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ЕЖЕМЕСЯЧНЫЙ НАУЧНЫЙ ЖУРНАЛ

Медицинские новости Грузии

საქართველოს სამედიცინო სიახლენი

GEORGIAN MEDICAL NEWS

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GMN: Georgian Medical News is peer-reviewed, published monthly journal committed to promoting the science and art of medicine and the betterment of public health, published by the GMN Editorial Board since 1994. GMN carries original scientific articles on medicine, biology and pharmacy, which are of experimental, theoretical and practical character; publishes original research, reviews, commentaries, editorials, essays, medical news, and correspondence in English and Russian.

GMN is indexed in MEDLINE, SCOPUS, PubMed and VINITI Russian Academy of Sciences. The full text content is available through EBSCO databases.

GMN: Медицинские новости Грузии - ежемесячный рецензируемый научный журнал, издаётся Редакционной коллегией с 1994 года на русском и английском языках в целях поддержки медицинской науки и улучшения здравоохранения. В журнале публикуются оригинальные научные статьи в области медицины, биологии и фармации, статьи обзорного характера, научные сообщения, новости медицины и здравоохранения. Журнал индексируется в MEDLINE, отражён в базе данных SCOPUS, PubMed и ВИНИТИ РАН. Полнотекстовые статьи журнала доступны через БД EBSCO.

GMN: Georgian Medical News – საქართველოს სამედიცინო სიახლენი – არის ყოველთვიური სამეცნიერო სამედიცინო რეცენზირებადი ჟურნალი, გამოიცემა 1994 წლიდან, წარმოადგენს სარედაქციო კოლეგიისა და აშშ-ის მეცნიერების, განათლების, ინდუსტრიის, ხელოვნებისა და ბუნებისმეტყველების საერთაშორისო აკადემიის ერთობლივ გამოცემას. GMN-ში რუსულ და ინგლისურ ენებზე ქვეყნდება ექსპერიმენტული, თეორიული და პრაქტიკული ხასიათის ორიგინალური სამეცნიერო სტატიები მედიცინის, ბიოლოგიისა და ფარმაციის სფეროში, მიმოხილვითი ხასიათის სტატიები.

ჟურნალი ინდექსირებულია MEDLINE-ის საერთაშორისო სისტემაში, ასახულია SCOPUS-ის, PubMed-ის და ВИНИТИ РАН-ის მონაცემთა ბაზებში. სტატიების სრული ტექსტი ხელმისაწვდომია EBSCO-ს მონაცემთა ბაზებიდან.

WEBSITE

www.geomednews.com

К СВЕДЕНИЮ АВТОРОВ!

При направлении статьи в редакцию необходимо соблюдать следующие правила:

1. Статья должна быть представлена в двух экземплярах, на русском или английском языках, напечатанная через **полтора интервала на одной стороне стандартного листа с шириной левого поля в три сантиметра**. Используемый компьютерный шрифт для текста на русском и английском языках - **Times New Roman** (**Кириллица**), для текста на грузинском языке следует использовать **AcadNusx.** Размер шрифта - **12**. К рукописи, напечатанной на компьютере, должен быть приложен CD со статьей.

2. Размер статьи должен быть не менее десяти и не более двадцати страниц машинописи, включая указатель литературы и резюме на английском, русском и грузинском языках.

3. В статье должны быть освещены актуальность данного материала, методы и результаты исследования и их обсуждение.

При представлении в печать научных экспериментальных работ авторы должны указывать вид и количество экспериментальных животных, применявшиеся методы обезболивания и усыпления (в ходе острых опытов).

4. К статье должны быть приложены краткое (на полстраницы) резюме на английском, русском и грузинском языках (включающее следующие разделы: цель исследования, материал и методы, результаты и заключение) и список ключевых слов (key words).

5. Таблицы необходимо представлять в печатной форме. Фотокопии не принимаются. **Все цифровые, итоговые и процентные данные в таблицах должны соответствовать таковым в тексте статьи**. Таблицы и графики должны быть озаглавлены.

6. Фотографии должны быть контрастными, фотокопии с рентгенограмм - в позитивном изображении. Рисунки, чертежи и диаграммы следует озаглавить, пронумеровать и вставить в соответствующее место текста **в tiff формате**.

В подписях к микрофотографиям следует указывать степень увеличения через окуляр или объектив и метод окраски или импрегнации срезов.

7. Фамилии отечественных авторов приводятся в оригинальной транскрипции.

8. При оформлении и направлении статей в журнал МНГ просим авторов соблюдать правила, изложенные в «Единых требованиях к рукописям, представляемым в биомедицинские журналы», принятых Международным комитетом редакторов медицинских журналов - http://www.spinesurgery.ru/files/publish.pdf и http://www.nlm.nih.gov/bsd/uniform_requirements.html
В конце каждой оригинальной статьи приводится библиографический список. В список литературы включаются все материалы, на которые имеются ссылки в тексте. Список составляется в алфавитном порядке и нумеруется. Литературный источник приводится на языке оригинала. В списке литературы сначала приводятся работы, написанные знаками грузинского алфавита, затем кириллицей и латиницей. Ссылки на цитируемые работы в тексте статьи даются в квадратных скобках в виде номера, соответствующего номеру данной работы в списке литературы. Большинство цитированных источников должны быть за последние 5-7 лет.

9. Для получения права на публикацию статья должна иметь от руководителя работы или учреждения визу и сопроводительное отношение, написанные или напечатанные на бланке и заверенные подписью и печатью.

10. В конце статьи должны быть подписи всех авторов, полностью приведены их фамилии, имена и отчества, указаны служебный и домашний номера телефонов и адреса или иные координаты. Количество авторов (соавторов) не должно превышать пяти человек.

11. Редакция оставляет за собой право сокращать и исправлять статьи. Корректура авторам не высылается, вся работа и сверка проводится по авторскому оригиналу.

12. Недопустимо направление в редакцию работ, представленных к печати в иных издательствах или опубликованных в других изданиях.

При нарушении указанных правил статьи не рассматриваются.

REQUIREMENTS

Please note, materials submitted to the Editorial Office Staff are supposed to meet the following requirements:

1. Articles must be provided with a double copy, in English or Russian languages and typed or compu-ter-printed on a single side of standard typing paper, with the left margin of **3** centimeters width, and **1.5** spacing between the lines, typeface - **Times New Roman** (**Cyrillic**), print size - **12** (referring to Georgian and Russian materials). With computer-printed texts please enclose a CD carrying the same file titled with Latin symbols.

2. Size of the article, including index and resume in English, Russian and Georgian languages must be at least 10 pages and not exceed the limit of 20 pages of typed or computer-printed text.

3. Submitted material must include a coverage of a topical subject, research methods, results, and review.

Authors of the scientific-research works must indicate the number of experimental biological species drawn in, list the employed methods of anesthetization and soporific means used during acute tests.

4. Articles must have a short (half page) abstract in English, Russian and Georgian (including the following sections: aim of study, material and methods, results and conclusions) and a list of key words.

5. Tables must be presented in an original typed or computer-printed form, instead of a photocopied version. **Numbers, totals, percentile data on the tables must coincide with those in the texts of the articles.** Tables and graphs must be headed.

6. Photographs are required to be contrasted and must be submitted with doubles. Please number each photograph with a pencil on its back, indicate author's name, title of the article (short version), and mark out its top and bottom parts. Drawings must be accurate, drafts and diagrams drawn in Indian ink (or black ink). Photocopies of the X-ray photographs must be presented in a positive image in **tiff format**.

Accurately numbered subtitles for each illustration must be listed on a separate sheet of paper. In the subtitles for the microphotographs please indicate the ocular and objective lens magnification power, method of coloring or impregnation of the microscopic sections (preparations).

7. Please indicate last names, first and middle initials of the native authors, present names and initials of the foreign authors in the transcription of the original language, enclose in parenthesis corresponding number under which the author is listed in the reference materials.

8. Please follow guidance offered to authors by The International Committee of Medical Journal Editors guidance in its Uniform Requirements for Manuscripts Submitted to Biomedical Journals publication available online at: http://www.nlm.nih.gov/bsd/uniform_requirements.html
http://www.icmje.org/urm_full.pdf
In GMN style for each work cited in the text, a bibliographic reference is given, and this is located at the end of the article under the title "References". All references cited in the text must be listed. The list of references should be arranged alphabetically and then numbered. References are numbered in the text [numbers in square brackets] and in the reference list and numbers are repeated throughout the text as needed. The bibliographic description is given in the language of publication (citations in Georgian script are followed by Cyrillic and Latin).

9. To obtain the rights of publication articles must be accompanied by a visa from the project instructor or the establishment, where the work has been performed, and a reference letter, both written or typed on a special signed form, certified by a stamp or a seal.

10. Articles must be signed by all of the authors at the end, and they must be provided with a list of full names, office and home phone numbers and addresses or other non-office locations where the authors could be reached. The number of the authors (co-authors) must not exceed the limit of 5 people.

