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Study of biochemical markers of osteogenesis in case of bone allografts incorporation in rats with followed after surgery administration of cisplatin at the different methods of implant sterilization

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Bone allografts are commonly used for surgical treatment of cancer patients. However, such complications as violation of allograft fusion, its lysis and fractures, infection lead to additional research in this field of medicine. Objective. To study changes in biochemical osteogenesis markers under the action of cytostatics on the process of incorporation of bone allografts. Methods. The work was performed on 20 male white rats (age at the beginning of the experiment 5–6 months). All animals have a perforated defect in the distal metaphysis of the femur filled with bone allograft (diameter 2 mm, height 3 mm), γ-radiation sterilized (Control-1 and Experiment-1) or saturation of the antibiotics sterilized (Control-2 and Experiment-2). In groups «Control» 14 days after implantation intraperitoneally injected 2.0–2.4 ml of 0.9 % sodium chloride solution, in the groups «Experiment» — cisplatin at a dose of 2.5 mg/kg once. 30 days after surgery, blood glycoproteins, total protein, Ca, chondroitin sulfates, acidic and alkaline phosphatase activity were evaluated. The index of mineralization (ratio of alkaline to acid phosphatases), degree is analyzed mineralization (ratio of calcium to protein). Results. In the experimental groups, compared with the control, a significant decrease in total protein and values was determined: total calcium, which indicates the suppression of processes mineralization during remodeling of bone tissue of the recipient and allograft. The highest indicators of activity acid phosphatase were recorded in groups Experiment-1 and Experiment-2, reflecting the predominance of resorption over bone formation. The degree of mineralization in the experimental groups was higher than in the control, and the mineralization index was significantly smaller. Conclusions. The detected changes in the values of biochemical markers of bone metabolism reflect the negative effect of cisplatin on osteogenesis under the conditions of allograft implantation, which leads to the lack of their fusion with the recipient bone. Key words. Biochemical markers of bone metabolism, remodelling of bone allografts, sterilisation, γ-ionisation, cisplatyn, rats.

Кісткова алопластика — часто вживана методика хірургічного лікування онкологічних пацієнтів. Проте ускладнення в разі її використання (порушення зрощення алоімпланта та кістки реципієнта, його лізис і переломи, інфекція) обумовлюють проведення досліджень у цьому напряму. Мета. Дослідити зміни біохімічних маркерів остеогенезу за умов дії цитостатиків на процес інкорпорації кісткових алоімплантатів. Методи. Роботу виконано на 20 самцях білих щурів (вік на початок експерименту 5–6 міс.). Усім тваринам дірчастий дефект у дистальному метафізі стегнової кістки заповнили алогенним кістковим матеріалом (діаметр 2 мм, висота 3 мм), стерилізованим за допомогою γ-випромінювання (Контроль-1 і Дослід-1) або насиченням антибіотика (Контроль-2 та Дослід-2). У групах «Контроль» через 14 днів після імплантації внутрішньоочеревинно вводили 2,0–2,4 мл розчину 0,9 % натрію хлориду, у групах «Дослід» — цисплатин у дозі 2,5 мг/кг одноразово. Через 30 днів після операції в крові оцінювали глікопротеїни, загальний білок, Са, хондроїтинсульфати, активність кислої та лужної фосфатаз. Проаналізовано індекс мінералізації (співвідношення лужної до кислої фосфатаз), ступінь мінералізованості (співвідношення вмісту кальцію до білка). Результати. У дослідних групах порівняно з контрольними визначено суттєве зниження загального білка, значень загального кальцію, що свідчить про пригнічення процесів мінералізації під час ремоделювання кісткової тканини реципієнта й алоімплантата. Найбільші показники активності кислої фосфатази зафіксовано в групах Дослід-1 і Дослід-2, що відображує переважання резорбції над кісткоутворенням. Ступінь мінералізованості в дослідних групах був вищим, ніж у контрольних, а індекс мінералізації — суттєво меншим. Висновки. Виявлені зміни значень біохімічних маркерів кісткового метаболізму відображують негативний вплив цисплатину на остеогенез за умов імплантації алоімплантатів, що призводить до відсутності їхнього зрощення з кісткою реципієнта. Ключові слова. Біохімічні маркери кісткового метаболізму, ремоделювання кісткових алоімплантатів, стерилізація, γ-випромінювання, цисплатин, щури.

