



Dynamics of bone remodeling markers under osteoplastic materials in experiment

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Abstract. Molecular regulation of the balance between bone resorption and formation is primarily mediated by the RANKL/RANK/OPG signaling pathway and the Wnt-mediated pathway, the key inhibitor of which is sclerostin. A comprehensive assessment of these regulatory markers allows elucidation of the mechanisms underlying the observed changes in bone resorption and formation during the application of osteoplastic materials. The aim of the study was to investigate the dynamics of serum concentrations of RANKL, OPG and sclerostin, as well as the RANKL/OPG ratio, on days 5, 14, 30 and 60 of experimental femoral bone defect healing under the application of collagen-free, collagen-containing osteoplastic materials and demineralized bone matrix (DBM) compared with an untreated defect. The study was performed on 170 Wistar rats. Serum RANKL and OPG concentrations were determined by sandwich ELISA (Thermo Fisher Scientific); sclerostin was measured by ELISA using commercial kits. Statistical analysis was performed using STATISTICA 10.0 (StatSoft). In the untreated defect group on day 5, RANKL exceeded the control level by 163.6 %, OPG decreased by 28.5 %, and sclerostin increased by 100.4 %. In the DBM group, a pro-osteogenic profile was already evident from day 5, with OPG exceeding the control by 25.6 %, and from day 14 RANKL fell below the control level. The RANKL/OPG ratio in the DBM group remained below the control throughout the observation period. A gradient of regulatory efficacy of the materials was identified (DBM > collagen-containing implant > collagen-free implant > untreated defect), which is consistent with the data on resorption and formation markers and confirms the realization of the osteoreparative effect through modulation of the RANKL/RANK/OPG signaling pathway and suppression of sclerostin-mediated blockade of Wnt signaling. The obtained results substantiate the preferential use of demineralized bone matrix for accelerating reparative osteogenesis at the molecular regulatory level.

Keywords: osteoplastic materials; RANKL; osteoprotegerin; sclerostin; bone remodeling; demineralized bone matrix; reparative osteogenesis.

Introduction

The molecular balance between resorption and formation of bone tissue is ensured by the coordinated action of regulatory signaling systems, the key among which is the RANKL/RANK/OPG axis. Receptor activator of nuclear factor κ B ligand (RANKL) is expressed on the surface of osteoblasts, osteocytes and bone marrow stromal cells and, upon binding to the RANK receptor on osteoclast precursors, initiates

their differentiation, activation and prolongs the survival of mature osteoclasts [1]. Osteoprotegerin (OPG) is a soluble decoy receptor of the TNF family that competitively binds RANKL and prevents its interaction with RANK, thereby inhibiting osteoclastogenesis [1, 2]. The RANKL/OPG ratio is a recognized integral indicator of the bone remodeling balance: its increase indicates a shift toward resorption, while a decrease indicates predominance of formation [1, 3].

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In parallel with the RANKL/RANK/OPG axis, bone formation is regulated by the Wnt/ β -catenin signaling pathway, the key physiological inhibitor of which is sclerostin – a glycoprotein secreted by osteocytes that binds to the LRP5/6 co-receptors, blocking the Wnt cascade and suppressing osteoblastic differentiation and activity [4]. Since RANKL, OPG and sclerostin are produced predominantly by cells of the osteoblastic lineage and osteocytes, they constitute a single regulatory network that links the processes of bone resorption and formation within one remodeling cycle [5].

The dynamics of this regulatory triad have been studied in models of physiological remodeling and under osteoporotic changes [3, 5]; however, data on its behavior under implantation of osteoplastic materials of different composition – collagen-free, collagen-containing and demineralized bone matrix – remain limited. This complicates the molecular interpretation of the observed differences in the dynamics of bone resorption and formation markers.

