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Review

In vivo application of prevascularized bone scaffolds: A literature review

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ABSTRACT

BACKGROUND: Despite expanding research, the development of materials for replacing bone defects remains an urgent problem in orthopedics and traumatology. Thus, one of the most important tasks is to create conditions for proper trophicity of the bone implant.

AIM: To analyze modern approaches to bone scaffold vascularization and evaluate their adequacy in *in vivo* models.

MATERIALS AND METHODS: The article presents a literature review dedicated to the methods of vascularization of bone scaffolds. A literature search was performed in PubMed, ScienceDirect, eLibrary, and Google Scholar databases from 2013 to 2023 using keywords, and 271 sources were identified. After exclusion, 95 articles were analyzed, and the results of 38 original studies and one literature review were presented.

RESULTS: Regardless of the initial vascularization method of scaffolds, bone implants show distinct osteoinductive features and promote advanced bone tissue regeneration. Constructs based on solid polymers and calcium–phosphate compositions also perform osteoconductive functions. Mesenchymal stem cells are used as the main cell type, as well as vessel-type cells, which in cooperation also have a positive effect on bone-defect remodeling. Bone morphogenetic proteins are used for directed differentiation in the osteogenic direction, and vascular endothelial growth factor is used for differentiation in the vascular pathway.

CONCLUSIONS: At present, no method for vascularization of scaffolds has been approved universally. In addition, no evidence supported the comparative effectiveness of vascularization methods, whereas animal model studies have demonstrated a positive effect of prevascularized patterns on the recovery rate of minor and critical defects.

Keywords: prevascularized bone scaffolds; arteriovenous loops; 3D bioprinting; cell sheets.

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Научный обзор

Применение преваскуляризированных костных скаффолдов *in vivo* (обзор литературы)

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АННОТАЦИЯ

Обоснование. Создание материалов, замещающих костные дефекты, несмотря на множество работ, остается актуальной проблемой в ортопедии и травматологии. Одна из наиболее важных задач — создание условий для адекватной трофики костного имплантата.

Цель — проанализировать современные подходы к васкуляризации костных скаффолдов и оценить их адекватность в моделях *in vivo.*

Материалы и методы. Представлен обзор литературных данных, посвященный методам васкуляризации костных скаффолдов. Поиск литературы осуществляли в базах данных PubMed, ScienceDirect, eLibrary, Google Scholar в период с 2013 по 2023 г. по ключевым словам. Выявлен 271 источник. После исключения проанализированы 95 статей, результаты 38 оригинальных исследований и одного обзора литературы.

Результаты. Вне зависимости от метода предварительной васкуляризации скаффолдов костные имплантаты проявляют выраженные остеоиндуктивные свойства и способствуют ускоренному восстановлению костной ткани. Конструкции на основе твердых полимеров и кальций-фосфатных соединений также выполняют остеокондуктивную функцию. В качестве основного типа клеток используют мезенхимные стволовые клетки, а также клетки сосудистого типа, которые в синергии оказывают положительный эффект на ремоделирование костного дефекта. Для направленной дифференцировки в остеогенном направлении применяют костный морфогенетический белок, а для дифференцировки в сосудистом направлении — фактор роста эндотелия сосудов.

Заключение. В настоящее время не существует общепринятого метода васкуляризации скаффолдов, отсутствуют и данные о сравнительной эффективности методов васкуляризации, при этом в исследованиях на животных моделях продемонстрировано положительное влияние преваскуляризованных образцов на скорость восстановления как незначительных, так и критических дефектов.

Ключевые слова: преваскуляризованные костные скаффолды; артериовенозные петли; 3D-биопечать; клеточные листы.

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BACKGROUND

Nowadays, tissue engineering and regenerative medicine are among the most promising fields of healthcare research. Their popularity is attributed to great prospects in treating patients with loss of organs and tissues. The relevance of replacing large bone defects in the practice of an orthopedic traumatologist is beyond doubt. Critical diastasis as a result of fragmentation, mine blast wounds, osteomyelitis, post-resection defects, limb malformations characterized by hemimelia, control over regenerates under conditions of compression–distraction osteosynthesis, and pseudarthrosis requires replacement of a large volume of bone mass, particularly in combination with peripheral arteriopathy.

