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ORIGINAL ARTICLE

CULTIVATION OF CELLS IN ALGINATE DROPS AS A HIGH-PERFORMANCE METHOD OF OBTAINING CELL SPHEROIDS FOR BIOPRINTING

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ABSTRACT

Purpose of the study. Testing the protocol of obtaining cell spheroids of breast cancer cell cultures for bioprinting by growing in alginate drops.

Materials and methods. Cells of breast cancer cell lines BT-20 and MDA-MB-453 were cultured in DMEM medium supplemented with 10 % FBS. Next, the cells were removed from the plastic using a trypsin-Versene solution and resuspended in a sterile 2 % alginate solution in DPBS to the concentration of 10^5 cells/ml. Then the alginate solution with the cells was slowly dripped through a 30G needle into a sterile cooled solution of calcium chloride (100 mM) from a height of 10 cm. After polymerization, alginate drops were washed in DMEM and cultured for two weeks in DMEM with the addition of 10 % FBS at 37 °C and 5.0 % CO_2.The spheroids formed in the alginate were photographed on the 3rd, 7th, 10th, and 14th days of cultivation, after which they were removed from the alginate by keeping in 55 mM sodium citrate solution with the addition of 20mM ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin blocks according to the standard method, followed by histological examination.

Results. Cellular spheroids were formed in both cell cultures already on the 3rd day of cultivation. From the 3rd to the 10th day in both cultures, a uniform growth of cell spheroids was observed with a gradual slowdown in the increase in the size of spheroids by the 14th day of cultivation. On the 10th day the proportion of cells that formed clones (more than 500 μm^2 in size) was 25.2 % ± 7.1 % (n = 25) in the BT-20 culture and 38.5 % ± 9.9 % (n = 25) in MDA-MB-453 culture. On the 14th day, BT-20 culture was characterized by spheroids varying little in size and shape, with an average area of 1652 ± 175 μm^2, having a dense structure with smooth edges. The spheroids in MDA-MB-453 culture turned out to be more loose and easily deformed, their size and shape varied noticeably, the average area of the spheroids was 2785 ± 345 μm^2.

Conclusion. The production of spheroids in alginate drops is inferior in speed to the methods of forming cell conglomerates in hanging drops or on microwells, but it surpasses these methods in productivity, which is comparable to the production of spheroids by constant medium stirring on low-adhesive substrates. In addition, the clonal nature of the obtained spheroids leads to an increase in research costs and thus limits their scalability.

Keywords: 3D cell culture, cell spheroid, alginate, bioprinting

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ОРИГИНАЛЬНАЯ СТАТЬЯ

КУЛЬТИВИРОВАНИЕ КЛЕТОК В АЛЬГИНАТНЫХ КАПЛЯХ, КАК ВЫСОКОПРОИЗВОДИТЕЛЬНЫЙ МЕТОД ПОЛУЧЕНИЯ КЛЕТОЧНЫХ СФЕРОИДОВ ДЛЯ БИОПЕЧАТИ

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РЕЗЮМЕ

Цель исследования. Тестирование протокола получения клеточных сфероидов культур рака молочной железы (РМЖ) для биопечати путём наращивания в альгинатных каплях.

Материалы и методы. Клетки культур BT-20 и MDA-MB-453 культивировали в среде DMEM с добавлением 10 % FBS. Далее клетки снимали с пластика при помощи раствора трипсин-Версена и ресуспендировали в стерильном 2 % растворе альгината, приготовленном на DPBS, до концентрации 10^5 кл/мл. Раствор альгината с клетками исследуемых культур РМЖ медленно капали через иглу 30G в стерильный охлажденный раствор хлорида кальция (100 мМ) с высоты 10 см. После полимеризации альгинатные капли отмывали в среде DMEM и культивировали в течение двух недель в среде DMEM с добавлением 10 % FBS при 37 °C и 5,0 % CO_2. Образующиеся в альгинате сфероиды фотографировали на 3-, 7-, 10- и 14-е сутки культивирования, после чего их извлекали из альгината путём выдерживания в 55мМ растворе цитрата натрия с добавлением 20мМ этилендиаминтетрауксусной кислоты (ЭДТА) и заключали в парафиновые блоки по стандартной методике с последующим гистологическим исследованием.

