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ORIGINAL ARTICLE

APPLICATION OF SILICONE COATING TO OPTIMIZE THE PROCESS OF OBTAINING CELLULAR SPHEROIDS BY THE HANGING DROP METHOD

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ABSTRACT

Purpose of the study. To study the effect of SIEL 159–330 coating on the cell clusters formation rate in a hanging drop method in combination with the use of methylcellulose (MC) and collagen as cell aggregation improving agents.
Materials and methods. BT20 breast cancer cells were cultured in drops of 20 µL (10^4 cells per drop) on the lid of a polystyrene Petri dish coated with SIEL 159–330 silicone elastomer (GNIIKHTEOS, Moscow, Russia) or without coating. The study tested three concentrations of MC (0.1 %, 0.25 % and 0.4 %) and collagen (150 µg/ml, 300 µg/ml and 600 µg/ml). The rate of formation of cell conglomerates was assessed by evaluating their area after 4, 24, 48, and 72 hours of cultivation.
Results. The use of SIEL 159–330 coating made it possible to obtain spheroids of the same size as the addition of 0.4 % MC over a time interval of 72 hours. The silicone coating additionally reduced the size of cell spheroids in the medium with 0.1 % MC at all time points; however, this effect disappeared with increasing concentration of MC. In addition, the use of SIEL 159–330 reduced the relationship between the size of cellular spheroids and the concentration of MC, which allows us to consider the use of this coating as an alternative to MC or a way to reduce its concentration. In the experiment with the addition of collagen to the culture medium, the sizes of cell conglomerates formed on the silicone coating were significantly smaller than on uncoated plastic in all variants of the experiment and time points. The effect was more pronounced for a collagen concentration of 600 µg/ml. The use of SIEL 159–330 coating, in addition, reduced the variability in the size and shape of the resulting cell conglomerates.
Conclusion. Accelerated aggregation of cells and fibers of the extracellular matrix in hanging drops, as well as a reduction in the variability in the size and shape of the resulting cell clusters on SIEL 159–330, allows us to reduce the time of experiments and material costs, as in experiments with the addition of substances that accelerate the formation of spheroids (MC and collagen), as well as in their absence.

Keywords:
3D cell culture, cell spheroid, hanging drop method, methylcellulose, collagen, silicone elastomer

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ПРИМЕНЕНИЕ СИЛИКОНОВОГО ПОКРЫТИЯ ДЛЯ ОПТИМИЗАЦИИ ПРОЦЕССА ПОЛУЧЕНИЯ КЛЕТОЧНЫХ СФЕРОИДОВ МЕТОДОМ ВИСЯЧЕЙ КАПЛИ

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РЕЗЮМЕ

Цель исследования. Исследовать влияние покрытия из СИЭЛ 159–330 на скорость и характер образования клеточных скоплений в висячей капле в сочетании с применением метилцеллюлозы (МЦ) и коллагена в качестве агентов, улучшающих агрегацию клеток.

Материалы и методы. Клетки культуры рака молочной железы ВТ20 в количестве 10^4 помещали в каплях объёмом 20 мкл на крышку полистироловой чашки Петри с покрытием из силиконового эластомера СИЭЛ 159–330 (АО «ГНИИХТЭОС», г. Москва, Россия) или без покрытия. В исследовании тестировали по три концентрации МЦ (0,1 %, 0,25 % и 0,4 %) и коллагена (150 мкг/мл, 300 мкг/мл и 600 мкг/мл). Скорость формирования клеточных конгломератов оценивали через изменение их площади спустя 4, 24, 48 и 72 часа культивирования.