Reducing the duration of hospitalization and early initiation of patient rehabilitation is the most important issue for practical healthcare. This can be solved using synthetic and semisynthetic materials to replace defects. However, the adequacy of trophism of the bone implant remains unresolved. Based on publications on cell survival in the center of large tissue-engineered structures, the initial vascularization of the developed structures is suboptimal [1].

This study aimed to review current approaches to the vascularization of bone scaffolds and evaluate their adequacy in models *in vivo*.

MATERIALS AND METHODS

This study presents a review of literature data on the vascularization methods of bone scaffolds. The literature search was performed in PubMed, ScienceDirect, eLibrary, and Google Scholar databases from 2017 to 2023 using the following keywords: "prevascularized bone scaffolds," "arteriovenous loops," "3D bioprinting," and "cell sheets." The search yielded 128 sources. After exclusion, 95 articles, 38 original studies, and 1 literature review were analyzed.

The specific inclusion criteria were as follows: full-text materials, experimental studies on animal models of prevascularized bone scaffolds, and studies presenting comprehensive data based on which a definite conclusion can be drawn about the effect of prevascularization on bone defect restoration. Duplicate publications, including similar research protocols, conducted by different teams, use of the same materials, cells, and growth and differentiation factors, and studies by a similar team of authors were excluded from the review. If such studies were extracted, only the most recent ones by publication date were analyzed.

RESULTS AND DISCUSSION

The duration of bone tissue regeneration depends on various factors, such as damage severity, size of the diastasis between fragments, presence of concomitant damage to soft tissues and blood vessels, and premorbid background of the patient. The treatment approach plays a significant role. Bone tissue regeneration processes are divided into primary and secondary healing mechanisms. Primary healing of a damaged area required conditions, such as continuous integrity of the periosteum and stable contact of bone fragments. Secondary healing is registered in cases of periosteal damage, compact substance, bone marrow, and insufficient contact between bone surfaces caused by the lack of immobilization or relative stability in the fracture zone. Blood extravasation and hematoma formation in the fracture area occur. During blood coagulation, callus is formed from the fibrocartilaginous tissue as a complex of undifferentiated cells with multipotency. Osteoblasts are activated, which causes gradual ossification until regeneration was completed [2].

Extensive bone tissue defects have traditionally been a challenge for orthopedic traumatologists because natural regeneration is not only limited but also variable and specific to each case. Currently, the generally accepted clinical standard is the use of compression–distraction devices using Ilizarov principles. Although the long-term results of treatment are more than satisfactory, this method is not economically profitable because it involves extended hospital stay and is associated with the risks of iatrogenic and infectious complications.

Free bone grafting with blood-supplied cortical–periosteal grafts requires an operator with a high level of knowledge of microsurgical techniques. In addition, this method cannot be used in all cases, particularly in the case of massive foci of bone loss that cannot be replaced with an autograft because of its limited volume. Similar difficulties may arise when using free bone autografts. Bone allografts can unlimitedly increase the volume of the donor material and match the biomechanical requirements of the replaced site; however, its immunogenicity and survival rate are still debatable. The introduction into clinical practice of the results of research in tissue engineering can eliminate all the disadvantages of auto- and allotransplantation methods in relation to the restoration of extensive and volumetric bone tissue defects. This field aims to develop tissue-engineered structures that include a carrier material with cells and factors that can direct and accelerate cell growth and, as a result, hasten defect repair.

Cell sheets

Cell sheets are technologies that can be used to obtain a cell monolayer from the surface of a culture plastic and several such "sheets" together. Obtaining tissue-engineered structures using the layer-by-layer method is one of the first methods based specifically on cell cultures. This methodology includes several main stages:

1) Inoculation of cells and cultivation on the surface of a Petri dish until a uniform monolayer is created.
2) Separation of the layer from the surface.
3) Connecting several layers to obtain a three-dimensional structure.

