



ARTÍCULOS ORIGINALES / Originals

INTERCELLULAR MEDIATORS IN BONE REMODELING REGULATION IN THE EXPERIMENTAL RENAL PATHOLOGY

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Abstract

Bone metabolism disorders are characterized by an imbalance of bone resorption and formation in the bone remodeling process. Glucocorticoids that are used to treat kidney diseases exacerbate these disorders. P-selectin and galectin-3 are molecules involved in the sclerotic process in kidney, whereas bone resorption is regulated by the interaction between the nuclear factor activator kappa β receptor (RANK), its ligand (RANKL) and the RANKL decoy receptor osteoprotegerin (OPG).

The aim of this study was to investigate the cellular and molecular mechanisms of disruption of bone remodeling regulation processes, reflected by intercellular mediators (RANKL, OPG, P-selectin and galectin-3) in chronic kidney disease experimental model treated with glucocorticoids.

Rats were divided into four groups of 10 animals each. The first group, the control group, included intact animals. The second group consisted of rats with impaired bone remodeling resulting from chronic kidney disease (experimental group (CKD)). The

third group was a group of animals with impaired bone remodeling due to exposure to glucocorticoids (experimental group (GCs)). The fourth group consisted of rats with impaired bone remodeling in chronic kidney disease, followed by exposure to glucocorticoids (experimental group (CKD + GCs)). The effects of CKD and glucocorticoid were evaluated biochemically, histologically and by measuring bone density. An enzyme-linked immunoassay was used to measure intercellular mediator levels in the serum.

The bone density in the experimental groups was reduced compared to the control group. RANKL levels in animals of three experimental groups were higher than in intact animals. Serum levels of OPG were higher in CKD and GCs groups than in intact animals. At the same time, in the animals' blood serum of the CKD + GCs group, the levels of OPG were lower, than those in animals from the control group. The levels of galectin-3 in the serum of the experimental groups GCs and CKD + GCs were lower than in intact animals. The serum levels of galectin-3 in animals of the CKD group

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were higher than those in animals from the control group. The levels of P-selectin were lower in the serum of the GCs group than in intact animals. At the same time, the levels of P-selectin were higher in the CKD and CKD + GCs groups, than those in animals from the control group.

In conclusion, the study of the complex system of bone remodeling regulation, which

includes many factors and their interactions, may lead to the development of new methods for treating patients with chronic kidney disease in order to prevent osteoporosis in the future.

Keywords: boneremodeling, renalinsufficiency, chronic, glucocorticoids, cytokines.

Resumen

Las enfermedades metabólicas óseas se caracterizan por un desequilibrio en el proceso de remodelación ósea en los que participan mediadores tales como receptor del activador del factor nuclear- kappa- β (RANK), su ligando (RANKL) y la osteoprotegerina (OPG). Los glucocorticoides, frecuentemente empleados en el tratamiento de la enfermedad renal crónica, exacerbaban este desequilibrio. En la enfermedad esclerótica renal, las moléculas de adhesión celular P-selectina and galectina-3 tienen un rol fundamental.

El objetivo de esta trabajo fue estudiar las alteraciones en los mediadores de la remodelación ósea (RANKL, OPG, P-selectina and galectina-3) en un modelo de enfermedad renal crónica con tratamiento glucocorticoideo.

Rratas Wistar hembras fueron divididos en 4 grupos: control (C); enfermedad renal crónica con afección de la remodelación ósea (ERC); animales con afección de la remodelación ósea expuestos a glucocorticoides (GC); enfermedad renal crónica con afección de la remodelación ósea tratados con glucocorticoides (ERC+GC). Los efectos de la ERC y los GC fueron evaluados bioquímicamente, histológicamente y por me-

dición de la densidad ósea. RANKL, OPG, P-selectina and galectina-3 se cuantificaron en muestras de sangre venosa empleando enzimoimmuno análisis.