11. Editorial Staff reserves the rights to cut down in size and correct the articles. Proof-sheets are not sent out to the authors. The entire editorial and collation work is performed according to the author's original text.

12. Sending in the works that have already been assigned to the press by other Editorial Staffs or have been printed by other publishers is not permissible.

Articles that Fail to Meet the Aforementioned Requirements are not Assigned to be Reviewed.

ავტორთა საყურადღებოდ!

რედაქციაში სტატიის წარმოდგენისას საჭიროა დავიცვათ შემდეგი წესები:

1. სტატია უნდა წარმოადგინოთ 2 ცალად, რუსულ ან ინგლისურ ენებზე,დაბეჭდილი სტანდარტული ფურცლის 1 გვერდზე, 3 სმ სიგანის მარცხენა ველისა და სტრიქონებს შორის 1,5 ინტერვალის დაცვით. გამოყენებული კომპიუტერული შრიფტი რუსულ და ინგლისურენოვან ტექსტებში - **Times New Roman (Кириллица)**, ხოლო ქართულენოვან ტექსტში საჭიროა გამოვიყენოთ **AcadNusx.** შრიფტის ზომა – 12. სტატიას თან უნდა ახლდეს CD სტატიით.

2. სტატიის მოცულობა არ უნდა შეადგენდეს 10 გვერდზე ნაკლებს და 20 გვერდზე მეტს ლიტერატურის სიის და რეზიუმეების (ინგლისურ, რუსულ და ქართულ ენებზე) ჩათვლით.

3. სტატიაში საჭიროა გაშუქდეს: საკითხის აქტუალობა; კვლევის მიზანი; საკვლევი მასალა და გამოყენებული მეთოდები; მიღებული შედეგები და მათი განსჯა. ექსპერიმენტული ხასიათის სტატიების წარმოდგენისას ავტორებმა უნდა მიუთითონ საექსპერიმენტო ცხოველების სახეობა და რაოდენობა; გაუტკივარებისა და დაძინების მეთოდები (მწვავე ცდების პირობებში).

4. სტატიას თან უნდა ახლდეს რეზიუმე ინგლისურ, რუსულ და ქართულ ენებზე არანაკლებ ნახევარი გვერდის მოცულობისა (სათაურის, ავტორების, დაწესებულების მითითებით და უნდა შეიცავდეს შემდეგ განყოფილებებს: მიზანი, მასალა და მეთოდები, შედეგები და დასკვნები; ტექსტუალური ნაწილი არ უნდა იყოს 15 სტრიქონზე ნაკლები) და საკვანძო სიტყვების ჩამონათვალი (key words).

5. ცხრილები საჭიროა წარმოადგინოთ ნაბეჭდი სახით. ყველა ციფრული, შემაჯამებელი და პროცენტული მონაცემები უნდა შეესაბამებოდეს ტექსტში მოყვანილს.

6. ფოტოსურათები უნდა იყოს კონტრასტული; სურათები, ნახაზები, დიაგრამები - დასათაურებული, დანომრილი და სათანადო ადგილას ჩასმული. რენტგენოგრამების ფოტოასლები წარმოადგინეთ პოზიტიური გამოსახულებით **tiff** ფორმატში. მიკროფოტოსურათების წარწერებში საჭიროა მიუთითოთ ოკულარის ან ობიექტივის საშუალებით გადიდების ხარისხი, ანათალების შეღებვის ან იმპრეგნაციის მეთოდი და აღნიშნოთ სურათის ზედა და ქვედა ნაწილები.

7. სამამულო ავტორების გვარები სტატიაში აღინიშნება ინიციალების თანდართვით, უცხოურისა – უცხოური ტრანსკრიპციით.

8. სტატიას თან უნდა ახლდეს ავტორის მიერ გამოყენებული სამამულო და უცხოური შრომების ბიბლიოგრაფიული სია (ბოლო 5-8 წლის სიღრმით). ანბანური წყობით წარმოდგენილ ბიბლიოგრაფიულ სიაში მიუთითეთ ჯერ სამამულო, შემდეგ უცხოელი ავტორები (გვარი, ინიციალები, სტატიის სათაური, ჟურნალის დასახელება, გამოცემის ადგილი, წელი, ჟურნალის №, პირველი და ბოლო გვერდები). მონოგრაფიის შემთხვევაში მიუთითეთ გამოცემის წელი, ადგილი და გვერდების საერთო რაოდენობა. ტექსტში კვადრატულ ფჩხილებში უნდა მიუთითოთ ავტორის შესაბამისი N ლიტერატურის სიის მიხედვით. მიზანშეწონილია, რომ ციტირებული წყაროების უმეტესი ნაწილი იყოს 5-6 წლის სიღრმის.

9. სტატიას თან უნდა ახლდეს: ა) დაწესებულების ან სამეცნიერო ხელმძღვანელის წარდგინება, დამოწმებული ხელმოწერითა და ბეჭდით; ბ) დარგის სპეციალისტის დამოწმებული რეცენზია, რომელშიც მითითებული იქნება საკითხის აქტუალობა, მასალის საკმაობა, მეთოდის სანდოობა, შედეგების სამეცნიერო-პრაქტიკული მნიშვნელობა.

10. სტატიის ბოლოს საჭიროა ყველა ავტორის ხელმოწერა, რომელთა რაოდენობა არ უნდა აღემატებოდეს 5-ს.

11. რედაქცია იტოვებს უფლებას შეასწოროს სტატია. ტექსტზე მუშაობა და შეჯერება ხდება საავტორო ორიგინალის მიხედვით.

12. დაუშვებელია რედაქციაში ისეთი სტატიის წარდგენა, რომელიც დასაბეჭდად წარდგენილი იყო სხვა რედაქციაში ან გამოქვეყნებული იყო სხვა გამოცემებში.

აღნიშნული წესების დარღვევის შემთხვევაში სტატიები არ განიხილება.

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OSTEOREGENERATIVE POTENTIAL AND REMODELING OF A COMPOSITE BASED ON NANOFIBRILLATED CELLULOSE, XENOGRAFT, AND BUTVAR-PHENOLIC ADHESIVE: A HISTOLOGICAL STUDY UNDER NORMAL AND INFECTED BONE WOUND CONDITIONS

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Abstract.

Introduction: The present study aimed to conduct a comparative histomorphological evaluation of bone regeneration after implanting a biocomposite with nanofibrillated cellulose, xenograft, and butvar-phenolic glue into a rat femoral bone defect. The analysis focused on the influence of the packing method (mixed or multilayer) on bone tissue formation, remodeling, biodegradation, and graft migration.

Study Design: The experiment included 99 rats with a standardized femoral bone defect. The animals were divided into three groups: (1) negative control (defect without filling); (2) multilayer composite (layered packing of composite components); (3) mixed composite (implantation of a pre-mixed mass). Histological and histomorphometric evaluations were performed on days 30, 60, and 90 post-surgery. Additionally, an infected bone tissue wound was modeled; analogous groups, comprising 11 rats each, underwent histological evaluation on day 30.

Results: Both composite packing methods (multilayer and mixed composite) facilitated accelerated cortical plate defect closure compared to the negative control on days 30 and 60 ($p<0.01$). In the mixed composite group, composite remodeling was more uniform and involved both peripheral and central zones of the composite. Material migration in this group was significantly less frequent than in the multilayer composite group ($p<0.05$). However, the mixed composite group more frequently exhibited fibroblastic barrier formation in the defect zone than the multilayer composite group ($p<0.05$). Layered packing was associated with greater composite biodegradation and reduced composite positional stability (including intra- and extraosseous migration). Under conditions of bacterial contamination, composite implantation was accompanied by a reduction in inflammatory response intensity and a decrease in necrosis area compared to the infected control group without filling.

Conclusion:

This study demonstrated that mixed composites, including synthetic and biological components (nanofibrillated cellulose, xenograft, and butvar-phenolic adhesive), possess high osteoconductive and osteoinductive potential for bone tissue defect restoration. The obtained results indicate the material's biocompatibility and a low incidence of both early and late complications, including graft migration and degradation. Furthermore, under conditions of bacterial wound contamination, the composites exhibited pronounced barrier and antimicrobial properties, preventing bacterial colonization and the development of purulent-necrotic inflammation.

Key words. Bone defect, bone regeneration, composite, xenograft, butvar-phenolic adhesive, nanocellulose.

Introduction.

The restoration of anatomically normal bone tissue, while preserving its static strength and dynamic adaptability, remains a pressing task in modern orthopedics and traumatology. Regeneration of long bones is particularly challenging due to their pronounced anatomical heterogeneity, which includes dense compact and spongy bone, as well as the bone marrow containing hematopoietic and adipose tissue [1-3]. Successful osteoregeneration requires not only filling the bone defect but also restoring the bone's histotexture, ensuring the preservation of its mechanical properties, biological function, and ability to remodel, thereby reducing the risk of late complications.

