ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2023. Vol. 39. N 2. P 90–109
http://dx.doi.org/10.7124/bc.000A91

UDC 616.5:616-089.843

From mesenchymal stem cells to their secretoms as basic components of dermal coverings for the treatment of massive burns

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In the course of our long term research, we have created new biotechnological products — dermal coverings (dermis equivalents) with the inclusion of human MSCs or their secretomes (cells conditioned mediums which contain a complex of biologically active substances synthesized by them). Preclinical studies on the model animals and clinical trials of new dermal equivalents on the limited group of patients with massive burns have been conducted to determine their therapeutic effectiveness and well as safety. We proved at special *in vivo* experiments that new dermal coverings contribute positively to the healing severe deep burn wounds when applied to the wound surface and don't reveal any toxic effect in the studied organisms. The developed method of obtaining new biotechnological products has been patented.

Keywords: cellular biotechnology, dermal covering or dermis equivalent, mesenchymal stem cell (MSC), conditioned media (CM), secretome, burns.

Introduction

The burn injuries are one of the most common types of damages, affecting the lives of millions of people around the world. About 6 million people with burns need medical help every year. According to WHO, burns take the third place among all types of peacetime injuries and they are the cause of 180,000 deaths in the world annually, most of which occur in middle-income and low-income countries. Approximately half of severe burn injuries are the burns in children, with 50–80 % of cases occurring in children under 5 years of age. In Ukraine, before the Russian invasion burns were are registered annually in 80,000 people, 10 % of them are children. In total, 70 % of injuries are the burns received in domestic conditions. In

modern time the frequency and severity of thermal burns and other massive skin damages increased dramatically.

Over the past half century, the results of treatment of patients with burns have been improved significantly, but still, the therapy of massive burn wounds remains one of the most urgent problems of medicine throughout the world. In the burns of IIIb and IV degree, when not only the epidermal, but also the skin dermal layer is completely lost in some areas, it is necessary to use autodermoplasty or autodermotransplantation. However, if the total area of wounds exceeds 30–40 % of the body surface, this method does not allow closing the entire surface of the wound, even when using perforated autodermal grafts. This led to the introduction of various artificial skin substitutes into the arsenal of a combustiologist, among which the best ones are biotechnological wound coverings containing living cells of various types and origins, mainly differentiated fibroblasts and keratinocytes [1].

Such bioconstructions usually serve as temporary equivalents of the skin or its separate layers (dermis, epidermis), which provide the patient's damaged tissues with biologically active substances that stimulate skin regeneration. A huge number of requirements for an ideal skin substitute are listed in the review [1]. But perfect temporary skin substitutes have not yet been invented, so the efforts of biotechnologists around the world are aimed at finding new ways to solve this important task using new approaches and materials.

Therefore, the development and introduction into clinical practice of new biotechnological wound coverings with cellular components, among which the dermis equivalents used for the treatment of the most dangerous burn wounds, namely several burns of degree IIIb and IV, are highly demanded.

Starting from 2006 until nowadays, on the basis of experimental research data the world's scientists came to the conclusion that mesenchymal stem cells (MSCs) or multipotent mesenchymal stromal cells (MMSCs) and their derivatives, exosomes and secretomes (cultural mediums conditioned by MSCs), are really promising biologically active components for biotechnological wound coverings [2–16]. Taking this into account, it was interesting for us to compare the activity of human cells of different types and origin in the same experiments. We took into experimental work human cells of the established E8 line (embryonic germ cells in origin), the cells of the established 4BL line (MSCs obtained from peripheral blood of healthy donor) and the cells of the established A102 line (newborn skin fibroblasts) as the biologically active components of dermis equivalents [16–18].

Thus, we have provided special experiments using an animal model of burn wounds (ICR mice) for comparing the wound-healing properties of such biologically active components as human cells of three mentioned established lines (4BL, E8, A102) and cell-free conditioned mediums (CMs), which were produced using the cells of corresponding lines (CM-4BL, CM-E8, CM-A102) as a part of hydrogel plastic coatings [16]. It was concluded that among all six investigated dermis equivalents the most effective were those containing either MSCs of the 4BL line or their conditioned media CM-4BL. Using another animal model of burn wounds (Wistar rats), we confirmed the high wound-healing activity

of 4BL cells and the cultural mediums conditioned by them.

So the results of *in vivo* animal studies proved that both MSCs of the 4BL line and the samples of the corresponding CM-4BL had a positive effect on the healing process of IIIb degree thermal burns of skin. These experiments are described in the next part of this article in details.

Summarizing, we have obtained the following results [1–4, 12–16, 19–21]: a) new biotechnological products have been created, the dermal coatings with the inclusion of human MSCs or their secretomes, which contain a complex of biologically active substances synthesized by them; b) *in vivo* studies on the model animals and clinical trials on the limited contingent of patients with massive burns were conducted to determine therapeutic effectiveness of new dermis equivalents, as well as the safety at special *in vivo* experiments. And it was proven that new dermis equivalents contribute to the healing of severe burn wounds when applied to the wound surface and do not reveal toxic properties as well; c) the method of obtaining new biotechnological products has been patented.

The main purpose of this article is a more detailed consideration of the new biotechnological dermis equivalents with the inclusion of human cells of various origins or their secretomes, which have been developed by us for the treatment of severe skin burns in comparison with the literature data.