Результаты. Клеточные сфероиды-клоны образовывались в обеих культурах уже на 3 сутки культивирования. С 3 по 10-е сутки в обеих культурах наблюдался равномерный рост клеточных сфероидов с постепенным замедлением увеличения размеров сфероидов к 14-му дню культивирования. Доля клеток, образовавших клоны (размером более 500 мкм^2), на 10-е сутки составила 25,2 % ± 7,1 % (n = 25) в культуре BT-20 и 38,5 % ± 9,9 % (n = 25) в культуре MDA-MB-453. На 14-е сутки для BT-20 были характерны сфероиды, мало варьирующие по размеру и форме, средней площадью 1652 ± 175 мкм^2, обладающие плотной структурой с ровными краями. Сфероиды из клеток культуры MDA-MB-453 оказались более рыхлыми и легко деформирующимися, их размеры и форма заметно варьировали, средняя площадь сфероидов составила 2785 ± 345 мкм^2.

Заключение. Получение сфероидов в альгинатных каплях уступает по скорости методам формирования клеточных конгломератов в висячих каплях или на микроячейках, однако превосходит эти методы по производительности, которая сравнима с получением сфероидов на низкоадгезивных подложках путём постоянного перемешивания среды. Кроме того, клональная природа получаемых сфероидов приводит к увеличению трат на проведение исследований и ограничивает, тем самым, их масштабируемость.

Ключевые слова: трёхмерная клеточная культура, клеточный сфероид, альгинат, биопечать

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BACKGROUND

Breast cancer (breast cancer) remains an urgent problem of modern healthcare [1; 2]. In the structure of the incidence of malignant neoplasms of the female population in Russia, breast cancer occupies the first place [3; 4], its incidence in 2021 accounted for 22.1 % of new cases, and the average annual rate of increase in morbidity from 2011 to 2021 was 1.72 %. In terms of mortality, this pathology also ranks first among malignant diseases in women – breast cancer accounted for 15.8 % of all cancer deaths in 2021 [5]. The reduction of mortality depends on the development and introduction of new drugs and approaches to the treatment of tumors. Despite the progress in the discovery of new antitumor agents, only a small part of the substances that have demonstrated effectiveness in preclinical studies on cell cultures and animal models are successfully undergoing clinical trials. Scientists agree that the main reason for this state of affairs is the discrepancy between the biological characteristics of monolayer cell cultures and tumors grown in animal models and the characteristics of human tumors [6]. In particular, it is known that the cultivation of cancer cells in two-dimensional cultures leads to a change in their phenotype and loss of expression of molecules of key signaling pathways, which these cells demonstrate *in vivo* in the body of patients [7]. In this regard, it is urgent to create new models of tumor growth that would combine the mass and reproducibility characteristic of *in vitro* cell cultures and the complexity demonstrated by animal models. One of the promising directions of the search is to recreate the three-dimensional microenvironment of the tumor *in vitro* by combining various components using bioprinting methods [8]. Researchers use various materials and approaches to the construction of such models, but in general they are malignant cells and microenvironment cells enclosed in biogels of different chemical nature and origin. In this case, most often the cells are located in the thickness of the biochernils singly, at a distance significantly exceeding that observed in situ [8]. Such a structure of the model obviously does not allow to fully reflect the biological features of the tumor, so attempts are being made to introduce cellular spheroids into bioprinted designs – the simplest models of tumor nodes that have proven themselves well in the practice of preclinical studies [9]. As a rule, cell spheroids for bioprinting are obtained by methods of mechanical agglomeration of cells by suspending a cell suspension in droplets, using small-cell plates or special matrices, culturing on a low-adhesive substrate with constant mixing of the medium, as well as by directly imprinting a biogel with a high concentration of cells into the matrix model [10]. One of the least common approaches to obtaining cellular spheroids for bioprinting is their cultivation from single progenitor cells. However, spheroids obtained in this way, in our opinion, are more adequate models of micrometastases or early stages of tumor node formation than mechanical cell conglomerates, since each cellular spheroid in this case is a clone of one cell. In addition, the tumor model assembled from individual clones is more consistent with the structure observed in a heterogeneous tumor in situ. As a rule, spheroids-clones are obtained by culturing tumor cells in a biogel that does not support cell adhesion. The most popular biogel for producing spheroids is soft agar or agarose gels, which are traditionally used by researchers to estimate the number of tumor stem cells (TSC) in culture [11]. However, to obtain spheroids suitable for bioprinting, this method is not applicable, since the extraction of cells from agarose gel is associated with heating and mechanical action, which reduces their viability. We suggested that encapsulation of cells in alginate droplets could be considered as a promising method for obtaining spheroids for bioprinting. Previously, this approach was tested by us to study the properties of stemness in adhesive cell lines of colorectal cancer [12]. Alginate gel, like agarose, does not support adhesion, but its advantage is the ability to rapidly depolymerize under the action of agents chelating calcium ions, which allows you to quickly extract the resulting spheroids under physiological conditions and then use them in bioprinting. Analysis of the literature data has shown that the approach based on the build-up of spheroids in alginate, followed by purification and conclusion of biogel, has not yet been applied by anyone. In some studies, bioprinting of a tumor model with single cells in an alginate-gelatin biogel is found, followed by the formation of spheroids-clones directly in the resulting structure [13]. In contrast to our proposed approach, this method of including spheroids in the model does not make it possible to widely vary the