In this case, often, the cell layers do not adhere to each other independently. To connect several layers, various methods are used, including mechanical, thermal, and magnetic methods, which were described in detail by Q. You et al. [3].

J. Zhang et al. [4] used structures based on human amniotic stem cells that were subjected to osteogenic differentiation using bone morphogenetic protein 2 (BMP-2). The same cell culture was taken as the second layer, whose differentiation was regulated by endothelial growth factor (VEGF) in the direction of the vascular tissue. The cell layer was implanted in a cranial defect model in rats at weeks 8 and 12 of the study. The comparison groups included control animals without treatment and groups with implants based on cell monocultures. Cell cultures accelerated bone tissue restoration compared with the untreated control group. However, no significant differences were found when using cell monocultures. In comparison with all groups, the double layer of cells ensured rapid restoration of the bone tissue. A similar design was presented in the work on mice, in which the cell layers were obtained from bone marrow mesenchymal stem cells (MSCs) and human umbilical vein endothelial cells (HUVECs) [5] and as on a culture of adipose stem cells and umbilical vein endothelial cells [6].

With repetitions of these layers, volumetric osteo-like structures can be obtained. To obtain the structures, the approach described in the above works, in which the layers adhered using magnetic particles, was used [7]. The work involved subcutaneous implantation of a sample, followed by the assessment of the expression of protein markers of endothelial formation. The use of two types of cells leads to a multiple increase in vascular network density.

A similar result can be obtained in the monoculture of cells added with two growth factors. Compared with tissue-engineered structures without using differentiation factors, the use of BMP-2 and VEGF separately did not lead to significant differences in the rate of bone defect restoration. Moreover, the addition of both substances to the MSC culture to restore a critical cranial defect in mice significantly increased the rate of defect closure [8].

Since the bone structure is more complex than the simple layering of different cells, more complex manipulations may be needed to obtain artificial structures that can exhibit biomimical properties. The use of gels, including those based on alginate, gelatin, chitosan, or silk fibroin, is one of the most common ways to achieve more complex implant architecture.

Z. Lin et al. [9] incubated human bone marrow-derived MSCs for 10 days in a gelatin methacrylate (GelMA) gel supplemented with ascorbic acid to induce extracellular matrix formation. HUVECs were cultured in gel based on GelMA and VEGF-supplemented fibrin. After cultivation, both gels were dissolved in Hanks' solution, mixed with a photoinitiator, and cross-linked under ultraviolet radiation. The finished implant was cultured for 14 days in an osteogenic culture with a VEGF-supplemented medium. A cranial defect was created in mice, which were divided into three groups. In the untreated control group, the bone tissue was not recovered. In group 1, a fresh gel was used, which was not subjected to additional cultivation; in group 2, a gel obtained using the aforementioned technology was used. The preliminary cultivation of implants ensures the growth of the vascular network and increased trypsin K level and alkaline phosphatase activity, which determines a higher rate of bone tissue remodeling than with materials without cells and differentiation factors. During histological examination, many vessels were noted at the defect edge by the end of the experiment. The co-culture of cells before implantation allows for the enhanced differentiation and production of growth factors and thereby improves bone tissue restoration.

Notably, neither of the cited works included a critical defect in bone tissue. A critical defect is a defect whose size inhibits bone tissues from performing its functions, or a defect that does not recover independently throughout the organism's life. Cell sheets do not have the mechanical strength to fill a bone defect; therefore, other methods are used.

H. Zhang et al. [10] used hydroxyapatite modified with polylysine. The cell sheet contained two cell types obtained from a rabbit, which were subsequently implanted into a critical defect in the radius. Cell sheets were formed using MSCs, osteogenic differentiation factors, and endothelial cells with VEGF. The authors demonstrated accelerated bone tissue recovery using histological methods and computed tomography. The expression of factors indicating the formation of bone tissue and vasculature was revealed. Another scaffold was obtained using a similar scheme. Two formed sheets were applied to the surface of the β-tricalcium phosphate (β-TCP) scaffold. Implantation was performed in the skull of a rat [11].