Результаты. Применение покрытия из СИЭЛ 159–330 позволило получить сфероиды таких же размеров, что и добавление 0,4 % МЦ на временном промежутке 72 часа. Силиконовое покрытие дополнительно уменьшило размеры клеточных сфероидов в среде с 0,1 % МЦ во всех временных точках, однако с ростом концентрации МЦ данный эффект исчезал. Кроме того, использование СИЭЛ 159–330 уменьшило связь размеров клеточных сфероидов с концентрацией МЦ, что позволяет рассматривать применение данного покрытия, как альтернативу МЦ или способ сократить её концентрацию. В опыте с добавлением в среду культивирования коллагена размеры клеточных конгломератов, образующихся на силиконовом покрытии, были достоверно меньше, чем на пластике без покрытия во всех вариантах опыта и временных точках. При этом эффект был более выраженным для концентрации коллагена 600 мкг/мл. Применение покрытия из СИЭЛ 159–330, кроме того, сократило вариативность размеров и формы образующихся клеточных конгломератов.

Заключение. Ускоренная агрегация клеток и волокон внеклеточного матрикса в висячих каплях, а также сокращение вариативности в размерах и форме образующихся клеточных скоплений на СИЭЛ 159–330 позволяет сократить время проведения экспериментов и материальные затраты, как в опытах с добавлением веществ, ускоряющих формирование сфероидов (МЦ и коллаген), так и в их отсутствие.

Ключевые слова:
трёхмерная клеточная культура, клеточный сфероид, метод висячей капли, метилцеллюлоза, коллаген, силиконовый эластомер

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RELEVANCE

High-performance methods of drug screening traditionally rely on two-dimensional cell cultures grown on plastic in the wells of multipath tablets. Recently, the trend has shifted towards three-dimensional drug screening, especially in cancer therapy, due to the unique characteristics provided by these cultivation platforms. It is known that the cultivation of cancer cells in two-dimensional cultures leads to a change in their phenotype and a loss of the properties that these cells possess in vivo in the body of patients, in particular, to a loss of expression of molecules of key signaling pathways [1]. Nevertheless, the phenotype of tumor cells is able to recover in three-dimensional cultures due to increased adhesion and signaling interactions between cells and the extracellular matrix, a decrease in the rate of proliferation to the corresponding physiological growth rate, the formation of limited nonlinear metabolic gradients corresponding to the natural growth environment of tumor cells and other factors [2].

One of the most common types of three-dimensional cell culture are cellular spheroids. At the moment, there are many methods for obtaining spheroids, of which the "hanging drop" method is considered the most affordable, which does not require special equipment or consumables [3]. The essence of this method is that the cells are placed in a suspended drop of the medium, and as a result of the action of gravity and the meniscus that occurs at the air-liquid interface, the cells are localized at the bottom of the hanging drop [4]. One of the weak points of the culture of spheroids obtained by the "hanging drop" method is the variability of the morphology and structure of the resulting cellular conglomerates, which reduces the accuracy of reproduction of natural profiles of metabolic gradients and makes it difficult to compare the effectiveness of drugs. The introduction of additives that accelerate cell aggregation contributes to improving the reproducibility of compact round spheroids. Currently, both biological and synthetic additives are used, which are based on the ability to cross-link cells with each other, stimulate cell adhesion or modify the rheological properties of the medium to accelerate the sedimentation of cells in the lower part of the drop. Extracellular matrix components such as collagen, fibronectin or a preparation of the basement membrane formed by the culture of mouse sarcoma cells – Matrigel® are usually used as crosslinking agents [5]. Various cellulose derivatives, in particular, methylcellulose (MC), are most often used as additives that change the rheological properties of the media [6].

The disadvantages of MC and collagen include the possible effect on the interaction of cells with each other and other components of the medium. Thus, collagen is an active participant in cell signaling [7], so its presence can change the behavior of cells that interact with the extracellular matrix of a different chemical composition under natural conditions, such as brain tumor cells [8]. Methylcellulose, according to the general idea, is an inert agent that does not interact with cells, however, due to the large number of coordination bonds with other molecules of the medium, MC can lead to an unpredictable change in their properties [9].