composition of biochernils, and also does not allow for precise adjustment of the composition and location of other elements of the model.

The purpose of the study: development and testing of a protocol for obtaining spheroids of breast cancer cultures for bioprinting by building up in an alginate gel.

MATERIALS AND METHODS

BC cell cultures BT-20 and MDA-MB-453 served as the material for the study. The cells were cultured in DMEM (Gibco) medium with the addition of 10 % FBS (Hyclone). When the monolayer of cells reached 70 % confluence, they were removed from the plastic using a trypsin-Versene solution (1:1; Biolot, Russia). Next, the cells were resuspended in a sterile 2 % alginate solution (Sigma) prepared on DPBS (Biolot) to a concentration of 10^5 cells/ ml. The alginate solution with the cells of the studied breast cancer cultures was slowly dripped through a 30G needle into a sterile cooled calcium chloride solution (100 mM) from a height of 10 cm. Upon contact with a solution of calcium chloride, alginate droplets were instantly cured and beads with a diameter of 2.5–3 mm were formed (Fig. 1). The obtained beads were kept for additional polymerization in a solution of calcium chloride for another 5 minutes and washed once with a cooled DMEM medium, after which they were placed in a cultivation medium consisting of DMEM with the addition of 10 % FBS. Next, alginate beads with breast cancer culture cells enclosed in them were cultivated for 14 days with medium replacement every three days. Spheroids formed in alginate were photographed on the 3rd, 7th, 10th and 14th days of cultivation. In the obtained images, the size of the spheroids was determined using the ImageJ package. Statistical processing of the results was carried out using MS Excel software.

On the 14th day of cultivation, alginate was dissolved by holding beads in a 55mM sodium citrate solution with the addition of 20mM ethylenediaminetetraacetic acid (EDTA) for 3 minutes at room temperature. The isolated spheroids were washed twice in a culture medium and placed in a 5 % agarose gel, which, after curing, was enclosed in a paraffin block according to a standard procedure. The histological structure of the obtained cellular spheroids was studied on sections stained with hematoxylin-eosin.