3D printing

3D bioprinting, a more accurate and advanced method, involves the use of gels that include growth factors and cells and solid polymers such as hydroxyapatite or plastics. The main advantage of this method is complete control

over the geometry, internal architecture, and composition of the resulting scaffolds.

Printing technology enables the creation of a complex architecture. W. Zhu et al. [12] obtained hexagonal structures added with HUVECs. In this case, the authors used additional cells. Two types of structures were obtained with constant and gradient sizes of hexagonal structures. A vascular network was formed using cellular methods. However, this study did not present implantation tests.

W. Zhang et al. [13] studied an alginate-based material with the addition of ceramics ($Ca_7MgSi_4O_{16}$) on a model of a critical defect in the radial bone of a rabbit. Owing to the 3D printing technique, meshes consisting of hollow cylinders were created (Fig. 1). The cavities in the cylinder were actively involved in scaffold vascularization. Structures based on hollow cylinders have greater osteogenic potential and undergo greater vascularization than structures based on solid cylinders. Using a similar scheme, samples based on an acrylamide compound added with synthetic hydroxyapatite were obtained [14]. The authors separately noted the passivity of cells when penetrating deep into the channel. They proposed local hyperthermia, leading to an increase in the cylinder lumen with subsequent normalization of temperature, which creates an artificial pressure gradient that sucks cells into the channels. A possible solution to this problem is the use of bioreactors, which are widely used in biological activities.

The use of various types of ceramics, including calcium phosphate compounds, is justified by the biochemical composition of the bone tissue. Hydroxyapatite is the main inorganic element of bone tissue; therefore, ceramics bring the artificial scaffold closer to the native bone tissue.

Nevertheless, work was also performed without using gels. J. Xu et al. [15] obtained structures based on β-TCP. Samples were implanted into a critical defect in the tibia of rats. The animals were randomly divided into two groups; in group 1, prevascularized scaffolds were implanted, and in group 2, conventional scaffolds were used. Faster bone tissue restoration was noted in the group implanted with vascularized scaffolds. Similar methods are suitable for calcium phosphate cement used as a filling material and scaffold for subantral augmentation [16]. Increasing the period of preliminary cultivation of samples leads to the formation of a more extensive vascular network [17].

C. Buckley et al. created a complex structure, including the imitation of the trabecular and cortical zones [18]. Printed hollow cylinders were lined up around the trabecular part, hydroxyapatite was placed inside some of them, and the structure was combined by electrospinning a nonwoven material onto the scaffold surface. For prevascularization, human dermal microvascular endothelial cells cultured in a ready-made differentiation medium were used. Implantation tests were performed on female New Zealand white rabbits, from which an 8-mm fragment of the radial bone was removed. Comparisons were made with bone allografts. After 8 weeks, bone tissue began to form around the scaffold, and despite the load, the material structure was not disturbed; whereas in the comparison group, bone tissue growth was difficult to assess because of graft density, and an additional study showed a decrease in the timing of remodeling in the center of the defect.

In comparison with the layer-by-layer formation of tissue-engineered structures, 3D-printing technology enables the creation of objects for implantation in bone tissue areas subject to significant mechanical loads. Musculoskeletal function is one of the main functions of the skeleton; therefore, technologies for creating scaffolds that can replace a critical defect in a loaded area are of great