En los 3 grupos experimentales la densidad ósea se evidenció reducida y los niveles séricos de RANKL elevados respecto al grupo control. Los niveles de OPG en los grupos ERC y GC fueron superiores mientras que en el grupo ERC+GC menores respecto a los animales controles. Galectina 3 plasmática en GC y ERC+GC se encontró reducida y aumentada en los animales ERC, en comparación con los animales controles. La concentración sérica de P-selectina sérica fue mayor en los grupos ERC y ERC+GC, y menor en los animales GC respecto a los niveles plasmáticos de los animales intactos.

El avance del conocimiento sobre la regulación de la remodelación ósea a través de la interacción de mediadores sistémicos, en un futuro, puede conducir al desarrollo de nuevas estrategias terapéuticas para la prevención de la osteoporosis en pacientes con enfermedad renal crónica.

Palabras clave: remodelación ósea, enfermedad renal crónica, glucocorticoides; citoquinas.

Introduction

Chronic kidney disease (CKD) affects 10-15% of the population worldwide.¹ Reduction in renal function in CKD patients affects a number of interrelated secondary pathophysiological processes, including mineral and bone disorders.² The impaired bone metabolism in individuals with kidney function insufficiency determines the need for early detection and prevention of CKD and its associated complications. Thus, it is necessary to search for markers that reflect the presence of pathological changes in the renal tissue and determine their nature.

Glucocorticoids (GCs) are widely used to treat various inflammatory diseases, including kidney disease, due to their anti-inflammatory actions through the suppression of the production of pro-inflammatory cytokines. At the same time, GCs suppress bone formation due to both disruption of the functional activity of osteoblasts, as well as reduction of their number, and impaired precursor differentiation.³ Unfortunately, our knowledge about molecular regulators that modulate the differentiation and activity of osteoclasts and osteoblasts is still insufficient.⁴

Many intercellular mediators are involved in the processes of bone resorption and formation, as well as in the stages of kidney fibrosis. There may be interdependencies between bone remodeling disorders and kidney pathology, realized through cytokines, which simultaneously affect bone and kidney tissue.⁵

The cytokine system comprising the nuclear factor activator kappa β receptor (RANK), its ligand (RANKL) and the decoy receptor osteoprotegerin (OPG) play key roles in the regulation of bone remodeling. This cytokine system is also actively involved in the regulation of such processes as angiogenesis, neovascularization and remodeling of the vessel wall.⁶

Chronic kidney disease is a consequence of the interstitial extracellular matrix expansion, which leads to nephron loss.

Renal tissue remodeling disorder is caused by an imbalance between cell proliferation and apoptosis. The selectin and galectin family of proteins play an important role in these processes. Selectins mediate the migration of inflammatory cells to the renal interstitium, which, in turn, can cause apoptosis and tubular atrophy, and interstitial fibrosis.⁷ Galectin-3 is able to trigger apoptosis through the extracellular and mitochondrial pathways, exerting both pro-and anti-apoptotic actions.⁸ The aim of this study was to investigate the processes that lead to the regulation of bone remodeling by intercellular mediators (e.g., RANKL, OPG, P-selectin and galectin-3) in experimental chronic kidney disease subsequent exposure to glucocorticoids.

Materials and methods

An experimental study was conducted in four groups of female white Wistar rats aged 9 months and weighing 250 ± 30 g, in accordance with the principles of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986) and the rules for working with experimental animals approved by the Bioethics Committee of Kharkiv Medical Academy of Postgraduate Education.

The first group – the control group, included intact animals ($n = 10$). The second group ($n = 10$) consisted of rats with an impaired bone remodeling resulting from chronic kidney disease (experimental group (CKD)). The third group ($n = 10$) was a group of animals with an impaired bone remodeling under the influence of glucocorticoids (experimental group (GCs)). The creation of a model of experimental bone remodeling disorders under the influence of glucocorticoids was carried out by injecting dexamethasone phosphate at a dose of 6 mg/kg intramuscularly twice a week for a month.⁹ The fourth group ($n = 10$) consisted of rats with an impaired bone remodeling in chronic kidney disease followed by glucocorticoid exposure (experimental group (CKD + GCs)). The model