Due to the existing disadvantages of additives accelerating cell aggregation, researchers pay attention to the modification of the surface and giving it hydrophobic properties, which contributes to the creation of a higher curvature of the drop surface, leading to accelerated aggregation of cells in its lower part. For these purposes, Parafilm® laboratory film is most often used [10; 11] and a coating of polydimethylsiloxane (PDMS) [12] – one of the varieties of silicone. Earlier we showed that the coating made of biologically inert silicone elastomer SIEL 159–330, produced for medical purposes (GNIIKHTEOS, Moscow, Russia) [13] after modification of the curing mode for working with culture plastic does not have cytotoxic properties and is not inferior to Parafilm® film in its ability to accelerate the formation of cellular spheroids [14]. In this paper, we continued to study the possibilities of this silicone coating in the context of improving the protocol for obtaining spheroids by the hanging drop method.

The purpose of the study was to investigate the effect of SIEL 159–330 coating on the rate and nature of the formation of cell clusters in a hanging drop in combination with the use of methylcellulose and collagen as agents that improve cellular aggregation.

MATERIALS AND METHODS

BT20 breast cancer culture cells were grown in DMEM (Gibco, USA) with the addition of 10 % fetal cow serum (HyClone, USA) without the addition of antibiotics. Spheroids from BT20 culture cells were obtained by the hanging drop method, namely by

applying droplets of culture medium with cells to the inner surface of the Petri dish lid, followed by turning over and covering the bottom of the cup, into which a phosphate buffer was introduced to create a wet chamber and thereby prevent the droplets from drying out too quickly. The volume of applied drops was 20 µl, each drop contained 10^4 VT20 culture cells. The study tested the coating of culture plastic (polystyrene) with silicone elastomer SIEL 159–330, cured at a temperature of 60 °C for 18 hours, in combination with the addition of methylcellulose in 3 concentrations (0.1 %, 0.25 % and 0.4 %), as well as collagen in 3 concentrations (150 µg/ml, 300 µg/ml and 600 µg/ml). In total, 35 repetitions were laid for each version of the experience and controls. Petri dishes with applied droplets were kept in a CO_2 incubator at a temperature of 37 °C and a CO_2 content of 5.0 % without changing the medium for 72 hours and photofixation of the resulting cell conglomerates was performed after 4, 24, 48 and 72 hours using an inverted Axio Vert microscope. A1 (Carl Zeiss Microscopy, Germany). The resulting images were used to measure the area of the resulting conglomerates. The data is given as an average value of ± 95 % confidence interval for the average value.

RESEARCH RESULTS AND DISCUSSION

The use of a SIEL 159–330 coating by itself led to a reduction in the size of the formed cell conglomerates, which is clearly seen in the graphs in samples without MC (Fig. 1A, B) The result obtained corresponds to the data we previously published [14] and is confirmed by statistical analysis (Table 1). The addition of methylcellulose further reduced the size spheroids on both tested surfaces, which is also consistent with the literature data [6; 15]. Nevertheless, after 72 hours of cultivation on a silicone coating, the difference between the control and the medium with the addition of 0.4 % MC became statistically insignificant ($t = 0.68$, $t_{crit} = 1.995$, $df = 68$, $\alpha = 0.05$), therefore, at this time interval, SIEL 159–330 can be a replacement for MC.

The minimum size and stabilization of the size of the spheroid were not observed on uncoated plastic and without the addition of MC, up to 72 hours