The aim of the study: to investigate the dynamics of serum concentrations of the regulatory markers of bone remodeling RANKL, OPG and sclerostin, as well as the RANKL/OPG ratio, on days 5, 14, 30 and 60 of experimental healing of a femoral bone defect under the application of collagen-free, collagen-containing implantation materials and demineralized bone matrix compared with intact control and untreated defect.

Materials and Methods

The study was performed at the Departments of Functional and Laboratory Diagnostics, Pathological Anatomy with Sectional Course and Forensic Medicine, and at the Interdepartmental Educational and Research Laboratory Center of I. Horbachevsky Ternopil National Medical University of the Ministry of Health of Ukraine, in compliance with Directive 2010/63/EU of the European Parliament and of the Council, the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.03.1986) and the Resolution of the First National Congress on Bioethics (Kyiv, 2001), and was approved by the University Bioethics Commission.

The study was conducted on 170 linear white Wistar rats weighing 180–210 g, divided into five groups: Group I – intact animals without defect modeling (control); Group II – animals with a modeled femoral bone defect without correction; Group III – animals with implantation of a bone regeneration material without collagen; Group IV – animals with implantation of a bone regeneration material containing collagen; Group V – animals with application of demineralized bone matrix. The materials were obtained from ABM Technology LLC.

Animals of Groups II–V under sodium thiopental anesthesia (40 mg/kg body weight intraperitoneally) underwent osteoperforation of the femur with a bur under aseptic conditions, forming a standard defect 3 mm in diameter. After introduction of the tested material, the defect was covered with an ActiveBone collagen membrane, and the wound was sutured layer by layer. Animals were withdrawn from the experiment, and blood for biochemical analysis was collected on days 5, 14, 30 and 60 after surgery.

RANKL concentration was determined by the enzyme-linked immunosorbent assay using sandwich ELISA with monoclonal antibodies specific for soluble RANKL (Thermo Fisher Scientific) according to the manufacturer's instructions; the result was expressed in ng/mL. OPG concentration was determined by sandwich ELISA: monoclonal capture antibodies against OPG were pre-coated on the plate wells; after binding of the analyte from the test sample, detector antibodies conjugated with horseradish peroxidase were added; the optical density of the colored reaction product was measured at 450 nm (Thermo Fisher Scientific kits); the result was expressed in ng/mL. Sclerostin concentration was determined by the enzyme-linked immunosorbent assay using commercial test systems according to the manufacturer's instructions; the result was expressed in ng/mL.

Statistical processing of the results was performed using the Statistica 10.0 package (StatSoft Inc.). Data are presented as mean $\pm$ standard error of the mean ($M \pm m$). Normality of distribution was checked by the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by pairwise comparison using Student's t-test was applied to compare indicators between groups and observation periods; differences were considered statistically significant at $p < 0.05$.

Results

Control (baseline) values in the intact group were: RANKL – (0.22 ± 0.04) ng/mL, OPG – (89.7 ± 4.3) ng/mL, sclerostin – (0.483 ± 0.028) ng/mL (Table).

On day 5 in the untreated defect group, the most pronounced shifts were recorded: RANKL exceeded the control by 163.6 %, OPG decreased by 28.5 %, and sclerostin increased by 100.4 %, reflecting simultaneous hyperactivation of pro-resorptive signaling and suppression of osteoblastogenesis. Application of implantation materials was accompanied by a dose-dependent attenuation of this imbalance: in the collagen-free group RANKL +136.4 %, OPG -20.7 %, sclerostin +62.3 % relative to control; in the collagen-containing group – RANKL +59.1 %, OPG -5.6 %, sclerostin +35.4 %. The least pronounced shifts were observed in the DBM group: RANKL exceeded the control by only 9.1 %, while OPG already on day 5 exceeded the control level by 25.6 %, and sclerostin by 27.9 %.