Short description of the problem. Biotechnological dermal equivalents are of the particular importance for the treatment of massive burns. One of the main problems in the treatment of burn disease is the need to treat deep and extensive damage of the skin with further stimulation of tissue repair regeneration, as far as it is possible with the capacity of modern medicine [1]. Such wounds have a high risk of infection and often require expensive treatment by skin autotransplantation or autodermoplasty. Nowadays combustiologists theoretically (that is, in the presence of a significant amount of money resources) can use a variety of temporary wound coverings, which contain living cells of various types and origins. The existing overseas epidermal skin equivalents include Epicel, EPIBASE, EpiDex, MySkin, Laserskin, Bioseed S and CellSpray; the well-known foreign dermal equivalents are, for example, TransCyte and Hyalograft 3D, which are described in details [1]. All these biotechnological wound dressings are temporary. Despite the availability of various commercial products of this type abroad (skin equivalents, equivalents of epidermis or dermis), including those containing human cells, they are practically not available for Ukrainian patients due to high cost and logistic problems.

This way, under current conditions in the field of medicine, and, in particular, in the national combustiology, the need to create new biotechnological wound coverings, which include human stem cells or their derivatives, intended for the treatment of burn disease, is a demand of society. This led us to focus our efforts on the development of new equivalents of dermis using natural materials and human stem cells or their derivatives, that, on the one hand, would be quite effective for the treatment of burns, safe for the organism and would have improved operational properties, and on the other hand — would be inexpensive, easy

to manufacture and operate and available to Ukrainian patients.

Research and Discussion

1. Carriers for biologically active components of dermis equivalents. In our works [1–4, 12–16, 19–21] we are talking about the creation of new biotechnological dermal coatings: the features of the materials selection for the cellular carrier, as well as cellular components, cells and/or their derivatives, in comparison with known literature data. Polyacrylamide hydrogel was used by us as a first sample of cell carrier for temporary dermal equivalents [1–4, 12]. At the beginning of experimental research in the field of cellular technologies, polyacrylamide hydrogels were not used for the manufacturing of artificial wound coverings. However, later the works appeared, in which various manners of using polyacrylamide were proposed. The first one of those works was completed in Canada in 2014 [22], where the scientists proposed to use a thin polyacrylamide layer on the surface of the artificial wound covering closely contacting skin. Such layer was used to reduce the strength of the contact between the skin and the coating in order to avoid microtraumas and pain during further replacement with pieces of the patient's own skin. It was also assumed that the polyacrylamide hydrogel layer could carry some bioactive or antimicrobial agents, which would be gradually released and reach the wound bed. The authors of this publication, continuing work in this direction, two years later [23] proposed for consideration a wound covering with the same polyacrylamide layer, but with an additional load of silver in the form of nanoparticles or in the form of an additional silver layer.

Over the past few years (from 2017 to 2021), various groups of Chinese scientists have proposed different options for artificial wound coverings that include polyacrylamide. Other substances that were added to the proposed biological constructions (bioconstructions) as a composite material were:

- polydopamine, 2017 [24],
- a complex of dopamine and oxidized sodium alginate (dopamine-grafted oxidized sodium alginate), 2018 [25],
- hydroxypropylmethylcellulose, 2019 [26],
- polyacrylic acid and ferrocene (polyacrylicacid-functionalized with ferrocene), 2021 [27],
- polydopamine and bacterial cellulose (bacterial cellulose), 2021 [28],
- water-soluble polysaccharides from *Enteromorpha prolifera* (a species of green seaweed), cross-linked with boric acid (water-soluble polysaccharides from *Enteromorpha prolifera* cross-linked with boric acid), 2021 [29].

In one of the latest works of 2021, the proposed option proved to be the most effective among all those presented in the mentioned references [29]. The authors loaded the created hydrogel with human epidermal growth factor and found that such samples of wound coverage promoted cell proliferation and migration *in vitro* and significantly accelerated wound healing *in vivo* in the model rats.

Noteworthy that all variants of dense wound coverings, have been described earlier, significantly differed from the bio-constructions that became the object of our work [2–4, 12]. We intend to create new equivalents of the dermis and conduct their investigation — first

laboratory, then preclinical — using an adapted polyacrylamide hydrogel coating, on the surface of which the immobilized living cells are able to attach and multiply. The adult human MSCs approved for use in medical practice were offered for this purpose. This is the main difference between our work and the results described in the literary sources mentioned above.

One of the main tasks of our work was to optimize the production of bio-constructions based on dense hydrogel carriers (membranes) in order to improve the immobilization, adhesion and proliferation of cells on them. Nanoparticles of metals or metal oxides were modifying components that could be included during the production of polyacrylamide membranes.

Some data indicated that the biological response to the metal nanoparticles entering a living organism differs from the response to the metal ions. For example, it has been shown that certain doses of metal nanoparticles stimulate metabolic processes and also exhibit bacteriostatic and bactericidal activity [30].