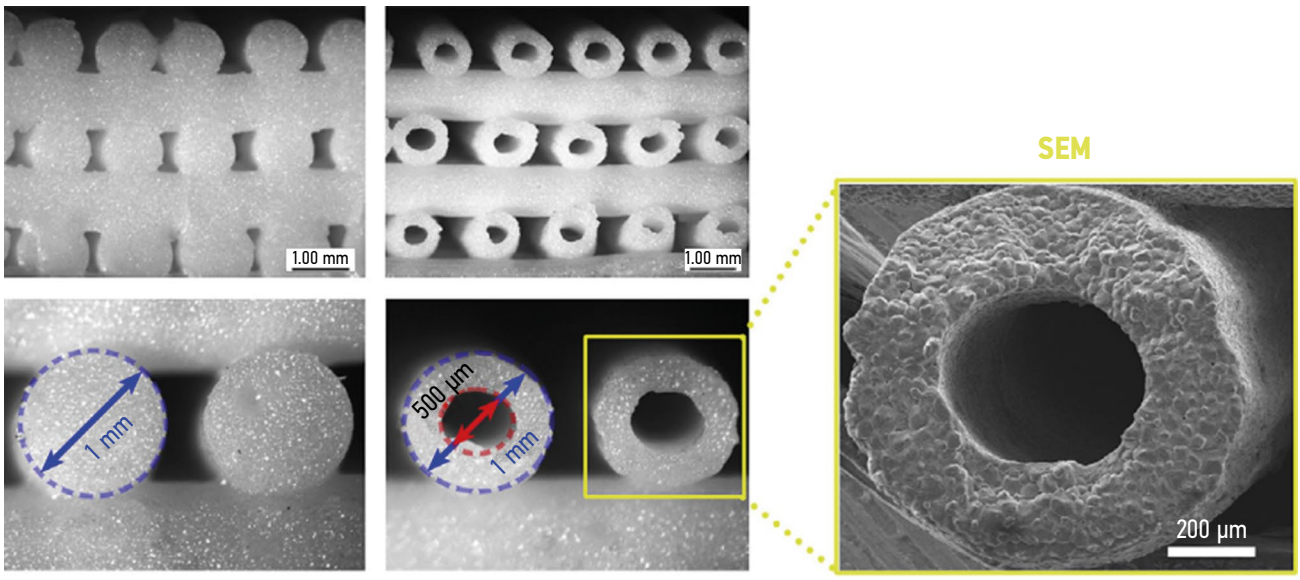

Fig. 1. A mesh structure based on hollow and blind cylinders obtained by 3D printing [13]

clinical demand. Printing technologies with plastics such as polylactide, polycaprolactone, and others enable the creation of more durable structures. Using 3D-modeling methods, a complex geometry can be obtained, which corresponds to the topology of the bone tissue defect and the mechanics of the implanted bone site, which can hypothetically allow the restoration of mobility in a short time and avoid shielding stress that destroys bone tissue.

To obtain scaffolds, J. Nulty et al. [19] used a polycaprolactone-printing method, which performed an osteoconductive function. The cylinders were filled with a gel with MSCs and endothelocytes, some scaffolds were cultured to form the native cell environment and extracellular matrix, and another part was subjected to vascularization. The samples were implanted in a rat femur fragment; parts of the bone above and below the implantation area were fixed with a plate to prevent displacement of the fragments. As in other studies, the use of two cell types, as well as prevascularization, accelerated bone remodeling compared with other samples. In addition, GelMA activated the growth of the vascular network in comparison with alginate- and fibrin-based gels. S.Y. Hann et al. used a similar approach [20].

In the study by C. Li et al. [21], the vessels were printed with a gel deposited in a $CaCl_2$ solution. The formed hollow microtubules were added to gel-like scaffolds and wrapped around the cells of solid blocks. Subcutaneous implantation was performed, which served as a method of *in vivo* prevascularization of the sample; as a result, the vascular network grew, and new vascular structures were formed.

According to T. Anada et al. [22], coaxial cylinders can be produced using 3D printing. The outer cylinder contains GelMA added with calcium phosphate and stem cells, whereas the inner, thinner one contains GelMA with VEGF and endothelial cells.