Despite the inherent self-regenerative capacity of bone tissue, extensive injuries often necessitate the use of bone grafts or artificial substitutes [4,5]. In clinical practice, the autograft remains the "gold standard," possessing osteoconductive and osteoinductive properties and not eliciting a rejection reaction. However, its application is limited by a deficit of donor material, the need for additional surgical trauma, and the risk of complications at the donor site [6]. Therefore, there is a growing interest in the development of composite materials that combine organic matrices and inorganic components to achieve a synergistic effect from the combination of their biological and mechanical properties [7-9].

A fundamental challenge remains in finding the appropriate balance between the requirements for mechanical strength and tissue regeneration. Most synthetic biomaterial scaffolds currently cannot provide sufficient mechanical strength for use in load-bearing areas. This creates a sort of "strength-regeneration paradox": materials strong enough for load-bearing often hinder biological integration or degradation, while highly regenerative materials often lack sufficient mechanical integrity. This suggests that the future development of biocomposites lies not only in improving individual properties but also in complex

engineering that allows for achieving dynamic mechanical properties (e.g., degradation at a rate commensurate with new bone formation) or creating materials adapted for a specific patient.

Among promising materials, particular attention is given to nanofibrillated cellulose (NFC) [10]. This biocompatible polymer possesses high strength and stability, is capable of forming structured matrices that support cellular migration and angiogenesis, and demonstrates the potential for prolonged osteoinduction due to gradual biodegradation [11]. Another important component of composites is bone allograft—an accessible osteoconductive material widely used in clinical practice due to its relatively low cost and the absence of the need for additional surgery to harvest an autograft. However, it has several drawbacks: insufficient mechanical strength, the risk of bacterial adhesion, weak osteoinductivity, and slow resorption, which limits its application as a standalone graft [12].

Modern approaches aim to create multi-component composites that combine the osteoconductive properties of allograft, the structural and mechanical advantages of NFC, and additional components that enhance composite stability. One such component is a medical adhesive based on a butvar-phenolic adhesive (BF-adhesive), which possesses high adhesive capacity, can rapidly polymerize upon contact with biological tissues, creating a temporary barrier against particle displacement and fixing the composite in a desired configuration [13]. This finding is particularly relevant when using fragmented composites prone to migration beyond the defect boundary.

Polyvinyl butyral (PVB) is a well-characterized polymer with established in vitro biocompatibility and minimal cytotoxicity. However, the application of phenol-formaldehyde resins in orthopedic practice remains constrained by the risk of localized histotoxicity resulting from the potential release of residual formaldehyde. Consequently, composites incorporating phenolic components necessitate rigorous assessment of their concentration levels, degree of polymerization, and exposure conditions.

It should be considered that the use of both combined and single-component composites is associated with a number of risks, including early complications such as material migration [14], ischemic-necrotic and degenerative changes, and low resistance to infectious-inflammatory complications [15]. In the long term, there is a possibility of deterioration in the mechanical properties of regenerated bone tissue and the need for repeated surgical interventions. These limitations underscore the relevance of searching for new multi-component composites with high biological activity and stability in bone defect conditions.

Despite significant advances in the development of combined composites, the question of how the spatial organization of composite components influences the regenerative potential of the material remains underexplored. In particular, there is insufficient data on how the method of component placement (as a mixed mass or a structured layered placement) affects osteoregeneration, vascularization, and material remodeling, as well as composite migration and its integration with bone.

The purpose of the present study is a comparative histological and histomorphometric evaluation of osteoregeneration upon implantation of a multi-component composite based on NFC, bone xenograft, and BF-adhesive into a rat femoral defect using two methods: as a mixed mass (mixed composite) and as a structured layered placement (multilayer composite), compared to a group without filling (negative control).

Materials and Methods.

Preparation of the Composite based on Nanofibrillated Cellulose, Bone Xenograft, and Butvar-phenolic adhesive (BPA).

Nanofibrillated cellulose (NFC) is produced through a multi-stage chemical and mechanical process applied to nanocellulose. This results in the formation of nanofibers with diameters ranging from 5 to 100 nm and lengths from 200 nm to several tens of microns. These nanofibers exhibit a significant specific surface area of 100–200 m^2/g. NFC is essentially a nanoscale cellulose fiber, characterized by both amorphous and crystalline regions and a high length-to-diameter ratio [1].

For this study, a nanocellulose-based biocomposite was utilized. It was synthesized from nanocellulose and dicalcium phosphate dihydrate (CaHPO4·2H2O) through the following steps: initially, bleached cellulose, in the form of white fibers, underwent mechanical disintegration using a Masuko MKCA supermass 6-5 collider for eight cycles. The resulting NFC possessed an average fiber (fibril) diameter of 12 nm and an average length of 1.5 μm. Subsequently, the synthesis of the composite, comprising NFC and dicalcium phosphate dihydrate, commenced.

To achieve this, two distinct solutions were prepared. The first solution was formulated by dissolving 0.05 mol of Na2HPO4·2H2O in 300 mL of an aqueous NFC suspension. The second solution involved dissolving 0.05 mol of Ca(NO3)2·4H2O in 100 mL of distilled water. The second solution was then carefully added dropwise to the sodium phosphate solution containing the NFC suspension, while maintaining a pH between 6 and 6.5 using a 25% ammonia solution. Following the addition, the combined solutions were stirred at 400 rpm at room temperature for 1 hour. The resulting precipitate was isolated by vacuum filtration through a Buchner funnel on filter paper, washed three times with deionized water, and dried overnight at 40°C in a drying oven.

The butvar-phenolic adhesive was provided as a viscous solution, containing 1.65 g of butvar-phenolic adhesive per 100 g of adhesive. This type of medical adhesive previously underwent preclinical and clinical trials in accordance with the Canadian Environmental Protection Act, 1999 (CEPA 1999). The results concluded that the substances within the adhesive were not harmful to human health and were not released into the environment at levels detrimental to the ecosystem. We are unable to disclose the commercial name of the final product to avoid potential conflicts of interest with the manufacturer.

The bone xenograft was procured from the bone tissue bank at Professor H.Z. Makazhanov Multidisciplinary Hospital in Karaganda, utilizing the certified technology of the Marburg Bone Bank System, sourced from human femoral heads.

Our research team has previously presented the comparative

characteristics of the individual components of this biocomposite in several publications, where the efficacy of nanofibrillated cellulose and bone xenograft was demonstrated independently [11]. The results of these prior studies allowed us to shift our focus away from the performance of individual components and toward identifying the advantages and disadvantages of two distinct implantation techniques for multicomponent composites.

The combined biocomposite was prepared under absolute sterile conditions in the vivarium's operating room immediately before implantation.

For the infected bone wound group, Staphylococcus aureus (ATCC 43300) was used as the infectious agent, as it is the most frequently identified wound flora in patients with chronic osteomyelitis. The inoculum was prepared from the third passage of a Staphylococcus aureus test culture on soy broth (trypticase soy broth) and casein digest soy agar (trypticase soy agar). The suspension density was adjusted to 5 x 10^9 CFU/mL. A buffered saline solution with 15% gelatin added to increase viscosity was used as the diluent. This form of inoculum ensured the stability of bacterial content in the experimental focus for a sufficiently long period. The medium was stored at 25°C in a sterile, airtight container.

Animals and Surgical Procedures.

The animals were randomly divided into three equal groups of 33 rats each. In Group 1, the surgical procedure was performed without filling the defect. In Group 2, the defect was filled with the multicomponent biocomposite using the mixing technique, and in Group 3, the same composite was implanted using the layered placement method.

This grouping was established to comparatively characterize the advantages and disadvantages of the multicomponent biocomposite under different implantation techniques. The inclusion of the first group (empty defect) served to demonstrate that the surgically created bone defect was of critical size, meaning it was incapable of rapid spontaneous healing.

Furthermore, it is important to emphasize that the study design was specifically aimed at minimizing the number of animals used, in accordance with the 3Rs principle (Replacement, Reduction, and Refinement). This was achieved by excluding experimental groups that were deemed redundant based on our comprehensive preliminary data.

The surgical technique followed a previously developed method successfully applied in earlier research [11]. Under Zoletil anesthesia at a dose of 0.1 mg/kg (Virbac), after hair removal and three applications of antiseptic solutions to the thigh, a surgical incision approximately 20 mm long was made on the outer surface of the thigh, exposing the femoral bone. A round bone defect 2 mm in diameter and 1 mm deep was created in the mid-diaphysis of the femur using a PIT PMG150-C engraver with a 2 mm drill bit [16,17].

In the first group, the defect was not filled.

In the multilayer composite group, components were applied in layers, with nanofibrillated cellulose (NFC), xenograft, and butvar-phenolic adhesive applied sequentially to form an ordered structure. Immediately before component implantation, the xenograft was comminuted into bone chips of variable sizes, not exceeding the size of the bone defect, using Lisson bone rongeurs and a surgical chisel. The bone defect was initially filled with nanocellulose up to contact with the intramedullary space, but without filling the latter. Then, bone chips were placed into the defect. Butvar-phenolic glue (0.02 mL) was then applied over the bone defect area to completely cover the perforated opening. Layered suturing of the soft tissues and skin followed.