The antibacterial properties of silver are widely known. This effect, its manifestations and mechanisms are the subject of research by many scientists. A search for the papers related to this phenomenon in the electronic database PubMed gives us a list of more than two thousand articles, in which the bactericidal properties of silver in one form or another are investigated. Additionally, silver can affect the proliferation of mammalian cells. Back in 2007, scientists from Hong Kong [31] studied the wound-healing properties of silver nanoparticles in an animal model (BALB/C mice) and found that the speed of wound healing and cosmetic improvements of the wound area is a dose-dependent effect. The authors also showed that silver nanoparticles have a positive effect due to their antimicrobial properties, reduction of the inflammatory process in the wound and modulation of fibrogenic cytokines. German scientists Greulich *et al.* [32] and Hackenberg *et al.* [33] conducted the studies with human mesenchymal stem cells (hMSCs) and showed that silver nanoparticles in high concentrations can exert a cytotoxic effect on hMSCs, but at subtoxic concentrations of silver the cell activation is induced.

The effect of nanoparticles of iron, iron oxide and some iron-containing compounds on living cells has also been the subject of study in many works. Magnetic nanoparticles of magnetite, i.e. iron (II, III) oxide, are recognized as a promising tool for performing many medical tasks. They acquired this status, in particular, due to their magnetic properties. These properties are used, for example, in the work of British scientists [34], who invented an interesting approach using superparamagnetic iron oxide nanoparticles (II, III) (superparamagnetic iron oxide nanoparticles, SPIONs), coated with a thermosensitive polymer. Such nanoparticles were found to be able to capture proteins, and then, after being heated using magnetic field, the proteins were released into the environment. It has also been shown that magnetite nanoparticles, like silver nanoparticles, have antibacterial properties [35]. The effect of nanoparticles of iron oxide (II, III) on cells depends on the cell type, concentration and duration of nanoparticles action [36, 37].

Therefore, taking into account the known literature data, we checked how the presence

of silver nanoparticles and iron oxide (II, III) affected the cellular component of polyacrylamide hydrogel coatings: immobilization, adhesion and proliferation of MSCs stem cells of the 4BL line [17, 18]. It was shown that the introduction of 30 nm silver (Ag) nanoparticles at a concentration of 25 мg/g into the hydrogel composition or the introduction of 20 nm iron (II, III) (Fe_3O_4) nanoparticles into the hydrogel composition at a concentration of 5–10 mg/ml stimulates the metabolic activity of cells, immobilized in bio-constructions based on polyacrylamide hydrogel matrices. However, the addition of Ag or Fe_3O_4 nanoparticles to the composition of polyacrylamide hydrogels reduces their transparency, which not only interferes with the monitoring of the state of burn wounds (which is especially important when using such hydrogel coatings in the clinic), but also with the quality and behavior of the cells (morphology and proliferation) during trials in the laboratory.

Thus, the significant disadvantages of polyacrylamide carriers with silver nanoparticles in certain concentrations were represented by: 1) a decrease in the transparency of the samples due to the introduction of inorganic nanoparticles into their composition, and 2) increasing of fragility of hydrated flat hydrogel fragments that was revealed during the experiments. That is why finally we chose polyacrylamide carriers without silver nanoparticles for creation of dermal equivalents.

The next trial sample for a carrier of cells among our temporary equivalents of dermis was the biofilm of the bacterial-yeast group "kombucha" (*Medusomyces gisevii*). The basis of this biofilm is bacterial cellulose, which is already used to create biodegradable antimicrobial composites for wide application [38]. It is important that the cell membrane of yeast contains chitin in the amount of 1–3 %, which is able to promote the regeneration of the dermal layer of skin [39]. Although most of the 4BL cells that we used remained alive on the surface of such matrix for 6 days (which is much longer than the possible clinical use of a single wound covering sample), only a small proportion of cells acquired the flattened shape that is typical for viable MSCs on the surface of solid media. So we cannot say for sure that the vital activity of cells in a bio-construction of this type would be similar to that under normal conditions *in vitro*.

Therefore, we concluded that the biofilm of the fungus Medusomyces gisevi may be considered as a material of a low-cost that can serve as a carrier for human stem cells in the composition of new biotechnological dermal equivalents. However, further experimental studies with this material should be conducted in order to learn about the peculiarities of the metabolic activity of cells, particularly in relation to the secretion of growth factors and cytokines in the composition of such bio-constructions.

The third type of matrix or cell carrier that we studied was the collagen film "Bilkozyn", which has been produced on an industrial scale for a long time. In general, attempts of using collagen to produce dermal or dermal-epidermal equivalents have been repeatedly encountered in biotechnological research. Human collagen is not used as compound of commercial dermal coatings with cells inclusion that already exist and are widely used in medical practice, as far as the technological capabilities of manufacturers and financial capabilities of

hospitals allow. However, other biotechnological dermo-epidermal equivalents (Apligraf Organogenesis Inc., USA) and OrCell (OrCel Ortec International, Inc., USA) contain bovine collagen type I hydrogel [1]. Both biotechnological products carry two types of cells: fibroblasts and keratinocytes, allogeneic in relation to the patients.