Despite the exact coincidence of the scaffold preparation method in the work by J. Nulty et al. [19], M.A. Kuss et al. [23] used three types of controls, including gel-based scaffolds with single cells, cell spheroids, and scaffolds without cells. Compared with spheroids, the scaffold with two cell types showed significant positive results, with better results in samples with non-encapsulated cells, which can be due to free migration, adhesion, and proliferation. However, in the case of spheroids, a larger number of vessels are formed with an area of >200 μm^2 compared with other types of scaffolds.

Arteriovenous (AV) loops

To solve the problem of trophism and vascular network formation, blind-closed and through AV bundles and shunted AV loops can be used [24]. AV loops are used to vascularize scaffolds when placed in direct contact with the vessel. In this case, the structure is placed in an isolating chamber, which neutralizes the influence of adjacent tissues on the bone scaffold.

A. Weigand et al. [25] used an AV loop model in sheep. By week 12, microvascular network formation was noted; by week 18, the number of vessels decreased, whereas the vessels formed in the scaffold grew. The AV loop was formed to pass through the interior of the material, ensuring vascular ingrowth from the inside, and touching the outer portion, promoting vascular ingrowth into the scaffold.

Studies have presented data on the use of an AV loop in combination with cell-containing materials. D. Steiner et al. [26] used calcium compound scaffolds added with endothelial progenitor cells, MSCs, and both cells simultaneously, which were placed in an AV loop created in a rat model. Vascular network formation was detected in all samples, and matrices containing both cell types showed better results than the use of cell monocultures. S. Kratzer et al. [27] presented similar data in a similar model; they used polylactide as a scaffold material and a 3D printing method. Moreover, S. Kratzer et al. and A. Eweida et al. [28, 29] studied a scaffold made of caprolactone and type 1 collagen, obtained by electrospinning, with a fibrin gel layer (Fig. 2).

Different gels in AV loop models have different effects. Thus, upon GelMA implantation, microvessels were formed on day 14, and signs of their restructuring and maturation were registered on day 21. The fibrin gel did not show significant vascular growth, as noted by J. Nulty et al. [19]; in addition, blood clots formed in the loop using fibrin, which R. Vaghela et al. [30] associated with the fibrin scaffold resorption.

Because of natural factors, AV loops cannot regulate processes occurring in the scaffold. A fibrin gel with stromal vascular fraction increases the production of fibroblast growth factor (FGF), VEGF and TGF, which regulate cell proliferation and differentiation. B.C. Kim et al. [31] obtained different results, which indicated vasculature formation 2 weeks after implantation. Despite the use of a fibrin gel, the authors did not reveal thrombogenesis in the AV loop. In a similar model, dimethylglyoxime was injected, which was a factor that accelerates the vascular network formation [32].

J. Biggemann et al. [33] used a 3D printing method of a scaffold, which enabled the creation of a complex architecture of samples with different shapes and pore sizes, which were controlled manually. According to the authors, such a complex geometry will allow the formation of vessels of various diameters, which corresponds to native tissues of the body, including many collateral branches, in addition to the main ones.

A. Kengelbach–Weigand et al. used similar designs in sheep [34]. A tibial fragment was removed from the sheep. The material was surrounded by an AV loop and incubated in