RESEARCH RESULTS AND DISCUSSION

Both cell cultures in alginate drops showed growth already on the third day of cultivation. At the same time, the BT-20 culture formed approximately the same rounded spheroids in size, while the cells of the MDA-MB-453 culture formed spheroids of different shapes and sizes (Fig. 2a, b). The difference in the

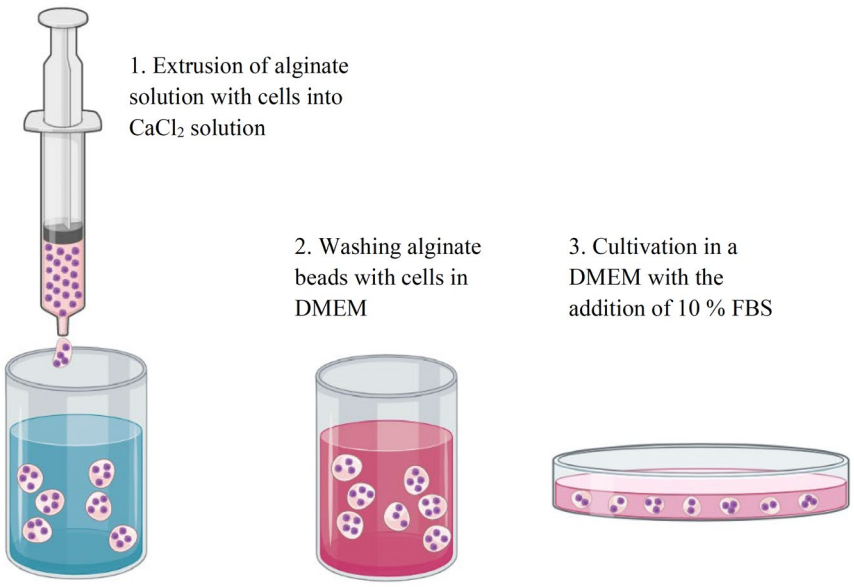

Fig. 1. Schematic representation of the protocol for obtaining cellular spheroids in alginate droplets.

growth pattern of spheroids remained on the 10th day of cultivation (Fig. 2b, d).

Variability in the size and shape of spheroids can be a negative phenomenon for those bioprinting applications in which measurement of the size of spheroids in the composition of the resulting contruct is required in response to various influences. As, for example, it is required in the case of bioprinting a tumor growth model. If compared with other methods of spheroid formation, the proposed method is inferior in this indicator to methods of direct imprinting of a thick suspension of cells into the matrix model and methods of controlled agglomeration of cells in hanging droplets or microlunks, but comparable to free-floating spheroids-conglomerates on low-adhesive substrates [4]. To overcome this disadvantage, it is possible to propose the use of various methods of separation (filtration, centrifugation) of spheroids-clones in size before printing or the use of automatic microscopes with precise positioning of the slide table for the analysis of the resulting models, which will allow taking into account the behavior of each spheroid separately.

From the 3rd to the 10th day of cultivation, uniform growth of cellular spheroids was observed in both cultures with a gradual slowdown in the increase in the size of spheroids by the 14th day of cultivation. At the same time, on average, the size of the MDA-MB-453 spheroids was larger than that of BT-20 (Fig. 3). For BT-20, spheroids with an average area of 1652 ± 175 μm^2 were characteristic, and the average area of spheroids formed by MDA-MB-453 culture cells was 2785 ± 345 μm^2 . This phenomenon can be explained by the semi-adhesive nature of the growth of the MDA-MB-453 culture on culture plastic, which gives the cells of this culture an advantage even under growing conditions in an alginate gel that does not support cell adhesion.

Thus, the optimal time for collecting spheroids for the purpose of further bioprinting for these crops is 7–10 days of cultivation. This rate of spheroid formation is comparable to free-floating spheroids-