In the mixed composite group, a homogeneous mixture was prepared by pre-mixing the components into a uniform mass in a sterile beaker. This homogeneous, paste-like material was then implanted into the defect cavity, filling the perforated opening to the level of the periosteum.

After surgery, wounds were closed with Vicryl 5.0. Post-surgery, each animal received intramuscular Ketonal at 0.04 mL/kg (Sandoz) for 3 days. The clinical condition of the rats was assessed by measuring temperature, weight, general motor activity, and the degree of wound healing. Wound healing was considered satisfactory if there were no signs of inflammation, marginal necrosis, and the sutures remained intact.

Animals were euthanized by anesthetic overdose on days 30, 60, and 90 after surgery. The 30-day time point was chosen as optimal for evaluating primary osseointegration. By day 30, a significant amount of newly formed bone develops on the implant surface, allowing for a reliable assessment of the bone-implant contact (BIC) quality.

The 60-day time point was established because significant bone tissue remodeling occurs around the implant by this time. It allows for the evaluation of functional integration and the mechanical properties of the bone-implant connection.

The 90-day time point was selected as the final period for histomorphological assessment because it allows for the evaluation of the completion of the osseointegration process and the formation of mature bone tissue around the implant. It is considered the "gold standard" for assessing the long-term biocompatibility of materials. After euthanasia, the operated limb was dissected, and the femoral bone block containing the defect area and surrounding soft tissues was removed. The excised material was fixed in a 10% neutral formalin solution for histological analysis.

For the bacterial contamination groups, animals were randomly divided into three equal groups of 11 rats each. In the first group, the defect was infected with a microbiological culture of Staphylococcus aureus without composite filling. In the second and third groups, the cavity was inoculated and subsequently filled with multilayer composite and mixed composite, respectively, using the previously described technique.

After defect formation, the surgical field was bacterially inoculated with a suspension of bacterial cells. A laboratory culture of Staphylococcus aureus, provided by the Department of Microbiology of NPJSC "Karaganda Medical University," was used as the infectious agent. The bacterial suspension, with a concentration of 5×10^9 CFU/mL, was applied in a volume of 2 mL directly into the defect cavity using a microbiological loop.

On day 30, animals were euthanized similarly to those described above.

The study design is presented in Figure 1.

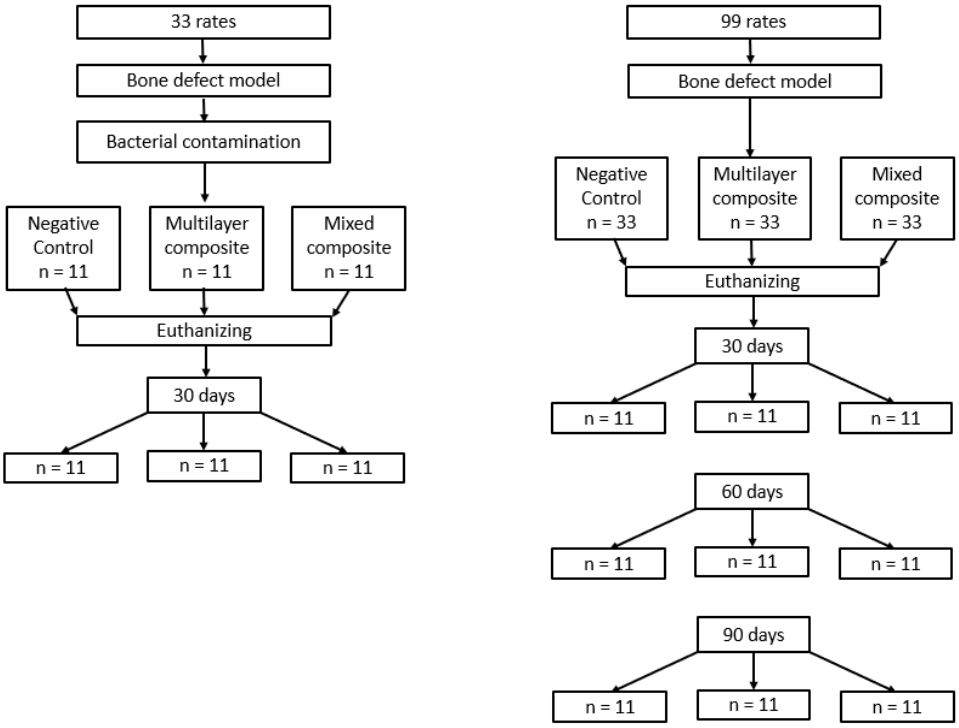

Figure 1. *Design of study.*

Histopathological Examination.

Prior to histological analysis, samples were fixed in 10% neutral buffered formalin for 24 hours, followed by decalcification in a Biodec R solution (Bio-Optica Milano SPA) for an additional 24 hours. Samples were then washed in phosphate buffer (pH 7.4). After achieving optimal softening of the bone tissue (decalcification), a bone section was taken and oriented in a histological block.

For further processing, the tissue was fixed in 10% formalin at 4°C for 24 hours, washed with tap water, and dehydrated using a series of alcohols of increasing concentration (70%, 90%, 95%, and 100%). Subsequently, the samples were immersed in xylene and embedded in paraffin blocks.

Five-micrometer-thick sections were cut using a Leica SM 2000R sliding microtome. Following preparation, tissue sections were stained with hematoxylin and eosin (H&E) for general morphological analysis, detection of inflammatory infiltration and necrosis, and Masson's trichrome for assessment of bone graft remodeling and new bone formation [18].

Hematoxylin and Eosin (H&E) Staining Procedure.

Tissue sections were immersed in hematoxylin-Eosin Mayer's reagent for 15 minutes, followed by washing with tap water for 5 minutes. Subsequently, the sections were stained with eosin for one minute.

Masson's Trichrome Staining Procedure.

For Masson's trichrome staining, a commercial kit (Masson's Trichrome BioVitrum TU 9398-001-89079081-2012) was used. After deparaffinization and rehydration, slides were placed in Bouin's solution at 56°C for 15 minutes. They were then washed in tap water for 5 minutes. Weigert's hematoxylin was applied for 5 minutes, followed by another 5 minutes of washing in tap water and a rapid wash in distilled water. The slides were then stained with Bibrich's crimson fuchsin for 5 minutes, washed in distilled water, and immersed in phosphotungstic acid-phosphomolybdic acid for an additional 5 minutes. Following this, aniline blue was applied for 5 minutes, and finally, the slides were fixed in 1% acetic acid for 2 minutes. Microscopic examination of the slides was performed on a Zeiss AxioLab 4.0 microscope at 400x magnification. Axiovision 7.2 for Windows software was used for the acquisition and analysis of micrographs of the sections.

Morphometric Examination.

H&E staining was used for visualization of overall cell morphology and tissue structure. Masson's trichrome staining was used for identification of newly formed bone tissue at different stages of bone defect repair and for assessment of bone remodeling.

Two independent observers, experienced in working with animal models, performed the morphometric examination, blinded to the group assignment of each animal. The terminology used in the histomorphometric analysis conformed to the recommendations of the Nomenclature Committee on Histomorphometry of the American Association of Mineral and Bone Scientists [19].

The following parameters were defined:

- Closure of the defect by newly formed bone tissue, expressed as a percentage;
- Composite migration;
- Composite repair;
- Graft degradation.

Histomorphometric Evaluation of Cortical Plate Tissue.

Histomorphometric evaluation of the cortical plate tissue

was performed as previously described [11]. The evaluation was conducted within an area radially bounded by the defect margins and laterally by the native femoral bone, as well as the outer boundary of the bone graft and/or newly formed bone tissue. This evaluation was presented as a percentage of the total defect area. For each bone defect, three histological sections were analyzed, and their mean value was calculated. Tissues indicating a nonspecific reparative process, such as vessels or Haversian canals, were not included in the quantification and constituted the smallest percentage in the callus area [20,21].

Composite migration was defined as the displacement of the composite beyond the cortical plate defect margins into the intramedullary space (intramedullary migration) or beyond the bone tissue into the surrounding soft tissues (external/ extraosseous migration).

Intramedullary composite migration was assessed according to the following scale:

- "0 points" - the composite is completely localized within the bone defect: no signs of displacement or scattering of the material.
- "1 point" - local displacement: ≤25% of the material volume extends beyond the defect zone.
- "2 points" - moderate migration: 25–50% of the composite is found outside the defect zone.
- "3 points" - pronounced migration: >50% of the material is detected outside the defect.

Extraosseous migration was defined as the presence of the composite outside the bone tissue in the surrounding soft tissues and was assessed on a binary scale: presence/absence.

Composite reparation referred to remineralization and remodeling with replacement of the composite by newly formed bone tissue. Composite reparation was qualitatively assessed (presence/absence) in the central and peripheral parts of the composite, as well as as a percentage of the composite area in the histological section.