of kidney damage in CKD and CKD + GCs groups was performed by a single injection of 50% glycerol solution at a dose of 10 ml/kg of animal body weight. The development of CKD was controlled in accordance with the methodology of the model's authors.¹⁰ Glomerular filtration rate and morphological changes in kidney tissue were evaluated. Six weeks after the injection of glycerol, animals were injected with dexamethasone phosphate at a dose of 6 mg/kg intramuscularly twice a week for a month.⁹ Animals were euthanized by inhalation of chloroform in a confined space. Bone density was measured as the ratio of bone mass (grams) to its volume (cubic centimeters).¹¹ The femora were separated, cleaned of soft tissues and weighed. Since the study was focused only in changes in bone density, not all organic components of the bone (such as collagen fibrils, components of the bone marrow) were removed before measurement. The error associated with the presence of organic component was considered negligible. The volume of the femur was determined by the displaced fluid volume. For each animal, the average value of the femoral parameters was determined, consisting of the obtained values for the right and left femur. Based on the measurement results, bone density was calculated.

Histology of the kidneys was performed in samples fixed in 10% neutral formalin, and then dehydrated in increasing strength of alcohols (50°, 70° and twice 96°), then alcohol with chloroform was used, then chloroform, followed by paraffin embedding.¹² Sections, 5-7 microns thick, were stained with hematoxylin and eosin, or picric acid/acid fuchsin, following the Van Gieson's method.

For histological examination, the thoracic and lumbar spine vertebrae of the rats were isolated. The material was fixed in 10% neutral formalin, decalcified in 5% nitric acid, embedded in paraffin according to a conventional technique.¹² Sections, 7-10 μ m thick, were stained with hematoxylin and

eosin, or picric acid/acid fuchsin, following the Van Gieson's method.

The sections were visualized using a "Primo Star" (Carl Zeiss). Photomicrographs of the preparations were obtained using a Microocular digital camera.

Studies of the cytokine level were performed in serum by enzyme immunoassay. Blood samples were collected from the heart. The levels of RANKL were measured using the «ampli-sRANKL» kit (BIOMEDICA, Austria). OPG levels were determined using the «Human Osteoprotegerin Instant» kit (eBioscience, Austria), and the P-selectin levels were determined using the «Human sP-selectin Platinum ELISA» kit (eBioscience, Austria). The levels of galectin-3 were determined using the «Human Galectin-3 Platinum ELISA» kit (eBioscience, Austria).

Results are presented as mean \pm SE (standard error of the arithmetic average). Statistical analyses of the results were performed using the Statistica 6.0 software package, using the non-parametric Kruskal-Wallis test for independent samples and correlation analysis. Differences were considered statistically significant with p values < 0.05.

Results

The measured bone density of animals of the experimental groups was significantly reduced compared with control group (Table). The kidneys of experimental rats of CKD and CKD + GCs groups revealed significant structural changes, suggesting the disruption of the excretory function of the organ. Thus, diffuse venous-capillary plethora is noted in all the specimens, blood separation into plasma and uniform elements, erythrostasis is observed in the dilated vessels, which is a manifestation of a disruption of the blood supply to the organ and the rheological properties of the blood (Fig. 1).

The structure and shape of the renal glomeruli are preserved. Also there are glomeruli of a "branched" form, which can be a manifestation of microcirculatory disorders.

Table. Changes in bone density, cytokines and lectins in the control and experimental groups.

Parameter	Control group	Group with impaired bone remodeling in CKD	Group with impaired bone remodeling in GCs	Group with impaired bone remodeling in CKD + GCs
Bone density (g/cm)	1.62 ± 0.032	1.43 ± 0.032*	1.37 ± 0.041*	1.53 ± 0.026*
RANKL, pmol/l	0.131 ± 0.020	0.184 ± 0.041	0.167 ± 0.046	0.158 ± 0.043
OPG, pg/ml	21.588 ± 2.015	28.338 ± 2.431	27.177 ± 5,386	16.588 ± 1.633
Galectin-3, ng/ml	1.151 ± 0.075	1.208 ± 0,095	1.117 ± 0.086	0.592 ± 0.037*
P-selectin, ng/ml	2.231 ± 0.080	2.956 ± 0,060*	1.656 ± 0.107*	3.380 ± 0.062*