Table. Markers of bone regeneration regulation in the serum of animals with a perforating femoral bone defect under the application of different osteoplastic materials

Indicator / Term	RANKL, ng/mL	OPG, ng/mL	Sclerostin, ng/mL
Intact control, n=10	0.22±0.04	89.7±4.3	0.483±0.028
Untreated defect			
Day 5, n=10	0.58±0.07*	64.1±3.6*	0.968±0.048*
Day 14, n=10	0.41±0.06*	68.3±4.8*	0.831±0.036*
Day 30, n=10	0.39±0.05*	72.3±6.5*	0.655±0.034*
Day 60, n=10	0.27±0.06	78.4±7.1	0.561±0.025
Collagen-free implant			
Day 5, n=10	0.52±0.05*	71.1±6.7*	0.784±0.042**
Day 14, n=10	0.35±0.03*	76.4±6.3	0.726±0.038*
Day 30, n=10	0.27±0.04	75.6±7.1	0.621±0.046*
Day 60, n=10	0.24±0.05	81.3±7.8	0.543±0.031
Collagen-containing implant			
Day 5, n=10	0.35±0.02**^	84.7±7.3	0.654±0.042**^
Day 14, n=10	0.31±0.02	87.2±8.3	0.566±0.039#^
Day 30, n=10	0.28±0.03	93.6±9.2	0.529±0.033#
Day 60, n=10	0.20±0.01	97.5±8.8	0.492±0.030
Demineralized bone matrix			
Day 5, n=10	0.24±0.02**^&	112.7±9.1**^&	0.618±0.036**^
Day 14, n=10	0.16±0.01**^&	126.2±10.4**^&	0.514±0.028#^
Day 30, n=10	0.14±0.01**^&	118.9±9.7**^	0.495±0.031#
Day 60, n=10	0.19±0.02	99.3±8.8	0.476±0.024#

Notes: 1. * – statistically significant difference compared with animals without bone defect modeling;
 2. ^ – statistically significant difference compared with animals with bone defect without correction;
 3. # – statistically significant difference compared with animals with bone defect and correction without collagen;
 4. & – statistically significant difference compared with animals with bone defect and correction with collagen.

On day 14 in the DBM group, the RANKL level fell below the control by 27.3 %, while OPG reached its peak value ((126.2±10.4) ng/mL; +40.7 % relative to control). In the collagen-containing group, OPG practically normalized on this term (-2.8 % relative to control). In the untreated group the imbalance persisted: RANKL +86.4 %, OPG -23.9 %, sclerostin +72.0 % relative to control. By day 30 in the DBM group, RANKL reached its minimum (-36.4 % relative to control), while OPG remained elevated by 32.6 %.

By day 60, the indicators in all groups approached the control values while retaining the intergroup gradient: in the untreated group RANKL remained elevated by 22.7 %, OPG was decreased by 12.6 %, and sclerostin by 16.1 %; in the DBM group RANKL was lower than control by 13.6 %, OPG was higher by 10.7 %, and sclerostin practically reached the baseline level (-1.4 %) (Fig. 1).

The integral indicator of osteoclastogenic potential is the RANKL/OPG ratio (Fig. 2). In the untreated