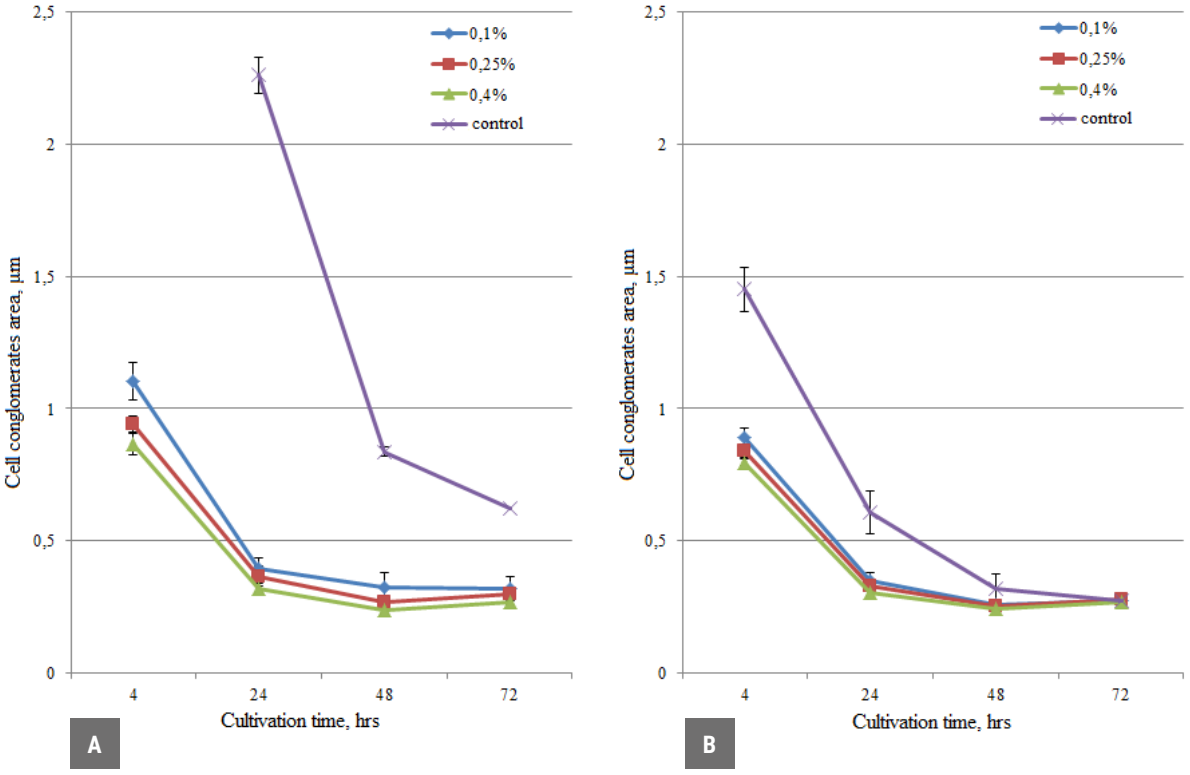

Fig. 1. Size of cell conglomerates with the addition of various concentrations of methylcellulose. A – uncoated polystyrene. B – coated with SIEL 159-330. Mean ± 95 % conf. interval.

of cultivatio (Fig. 1A, control). The use of a coating from SIEL 159–330 reduced the formation time of a compact spheroid to 48 hours (Fig. 1 B, control), and the additional introduction of MC accelerated the stabilization of the sizes of spheroids to 24 hours. At the same time, the addition of a coating from SIEL 159–330 did not affect the final size of the spheroids – after 72 hours of cultivation, there was no statistically significant difference in the values of the area of the spheroids between the two coatings (Table 1).

The data obtained by us also indicate that the silicone coating makes a significant contribution to accelerating the formation of spheroids at low concentrations of MC. This can be judged by the difference between the average values of the area of spheroids formed on uncoated plastic and silicone coating after 4 hours of cultivation (t = 6.53) and after 48 hours of cultivation (t = 2.32) at a concentration of 0.1 % MC. At other time points, the Student's criterion value, although close to the critical value (t_{crit} = 1.995, df = 68), nevertheless did not exceed it (Table 1). For concentrations of 0.25 % and 0.4 % MC, significantly significant differences in the average sizes of spheroids were observed only after 4 hours of cultivation ($t_{0.25\,\%}$ = 2.48, $t_{0.4\,\%}$ = 3.09), with a sharp decrease in the Student's criterion value at subsequent time points. Shortening the time for the formation of spheroids in the hanging drop method would make it possible to move earlier to manipulations with them – transfer, replacement of the medium, introduction of additional components, etc.