Composite degradation referred to pathological changes in synthetic nanocellulose with myxoid-like biodegradation of nanofibrillated cellulose. the presence of morphological signs of necrosis, ischemic damage, and destruction of the material's structure with the formation of an amorphous mass. Composite degradation was assessed as a percentage of the composite area.

Semiquantitative Evaluation of Inflammatory Infiltrate. A semiquantitative histological analysis was performed on each histological section, from which 10 fields of interest were scanned without any overlap and captured using high-resolution software with a 400x objective.

Hematoxylin and eosin staining was used to assess the inflammatory infiltrate. Parameters of the biological response were evaluated according to the presence or absence of histological signs of osteomyelitis in the form of polymorphonuclear leukocyte infiltration and bone necrosis [22,23] and were assessed as follows:

Presence of necrosis was evaluated on a scoring scale:

- "0 points" - absent
- "1 point" - minimal (focal necrosis less than 10%)
- "2 points" - focal (focal necrotic fields occupying 11-30% of the defect area)
- "3 points" - diffuse (extensive fields of necrosis occupying more than 30% of the defect area).

Quantity and distribution of inflammatory cells present at the tissue-material interface. Polymorphonuclear leukocytes were classified as:

- Absent (0 points)
- Rare or 1–5 per high-power field (400x) (1 point)
- Moderate infiltration (2 points)
- Diffuse infiltration (3 points).

Reparative reactive lympho-macrophage environment in the defect zone was assessed as:

- "0 points" - absent
- "1 point" - lymphocytes and macrophages surround less than 10% of graft fragments
- "2 points" - 10-30%
- "3 points" - more than 30% of graft fragments.

Statistical Analysis.

Statistical analysis was performed using Statistica 10.0 and IBM SPSS Statistics 27.0 (IBM Corp., Armonk, NY, USA) software.

Quantitative data are presented as means and standard deviations (M ± SD). Categorical data are presented as absolute values and percentages of the total group size, n(%).

The $\chi 2$ statistical test with Yates' continuity correction was applied to analyze differences between groups for qualitative characteristics. The normality of the distribution of quantitative data was assessed using the Shapiro-Wilk test.

For normally distributed data, Student's t-test for independent samples was used. For non-normally distributed data, group comparisons at each experimental time point were performed using the Mann–Whitney U test (for two groups) or the Kruskal–Wallis test (for > 2 groups). Where applicable, these were followed by the Bonferroni correction for multiple testing in pairwise comparisons.

Differences were considered statistically significant at $p<0.05$.

Results.

Histological and Morphometric Analysis of Cortical Plate Defect Closure by Bone Tissue.

30 Days. At 30 days (Table 1, Figure 1), the control group (without filling) showed uneven formation of scattered bundles of heterogeneous bone tissue, predominantly thin bone trabeculae, localized at the edges of the defect. The central part of the defect remained unfilled in all cases and was represented by loose vascularized connective tissue. The average area of newly formed bone tissue was 50.8%. In 5 (45.5%) animals, the defect filling area with bone tissue was less than 50%, while in 6 (54.5%) animals, it ranged from 51% to 80%.

In the multilayer composite group, newly formed bone tissue occupied an average of 66.4% of the cortical plate defect. Histologically, bone trabeculae formed an uneven but interconnected network, partially bridging the defect. The bone tract was characterized by pronounced mineralization and active longitudinal growth in both directions from the defect edges, with signs of early remodeling leading to the formation of mature bone tissue. In 1 (9.1%) animal, defect filling was less than 50%, while in 10 (90.9%) animals, it ranged from 51% to

Table 1. *Histopathological Evaluation of Bone Defect Healing and Composite/Xenograft Resorption and Remodeling at 30 Days.*

	Group			**p-value**
	Control (without healing)	**Multilayer composite**	**Mixed composite**	
	n = 11	n = 11	n = 11	
Closure of Cortical Plate Defect by Bone Tissue, %				
<30	0 (0)	0 (0)	0 (0)	p_1=0.0001 p_2=0.001 p_3=0.365
30-50	5 (45.5)	1 (9.1)	1 (9.1)	
51-80	6 (54.5)	10 (90.1)	9 (81.8)	
>80	0 (0)	0 (0)	1 (9.1)	
Fibroplastic barier				
No	11 (100)	10 (90.9)	5 (45.5)	p_1=0.307
Yes	0 (0)	1 (9.1)	6 (54.5)	p_2=**0.005** p_3=**0.023**
Migration of composite				
Intramedullary	-	1.0 ± 0.8	0.4 ± 0.5	p_1=**0.034**
No displacement	-	3 (27.3)	8 (72.7)	
Local displacement	-	5 (45.5)	3 (27.3)	
Moderate migration	-	3 (27.3)	0 (0)	
Pronounced migration	-	0 (0)	0 (0)	
Extraosseous migration	-	0.4 ± 0.5	0.3 ± 0.5	
absence	-	7 (63.6)	8 (72.7)	p_1=0.648
presence	-	4 (36.4)	3 (27.3)	
Composite reparation				
central	-	-	11 (100)	p_1= **0.001**
peripheral parts	-	11 (100)	11 (100)	p_1=1.00
Total %		8.4 ± 4.5	17.9 ± 7.8	p_1= **0.002**
Composite degradation				
Total %		60.5 ± 5.3	52.3 ± 8.3	p_1= **0.013**

Note: p is the significance level:
$p_1 < 0.05$ - statistically significant difference between Control and Multilayer composite;
$p_2 < 0.05$ - statistically significant difference between Control and Mixed composite,
$p_3 < 0.05$ - statistically significant difference between Multilayer composite and Mixed composite

80% of the cortical plate defect area.

In the mixed composite group, defect closure reached 69.2% of the cortical plate. In some areas of the defect, bone bridges were observed, completely spanning the defect lumen, accompanied by active osteoblastic activity at the periphery of the trabeculae and a pronounced osteoid matrix. In 1 (9.1%) animal, defect closure did not exceed 50%, while in 9 (81.8%) animals, it ranged from 51-80%, and in 1 (9.1%) animal, it was more than 80% of the cortical plate defect area.

60 Days. At 60 days (Table 2, Figure 2), in the control group (without filling), the newly formed bone tissue was characterized by predominantly thin, unevenly distributed bone beams forming focal bridge-like structures with contact zones mainly located at the poles of the beams and areas of dense mineralized bone tissue containing Haversian canals of various sizes. Cortical plate defect closure averaged 63.8%. In 1 (9.1%) animal, the defect filling area was less than 50%, while in 10 (90.9%) animals, it ranged from 51% to 80%.

In the multilayer composite group, newly formed bone tissue occupied 79.7% of the defect and was represented by chaotically arranged bone trabeculae merging into lamellar structures in the cortical plate defect area. In most cases, 6 (54.5%) animals showed defect filling of 51-80%, and in 5 (45.5%) animals, it was more than 80% (Figure 3, a).

In the mixed composite group, the formation of mature trabecular bone tissue was observed, with properly organized, well-mineralized bone beams having normal thickness and spatial organization. Defect closure by newly formed bone tissue reached 82% of the cortical plate defect: in 5 (45.5%) animals, defect filling was 51-80%, and in 6 (45.5%) animals, it was more than 80%.

90 Days. At 90 days (Table 3, Figure 2), in the control group (without filling), the cortical plate defect was closed by 90.4% with bone tissue. Histologically, in 1 (9.1%) case, areas of incomplete remodeling persisted, but in most samples, 10 (90.1%), mature, uniformly mineralized bone tissue was identified, completely closing the defect area.

In the multilayer composite group, newly formed bone tissue

Table 2. *Histopathological Evaluation of Bone Defect Healing and Composite/Xenograft Resorption and Remodeling at 60 Days.*

	Group			**p-value**
	Control (without healing)	**Multilayer composite**	**Mixed composite**	
	n = 11	n = 11	n = 11	
Closure of Cortical Plate Defect by Bone Tissue, %				
<30	0 (0)	0 (0)	0 (0)	p_1=**0.0001** p_2=**0.0001** p_3=0.270
30-50	1 (9.1)	0 (0)	0 (0)	
51-80	10 (90.9)	6 (54.5)	5 (45.5)	
>80	0 (0)	5 (45.5)	6 (54.5)	
Fibroplastic barier				
No	11 (100)	11 (100)	5 (45.5)	p_1=1.000 p_2=**0.011** p_3=1.000
Yes	0 (0)	0 (0)	6 (54.5)	
Migration of composite				
Intramedullary	-	1.2 ± 1.0	0.4 ± 0.5	p_1=0.047
No displacement	-	3 (27.3)	7 (63.6)	
Local displacement	-	4 (36.4)	4 (36.4)	
Moderate migration	-	3 (27.3)	0 (0)	
Pronounced migration	-	1 (9.1)	0 (0)	
Extraosseous migration	-	0.2 ± 0.4	0.1 ± 0.3	
absence	-	9 (81.8)	10 (90.9)	p_1=0.535
presence	-	2 (18.2)	1 (9.1)	
Composite reparation				
central	-	1 (9.1)	11 (100)	p_1= **0.001**
peripheral parts	-	11 (100)	11 (100)	p_1=1.00
Total %		11.1 ± 5.5	21.3 ± 6.7	p_1= **0.001**
Composite degradation				
Total %	-	61.2 ± 8.1	48.6 ± 8.8	p_1= 0.599

