalcidiol (Alpha D_3, "Zdravlje Actavis Co.", Serbia), *per os* 1μL/g body mass of animal every second day. In the group **III** after 12 weeks of GC treatment rats were treated as follows: Thymus extract (Thymex-L, Thymoorgan-GmbH Pharmazie, Germany) was applied *i.m.* at a dose of 2 mg/kg of body mass, Calcium gluconate (Calcium Sandoz, Novartis), at a dose of 0.05 mL per animal daily, as well as Alphacalcidiol (Alpha D_3, "Zdravlje Actavis Co.", Serbia), *per os* 1 μL/g body mass of animal every second day. In the group **IV** after 12 weeks of GC treatment rats were left to spontaneously recover without any treatment for 3 weeks, but in standard maintaining conditions, with food and water supplying *ad libitum*. The group labeled as **0** served as a control (no treatment group) because the animals were without any treatment, but maintained in the standard conditions, with food and water supplying *ad libitum*.

Sample preparation

After the experiments' completion the animals were sacrificed, and for physicochemical and spectroscopic analysis mandible samples were taken. The samples were dried then the samples were divided into two parts, one of which was used in the analysis of Ca, Mg and P content, and the other one in FTIR spectroscopic analysis of bone tissue.

AAS and ICP-AES analyses

The part of bone samples that was taken in aforementioned way was put into saline solution. After being in saline solution the samples of bone tissue were dried to constant mass, cut and measured using an analytical balance with a precision of ±0.1 mg. The samples of precisely known mass were burnt in furnaces at a temperature of 900 °C for 4 h. Scorching of the samples was done to remove possibly present volatile impurities, as well as to remove a part of the sample organic matter. After scorching, the bone organic matter was completely destroyed by dissolution in concentrated nitric acid (HNO_3), and inorganic components were left in the solution at their highest oxidation states and in completely soluble form as Ca^{2+}, Mg^{2+}, PO_4^{3-}. After that, concentrated hydrochloric acid (HCl) was added and subjected to evaporation until dry was done to eliminate retained nitrogen compounds. Bone samples were dissolved in deionized water to adequate aliquots that are appropriate for Ca and Mg determination. An increase of sensitivity in determining Ca^{2+} requires high flame temperature that is obtained by the gas mixture of acetylene/N_2O. The content of Ca^{2+} and Mg^{2+} was determined by atomic absorption spectrometer AAS Philips Pye Unicam SP 9 (Ca-lamp $\lambda = 422.7$ nm, Mg-lamp $\lambda = 285.21$ nm, slot width 0.5 nm). ICP-AES method was used to simultaneously determine phosphorous in bone tissue samples, and wavelength of 214.9 nm was selected for the measuring of the phosphorus content.

FTIR spectroscopy

Recording of the IR spectra of solid samples was done by KBr disc method. The IR spectra of solid samples were recorded by FTIR spectrometer BOMEM MB-100 (Hartmann&Braun, Canada), equipped with standard DTGS/KBr detector, within the wavenumbers 4000–400 cm^{-1} and at resolution of 2 cm^{-1}. The spectral analyses, following the FTIR spectroscopic measurements were performed with Win-Bomem Easy 3.04 software (Galactic Industries Corporation).