* p < 0.05 in comparison with the control group

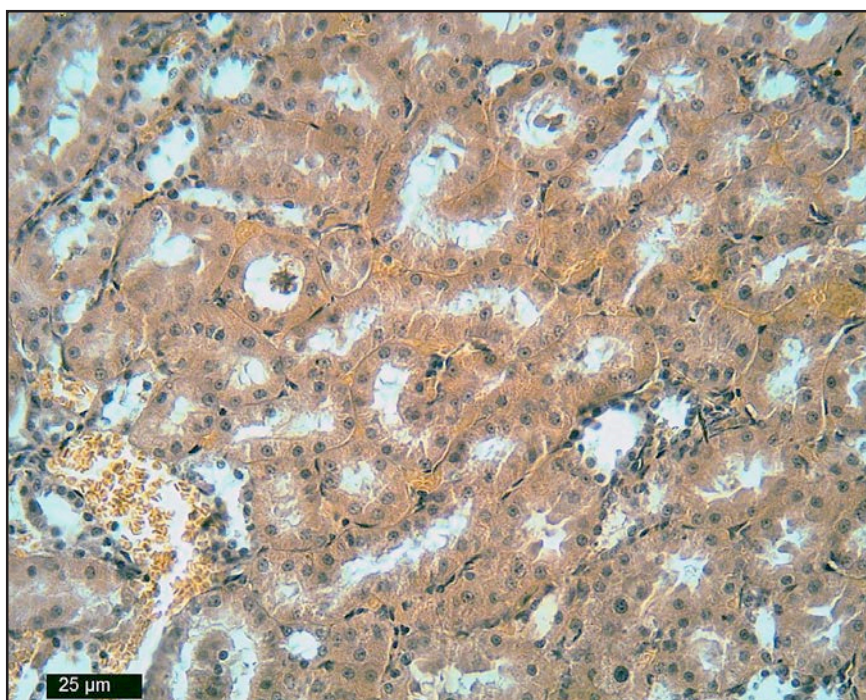


Figure 1. Section of rat renal cortex. Venous-capillary plethora Dystrophy of the epithelium. Hematoxylin and staining.

Polygonal and wrinkled glomeruli with enlarged capsule lumen, indicative of edema and atrophy were observed. Furthermore, nephrosclerosis centers in the cortex were noted. Connective

tissue with a large number of fibroblasts with large, brightly colored functionally active nuclei, were seen in a destructively altered renal epithelium, gradually replacing it (Fig. 2).