Key words. Biochemical markers of bone metabolism, remodelling of bone allografts, sterilisation, γ-ionisation, cisplatyn, rats

Introduction

Replacement of post-resection defects of long bones in case of their tumor lesion is one of the most important tasks of onco-orthopedics. Adequate and complete recovery of the affected limb is the key to a good orthopedic result. For many decades, bone alloplasty has been a commonly used method of surgical treatment of cancer patients [1–3]. Surgical techniques and methods of preparation of alloplastic material are constantly being improved. This is due to the difficulties encountered in the treatment of this category of patients. The main complications in the case of bone alloplasty are impaired fusion of the alloimplant and the recipient's bone, lysis and fractures of the alloimplant, infectious exacerbations [4, 5]. The processes of reconstruction of bone alloimplants are influenced by the methods of their sterilization and manufacture, as well as the age and disease of bone donors. It is known that the use of γ-radiation reduces the activity of osteoclasts and, as a consequence, impaired bone remodeling, and alloimplants become more fragile. Fresh-frozen alloimplants are associated with a higher percentage of infectious complications compared to the material treated with γ-radiation [5, 6].

Morphological methods and studies of biochemical parameters of blood of patients and experimental animals are used to study the quality of osteogenesis and remodeling of bone tissue, as well as the incorporation of bone alloimplants.

Currently, biochemical markers of bone remodeling have been widely studied under conditions of various bone abnormalities and, especially, during disorders of bone repair processes of traumatic origin and in the case of osteoporosis [7].

Main biochemical markers of osteogenesis and mineralization of bone tissue include acid and alkaline phosphatase, total calcium, osteocalcin, bone morphogenetic proteins, glycoproteins and more. Bone remodeling occurs due to two main processes that constantly take place in the body, namely resorption and bone formation. These processes are interconnected and ensure the balance of osteoclasts and osteoblasts. The main phases of bone remodeling are initiation, resorption, reversion, osteogenesis and rest. During the resorption phase, the activity of osteoclasts, which secrete acidic proteases, is initiated and then osteogenic cells are involved in the process, which are transformed into osteoblasts. Incorporation of hydroxyapatite crystals into the organic matrix provides the mineralization process. This physiological process allows the body to adapt to different loads by updating the microarchitectonics of bone tissue. As a result of its remodeling, free amino acids and high-molecular fragments of proteins enter the blood, which are later found in biological fluids of the body. To assess the quality of the remodeling process, the mineralization index (ratio of alkaline and acid phosphatases) and the degree of mineralization (ratio of calcium and protein) are analyzed [7]. Thus, by identifying these biochemical markers, it is possible to study various pathological processes that occur in bone tissue.

The aim of the study: to investigate changes in biochemical markers of osteogenesis under the action of cytostatics on the incorporation of bone alloimplants.

Material and methods

The study was performed on 20 laboratory white male rats (5 months old, body weight 300–440 g) of the population of the experimental biological clinic of the State Institution «Professor M. I. Sytenko Institute of Abnormalities of the Spine and Joints of the National Academy of Medical Sciences of Ukraine». The experiment on rats was conducted in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986) and the Law of Ukraine «On Protection of Animals from Cruelty» (Article 26) [8, 9]. All surgeries were performed in aseptic and antiseptic conditions under general anesthesia: aminazine (10 mg/kg body weight, intramuscular) and ketamine (50 mg/kg body weight, intramuscular). The plan of the experiment was approved at a meeting of the Committee on Bioethics of the Institute (Minutes No. 204 of 15.06.2020).

Animals were divided into 4 groups of 5 rats each:

– Control-1 (n = 5) and Experiment-1 (n = 5) – allocyst implants (diameter 2 mm, height 3 mm), sterilized using γ-radiation (γ-rays);

– Control-2 (n = 5) and Experiment-2 (n = 5) — allocyst implants (diameter 2 mm, height 3 mm), impregnated with a solution of the antibiotic ceftriaxone.

Sterilization of alloimplants was performed by radiation γ-radiation at a dose of 15 to 25 kGy or by immersion for 24 h at a temperature of +4°C in a solution of the antibiotic ceftriaxone, solvent — 0.9 % sodium chloride, solution concentration 1 g / 10 ml.