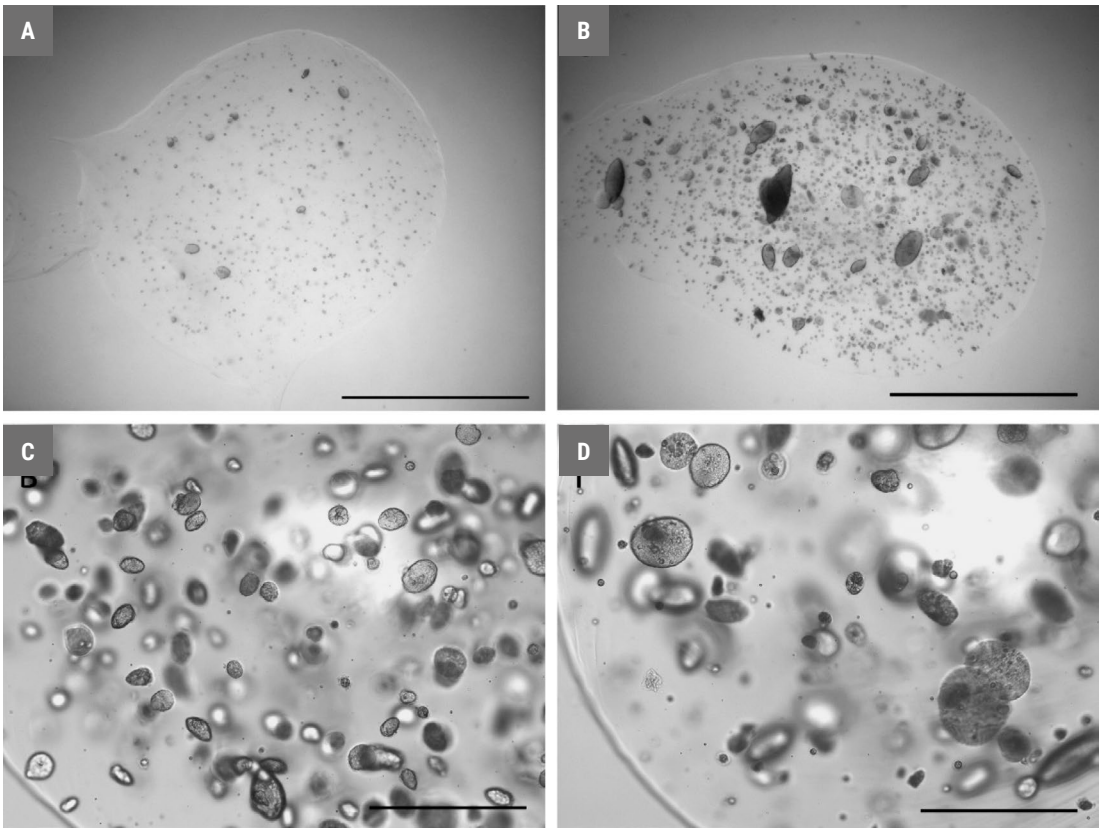

Fig. 2. Beads made of 2 % alginate gel with breast cancer culture cells enclosed in them. a – culture BT-20 for the 3rd cultivation knocks; b – culture MDA-MB-453 for the 3rd cultivation knocks; c – culture BT-20 for the 10th cultivation knocks; d- culture MDA-MB-453 for the 10th cultivation knocks. The size of the scale lines: a, b – 3 mm; c, d – 0.5 mm.

conglomerates on low-adhesive substrates, but significantly inferior to the rate of formation of cellular spheroids with controlled agglomeration of cells in hanging droplets or microlunks, where this indicator can range from 3 to 48 hours, depending on the materials used [10].

On the 10th day of cultivation, when the increase in the size of the spheroids begins to stabilize and it is possible to proceed to bioprinting, we measured the proportion of cells that formed clones (larger than 500 microns). Data analysis showed that this indicator was 25.2 % ± 7.1 % (n = 25) in BT-20 culture and 38.5 % ± 9.9 % (n = 25) in MDA-MB-453 culture. Thus, at a concentration of 100 thousand cells/ml, from one milliliter of alginate gel with BT-20 and MDA-MB-453 culture cells, 25–38 thousand spheroids suitable for further bioprinting can be obtained, which is orders of magnitude greater than the capabilities of even high-performance methods of spheroid formation in microlunks and hanging droplets and comparable to the formation performance free-floating spheroids-conglomerates on low-adhesive substrates [10]. The yield of cellular spheroids and their growth rate can be increased even more if growth factors such as EGF and FGF, which enhance cell division under conditions of reduced adhesion, are used [12].