We purposely note the presence of differentiated cells in commercial products, since the goal of our research is the equivalent of the skin dermal layer, containing human stem cells. There are also many experimental works on the creation of composite cell-containing substitutes for skin and dermis, in which the basis or one of the main components is collagen of one form or another (hydrogel, fibrous collagen matrix produced by "electrospinning", *etc.*). These various attempts to create composite cell-containing substitutes for the dermis and skin are described in details [1, 40, 41]. If we assume that all these experiments with collagen matrices will result in the industrial release of appropriate bioengineered cell-containing dermal or dermal-epidermal equivalents, then the deployment of production will require the establishment of a large-scale process of the actual collagen-containing cell carrier. With this in mind, we decided to try using a commercial collagen film as a matrix for the human stem cells, which is already commercially produced. This solution will allow, if necessary, significant simplifying and reducing the costs of the process of setting up industrial production.

Therefore, the new bio-construction "collagen+living cells in gelatin hydrogel" created by us was suitable for conducting preclinical studies: preparing applications for treatment of thermal burn wounds induced in model animals and monitoring the wound healing process [1, 14, 15]. We prepared the samples for *in vivo* studies, based on the results of experiments on the determination of the cells survival in gelatin hydrogel. These studies showed that such biotechnological wound coverings can be preserved for two to three days; however, the time of storage of ready-made samples of dermal coatings before the start of surgical procedures should be as short as possible to prevent the death of cells immobilized in the hydrogel.

The results of a previous experiment involving model animals (ICR mice) indicated the beneficial effect of the application of the temporary dermal equivalent created by us on the wound healing process. This was demonstrated in the stimulation of the regeneration processes (vascularization and epithelization) of the derma of model animals with burn wounds [14, 15].

We managed to conduct successful clinical studies of this model on a limited group of patients with massive burns at the Center for Thermal Trauma and Plastic Surgery at Kyiv Clinical Hospital No.2 [1, 4]. Namely, the dermis equivalents based on collagen or polyacrylamide membranes as carriers for MSCs preserved during a few days, have been applied for clinical trials on the limited contingent of patients with massive burn wounds and have shown high effectiveness for burn wounds healing [1, 4]. The use of new dermal equivalents with the inclusion of MSCs almost doubled the speed of wound healing, and no scarring was observed. Such observations have been supported by other authors [11].

We hypothesized that the effect of new dermis equivalents on the course of the burn disease could be explained by: a) a consequence of the beneficial effect of the complex of biologically active substances secreted by stem cells in the composition of the dermal coverings on the regeneration of the skin dermis layer, and b) a result of the partial absorption of toxic products of cell decay. All these issues were studied by us in the *in vivo* experiments.

We chose a collagen membrane as a carrier or solid base of our temporary cell-containing dermal equivalents. However, it was important to evaluate the effectiveness of the various components of the dermal equivalents developed by us in preclinical studies using a sufficient number of model animals. Therefore, we conducted the experiments involving small laboratory rodents (Wistar rats and ICR mice) on a larger scale than in the above-mentioned experiments.

Noteworthy, with a significant increase in the number of experimental animals, we faced the following problem: it was practically impossible to fix the fragments of dense dermal equivalents on rodents' integuments. If such fragments are mechanically fixed on the wound, for example, by tissue bandages, then, firstly, the animals will experience constant significant stress from manipulations of such a kind, and, secondly, both the additional traumatization or bandage loosening is possible.

Ensuring reliable fixation of the cell suspension on the wound surface could be achieved due to the fact that the cells will be immobilized on the surface of a film-like wound covering (for example, on polyacrylamide or collagen membranes) or distributed in a soft pharmaceutical form such as an ointment [16, 19–21]. In our opinion, the advantages of ointment-like substances for closing wound surfaces are:

- the convenience of their usage in the case when the burn wound occupies the surface of the body with a complex shape;
- the ability to control freely the thickness of the applied ointment layer, which will result in control of the amount of biologically active substances per unit area of the wound surface;
- the possibility, at the request of the doctor or patient, to reduce the amount of ointment per unit area of the wound surface in certain cases (for example, when there is a lack of necessary substances due to the fact that the treatment is carried out on the basis of small medical centers with a small supply of medicinal substances, or due to a large number of patients in the burn center).

Therefore, due to use of *in vivo* animal models later we decided to change slightly the direction of our research, focusing on the use of soft pharmaceutical forms of the ointment type separately from the solid carrier. The analysis of literature data led us to draw the following conclusion: it is the carbopol hydrogel based on the gelling agent Carbopol 980 with a carbopol concentration of 1.5 % that will serve as a soft pharmaceutical form in our experiments with model animals [16, 19–21].

When developing a cell-containing preparation based on a soft ointment-like hydrogel (carbopol hydrogel, which is used in medicine), the first priority was finding the solution to the question of how long the cells would

maintain their viability in the presence of such a pharmaceutical form. This is important not only for the creation of the cell-containing coating itself, but also for solving practical issues regarding the permissible time interval between its preparation and application, as well as regarding the possibility of its storing. The analysis of the data obtained during the monitoring of changes in the viability of cells incorporated into the carbopol hydrogel indicated a gradual decrease of viable cells number depending on the exposure time of the cell-containing preparation. Statistical processing of the data showed that the decrease in viability is quite low (~50 % of live cells after 24 hours storing) and not random: at room temperature, the influence factor was $\eta^2 = 0.36$ ($p \leq 0.05$), and at the temperature that is usually provided to cells during their *in vitro* cultivation and is natural for them, viability decreases rather quickly, and the impact factor was $\eta^2 = 0.26$ ($p \leq 0.05$) [20].