The graphs show that on uncoated plastic, the size of the spheroid clearly correlates with the concentration of MC – the higher the concentration, the smaller the size of the spheroids at each time point (Fig. 1A). At the same time, the difference was statistically significant, since the values of the Fisher criterion for all variants exceeded the critical value ($F_{crit.}$ = 3.087, k_1 = 2, k_2 = 102, α = 0.05) with a gradual decrease by 72 hours (Table 2). On the SIEL 159–330 coating, the difference between variants with different concentrations of MC is reduced, but also remains reliably significant.

The observed pattern corresponds to the data known from the literature [15]. These observations

Table 1. The Student's t test value of for pairwise comparison of the average values of the area of spheroids formed on the plastic coated with SIEL 159-330 and uncoated plastic

Cultivation media	Cultivation time, hrs.			
	4	24	48	72
Control	n/a	**71.03**	**11.4**	**31.18**
MC 0.1 %	**6.53**	1.91	**2.32**	1.98
MC 0.25 %	**2.48**	1.87	0.75	1.86
MC 0.4 %	**3.09**	1.56	0.66	0.37
Collagen 150 μg /ml	**6.46**	**10.55**	**10.41**	**9.05**
Collagen 300 μg/ml	**6.0**	**12.57**	**3.15**	**3.02**
Collagen 600 μg/m	**4.17**	**13.58**	**8.31**	**5.1**

Note: values of the t criterion exceeding the critical value for the significance level adopted in the study α = 0.05 (t_{crit} = 1.995, df = 68) are highlighted in bold.

Table 2. The Fisher F criterion values of the analysis of variance of the average values of the area of spheroids formed at three concentrations of methylcellulose for each time point

Coating type	Cultivation time, hrs.			
	4	24	48	72
Polystyrene without coating	112.76	26.09	29.8	14.29
SIEL 159-330	67.86	26.94	3.71	8.12

allow us to conclude that the use of a coating from SIEL 159–330 can contribute to a decrease in the concentration of MC for short exposures and a complete rejection of MC during cultivation for 72 or more hours in cases where MC can interfere with the experiment.

In addition to accelerating the formation of spheroids at the early stages of the experiment, the SIEL 159–330 coating had a noticeable effect on the shape of the formed cellular conglomerates in the experiment with MC. So, after 72 hours of cultivation, the cellular spheroids obtained on SIEL 159–330 had a more even contour, which is especially noticeable in the control samples (Fig. 2). Smooth contours indicate uniform formation of the spheroid and, as a consequence, its homogeneous structure. The homogeneous structure of spheroids, in turn, is the key to a smaller spread of data obtained on such cellular models when testing various physical and chemical influences.

In the experiment with the addition of collagen to the culture medium, the sizes of cellular conglomerates formed on silicone coating were significantly smaller than on uncoated plastic in all variants of the experiment and time points (Table 1). Compared with the control, the addition of collagen led to the formation of conglomerates, the size of which directly depended on the concentration of collagen (Fig. 3A, B). First of all, the observed effect is due to the fact that the resulting fibers of the extracellular matrix are themselves the material of the conglomerate, and, therefore, directly determine its volume. To an even greater extent, the concentration of collagen determines the observed area of conglomerates, since the fibers are collected on the surface of the drop and the cluster formed in this case is a disk, not a ball.

In contrast to the MC experiment, the addition of a silicone coating led to a significant reduction in the size of cell clusters at all time points and in all samples with the addition of collagen. Attention is also drawn to the change in the regularity of the formation of cell clusters when using a coating from SIEL 159–330. Thus, in uncoated plastic samples, the size and concentration are linearly related, as