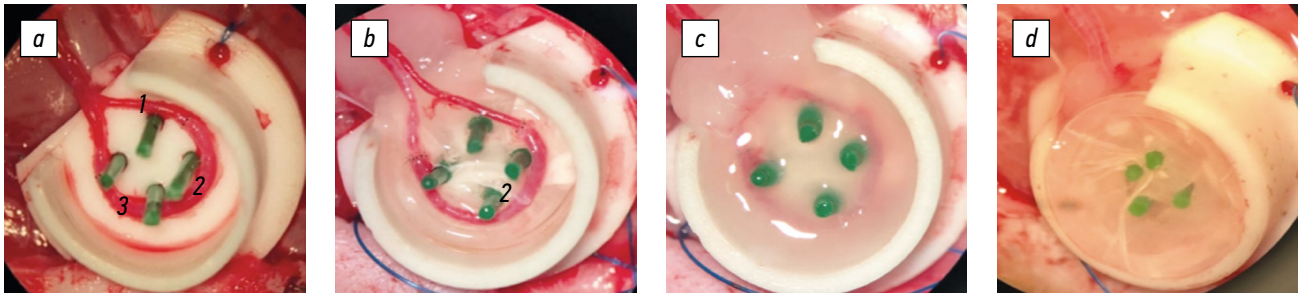

Fig. 2. Arteriovenous loop, presented by S. Kratzer et al. [28]: *a*, formation of an arteriovenous loop in the chamber; *b*, chamber made of polyethylene terephthalate (PET), including four holders and two layers of scaffold; *c*, fibrin gel on the scaffold surface; *d*, two additional layers of nanofiber scaffold. *1*, inferior epigastric artery; *2*, joint; *3*, inferior epigastric vein

the same animal that subsequently underwent implantation. According to the results of computed tomography and histological examination, bone tissue formation was noted 12 weeks after implantation. Angiographic and pathological findings also indicated that the AV loop samples had more extensive vasculature and that the VEGF samples had greater potential than the samples without growth factors.

Methods similar to the AV loop include the vascular bundle method, where a blind closure of the vessel feeds the implanted scaffold. In canine models, this approach can accelerate the restoration of bone tissue due to the active trophism of cells placed in the implanted sample. With this approach, a much smaller number of vessels were formed than with the complete AV loop [35].

Y.P. Yang et al. used the AV loop analog [36]. In a sheep model of the iliac bone defect, the authors implanted a specimen that partially protruded from the defect area. In a sample 3D-printed using polycaprolactone with the addition of β-TCP, two cylindrical recesses were made, behind which the deep circumflex iliac artery and accompanying vein were removed. This technique does not require separate preliminary exposure of the scaffold in an isolation chamber. Using a similar method, results were obtained on a 5-cm sheep hind limb defect model [37]. L. Vidal et al. presented a similar approach without using an isolation chamber in a rabbit model [38]. T. Kawai et al. [39] placed the formed AV loop in a hollow cylinder, which was implanted instead of a femoral fragment.

Thus, AV loops create adequate, close-to-native trophism in the material area, which promotes cell adhesion and proliferation. Vascular cells can induce the differentiation and growth factors that influence cells implanted with the matrix and accelerate vasculature formation without using third-party agents.

CONCLUSIONS

Despite the successes achieved by the authors of the analyzed works, currently, no ready-made technologies have been established for the prevascularization of implants of any type that would be used in clinical practice. Nevertheless, bone scaffolds subjected to preliminary vascularization accelerate the restoration of defects, which is of interest in pediatric traumatology and orthopedics, where autograft collection is extremely difficult.

Solid structures based on plastic or calcium phosphates contribute to the osteoinductive properties of tissue-engineered structures. Because various solid structures are available, approximating the developed material closer in mechanical properties to native bone tissue is possible, which will subsequently avoid the effect of stress shielding that destroys the bone tissue adjacent to the implanted sample.

Carrier gels added with cells are an important part of bone plastic materials being developed. Collagens and GelMA improve cell proliferation and help overcome possible difficulties with cell adhesion to the surface of a rigid scaffold. In the combination of several cell types, mainly MSCs and HUVEC, significantly more pronounced positive effects were noted in the formation of both the vascular network and bone tissue.

The use of differentiation factors can help replace the use of two cell types or increase the rate of bone repair and vascular network formation. The most promising combination is that of several cell types with the addition of BMP-2, which is involved in the differentiation in the osteoblastic direction, and VEGF, which promotes vascular network formation.

ADDITIONAL INFORMATION

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Author contributions. *Yu.A. Novosad* and *P.A. Pershina* analyzed literary data and wrote the article. *A.S. Shabunin* and *M.S. Asadulaev* edited the text of the article. *O.L. Vlasova* and *S.V. Vissarionov* created the concept and design of the study.

All authors made significant contributions to the study and preparation of the article, and read and approved the final version before its publication.

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