The histological structure of spheroids after extraction from alginate gel differed between cultures. BT-20 was characterized by spheroids of dense structure with smooth edges, retaining their shape after extraction from the gel, double washing and inclusion in the agarose block (Fig. 4a). Spheroids from the cells of the MDA-MB-453 culture turned out to be more loose, easily deformed with the loss of part of the cells from the surface layers after washing in the culture medium (Fig. 4b). The observed differences may be associated with a smaller amount of extracellular matrix formed by cells of the MDA-MB-453 culture in comparison with the BT-20 culture.

The structure of spheroids plays an important role in bioprinting. Loose decaying cellular conglomerates lose their shape during extrusion through the nozzle, which negatively affects the quality of measurement results carried out on a 3d model of tumor growth obtained by bioprinting using such spheroids. Cellular conglomerates obtained by aggregation in a hanging drop, as a rule, have a very dense structure and mechanical strength [14], as well as spheroids obtained in microplates [10], which favorably distinguishes these approaches from our proposed one. At the same time, free-floating spheroids-conglomerates on low-adhesive substrates, are comparable or even more inferior in strength to the spheroids-clones obtained by us. The selection of cell cultures that secrete a large number of extracellular matrix molecules (collagen, laminin, hyaluronic acid, and others) is likely to improve the practice of obtaining spheroids in alginate drops with the density and strength characteristics necessary for the needs of bioprinting.

A characteristic feature of the spheroids obtained by us from the cells of both cultures was the absence of a central apoptosis/necrosis region, which is probably explained by their small size. It is known that the first signs of cellular damage under the influ-

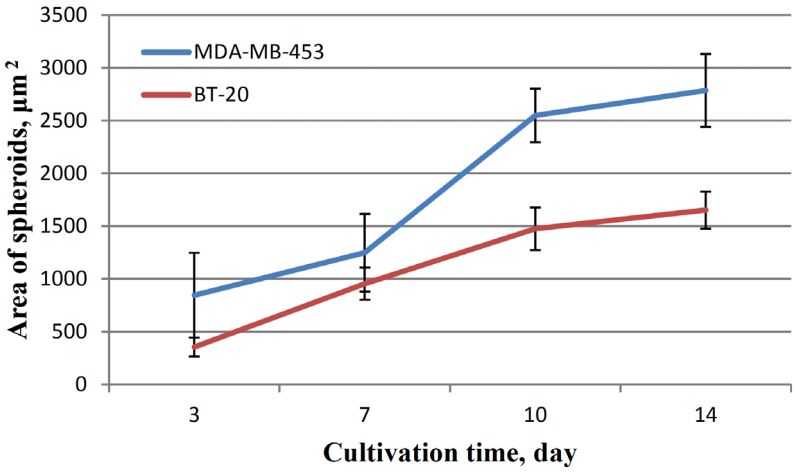

Fig. 3. Dynamics of growth of cellular spheroids formed in 2 % alginate gel by cells of BT-20 and MDA-MB-453 cultures. The data are presented as a sample mean value ± 95 % confidence interval of the general mean.

ence of hypoxia and lack of nutrients begin to manifest themselves in the center of the spheroids upon reaching their radius of 100 microns or more [15], in our case, the radius of even the largest cellular spheroids did not exceed 30 microns. Currently, it has been established that the adaptation of malignant cells and microenvironment cells to the lack of nutrients and oxygen observed in the tumor contributes to the development of chemo- and radioresistance, immunosuppression, invasion and metastasis, being one of the most important obstacles to cancer treatment [16]. In this regard, the reproduction of hypoxia and nutrient deficiency in a bioprinted cancer model has a special value. Large cellular spheroids are the main material on which hypoxia is studied *in vitro*, however, due to their size, bioprinting by extrusion through a thin nozzle with such structures is not possible. Therefore, in such models, direct imprinting of a suspension of cells into a biogel or a combination of methods of preforming sufficiently large cell conglomerates with subsequent pouring into a biogel is more often used [15]. Small spheroids, like those that we obtained by culturing cells of MDA-MB-453 and BT-20 cultures in alginate droplets, serve as good material for bioprinting, but do not show signs of hypoxia. Therefore, in this case, modeling of natural deficits existing in the tumor will be carried out not at the level of individual spheroids, but at the level of the entire model, where various gradients can be created by controlling the composition of the model components and their precise positioning relative to each other and sources of nutrients and gases.