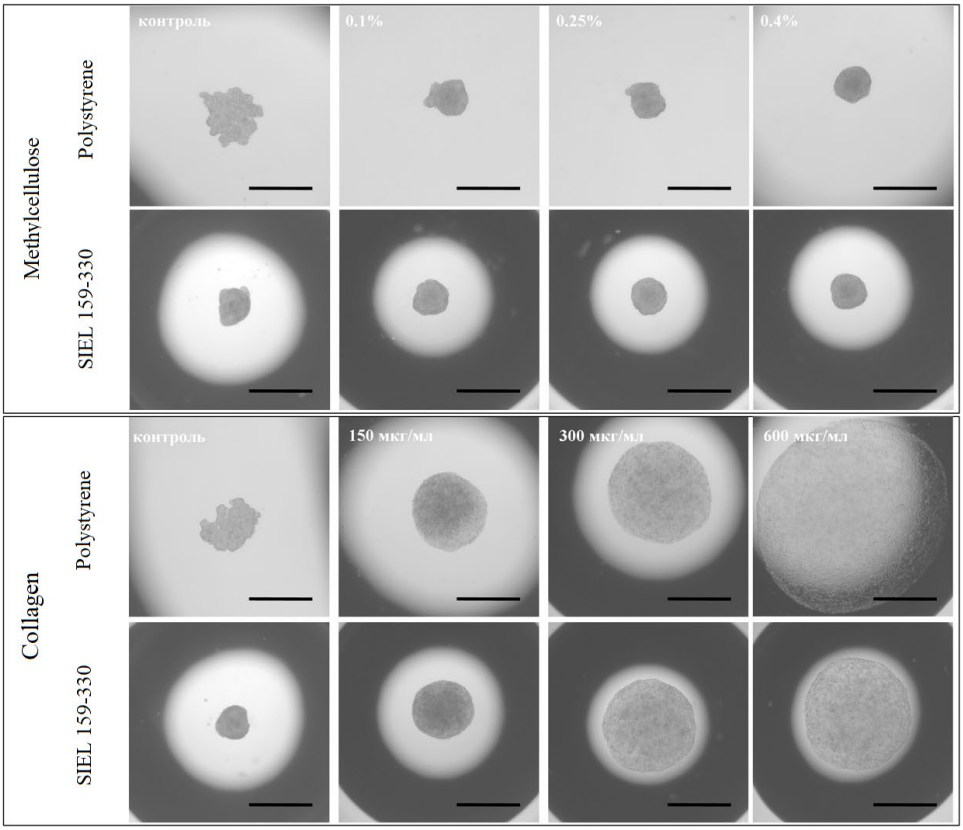

Fig. 2. View of cell spheroids obtained with the addition of methylcellulose or collagen to the medium after 72 hours of cultivation on polystyrene coated with SIEL 159-330 and uncoated polystyrene. Magnification ×20. Scale bar 1000 μm.

indicated by the increase in the size of the disc in proportion to the increase in the amount of collagen, which is most clearly seen after 72 hours of cultivation (Fig. 3A). At the same time, when cultured on a silicone coating, the gap between 300 μg/ml and 600 μg/ml was significantly reduced, with an almost unchanged difference between 150 μg/ml and 300 μg/ml (Fig. 3B). This behavior of cellular conglomerates most likely indicates the accelerated formation of conglomerates in the early hours after the experiment was started, which allowed a more compact cluster to form before the completion of collagen polymerization, all other things being equal. This is also evidenced by the high optical density of the clusters formed on the SIEL 159–330 coating compared to uncoated plastic at the same collagen concentrations (Fig. 2).

One of the disadvantages of collagen observed in our experiment is the long-term stabilization of the size of spheroids. Regardless of the concentration of collagen, the area of aggregates formed on uncoated plastic continues to decrease between 48 and 72 hours of cultivation (Fig. 3A), which increases the time for experiments with such 3-dimensional cell models. However, the use of silicone coating led to a reduction in the stabilization time of the size of aggregates from cells and fibers of the extracellular matrix up to 48 hours, regardless of the concentration of collagen (Fig. 3B).

Attention is also drawn to a sharp decrease in the variation in the size of cellular conglomerates when using a coating from SIEL 159–330 for all time points. Thus, the standard deviation on uncoated plastic averaged 0.9 compared to 0.18 on SIEL 159–330 in all variants of the experiment with the addition of collagen and time points. As in the case of a uniform surface and structure of spheroids, the reduced variability in the size of 3-dimensional cell models makes it possible to reduce the spread of experimental data in subsequent studies.