In the Control groups, 2.0–2.4 ml of 0.9 % sodium chloride solution was administered intraperitoneally 14 days after implantation of allocyst material, and in the Experiment groups, cisplatin at a dose of 2.5 mg/kg was administered once.

Euthanasia of rats was performed 30 days after surgery by administering a lethal dose of anesthetic (sodium thiopental, 90 mg/kg intramuscularly) and decapitation followed by blood collection for biochemical studies.

We do not recount the technique of surgical interventions on rats, as it has been described in detail in the previous article [10].

To assess the processes of bone regeneration and inflammation, we have selected the following biochemical indicators of blood: glycoproteins, total protein, Ca, chondroitin sulfates, acidic and alkaline phosphatases. Indicators such as the mineralization index (ratio of alkaline to acid phosphatase), the degree of mineralization (the ratio of calcium to protein, which is increased by 1,000 times for easier presentation) were also analyzed.

The content of glycoproteins in the blood serum was determined by the method of Steinberg and Dotsenko in the reaction with ammonium molybdate [11], total chondroitin sulfates by the reaction with rivanol [12]. The level of total protein was determined by the biuret reaction according to the instructions for the reagent kit. The activity of alkaline and acid phosphatases was studied by reaction with diethanolamine by kinetic methods according to the instructions for the kits «Alkaline phosphatase — kin Sp.L» and «Acid phosphatase — kin Sp.L». The study was performed on a semi-automatic biochemical analyzer GBG Statfax 1904 plus.

The content of total and ionized calcium in serum was determined by potentiometric method [13] using an electrolyte analyzer AEK-01.

The results were statistically processed using methods of parametric and nonparametric analysis. Accumulation, adjustment, systematization of source information and visualization of the obtained indicators were carried out in Microsoft Office Excel 2016 spreadsheets. Statistical analysis was performed using STATISTICA 10 software (StatSoft.Inc).

In the case of describing quantitative indicators, the measurement results were calculated and they are presented as the median (Me), upper and lower quartiles (25 and 75 ‰). The Mann-Whitney U-test was used to compare independent populations in cases with a small number of observations and no signs of normal data distribution.

Results and discussion

In the study, each indicator of biochemical markers of rat blood was analyzed separately and compared in the groups «Experiment» – «Control» and «Control 1» – «Control 2».

Analyzing the levels of chondroitin sulfates in the blood of rats, we found that their median in the groups Experiment-1 and Experiment-2 was 0.19 g/l and 0.18 g/l with an interquartile range from 0.17 g/l to 0.23 g/l and from 0.15 g/l to 0.20 g/l, respectively. The values in the Control-1 and Control-2 groups were 0.18 g/l (0.17; 0.21) and 0.16 g/l (0.16; 0.17), respectively. The level of chondroitin sulfates in all groups of animals did not differ statistically ($p > 0.05$), which indicated the absence of significant changes in glycosaminoglycan metabolism.

Evaluation of blood glycoprotein levels in rats showed similar results. Namely, the median level of glycoproteins in rats of groups Experiment-1 and Experiment-2 was 0.74 units and 0.77 units with an interquartile range of 0.73 units up to 0.75 units and from 0.73 units up to 0.78 units, respectively. Similar figures in the control groups (Control-1 and Control-2) amounted to 0.80 units (0.79; 0.82) and 0.77 units (0.63; 0.81), respectively. There was no statically significant difference in the analysis of glycoprotein values in all groups ($p > 0.05$) (Table 1), which indicated the absence of inflammatory processes in animals.

The median level of total blood protein in rats of groups Experiment-1 and Experiment-2 was 69.80 g/l and 66.00 g/l with an interquartile range from 66.20 g/l to 72.90 g/l and from 65.50 g/l to 67.80 g/l, respectively. Indicators in the Control-1 and Control-2 groups were 80.40 g/l (70.70; 83.20) and 83.30 g/l (78.80; 88.70), respectively.

The total blood protein of rats in the experimental groups of rats was lower than in controls. A statistically significant decrease in total blood protein was found in the experimental groups compared with Control-2: in the case of pairwise comparison of groups Experiment-1 and Control-2 — $U = 0$; $Z = 2.51$; $p = 0.012$, Experiment-2 and Control-2 — $U = 0$; $Z = 2.51$; $p = 0.012$.