Thus, limitation of the storage period of cell-containing hydrogel bio-constructions based on carbopol and the period of application to the wound bed are the necessary conditions for avoiding the loss of therapeutic effectiveness of these drugs. The long-time storing of cellular component should be carried out separately, in cryobank conditions. It has been established that the preparation of plastic dermal coatings, which contain a combination of cryopreserved cells thawed according to a standard scheme and 2-fold (3 %) carbopol hydrogel, is optimal ex tempore, immediately before application. The finished product should be used within 2–3 hours after preparation.

2.Biologically active components of dermis equivalents. Having determined the possibility of the existence of viable cells inside the hydrogel, first of all we decided to compare the therapeutic possibilities of such two important components of our hydrogel constructions as living cells and cell lysate. It has been hypothesized that the cell lysate contains the same biologically active substances, including growth factors released by living cells into the environment, as the cellular debris that may be toxic. So, we assumed that the cell lysate is also capable of stimulating the regeneration of tissues in the wound bed, as well as living cells in the composition of the dermal equivalent.

Cell lysates. There are already described cases (although they are quite few) of studying the effect of cell lysate on the healing process of skin wounds (Table 1). Belgian scientists D.I.Roseeuw *et al.* back in 1995 [42] made an experimental full-thickness wound on the backs of mice and studied the wound-healing activity of lyophilized lysate from cultured human keratinocytes using such model. This lysate was prepared in phosphate-buffered saline. The authors showed certain positive changes in the wound healing process, an increase in the thickness of the dermal layer of the skin compared to the control group of animals. However, no effect on the restoration of the epidermal layer of the skin was found. At the same time, it should be noted that the cultivation of keratinocytes is a rather expensive process, and obtaining a suspension of these cells from the patient's own tissues is to some extent a traumatic procedure for him. As evidenced by the literature data, this group of scientists did not conduct further research with cell lysates, in particular, with keratinocyte lysates.

Table 1. **Examples of experimental wound healing activity studies of cell lysates, cells or cell-free conditioned mediums (CFCM) in different countries**

State	Year	Active (healing) factor	Some experimental effects	
Belgium	1995	Cell lysates originated from cultured human keratinocytes	Acceleration of full thickness wound healing (mice): positive effect on the healing of the dermis	[42]
United Kingdom	2010	CFCM originated from the human bone marrow derived MSCs	It has been shown in the scratch assays that CFCM accelerated fibroblasts and keratinocytes migration. Collagen-I, fibronectin, TGF-beta1, IL-6, IL-8, MCP-1 were identified in the CFCM	[52]
Canada	2012	Cell lysates originated from human red blood cells	Modulation of expression of key extracellular matrix components (collagen-I, α-smooth muscle actin, fibronectin) and expression of matrix metalloproteinases (MMPs)-1,2,3	[43]
Japan	2012	The Wharton's jelly derived MSCs and CFCM originated from those cells	Acceleration of full thickness wound healing (mice): attraction of anti-inflammatory M2 macro–phages into the wound bed, promotion of neovessel maturation, increasing for the expression of IL-10, TGF-β1, VEGF-1 and angiopoietin-1	[48]
P. R. China	2015	CFCM originated from the rat bone marrow derived MSCs	Acceleration of full thickness wound healing (rats): enhancement of the neovascularization and the epithelialization due to movement of paracrine factors in the wound bed	[49]
Ukraine	2017	4BL cells (original human MSCs line); E8 cells (original human embryonic germ cells line); A102 cells (human newborn skin fibroblasts line); CFCM originated from those cells	Acceleration of partial thickness burn wound healing (mice): positive effect on the healing of the dermis and the epithelialization	[16]
P. R. China	2017	CFCM originated from the Wharton's jelly derived MSCs	It has been shown in the scratch assays that CFCM increased the proliferation and migration of dermal fibroblasts. Acceleration of full thickness wound healing (mice) with fewer scars compared with control groups	[56]
Ukraine	2018	Human MSCs line 4BL and cell lysates originated from it	Slowing down of partial thickness burn wound healing or no effect (mice)	[19]
Malaysia	2019	CFCM originated from the cultured human skin fibroblasts	Acceleration of full thickness wound healing (mice): positive effect on the expression of cytokeratin-14 and collagen-I in the epidermal and dermal layer, respectively	[46]
Romania	2020	CFCM originated from the human bone marrow derived MSC	It has been shown *in vitro* that CFCM sustained the adherence of keratinocytes and fibroblasts as well as the proliferation of keratinocytes, had chemo–attractant properties for keratinocytes and endothe–lial cells, increased for the expression of α-smooth muscle actin, tissue inhibitor of metalloproteinase-1,2 and matrix metalloproteinase-14	[50]

In 2012, Canadian scientists A. Akbari *et al.* [43] studied the effect of erythrocyte lysate on the expression of extracellular matrix components. As the authors wrote, erythrocytes are considered "inert observers" in the early and inflammatory phase of wound healing. However, under the influence of erythrocyte lysate, a significant increase in the level of metal-proteinases MMP-1, 2, 3 and a decrease in the expression of type I collagen and smooth muscle б-actin were demonstrated, as well as an increase in the expression of fibronectin in skin fibroblasts.