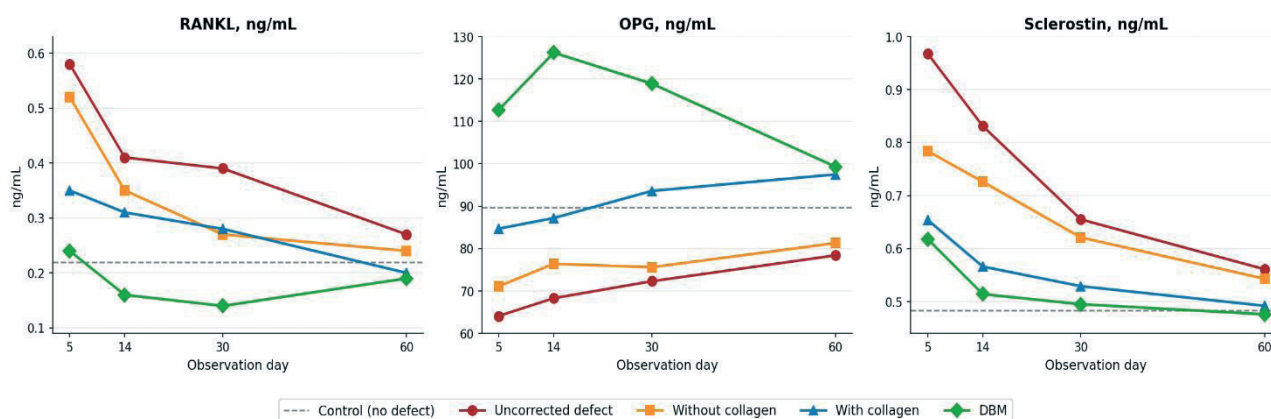


Fig. 1. Dynamics of serum concentrations of RANKL, OPG and sclerostin in the study groups on days 5, 14, 30 and 60 of observation.

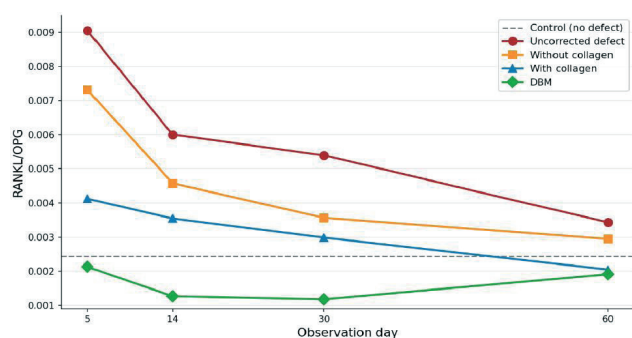


Fig. 2. Dynamics of the RANKL/OPG ratio in the study groups.

group it exceeded the control value 3.7-fold on day 5 and remained elevated even on day 60 (1.4-fold). In the collagen-containing group the ratio fell below the control level already on day 60 (0.84-fold), while in the DBM group it remained below the control throughout the entire observation period (from 0.87 on day 5 to 0.48 on day 30).

Discussion

The obtained results demonstrate that osteoplastic materials exert a regulatory effect at the molecular level, modulating the RANKL/OPG/sclerostin balance in a direction favorable for osteogenesis. The dynamics of RANKL and OPG in the untreated defect correspond to the pattern described in the literature: an increase in RANKL and a decrease in OPG in the acute phase of injury with subsequent activation of OPG in the bone formation phase, when osteoblasts acquire a dominant influence on the local microenvironment [6]. Notably, in the DBM group this sequence was substantially accelerated – a pro-osteogenic profile (elevated OPG, reduced RANKL) was already registered on days 5–14, whereas in the untreated defect analogous changes were only beginning.

The identified gradient of regulatory efficacy of the materials (DBM > collagen-containing implant > collagen-free implant > untreated defect) fully agrees with previously established data on resorption markers (CTX-I, TRAP-5b) and formation markers (PINP, BALP, osteocalcin): suppression of RANKL and an increase in OPG correlate with a decrease in resorption markers and an increase in bone formation markers. This confirms the hypothesis that the organic component of the materials (collagen, demineralized matrix) realizes the osteoreparative effect through modulation of the RANKL/RANK/OPG signaling pathway, approaching the physiological balance already in the early stages of healing [1, 2].

The parallel decrease in sclerostin in the groups with material implantation, most pronounced in the DBM group, is consistent with the role of sclerostin as an inhibitor of Wnt/ β -catenin signaling, the removal of which is a necessary condition for activation of osteoblastic differentiation [4]. Thus, the applied materials probably realize a dual mechanism of action – suppression of osteoclastogenesis via the RANKL/RANK/OPG axis and unblocking of osteoblastogenesis via sclerostin inhibition, which together ensure a coordinated and accelerated course of reparative osteogenesis compared with natural healing of the defect without correction.