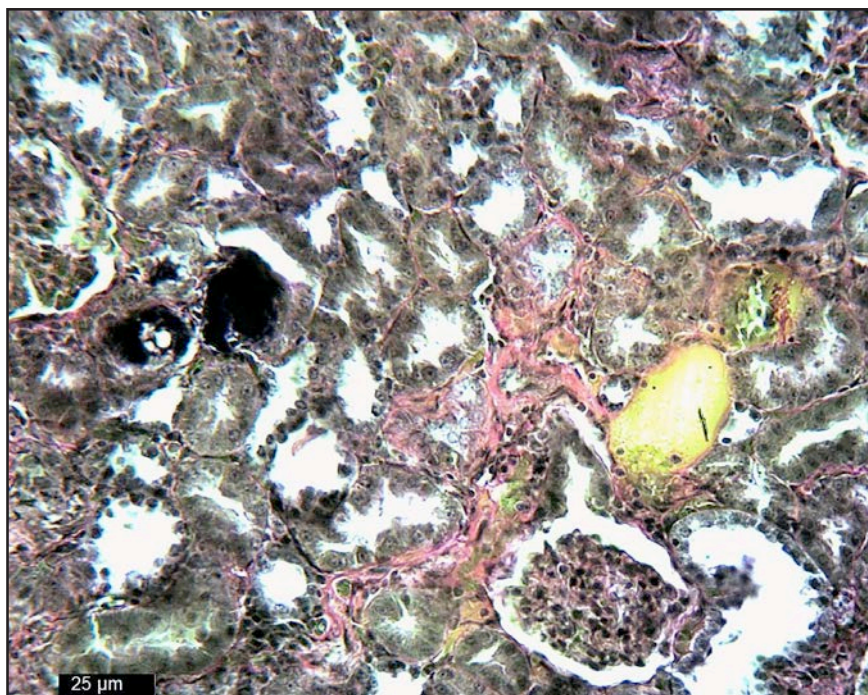


Figure 2. Histological section of the rat renal cortex. There are nephrosclerosis centers. The shape of glomeruli is polygonal. Calcification and colloid-like substance in the lumens of the tubules. Van Gieson`s stain.

Thus, histological examination confirms that a single injection of a glycerol solution in experimental animals of the CKD and CKD + GCs groups leads to dystrophic and necrotic changes in the kidney tubular apparatus, resulting in CKD.

Microscopic examination of histological preparations of vertebral bodies in the control group rats showed a typical structure of bone tissue. Spongy bone consisted of wide anastomosing trabeculae, separated by medullary spaces, which contained red bone marrow. Lacunae with osteocytes were located in the bone tissue of trabeculae and dark blue, slightly wavy cement lines were clearly visible. The cortex, represented by a compact bone, had enough width along the entire length.

Alteration of bone tissue histology was revealed by microscopic examination of the

vertebral bodies of rats of three experimental groups (Fig. 3).

In the cancellous bone, these disorders were associated with a decrease in the trabeculae thickness and the trabecular meshwork density reduction the number trabeculae and their contacts with each other and with the cortical plate were decreased. Most of the bone beams were thinned and had uneven edges and blind ends, which indicates the predominance of bone resorption processes. Compared to the control group, in histological preparations of experimental animals osteocytic lacunae of osteocytes containing cells at different stages of necrobiosis, uneven staining of the main substance of bone tissue, basophilia, and thickening of cement lines were noted. The bone marrow contained a significant amount of adipocytes, i.e. it was mixed. The cortical plate of the vertebral bodies was uneven

in width and thinned. The compact bone density reduction process was confirmed by the presence of dilated osteocyte lacunae, vascular channels and single cavities filled with reticulo-fibrous tissue and red bone marrow.

There were various sizes of osteocytes with an uneven distribution. Severe basophilia of the lacunae walls part and mosaic-colored in the matrix areas reflected an alteration of the calcification process.