Note: p is the significance level:
p_1 < 0.05 - statistically significant difference between Control and Multilayer composite;
p_2 < 0.05 - statistically significant difference between Control and Mixed composite,
p_3 < 0.05 - statistically significant difference between Multilayer composite and Mixed composite

Table 3. *Histopathological Evaluation of Bone Defect Healing and Composite/Xenograft Resorption and Remodeling at 90 Days.*

	Group			**p-value**
	Control (without healing)	**Multilayer composite**	**Mixed composite**	
	n = 11	n = 11	n = 11	
Closure of Cortical Plate Defect by Bone Tissue, %				
<30	0 (0)	0 (0)	0 (0)	p_1=0.088 p_2=0.243 p_3=0.699
30-50	0 (0)	0 (0)	0 (0)	
51-80	1 (9.1)	0 (0)	2 (18.2)	
>80	10 (90.9)	11 (100)	9 (81.8)	
Fibroplastic barier				
No	11 (100)	11 (100)	11 (100)	-
Yes	0 (0)	0 (0)	0 (0)	
Migration of composite				
Intramedullary	-	1.0 ± 0.9	0.3 ± 0.5	p_1=0.065
No displacement	-	4 (36.4)	8 (72.7)	
Local displacement	-	3 (27.3)	3 (27.3)	
Moderate migration	-	4 (36.4)	0 (0)	
Pronounced migration	-	0 (0)	0 (0)	

Extraosseous migration	-	0.1 ± 0.3	0	
absence	-	10 (90.9)	11 (100)	p_1=0.307
presence	-	1 (9.1)	0 (0)	
Composite reparation				
central	-	-	11 (100)	p_1= **0.001**
peripheral parts	-	11 (100)	11 (100)	p_1=1.00
Total %	-	21.7 ± 7.7	44.7 ± 15.4	p_1= **0.001**
Composite degradation				
Total %	-	48.4 ± 7.5	23.7 ± 13.0	p_1= **0.001**
Note: p is the significance level: p_1 < 0.05 - statistically significant difference between Control and Multilayer composite; p_2 < 0.05 - statistically significant difference between Control and Mixed composite, p_3 < 0.05 - statistically significant difference between Multilayer composite and Mixed composite				

Table 4. *Histological Evaluation of Resistance to Bacterial Inflammatory Damage.*

	Group			**p-value**
	Control (without healing)	**Multilayer composite**	**Mixed composite**	
	n = 11	n = 11	n = 11	
Ostonecrosis	1.4 ± 0.8	0.3 ± 0.5	0.4 ± 0.5	p_1=**0.002** p_2=**0.005** p_3=0.748
Polymorphonuclear leukocyte infiltration	2.0 ± 0.6	0.7 ± 0.6	0.9 ± 0.8	p_1=**0.001** p_2=**0.007** p_3=0.652
Eosinophils	0	0	0	-
Lymphocyte and macrophage infiltration	1.5 ± 0.9	0.5 ± 0.7	1.0 ± 0.6	p_1=**0.013** p_2=0.133 p_3=0.076
Bacterial contamination	0.1 ± 0.3	0	0	-
Note: p is the significance level: p_1 < 0.05 - statistically significant difference between Control and Multilayer composite; p_2 < 0.05 - statistically significant difference between Control and Mixed composite, p_3 < 0.05 - statistically significant difference between Multilayer composite and Mixed composite				

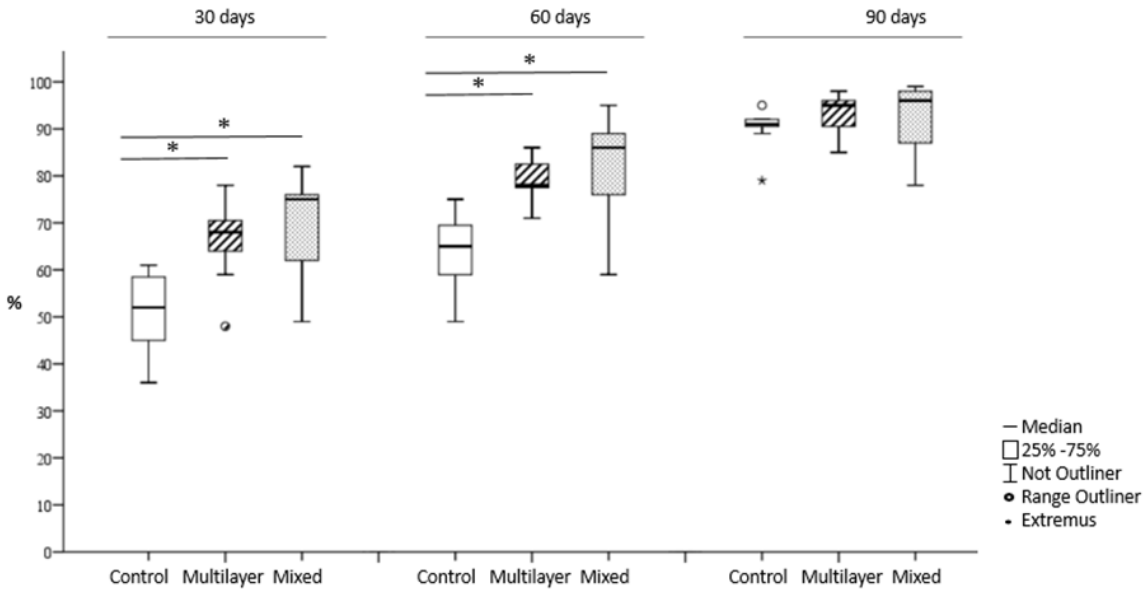

Figure 2. *Comparative histopathological assessment of the % of bone tissue in the cortical plate and intramedullary space on days 30, 60 and 90 in the study groups p < 0.05 - statistically significant difference between Control, Multilayer and Mixed composite.*

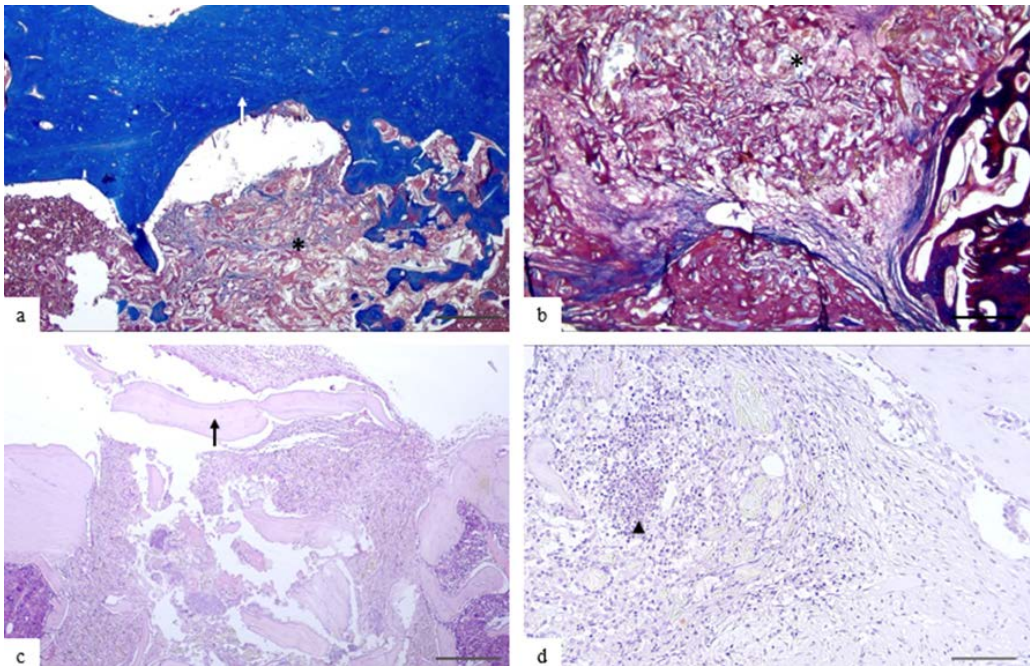

Figure 3. *Representative Microphotographs of the Cortical Plate Defect Area and Intramedullary Space Following Various Composite Filling Methods.*

a. *Multilayer Composite, Day 60. The defect zone exhibits active remineralizing remodeling: both the xenograft and synthetic matrix () are being actively replaced by newly formed bone tissue. The cortical plate is fully restored (arrow). The composite () is partially resorbed and replaced by bone regions with the formation of mature trabecular structures.*

b. *Mixed Composite, Day 30. Signs of myxoid degeneration of nanocellulose (*) are observed in the central part of the defect, resulting in the formation of an amorphous, glassy, gel-like mass.*

c. *Multilayer Composite, Day 30. Partial extraosseous migration of the bone component of the composite (arrow) is noted beyond the defect into the surrounding soft tissues. The synthetic portion of the composite remains within the bone window, completely covering the defect.*

d. *Multilayer Composite (Bacterial Contamination), Day 30. Focal inflammatory infiltration (▲) is detected in the implantation zone, predominantly composed of polymorphonuclear leukocytes, with areas of perivascular lympho-macrophage reaction. The inflammatory response is limited in scope and is accompanied by moderately expressed fibrosis and vascularization.*

(a-b) *Histochemical staining of collagen proteins with Masson's trichrome, 100× magnification, scale bar 500 µm.*

(c-d) *Hematoxylin and eosin staining, 100× magnification, scale bar 500 µm.*

in the cortical plate defect area occupied 93.3%. Histologically, mature trabecular bone tissue with normal histoarchitectonics and uniform mineralization was identified.