The median level of total calcium in the blood of rats in groups Experiment-1 and Experiment-2 was 0.0942 g/l and 0.0934 g/l with an interquartile range from 0.0934 g/l to 0.0954 g/l and from 0.0922 g/l to 0.0942 g/l, respectively. Indicators in the Control-1 and Control-2 groups were 0.0994 g/l (0.0954; 0.1006) and 0.0994 g/l (0.0982; 0.1006), respectively.

The total blood calcium of rats in the experimental groups of rats was lower than in controls. A statistically significant decrease in this indicator was recorded in the experimental groups compared with the Control-2 group: in the case of pairwise comparison of the Experiment-1 and Control-2 groups — $U = 0$; $Z = –2.51$; $p = 0.012$, and for groups Experiment-2 and Control-2 — $U = 0$; $Z = –2.51$; $p = 0.012$.

The median level of alkaline phosphatase in rats of Experiment-1 and Experiment-2 was 383 units and 290 units with an interquartile range of 276 units up to 446 units and from 255 units up to 299 units, respectively. Similar figures in the groups Control-1 and Control-2 amounted to 269 units (256; 290) and 313 units (262; 317), respectively. No statistically significant difference in the level of alkaline phosphatase was detected in pairs between all groups of animals ($p > 0.05$) (Table 3, Fig. 1).

The median level of acid phosphatase in rats of groups Experiment-1 and Experiment-2 was 35.30 units. and 35.00 units. with an interquartile range of 33.50 units. up to 40.50 units and from 33.70 units. up to 35.70 units in accordance. Similar figures in the groups Control-1 and Control-2 were equal to 6.80 units. (6.50; 7.80) and 18.50 units. (18.20; 20.20), respectively. After a pairwise comparison of acid phosphatase parameters between the experimental and control groups, a statistically significant increase was found in the groups Experiment-1 and Experiment-2 — $U = 0$; $Z = 2.51$; $p = 0.012$ (Table 3, Fig. 1).

The median degree of mineralization in rats of groups Experiment-1 and Experiment-2 was 1.35 and 1.41 with an interquartile range of 1.31 to 1.41 and 1.39 to 1.41, respectively. Similar values in the Control-1 and Control-2 groups were 1.24 (1.21; 1.35) and 1.21 (1.17; 1.25), respectively. A pairwise comparison of the degree of mineralization between the experimental and control groups showed a statistically significant increase in the groups Experiment-1 and Experiment-2 compared with the group Control-2 ($U = 0$; $Z = 2.51$; $p = 0.012$), as well as in the group Experiment-2 compared with the control group-1 ($U = 2.00$; $Z = 2.089$; $p = 0.037$).

Assessment of mineralization index (Table 4, Fig. 2) showed the following result: the median in rats of groups Experiment-1 and Experiment-2 was 9.36 and 8.54 units with an interquartile range of 7.82 units up to 12.22 units and from 7.14 units up to 8.61 units, respectively. Similar values in

Table 1

The value of glycoproteins and chondroitin sulfates in the blood of rats after the installation of bone alloimplants sterilized by various methods, and administration of a cytostatic drug

Group of animals	Glycoproteins, units			Chondroitin sulfates, g/l		
	median	25 ‰ quartile	75 ‰ quartile	median	25 ‰ quartile	75 ‰ quartile
Experiment-1 (γ-rays + cisplatin)	0.74	0.73	0.75	0.19	0.17	0.23
Experiment-2 (ceftriaxone + cisplatin)	0.77	0.73	0.78	0.18	0.15	0.20
Control-1 (γ-rays + NaCl 0.9 %)	0.80	0.79	0.82	0.18	0.17	0.21
Control-2 (ceftriaxone + NaCl 0.9 %)	0.77	0.63	0.81	0.16	0.16	0.17

Table 2

The value of total protein and total calcium in the blood of rats after the installation of bone alloimplants sterilized by various methods, and administration of a cytostatic drug

Group of animals	Total protein, g/l			Total calcium, g/l		
	median	25 ‰ quartile	75 ‰ quartile	median	25 ‰ quartile	75 ‰ quartile
Experiment-1 (γ-rays + cisplatin)	69,80	66.20	72.90	0.0942	0.0934	0.0954
Experiment-2 (ceftriaxone + cisplatin)	66,00	65.50	67.80	0.0934	0.0922	0.0942
Control-1 (γ-rays + NaCl 0.9 %)	80,40	70.70	83.20	0.0994	0.0954	0.1006
Control-2 (ceftriaxone + NaCl 0.9 %)	83,30	78.80	88.70	0.0994	0.0982	0.1006