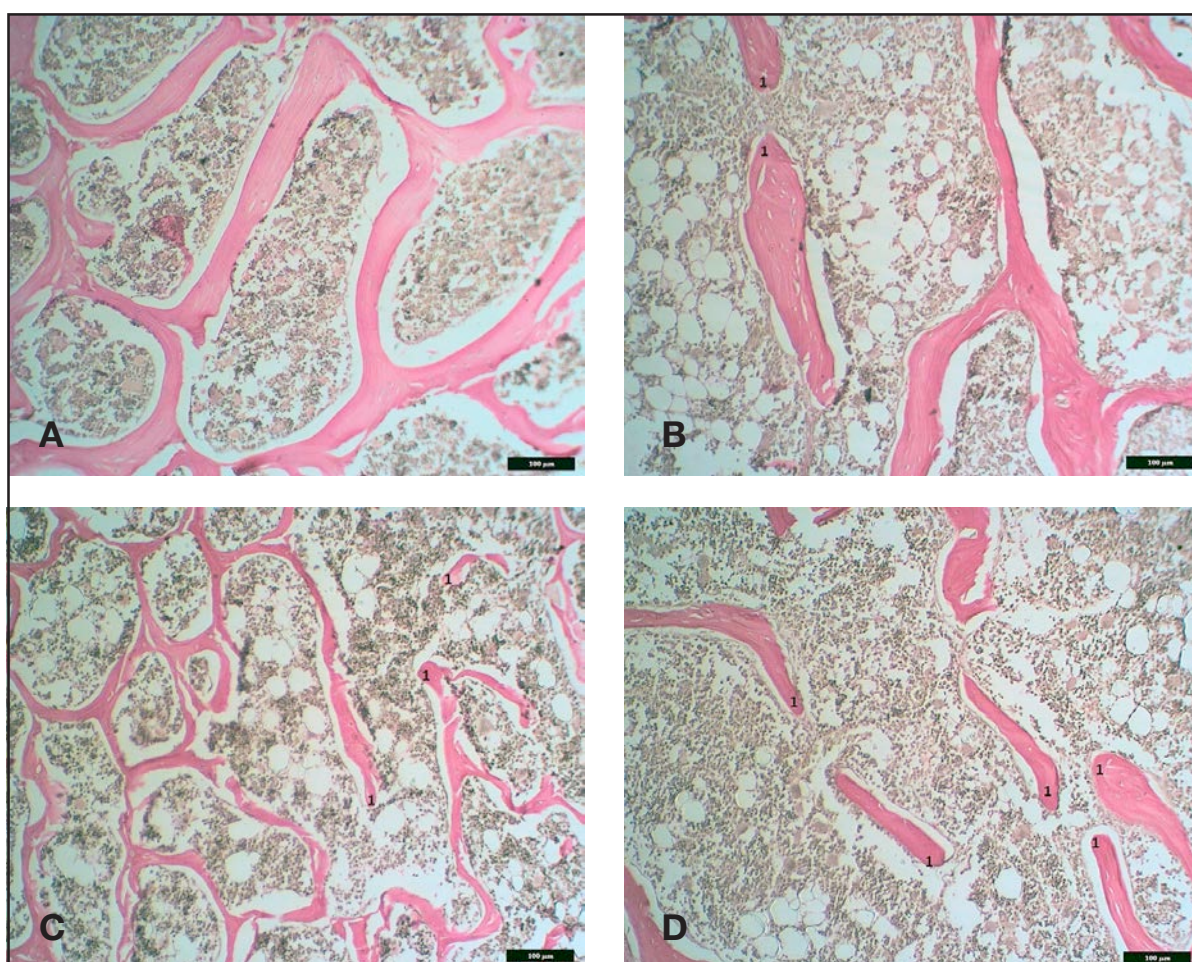


Figure 3. The section of rat lumbar spine vertebrae: **A:** the control group; **B:** group with an impaired bone remodeling in CKD; **C:** group with an impaired bone remodeling in GCs; **D:** group with an impaired bone remodeling in CKD + GCs: 1 – blind ends of trabeculae. Van Gieson staining.

The levels of RANKL in the serum of the three experimental groups were higher than in intact animals, but the difference did not reach statistical significance (Table). The levels of OPG in the serum of the CKD and GCs groups were higher than in intact animals. At the same time, in the serum of the CKD + GCs group, the levels of OPG were

lower than those in animals from the control group. The levels of galectin-3 in the serum of the experimental groups GCs and CKD + GCs were lower than in intact animals. The levels of galectin-3 in the serum of the CKD group were higher than those in animals from the control group. The levels of P-selectin in the animals' blood serum of the GCs group



were lower than in intact animals. At the same time, in the serum of the CKD and CKD + GCs groups, the levels of P-selectin were higher than those in animals from the control group (Table).

When conducting a correlation analysis in the control group, a direct strong correlation was found ($r = 0.683$, $p < 0.05$) between the content of RANKL and P-selectin. In the experimental groups of animals, this correlation was not significant. In the control group, strong negative correlations were found between P-selectin and bone density ($r = -0.766$, $p < 0.05$), RANKL and bone density ($r = -0.706$, $p < 0.05$). In the experimental groups, the correlation between the content of P-selectin and bone density changed direction. In the groups of CKD and GCs, the correlation between the content RANKL and bone density was not significant, while in CKD + GCs group the relationship between the content of RANKL and bone density remained inverse ($r = -0.407$, $p > 0.05$).