In the mixed composite group, complete bone tissue restoration was observed, with an average defect closure area of 91.9%.

Histological Evaluation of Composite Migration.

At 30 days (Table 1), in the multilayer composite group, intramedullary composite migration was observed in 8 (72.7%) cases: among these, local migration ($<$25%) was seen in 5 (62.5%) cases, and moderate migration (25-50%) in 3 (37.5%) cases; no pronounced migration was detected. Extraosseous composite migration was observed in 4 (36.4%) cases (Figure 3c).

In the mixed composite group, intramedullary local migration was observed in 3 (27.3%) cases; no moderate or pronounced migration was detected. Extraosseous composite migration was observed in 3 (27.3%) cases.

At 60 days (Table 2), in the multilayer composite group, intramedullary composite migration was observed in 8 (72.7%) cases: among these, local migration was recorded in 4 (50%) cases, moderate in 3 (37.5%) cases, and pronounced in 1 (12.5%) case. Extraosseous composite migration was detected in 2 (18.2%) cases.

In the mixed composite group, intramedullary local migration was observed in 4 (36.4%) cases; no moderate or pronounced migration was detected. Extraosseous composite migration was observed in 1 (9.1%) case.

At 90 days (Table 3), in the multilayer composite group, intramedullary composite migration was observed in 7 (63.6%) cases: among these, local migration was seen in 3 (42.9%) cases, and moderate in 4 (57.1%) cases. Extraosseous composite migration was detected in 1 (9.1%) case.

In the mixed composite group, intramedullary local composite migration was observed in 3 (27.3%) cases; no extraosseous migration was detected.

Histological Evaluation of Composite Remodeling and Degradation.

At 30 days (Table 1), in the multilayer composite group, no signs of composite remodeling were observed in the central part of the graft, but signs of remodeling were noted in the peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled composite was 8.4%. The average percentage of

composite biodegradation with the formation of an amorphous vitreous gel-like mass, was 60.5% (Figure 3b).

In the mixed composite group, signs of remodeling were observed in both central and peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled tissue was 17.9%. Composite biodegradation was 52.3%.

At 60 days (Table 2), in the multilayer composite group, signs of graft remodeling were observed in the central part in 1 (9.1%) case, and in the peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled graft was 11.1%. Composite biodegradation was 61.2%.

In the mixed composite group, signs of remodeling were observed in both central and peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled tissue was 21.3%. Composite biodegradation was 48.6%.

At 90 days (Table 3), in the multilayer composite group, no signs of graft remodeling were observed in the central part, but signs of remodeling were noted in the peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled graft was 21.7%. Composite biodegradation was 48.4%.

In the mixed composite group, signs of remodeling were observed in both central and peripheral parts of the graft in all 11 (100%) cases. The average percentage of remodeled tissue was 44.7%. Composite biodegradation was 23.7%.

Histological Evaluation of Resistance to Bacterial Inflammatory Damage.

At 30 days (Table 4), in the negative control group, signs of inflammatory damage with foci of necrosis were observed in 10 (90.9%) animals: among these, 1 (10%) showed diffuse damage with extensive necrotic areas occupying more than 30% of the defect area, while 3 (30%) and 6 (60%) showed moderate and minimal changes, respectively. Polymorphonuclear leukocyte infiltration was found in all 11 (100%) cases: among these, 2 (18.2%) had diffuse, 7 (63.6%) moderate, and 2 (18.2%) focal infiltration. Moderate persistence of lymphocytes and macrophages was noted, localized predominantly perivascularly and in the transition zone from granulation tissue to mature connective tissue. In one case (9.1%), clusters of bacterial colonies were found.

In the multilayer composite group, signs of inflammation with necrosis were detected in only 3 (27.3%) cases; all damage was focal, occupying less than 10% of the graft area. Polymorphonuclear leukocyte infiltration was observed in 7 (63.6%) cases: among these, 1 (14.3%) had moderate (Figure 3, d), and 6 (85.7%) had focal infiltration. Lymphocyte and macrophage infiltration was focal and minimal in severity. No signs of bacterial contamination were found.

In the mixed composite group, necrotic changes were observed in 4 (36.4%) cases, involving less than 10% of the defect area. Polymorphonuclear leukocyte infiltration was found in 7 (63.6%) cases: among these, 3 (42.9%) had moderate, and 4 (57.1%) had focal infiltration. Lymphocyte and macrophage infiltration was focal and moderate in severity. Bacterial colonies were absent.

Discussion.

This study evaluated the healing of a rat femoral diaphyseal defect using a composite based on nanofibrillated cellulose with xenograft and butvar-phenolic glue, implanted in two ways: as a mixed mass (mixed composite) and as a layered packing (multilayer composite). In the control group, the defect remained unfilled. The effectiveness of osteoregeneration was assessed based on histological and histomorphometric criteria, including the amount of newly formed bone tissue, the extent of composite remodeling and degradation processes, and the presence of signs of its component migration.

The objective of this study was to conduct a comparative assessment of how the spatial organization of a multicomponent composite (mixed versus layered placement) influences osteoregeneration, remodeling, material migration, and resistance to infectious damage, while maintaining a consistent base composition. A negative control was utilized to verify the overall biological activity of the composite, whereas the comparison between the two implantation methods allowed for an evaluation of the material's spatial organization on the regenerative process. Consequently, verifying the inherent quality of the material itself was not the primary objective of this study.

The results of the present study showed that the use of a combined multicomponent composite based on nanofibrillated cellulose, bone xenograft, and butvar-phenolic glue, both with layered packing (multilayer composite) and as a mixed mass (mixed composite), contributed to accelerated bone tissue formation in the cortical plate area compared to the unfilled control group. At 30 and 60 days, both experimental groups (multilayer and mixed composite) showed a statistically significant increase in the area of newly formed bone tissue within the cortical plate compared to the unfilled control group ($p < 0.01$). In the control group, the defect was predominantly filled with connective tissue with a moderate number of bone beams located at the defect edges, while in the groups with composite implantation, active bone formation occurred, with the formation of organized trabeculae, remineralization, and signs of remodeling characteristic of normal reparative osteogenesis. These findings are consistent with previous studies where the addition of nanofibrillated cellulose to composites facilitated earlier cortical plate restoration [11].

A comparative analysis of the two composite application methods (layered and mixed) revealed several interesting differences. While both groups demonstrated similar osteoinductive potential, the mixed composite showed more active composite remodeling with its replacement by bone tissue in both peripheral and central areas. In contrast, with layered packing, bone formation was observed predominantly at the periphery, while the central composite areas underwent biodegradation without complete replacement by bone tissue ($p<0.05$).

The more pronounced remodeling in the mixed composite group is also supported by a higher degree of graft resorption and degradation: the more homogeneous composite, mixed with finer bone particles, was replaced by newly formed bone tissue more quickly. With layered composite packing, a significant portion of the xenomaterial in the center underwent biodegradation without bone tissue replacement. We believe one reason for this difference could be the more uniform distribution of the xenograft within the cellulose matrix, which

provided better vascularization, access for microenvironment cells, and nutrients, promoting synchronous bone trabeculae formation throughout the entire composite thickness, including central deep areas.

Notably, the observed biodegradation of nanofibrillated cellulose (NFC) exerted no adverse effect on the healing process. Histological and histomorphometric analyses revealed sustained active osteogenesis and the progressive replacement of the composite with newly formed bone tissue, characterized by the absence of an intensified inflammatory response or tissue necrosis.

The analysis of composite migration deserves special attention. According to the data, with layered implantation (multilayer composite), the frequency of material migration into the intramedullary and extraosseous space and the degree of displacement were more pronounced at all observation periods compared to the mixed composite group ($p<0.05$). One possible explanation for this difference could be the structural heterogeneity of the multilayer composite, where alternating layers with different physicochemical properties (NNC, xenograft, BF-glue) create zones with varying adhesion and density. This, in turn, can lead to localized delamination and destabilization of the implant in the defect area. Conversely, the homogeneously mixed composite has a more uniform internal texture and, possibly, better adhesion between components, which contributes to its stable retention within the defect, minimizing both intra- and extraosseous migration.