Table 3

The value of alkaline and acid phosphatases activity in the blood of rats after the installation of bone alloimplants sterilized by various methods, and administration of a cytostatic drug

Group of animals	Alkaline phosphatase, units			Acid phosphatase, units		
	median	25 ‰ quartile	75 ‰ quartile	median	25 ‰ quartile	75 ‰ quartile
Experiment-1 (γ-rays + cisplatin)	383.00	276.00	446.00	35.30	33.50	40.50
Experiment-2 (ceftriaxone + cisplatin)	290.00	255.00	299.00	35.00	33.70	35.70
Control-1 (γ-rays + NaCl 0.9 %)	269.00	256.00	290.00	6.80	6.50	7.80
Control-2 (ceftriaxone + NaCl 0.9 %)	313.00	262.00	317.00	18.50	18.20	20.20

the groups Control-1 and Control-2 were 41.38 units (28.13; 42.65) and 17.14 units (13.13; 18.91), respectively. A pairwise comparison of the mineralization index between the experimental and control groups showed a statistically significant decrease in the Experiment-1 and Experiment-2 groups compared to the Control-1 group (U = 0; Z = 2.51; p = 0.012), as well as a statistically significant increase in the group Experiment-2 compared with Control-2 (U = 1.00; Z = –2.298; p = 0.022).

Discussion

The biology of bone graft engraftment has been studied by many experts [2, 4, 14–17], but the effect of γ-radiation on this process is unclear. Recovery and reconstruction of the allograft involves both osteoinduction and osteoconduction. Osteoinduction is associated with the adhesion of the recipient's mesenchymal cells to the graft surface, followed by their differentiation and bone formation. Osteoclasts play a crucial role in bone remodeling. Osteoclast activity is reduced by 57 % in the production of allografts (degreasing, freeze-drying and γ-radiation) compared to fresh-frozen bone. That is, γ-radiation affects the process of bone allograft remodeling. However, the level of this disorder differs [18], which depends on the dose and method of irradiation of implants. We found a negative effect of γ-radiation on osteogenesis as a result of histological studies [10], but the analysis of its biochemical markers did not show a significant effect. The effect of cytostatic chemotherapeutics on allograft remodeling processes has been insufficiently studied and no analytical review on this topic has been identified in the literature. There are reports that chemotherapy causes immunosuppression and therefore adversely affects (slows down or completely suppresses) the processes of allograft fusion with the recipient's bone [19].

There was a statistically significant decrease in total protein in the blood of animals of Experiment-1 and Experiment-2 groups (where cisplatin was administered 14 days after implantation) compared with control groups. This indicates that the selected dose of cytostatic drug (cisplatin) was adequate and caused a toxic reaction in the body. Thus, the experimental model for studying the effect of chemotherapeutics on the processes of bone remodeling in the conditions of bone alloplasty is reproduced as close as possible to that in patients with malignant bone tumors.

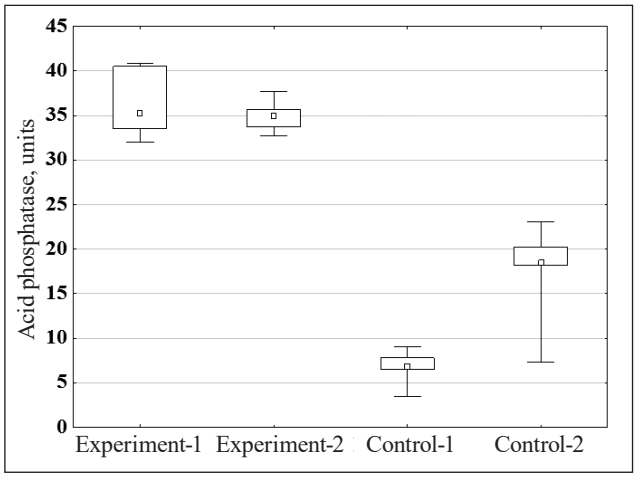

Fig. 1. Diagram of acid phosphatase activity. Statistically significant increase in groups Experiment-1 and Experiment-2