Consequently, we decided to test our hypothesis regarding the possibility of equal therapeutic potential in living cells and cell lysate [19]. For this purpose, we conducted two experiments with the involvement of two types of laboratory animals: the first, a pilot one — with the involvement of a small number of rats, and the next one — with the involvement of mice in a larger number. In the second experiment, we analyzed not only changes in the size of the wound in animals, but also the duration of healing of burn wounds and changes in body weight during 22 days of the experiment. Only in the experimental group where the hydrogel composition with the inclusion of living 4BL cells was applied, a total increase in the body weight of mice was observed, the effect size was 0.66 ($p \leq 0.05$). Although the average wound healing time in this group was not statistically significantly different from the similar indicator of the control group, but average wound condition was the best. As for the change in the size of the wound, here the greatest effectiveness was also observed with the preparation that carried living 4BL cells and did not contain cell lysate (impact index $\eta^2 = 0.60$, $p \leq 0.05$) [19].

Thus, at this stage of research, we concluded that the therapeutic effect is inherent to living cells in the composition of hydrogel constructions, and it significantly exceeds the positive effect of the use of cell lysates as the components of the studied dermal coatings.

Cells and their secretomes. Next, we set the goal of comparing the therapeutic properties of using human cells of different origin. We needed to decide which cells among the permanent cell lines available to us, that derived from healthy donors, should be used in further experiments (a priori we predicted that the therapeutic properties of different cell types would be different). Additionally, we believed that it is better to use the standardized established cell lines for the industrial production of dermal coatings than the primary cell cultures, which are expensive in terms of obtaining and maintaining procedures.

It was also necessary to test the hypothesis that the therapeutic effect may be associated with a complex of biologically active compounds secreted by cells into the culture medium. For this purpose, we used cell-free media (CM) conditioned by the cells of various lines, including stem cells *in vitro*. In the literature available to us at that time, we did not find any information about the creation of biotechnological dermal coatings using media conditioned by stem cells of the established lines.

However, the idea of using CMs, in particular, in order to improve the regeneration of skin tissues, has already been proposed by various scientists. First of all, it is worth ta-

king into account the works, in which the main cells of the skin, namely fibroblasts, were used. In 2017, scientists from South Korea [44] tried to stimulate the secretion of type I collagen by adipose-derived stem cells with the help of this environment. The result was positive, and in addition, the stimulated stem cells had a positive effect on the tissue regeneration using a full-thickness wound model in mice.

A group of Malaysian scientists has been studying the environment conditioned by human fibroblasts for several years [45–47]. The first works, published in 2018, demonstrated the data of proteomic analysis of the medium conditioned by human dermal fibroblasts in culture. The 2019 article describes the creation of a skin dermal substitute using a collagen hydrogel and a medium conditioned by human skin fibroblasts in culture. Testing in a full-thickness wound model in mice gave positive results. The 2021 article shows a continuation of the experiments described in the previous article, but chondroitin-4 sulfate was added to the structure of the carrier. The experiments with model animals are not mentioned in this article.

The culture medium conditioned by stem cells looks even more attractive from a therapeutic point of view, as it affects through paracrine mechanisms [48–50]. In addition, according to our data as well as according to other authors, the cultural medium conditioned by stem cells has antibacterial properties [19, 51]. Back in 2010, British scientists [52] using the "scratch assays" method tested the *in vitro* biological activity of a cell-free medium conditioned by MSCs. In this work, it is stated that collagen type I, fibronectin, as well as a number of mediators of wound healing were detected in the medium conditioned by MSCs. The authors suggest that the secretory activity of MSCs in the living organism may play a role in skin wound closure by influencing both the migration of skin fibroblasts and the migration of keratinocytes, along with contributing to the formation of the extracellular matrix.

Different groups of scientists indicate that the media conditioned by MSCs *in vitro* have an ability to positively influence the healing processes of burn wounds [53] and diabetic ulcers [54], to stimulate wound healing by accelerating the migration and proliferation of fibroblasts and keratinocytes [55], to prevent the formation of scars [56] and to improve angiogenesis [57, 58]. There are a few review articles devoted to discussing the therapeutic potential of MSCs and their secretomes [59, 60]. The examples of skin equivalents development using the cells of different types and their secretomes are presented in the Table 1.

Various sources of obtaining human MSCs are indicated in the literature [61–65]. MSCs obtained from adipose tissue are the most studied [11], although stem cells of other origins are also used. A new perspective source of MSCs is human placental tissue [58, 61–63]. In general, the positive effect of preparations with the inclusion of MSCs and their secretomes of various origins on the healing of skin lesions, including burn wounds, has been shown. As noted in the works of different authors [4–11, 14–16, 19–21, 63, 64, 66], the only drawback is non-standardized conditions for obtaining and storing MSCs and their secretomes.

Comparing cell-containing and cell-free preparations, the following can be attributed to the advantages of the latter:

- simplicity of storage procedures (in particular, cryopreservation);
- no need for special cryogenic protectors in the composition of the drug;
- facilitation of standardization of the manufacturing technology;
- easy control on pathogenic agents in the composition of the drugs;
- avoiding the potential risks associated with cell transplantation.