When interpreting the increased migration observed in the multilayer composite group, it is essential to consider not only the biological remodeling processes but also the differences in the material's handling properties and the surgical implantation technique. Layered placement requires the sequential application of components with distinct mechanical characteristics, which impacts intraoperative handling and stabilization, thereby increasing the risk of material displacement during the early postoperative period. Conversely, the pre-mixed composite behaves as a more cohesive, homogeneous, and easily moldable mass, ensuring more reliable primary fixation.

Also, in the layered composite group, dense central zones rich in xenomaterial may hinder uniform vascular ingrowth, reducing biological integration and promoting delamination and displacement of fragments. These results emphasize the importance of not only the composition but also the spatial organization and implantation method of the composite, especially in clinical settings where material stability within the defect cavity is crucial for preventing complications and successful osseointegration.

Furthermore, in the mixed composite group, the formation of a migrationblastic barrier in the cortical plate defect area was observed in a significant proportion of animals (over 50%) at 30 and 60 days, while in the multilayer composite group, the frequency of this phenomenon was statistically significantly lower ($p<0.05$). The more frequent formation of a fibrous barrier in the mixed composite group may be related to possible micromigration of nanocellulose fragments and their direct interaction with surrounding soft tissues and the periosteum. Such interaction can initiate a local fibroblastic response with the activation of collagen synthesis, leading to the formation of a connective tissue barrier that limits the penetration of osteogenic cells into the regeneration zone, as previously shown [11]. In the multilayer composite group, conversely, the layered organization of the material, including the presence of a continuous bone xenograft and an adhesive layer between the nanocellulose and the periosteum, could create a physical barrier preventing the development of a fibroblastic barrier.

Interestingly, under conditions of bacterial contamination of the defect, the composite also demonstrated advantages over the control. Specifically, in the infected wound model in rats without defect filling, pronounced purulent-necrotic inflammation was observed: necrosis occupied more than 30% of the defect area, and in some cases, bacterial colonies were identified. In contrast, in the composite groups, no pathological bacterial accumulations were found, and inflammatory changes were focal, of moderate to minimal severity. Such an effect may be associated with several factors: first, filling the defect creates a mechanical barrier, preventing bacterial colonization [24,25], while simultaneously accelerating its vascularization, closure with granulation tissue and bone tissue, and promoting the restoration of the structural barrier. Moreover, nanocellulose itself possesses high hydrophilicity and can sorb exudate, which may facilitate wound drainage and reduce the local microbial load. These observations confirm the potential effectiveness of the biomaterial not only as an osteoconductive scaffold but also as a component with barrier and possibly antiseptic functions.

Notably, the reduction in the severity of the inflammatory response in the infected model was not accompanied by signs of tissue damage or necrosis. This suggests that the observed effect is a consequence of the composite's barrier and stabilizing properties, rather than a manifestation of cytotoxic suppression of inflammation.

A key advantage of this study is the comparative evaluation of a composite based on nanofibrillated cellulose, xenograft, and butvar-phenolic glue implanted into a bone defect, compared to a negative control, at both late (60 days) and remote stages (90 days) of bone defect repair. This allowed for the assessment of not only early regenerative processes but also the long-term stability of the results. The use of a standardized rat femoral bone defect model, along with histological and morphometric analysis, improves the reproducibility and reliability of the study.

At the same time, it should be noted that the evaluation of bone regeneration was based solely on histological methods. While informative, these methods do not comprehensively characterize the biomechanical, molecular, and functional aspects of bone restoration. Furthermore, the safety assessment of the phenol-formaldehyde glue used in this study is limited by the observation periods and histological criteria, necessitating further research to evaluate potential long-term toxic effects. These limitations should be considered when interpreting the results and designing future studies aimed at filling potential knowledge gaps.

This study showed that a combined composite based on nanofibrillated cellulose, xenograft, and butvar-phenolic glue possesses osteoinductive and osteoconductive potential

when implanted into rat femoral bone defects. The bone xenograft performed an osteoconductive function, ensuring remineralization, angiogenesis, and directed bone tissue growth, which contributed to the remodeling of the synthetic matrix from both the periphery and the depth of the defect. Nanofibrillated cellulose performed an osteoinductive function, providing a favorable microenvironment for osteogenesis during its gradual biodegradation. Butvar-phenolic glue exhibited both adhesive properties (when pre-mixed), contributing to the stabilization and homogeneity of the mass, and limiting properties (when used in layers), preventing the formation of a fibroblastic barrier upon contact with periosteal tissues [26-30].

However, despite its adhesive properties, which ensure the stabilization of multicomponent systems in bone defects, the use of butvar-phenolic glue is associated with several limitations that require consideration in the development and clinical application of osteoregenerative composites. Its rapid polymerization and high mechanical strength make butvar-phenolic glue an effective fixing agent; however, it is a chemically inert polymer that persists in tissues for a long time. This dual property leads to both advantages (fixation, migration prevention, barrier protection against infection) and potential risks, especially when the glue is used in excess or as continuous, poorly perforated layers. The formation of such dense zones within the composite can hinder vascularization, infiltration of osteogenic and immune cells, thereby slowing down osseointegration and complete remodeling. Given this, a promising direction could be the creation of bioactive or biodegradable forms of glue that combine fixation with a beneficial effect on the microenvironment. Further comprehensive studies on bone regeneration models are required to evaluate such approaches.

A limitation of the present study is that a direct comparison between single-component and multicomponent composites was not conducted. While a head-to-head comparison between the standalone xenograft and the multicomponent composite would be ideal, the current comparison of different implantation techniques alongside a negative control nonetheless provides substantial novel data regarding the handling and biological interaction of the composite material [31-41].

Conclusion.

Mixed composites, containing synthetic and biological components (nanofibrillated cellulose, xenograft, and butvar-phenolic glue), demonstrate high osteoconductive and osteoinductive potential for restoring bone tissue defects. Our findings indicate the material's biocompatibility and a low incidence of both early and late complications, including graft migration and degradation. Furthermore, in the presence of bacterial wound contamination, these composites exhibited pronounced barrier and antimicrobial properties, effectively preventing bacterial colonization and the development of purulent-necrotic inflammation.

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Conflicts of Interest.

The authors declares that there is no conflict of interest regarding the publication of this paper.

Ethical Statement.

The study was conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (2010) and approved by the Local Ethics Committee of Karaganda Medical University (No.18 20.09.2022).

Data availability statement.

All data analyzed during this study are included in this article

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Абстракт

Целью: настоящего исследования была сравнительная гистоморфологическая оценка костной регенерации при имплантации композита, включающего нанофибриллированную целлюлозу, ксенографт и n-butyl-2-cyanoacrylate в дефект бедренной кости у крыс с анализом влияния метода укладки (смешанного или послойного) на формирование костной ткани, ремоделирование, биодеградацию и миграцию графта.

Дизайн исследования: В эксперимент включено 99 крыс с формированием стандартного дефекта бедренной кости.

Животные были разделены на три группы: (1) негативный контроль (дефект без заполнения); (2) multilayer composite (послойная укладка компонентов композита); (3) mixed composite (имплантация предварительно смешанной массы). Гистологическую и гистоморфометрическую оценку проводили на 30, 60 и 90 сутки после операции. Дополнительно была смоделирована инфицированная рана костной ткани; в аналогичных группах, включающих 11 крыс, с гистологической оценкой на 30 сутки.

Результаты: Оба метода укладки композита (multilayer и mixed composite) способствовали ускоренному закрытию дефекта кортикальной пластинки по сравнению с негативным контролем на 30-е и 60-е сутки ($p<0.01$). В группе mixed composite ремоделирование композита было более равномерным и вовлекало как периферические, так и центральные зоны композита. Миграция материала в данной группе регистрировалась достоверно реже, чем в группе multilayer composite ($p < 0.05$). Однако в группе mixed composite чаще наблюдалось формирование фибропластического барьера в зоне дефекта, чем в группе multilayer composite ($p < 0.05$). Послойная укладка сопровождалась большей биодеградацией композита и снижением стабильности положения композита (включая интра- и экстраоссальную миграцию). В условиях бактериальной контаминации имплантация композита сопровождалась снижением интенсивности воспалительного ответа и уменьшением площади некроза по сравнению с инфицированной контрольной группой без заполнения.

Заключение: Данное исследование показало, что смешанные композиты, включающие синтетические и биологические компоненты (нанофибриллированную целлюлозу, ксенографт и n-butyl-2-cyanoacrylate), обладают высоким остеокондуктивным и остеоиндуктивным потенциалом при восстановлении дефектов костной ткани. Полученные результаты свидетельствуют о биосовместимости материала и низкой частоте развития как ранних, так и отдалённых осложнений, включая миграцию и деградацию трансплантата. Кроме того, в условиях бактериального загрязнения раны композиты показали выраженные барьерные и антимикробные свойства, препятствуя бактериальной колонизации и развитию гнойно-некротического воспаления.

Ключевые слова: костный дефект, регенерация кости, композит, ксенографт, наноцеллюлоза.