Discussion

The observed decrease in bone density in animals of three experimental groups compared to bone density in animals of the control group can probably be due to the negative effect of inflammation and GCs on bone metabolism.^{13,14} Inflammation modulates bone resorption mainly due to the ability of proinflammatory cytokines to cause imbalances in the RANKL/OPG system, stimulating osteoclastogenesis.¹⁵

In animals of the CKD and GCs experimental groups, serum RANKL and its natural antagonist OPG were increased. RANKL is the main inducer of osteoclast maturation. An increase in RANKL expression leads to bone resorption, which corresponds to a decrease in bone density in these groups. The action of various factors controlling bone resorption is carried out through the influence on the synthesis of RANKL and OPG in osteoblasts, which activates osteoclastogenesis. At the

same time, glucocorticoids activate RANKL and inhibit OPG synthesis in osteoblasts. However, in our study, an increase in the concentration of OPG in the serum of animals of the GCs group was observed. Due to the influence of GCs, activation of differentiation of osteoclast progenitor cells can occur, which is characterized by increased expression of both RANKL and its RANK receptor. In response to increasing RANK levels, OPG production is enhanced. An increase in the concentration of serum OPG is also observed in animals of the group with CKD. It has been found that some pro-inflammatory cytokines, such as IL-1, increase the production of RANKL and OPG in osteoblasts.¹⁶ Thus, IL-1, by activating the expression of RANKL on the surface of osteoblasts, regulates bone metabolism, stimulating osteoclastogenesis. On the other hand, this cytokine inhibits the formation of osteoclasts, increasing the production of OPG. Thus, an elevation in the level of serum OPG in animals of the CKD group can be considered as a compensatory reaction to an increase in the activity of osteoclasts.

A significant increase in RANKL and a decrease in OPG in the blood serum of animals of the CKD + GCs group (table) compared to those in intact animals can be caused by the action of GCs simultaneously with the inflammatory effects in CKD. With CKD, there is an increase in the production of pro-inflammatory cytokines, which, in turn, stimulates the expression of RANKL and reduces the production of OPG, which stimulates the differentiation and activation of osteoclasts and helps to reduce bone density. GCs are able to act directly on osteoclasts, prolonging life span and reducing apoptosis of mature osteoclasts, despite the reduction in the number of their precursors,¹⁷ which ultimately contributes to the process of bone resorption. This is confirmed by the inverse relationship found by us between the level of RANKL and bone density in the CKD + GCs experimental group.

Our studies have shown a significant increase in the serum level of P-selectin, a protein that is expressed on the surface of activated endothelial cells and platelets, in the CKD group compared to intact animals (table). The elevation of this lectin appears to be an homeostatic response to inflammation in CKD,¹⁸ the development of which was facilitated by significant platelet activation and endothelial dysfunction. Currently, the role of endothelial dysfunction in the development of many chronic diseases, including CKD, has been demonstrated.¹⁹

The inflammatory response in CKD depends on the presence of both proinflammatory cytokines and adhesion molecules, which ensures the interaction of endothelial cells with circulating leukocytes and then leukocytes with elements of the extracellular matrix, which leads to the accumulation of leukocytes in the inflammatory foci.¹⁴ Uncontrolled leukocyte adhesion is of great importance in the pathogenesis of inflammation. Interacting with ligands on the membrane of circulating leukocytes, P-selectin mediates leukocyte adhesion to the activated endothelium in the process of inflammation. Thus, an increase in P-selectin expression in the CKD animal group is an important sign of endothelial cell activation associated with the development of inflammation in CKD.

A significant decrease in the level of P-selectin was observed in the GCs experimental group. GCs are able to inhibit endothelial expression of proinflammatory mediators, such as cytokines, chemokines, and adhesion molecules,²⁰ which reflects the level of P-selectin in animals of this group. At the same time, in the CKD + GCs group a significant increase in serum P-selectin level was observed compared to intact animals, which indicates endothelial-platelet dysfunction with CKD when exposed to GCs. Many authors point at to an increased level of P-selectin in CKD, but there is no consensus on the effect of GC on the expression of

P-selectin. According to the literature, along with the inhibitory effect of dexamethasone on the expression of this protein,²¹ high doses of dexamethasone increase the levels of P-selectin,²² or do not affect its content.²³ An increase in the level of P-selectin in the CKD + GCs group is probably associated with GCs-induced vascular endothelial dysfunction in addition to the effects of inflammation in CKD.