In our research we compared the effects of cells of different lines on the healing of burn wounds in rodents in the same experiments that allowed us to conclude that the most pronounced stimulation of tissue regeneration in the burn wounds was observed when using alive human MSCs of the 4BL line and the skin fibroblasts of A102 line [16]. This positive effect of cells on the healing of burn wounds in animals during xenotransplantation is apparently mediated by the biologically active compounds, which the cells synthesize and secrete into the cultural mediums, that probably leads to the stimulation of cell proliferation in the affected area of the burn wounds.

As mentioned above, the use of cell-free conditioned medium is more attractive from a practical point of view than the use of living cells. Therefore, we compared the therapeutic effect of cell-containing hydrogel constructions using the cells of each of the three lines and cell-free ones created using their secretomes [16]. It was shown that the secretome based on cells of the E8 line (embryonic germ cells in origin) was less effective than the cell-containing bio-construction. For the cell-free and cell-containing bio-constructions based on A102 skin fibroblasts, no difference in their wound-healing properties was found. And the bio-construction on the basis of the secretome CM-4BL was significantly more effective than the corresponding cell-containing product.

Noteworthy, the statistical analysis showed the following: the median duration of healing was the shortest in the group where the cell-free conditioned medium obtained using cells of the 4BL line was used while simultaneously comparing the duration of burn wounds healing in mice of six experimental groups (cells E8, A102, 4BL and CM-E8, CM-A102, CM-4BL) and in the control [16]. And CM-4BL was the most effective in burn wound healing among all variants (Fig. 1).

So, we have shown on the model of burn disease in ICR mice that the most effective among all six studied bio-constructions are those containing MSCs of the 4BL line or CM-4BL. As already noted, the peculiarity of this cell line is the method of obtaining it from the peripheral blood of an adult healthy donor and a long period of cultivation *in vitro* [17, 18]. Also, in this pair of hydrogel preparations, the cell-free bio-construction prepared using a conditioned medium was even more effective than the cell-containing ones at all stages of wound healing and, in general, the most effective of all investigated in the experiments with six types of therapeutic agents.

Having changed the type of laboratory animals (Wistar rats instead of mice) in the model of burn disease, we decided to test *in vivo* once again and compare the wound-healing activity of cells of the 4BL line and the medium conditioned by cells of the 4BL line [16, 19]. The

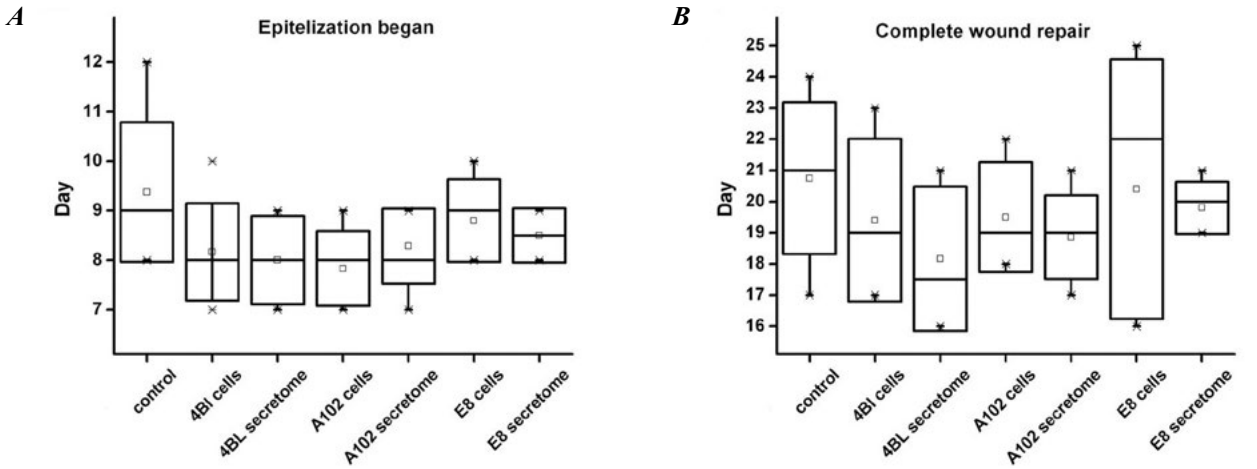

Fig. 1. Effectiveness of burn wounds regeneration in mice under the treatment by new dermis equivalents based on cell suspensions and cell-free secretomes: *A* — terms of wound epitelization beginning, *B* — terms of complete wound healing. Established cell lines have been used: 4BL — original human MSCs line derived from adult donor peripheral blood; A-102 — human newborn skin fibroblasts line; E8 — original human embryonic germ cells line.

effectiveness of both bio-constructions was again confirmed, and not only by evaluating the reduction of wound area, but also by microscopic observation of the state of vascularization of the wound surface and by examining the content of certain growth regulatory factors in the skin. Both live 4BL cells and CM-4BL were shown to stimulate blood vessel formation in the wound bed, with the stimulatory effect of cell-free conditioned medium being the most pronounced. In all animals treated with hydrogel constructions based on 4BL cells and CM-4BL, a statistically significant decrease in the content of regulatory proteins MMP2 and HIF-1ϐ was observed almost to the level characteristic for healthy animals, which may indicate inhibition of the inflammatory process and earlier completion of the first phase of wound healing. Similar data were obtained by other authors in the study of the influence of MSCs and especially their conditioned media on the healing of skin wounds in experimental animals [11, 65–67].