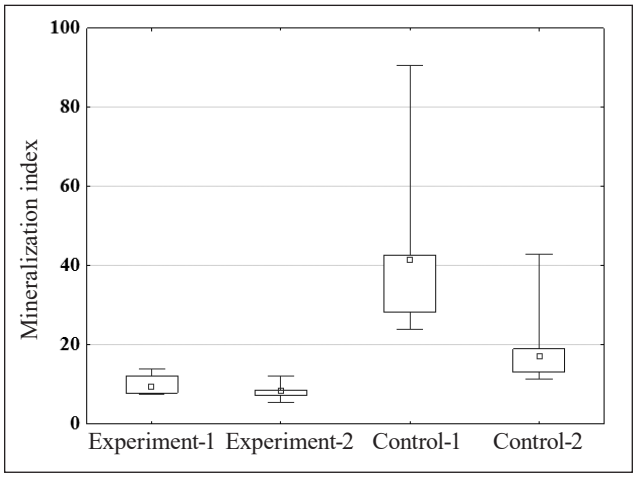

Fig. 2. Diagram of mineralization index indicators. Statistically significant decrease in the groups Experiment-1 and Experiment-2

Table 4

Evaluation of the degree of mineralization and bone mineralization index in rats after installation of bone alloimplants sterilized by various methods, and administration of a cytostatic drug

Group of animals	Degree of mineralization			Mineralization index		
	median	25 ‰ quartile	75 ‰ quartile	median	25 ‰ quartile	75 ‰ quartile
Experiment-1 (γ-rays + cisplatin)	1.35	1.31	1.41	9.36	7.82	12.22
Experiment-2 (ceftriaxone + cisplatin)	1.41	1.39	1.41	8.54	7.14	8.61
Control-1 (γ-rays + NaCl 0.9 %)	1.24	1.21	1.35	41.38	28.13	42.65
Control-2 (ceftriaxone + NaCl 0.9 %)	1.21	1.17	1.25	17.14	13.13	18.91

The content of chondroitin sulfates in the studied groups of rats did not differ significantly, which indicates the absence of the influence of both factors on the metabolism of glycosaminoglycans.

No statistically significant difference in the level of glycoproteins in the blood of rats of the studied groups was also observed, which reflects the absence of inflammatory processes. Accordingly, inflammation did not affect the course of osteogenesis, which allowed us to adequately assess other biochemical markers.

The decrease in the values of total calcium in the blood of animals of the experimental groups compared with the control indicates the inhibition of mineralization processes during the remodeling of bone tissue of the recipient and the reconstruction of the alloimplant.

The highest indicators of acid phosphatase activity in the groups Experiment-1 and Experiment-2, where cisplatin was used, showed violations of remodeling processes and the advantage of resorption over bone formation.

The degree of mineralization in the experimental groups was higher than in the control. There was also a significant decrease in the mineralization index in groups of rats treated with cisplatin.

After pairwise comparison of groups of biochemical markers of osteogenesis in the groups Experiment-1/Experiment-2, Control-1/Control-2 no significant differences were found. This suggests that the method of sterilization of bone alloimplants does not affect bone metabolism.

Thus, we can conclude that chemotherapeutic cytostatic drug (cisplatin) has a negative effect on osteogenesis, inhibits it and leads to a predominance of bone resorption. All this slows down the fusion of the bone alloimplant with the recipient's bone or completely prevents its incorporation and causes lysis.

Conclusions

Biochemical study of markers of osteogenesis showed that the toxic dose of cytostatic chemotherapy was selected adequately and no signs of inflammatory reactions were identified. That is, the experimental animals reproduced the situation as close as possible to the clinical situation in patients.

The detected changes in the values of biochemical markers of bone metabolism reflect the negative impact of cisplatin on osteogenesis under the conditions of implantation of alloimplants, which leads to the lack of their fusion with the bone of the recipient.

The method of sterilization of bone alloimplants does not affect bone metabolism.

Conflict of interest. The authors declare no conflict of interest.

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STUDY OF BIOCHEMICAL MARKERS OF OSTEOGENESIS IN CASE OF BONE ALLOGRAFTS INCORPORATION IN RATS WITH FOLLOWED AFTER SURGERY ADMINISTRATION OF CISPLATIN AT THE DIFFERENT METHODS OF IMPLANT STERILIZATION

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