The found correlations in groups of animals between P-selectin and RANKL may be due to the mutual influence of these intercellular mediators on bone metabolism. The correlations between the content of P-selectin and bone density indicate the complexity and ambiguity of the role of P-selectin in the regulation of bone metabolism and emphasize the involvement of adhesion molecules in bone remodeling processes. Features of the effect of GCs on endothelial function in case of inflammation require further study to develop and improve existing treatment strategies.

An important role in cell proliferation, adhesion, differentiation, angiogenesis, and apoptosis is played by galectin-3. Further, this pleiotropic lectin plays a key role in liver, kidney, lung and myocardial fibrogenesis.²⁴ Moreover, galectin-3 plays an important role in modulating the immune and inflammatory response.²⁵

Galectin-3 can affect bone homeostasis by regulating the function and interaction of osteoblasts and osteoclasts. Previous studies have shown that exogenous recombinant galectin-3 inhibits terminal differentiation of osteoblasts, which may indicate a different or even opposite effect of galectin-3 on osteoblastogenesis depending on its intracellular or extracellular localization.²⁶ At the same time, galectin-3, expressed on the surface of osteoclasts, is involved in the regulation process of bone resorption. However, data on the effect of this lectin on osteoclastogenesis are ambiguous.²⁵

An increase in serum galectin-3 was detected in the group of animals with CKD. This



is consistent with evidence that a decrease in renal function is associated with an increase in the level of this lectin.²⁷ On the other hand, a decrease in galectin-3 was observed in the GCs and CKD + GCs groups. In the CKD + GCs group, its decrease is even more significant. It should be noted that galectin-3 has a pro-inflammatory effect in acute conditions, while the anti-inflammatory effects of this lectin prevail in chronic inflammatory processes.²⁸

At the same time, a decrease in the expression of galectin-3 is induced by GCs, but the intensity of changes in the concentration of this lectin and the time of their appearance depend on the species, the concentration of GCs and the time of their exposure.²⁹ Probably, such a change in the expression of galectin-3 in the GCs and CKD + GCs groups is associated with the effects of glucocorticoids and the anti-inflammatory effects of galectin-3 in chronic inflammation. The reduction of bone density in the GCs and CKD + GCs groups, together with a decrease in the expression of galectin-3, which inhibits osteoclastogenesis in these groups, suggests a negative feedback mechanism, which might restrain excessive osteoclastogenesis.

Conclusion

Two different effects of the influence of glucocorticoids on the development of the pathological process in case of kidney disease are possible. GCs can be a treatment factor that reduces the intensity of inflammation in the kidneys and, accordingly, the risks of developing osteoporosis due to renal insufficiency. However, at the same time, GCs themselves are a risk factor for osteoporosis. We did not find confirmation of the additive, subtractive, or cumulative effects of GCs

acting simultaneously with CKD on metabolic processes in the bone. It was found that these relationships are changing significantly during the development of the pathological process. Further research is required to determine the optimal regimen for using GCs to minimize the activity of the pathological process, both in the kidneys and in the bone.

The studied profile of intercellular mediators and the revealed correlations suggest alterations in the regulatory pathways that lead to abnormalities in osteolytic processes activation with development of inflammation in chronic kidney disease. The imbalance between the levels of RANKL and OPG, resulting from the alteration of the feedback mechanism, contributes to bone resorption and, therefore, leads to altered bone remodeling.

Further studies will assess the role of extracellular mediators in the regulatory mechanisms of bone metabolic disturbances when exposed to glucocorticoids, both in renal diseases and in other chronic pathologies. The study of a complex system of regulation of bone remodeling, which includes many factors and their interactions, in the future may lead to the development of methods for treating patients with chronic kidney disease in order to prevent osteoporosis.

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