The comparison and generalization of the obtained results indicate that the most effective hydrogel construction among all investigated by us was the one containing a cell-free medium conditioned by MSCs of the 4BL line (CM-4BL) [16, 19–21]. We agree with the words of authors: “SC-CM is a promising novel cell-free therapy for wound healing in regenerative medicine and warrants further exploration” [11]. That is why it is necessary to investigate the dependence of the properties of the cell-free conditioned medium on the conditions of its development and storage. An experiment with varying the degree of confluency of 4BL cells *in vitro* showed that the density of cells in the monolayer of the producer culture is probably not critical for the quality of the obtained CM [19]. As already mentioned, there was a statistically significant

difference between the control and experimental groups, but the difference between the CM-4BL samples with different degrees of confluency was not statistically significant. We can make some assumptions about the reasons for the differences between the indicators. We assume that when the number of cells in the monolayer increases, both proliferation and general cell metabolism are inhibited, due to which the production of growth factors, that stimulate tissue regeneration in the wound bed, decreases. When the confluency approached 100 %, that is, the cells still proliferated, but occupied almost the entire area available to them, then some increase in the stimulating properties of CM, according to our assumptions, was a consequence of that the total mass of "micro-synthesizers", as cells could be tentatively called, was relatively large and, accordingly, they synthesized and secreted a sufficiently large amount of biologically active substances.

The storing conditions of the produced complex of biologically active substances in the composition of the hydrogel cell-free preparation are more significant for preserving its wound-healing activity than the degree of confluency of the cell mass [19]. Based on the experimental data, we consider it is optimal to deep freeze the obtained CM and store it at –80 °C (conditions provided by a laboratory kelvinator). But short-term storing in –20 °C is also allowable. A further increase in the storage temperature of the cell-free conditioned medium leads to a significant loss of its activity. So, although cell-free drugs, or rather their biologically active components, are much less picky about cryopreservation conditions than living cells, we still consider it necessary to continue the search for CM substances that would be less sensitive to storing conditions [19].

Thus, we have established that alive adult MSCs of the 4BL line, which were obtained in the Department of Human Genetics of the IMBG of the National Academy of Sciences of Ukraine from the peripheral blood of a healthy donor, as well as their cell-free culture medium (CM-4BL), can be combined with such a medicinal form, as a hydrogel based on the artificial polymer carbopol, and the resulting plastic bio-construction is able to stimulate tissue regeneration during the healing of burn wounds. It is shown that the practical use of CM-4BL is more beneficial both in terms of wound healing activity and in terms of the simplification of use and store. So the indicated carbopol hydrogel with components such as living cells of the 4BL line or their secretome can be used for the production of temporary skin substitutes or equivalents of the dermal layer, using carriers of natural (collagen) and artificial (polyacrylamide) origin.

Taking into account the results of our studies on model animals and preliminary data obtained in the clinic on a limited group of patients with massive burns, we can foresee the further usage of the temporary dermal equivalents that we have developed with the inclusion of MSCs of the original 4BL line and, especially, the cell-free CM-4BL preparation in the clinic. This will improve such vital indicators as stimulation of the regeneration of the skin dermal layer, increase the efficiency of allograft engraftment and, as a result, will lead to a significant improvement in the condition of health of patients with burn disease.

Conclusions

In our works using cellular technologies, we have created new artificial equivalents of the skin dermal layer on the basis of dense carriers (collagen and polyacrylamide membranes) and soft hydrogel substrates (gelatin, carbopol) with the inclusion of human stem cells or cultural mediums conditioned by these cells. It has been shown that the live cells of original 4BL line and, especially, the medium conditioned by these cells (CM-4BL) are the effective biologically active components of the studied bio-constructions that stimulate the healing of burn wounds in model animals and could be used for the production of biotechnological dermal equivalents for further application in medicine.

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Від мезенхімальних стовбурових клітин до їх секретомів як основних компонентів дермальних покриттів для лікування масивних опіків

О. Є. Папуга, Л. Л. Мацевич, Л. Л. Лукаш

У результаті проведення багаторічних досліджень нами створено нові біотехнологічні продукти, а саме — дермальні покриття (еквіваленти дерми) з включенням МСК людини або їхніх секретомів (кондиційованих клітинами культуральних середовищ, які містять комплекс синтезованих ними біологічно активних речовин). Проведено доклінічні дослідження нових еквівалентів дерми на модельних тваринах і клінічні випробування на обмеженому контингенті пацієнтів з масивними опіками з метою визначити їхню терапевтичну ефективність, а також безпечність. Ми довели в спеціальних експериментах *in vivo*, що нові дермальні еквіваленти позитивно впливають на загоєння масивних глибоких опікових ран при аплікації їх на ранову поверхню і не виявляють токсичних властивостей в досліджуваних організмах. Спосіб отримання нових біотехнологічних продуктів запатентовано.

Ключові слова: клітинна біотехнологія, дермальне покриття або еквівалент дерми, мезенхімальна стовбурова клітина (МСК), кондиційоване середовище (КС), секретом, опіки.

Received 29.04.2023