CONCLUSION

The use of a coating made of silicone elastomer

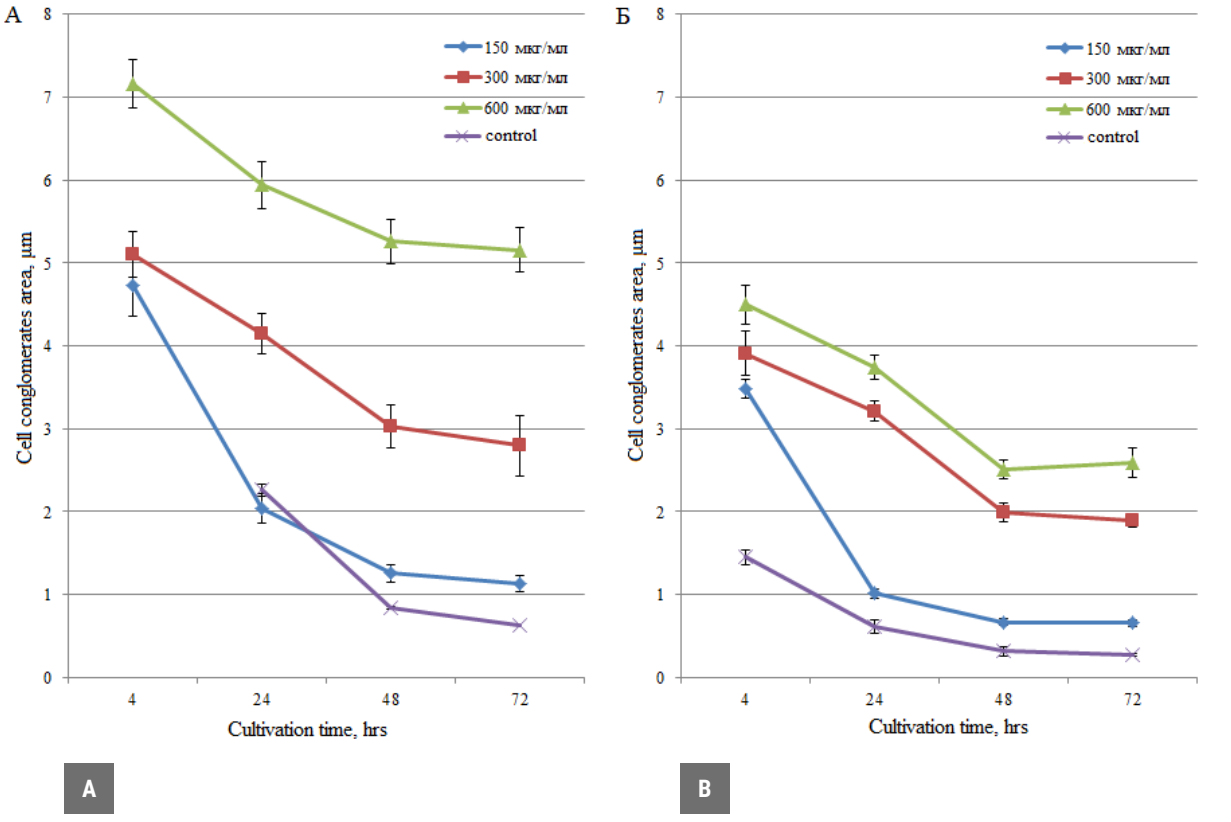

Fig. 3. The size of cell conglomerates with adding different concentrations of collagen. A – uncoated polystyrene. B – coated with SIEL 159-330. Mean ± 95 % conf. interval.

SIEL 159–330 makes it possible to improve the practice of obtaining cellular spheroids by the hanging drop method. Accelerated aggregation of cells and fibers of the extracellular matrix, which we observe in the first hours of cultivation of drops on SIEL 159–330, allows us to reduce the time of experiments, both with the addition of substances that accelerate the formation of spheroids (MC and collagen), and in their absence. In addition, the tested silicone coating also reduces the material costs of conducting studies in which collagen is used by reducing the variability in the size of the formed cellular conglomerates.

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