In the MDA-MB-453 cell culture grown in alginate droplets, individual spheroids of different sizes with signs of degradation of cell nuclei were found (Figure 4b, isolation), indicating the beginning of cell death processes. This phenomenon, along with the uneven distribution in size and shape of the spheroids formed, may indicate a pronounced heterogeneity of clones formed by the MDA-MB-453 culture under these cultivation conditions. Heterogeneity is fundamentally unavoidable when it comes to spheroids-clones, and is a definite challenge for modeling tumor growth *in vitro* using such structures. In the case of multicellular conglomerates obtained by mechanical connection of cells (the hanging drop method or the formation of spheroids in cells), each such spheroid combines cells with different biological characteristics, as a result of which the difference between such cellular conglomerates becomes insignificant. Therefore, each multicellular spheroid can be considered an experimental repeat. In the case of clone spheroids, not every spheroid should be considered a separate experimental repeat, but their totality, which must be taken into account when designing a model of tumor growth that includes such structures. Thus, in order to obtain reliable results of the experiment of spheroid clones, tens of times more is required, which increases the cost of conducting research and thereby limits their scalability.

CONCLUSION

Using encapsulation in an alginate gel makes it possible to obtain in a short time a large number of cellular spheroids-clones suitable for bioprinting.

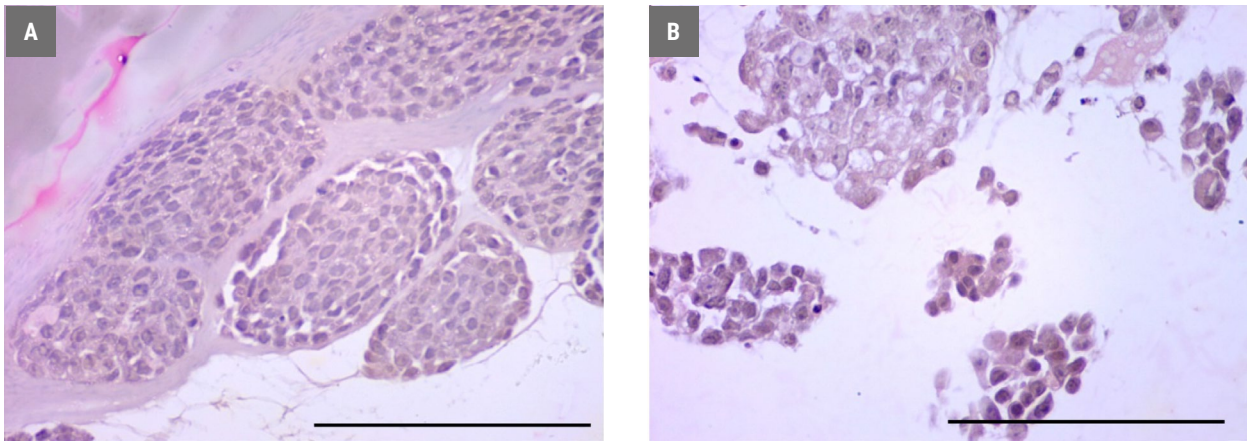

Fig. 4. Spheroids formed by cells of breast cancer cultures in a 2 % alginate gel on the 14th day of cultivation. a – culture BT-20, b – culture MDA-MB-453. Staining with hematoxylin-eosin.

The method we are testing is inferior to the methods of agglomeration of cells in a hanging drop and on microcells in terms of the speed of spheroid formation, as well as the density and uniformity of the resulting structures, but has an advantage over these methods in performance, which is comparable to the method of obtaining cellular spheroids by cultivation on low-adhesion substrates with constant mixing of the medium. The clonal nature of spheroids grown in alginate droplets somewhat increases the cost of conducting research using models derived from them compared to other approaches. However, such spheroids, in our opinion, are the best material for constructing, for example, bioprinted models of the development of micrometatstases, i.e. structures that by their nature are also clones of a single cell.

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