Conclusions

1. Modeling of a femoral bone defect is accompanied by an acute increase in RANKL and sclerostin and a decrease in OPG on day 5 with gradual normalization of the balance by day 60, which is slowest in the untreated defect group.

2. Application of osteoplastic materials is accompanied by a dose-dependent attenuation of the imbalance of regulatory markers, most pronounced in the demineralized bone matrix group, where a pro-osteogenic profile (elevated OPG, reduced RANKL) is registered already from days 5–14.

3. The RANKL/OPG ratio in the DBM group remains below the control throughout the entire observation period, indicating a stable shift of the remodeling balance toward osteogenesis.

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Conflict of interest

The authors declare no conflict of interest.

Information on the use of artificial intelligence

The authors of the manuscript confirm that no generative artificial intelligence tools were used to obtain scientific data, conduct the research, or analyze the results. During the preparation of this article, the authors used the artificial intelligence tool Claude (Anthropic) for creating charts and data visualization; all generated charts were re-checked by the authors. All scientific data, interpretation of results, and conclusions belong to the authors, who bear full responsibility for the content of the publication.

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Динаміка регуляторних маркерів кісткового ремоделювання за умов застосування остеопластичних матеріалів в експерименті

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Анотація. Молекулярна регуляція рівноваги між резорбцією та формуванням кісткової тканини переважно опосередковується сигнальним шляхом RANKL/RANK/OPG і Wnt-опосередкованим шляхом, ключовим інгібітором якого є склеростин. Комплексна оцінка цих регуляторних маркерів дає змогу з'ясувати механізми, що лежать в основі змін процесів резорбції та формування кісткової тканини під час застосування остеопластичних матеріалів. Метою дослідження було вивчення динаміки сироваткових концентрацій RANKL, OPG і склеростину, а також співвідношення RANKL/OPG на 5, 14, 30 та 60 доби загоєння експериментального дефекту стегнової кістки під впливом безколагенових і колагеновмісних остеопластичних матеріалів, а також демінералізованого кісткового матриксу (ДКМ) порівняно з нелікованим дефектом. Дослідження виконано на 170 щурах лінії Wistar. Концентрації RANKL та OPG у сироватці крові визначали за методом сендвіч-імуноферментного аналізу (Thermo Fisher Scientific); рівень склеростину – методом ІФА із використанням комерційних наборів. Статистичний аналіз проводили за допомогою програмного забезпечення Statistica 10.0 (StatSoft). У групі нелікованого дефекту на 5 добу рівень RANKL перевищував контрольне значення на 163,6 %, концентрація OPG знижувалася на 28,5 %, а рівень склеростину підвищувався на 100,4 %. У групі ДКМ уже на 5 добу виявляли прооостеогенний профіль: рівень OPG перевищував контрольне значення на 25,6 %, а починаючи з 14 доби, концентрація RANKL була нижчою за контрольний рівень. Співвідношення RANKL/OPG у групі ДКМ протягом усього періоду спостереження залишалося нижчим за контрольне значення. Встановлено градієнт регуляторної ефективності досліджуваних матеріалів: ДКМ > колагеновмісний імплантат > безколагеновий імплантат > нелікований дефект, що узгоджується з даними щодо маркерів резорбції та формування кісткової тканини і підтверджує реалізацію остеорепаративного ефекту шляхом модулювання сигнального шляху RANKL/RANK/OPG та пригнічення склеростин-опосередкованого блокування Wnt-сигналіну. Отримані результати обґрунтовують доцільність переважного застосування демінералізованого кісткового матриксу для пришвидшення репаративного остеогенезу на молекулярно-регуляторному рівні.

Ключові слова: остеопластичні матеріали; RANKL; остеопротегерин; склеростин; ремоделювання кісткової тканини; демінералізований кістковий матрикс; репаративний остеогенез.