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Original Article

Application of hydrogel wound dressings in cell therapy-approaches to assessment in vitro

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Abstract: Cell therapy is actively used to treat skin defects, particularly burn lesions. The effectiveness of its application may depend on the appropriate choice of wound dressings used together with any cellular material. The aim of the study was to investigate the interaction of 4 hydrogel dressings used in clinical practice with human cells in an in vitro model to determine if their use in combination with cell therapy is possible. The effect of the dressings on the growth medium was assessed by considering the changes caused in the medium's acid-base equilibrium (pH) and viscosity. Cytotoxicity was determined by applying an MTT-assay and by direct contact methods. Cell adhesion and viability on the dressing surfaces were analyzed using fluorescence microscopy. Proliferative and secretory cell activity were determined concurrently. Characterized human dermal fibroblast cultures were used as the test cultures. Results: The tested dressings interacted differently with the growth medium and the test cultures. 1-day extracts of all dressings had almost no effect on the acid-base balance, but, after 7 days, the pH of the dressing Type 2 extract had sharply acidified. The viscosity of the media under the influence of dressings of Types 2 and 3 had also markedly increased. MTT-assays showed nontoxicity of all the 1-day-incubated dressing extracts, while incubation for 7-days resulted in extracts with evident cytotoxicity, which was reduced upon dilution. Cell adhesion to the surfaces of the dressings differed, being observed occurring on dressings 2 and 3, and to a limited extent on dressing 4. Cells under dressing 1 showed evident proliferative and secretory activity whereas the other dressings impaired either proliferation or secretion processes. These effects indicate that, in general, comprehensive studies including a variety of methodological approaches at the in vitro stage are needed to allow the selection of appropriate dressings if they are to be used in combination with cell therapy to act as cell carriers. Of those investigated, the Type 1 dressing can be recommended as a protective dressing for use after transplantation of cells into a wound defect area by some other method.

Keywords: Wound dressings, cytotoxicity, biocompatibility, direct contact method, wound, human dermal fibroblasts

Introduction

Local treatment of skin defects cannot be taken into account without consideration of modern wound dressings. Such modern wound dressings should be multifunctional. For example, a moist environment should be maintained in the damage area to avoid cell dehydration and to provide appropriate conditions for regeneration. At the same time, a dressing needs to limit skin maceration as a result of the accumulation of exudate in the wound area, and therefore must act as a sorbent. In addition, it is valuable if the dressing can promote angiogenesis, stimulate collagen synthesis and the formation of granulation tissue, and prevent wound contamination, thereby preventing microorganisms from colonizing the damaged area [1, 2]. Currently, a wide range of wound dressings is available on the global market, ranging from the simplest dressings with basic protective functions to the so-called "smart" dressings with built-in sensors and panels controlling the wound repair process [3-8].

Hydrogel materials are some of the most in-demand modern types of wound dressing. They have become popular since 1960 due to sev-

eral unique properties that not only ensure wound protection, but also provide conditions for regeneration processes to advance. For instance, unlike many other dressings, hydrogel dressings are characterized by their high water content that allows them both to maintain an appropriate moisture level in the wound and to reduce the local temperature, thus decreasing the severity of the inflammatory process [9]. Such dressings can also absorb wound exudate, thereby lowering the risk of wound infection. Such wound dressings offer further advantages, including excellent biocompatibility, lack of cytotoxicity and the possibility of gas exchange with the environment [10, 11].

Thus, the general mechanism of action of hydrogel-based wound dressings is based on creating a specific microclimate in the wound area that promotes tissue regeneration due to the ability of such hydrogels to maintain wound hydration, facilitate exudate removal and stimulate cell migration and collagen production at different stages of the healing process [12-14]. The individual characteristics of hydrogel wound dressings, such as polymer type, their structure and the availability of biologically active molecules, provide additional functional activity [15]. For instance, providing an ability to serve as a matrix for the controlled release of antibiotics and drugs [16, 17]. It is also important to point out that, with hydrogel dressings being soft and elastic, this allows them to be easily applied and removed without damaging the wound after healing [18].

Clinical studies are confirming the efficacy of hydrogel wound dressings in assisting the repair of skin wound defects. E.g. R. Koivuniemi et al. [19] showed that hydrogel wound dressings based on nanofibrillar cellulose and used to treat donor skin areas in burn patients promoted vascularization and the formation of new skin with greater thickness and elasticity compared to control wound dressings. K. Velding [20] has demonstrated that the treatment of Buruli ulcer with hydrogel dressings is more effective than the classical treatment using gauze bandages. In particular, with a hydrogel wound dressing, the ulcer was cleared and the wound defect skinned over. It should be noted that the wound dressing used also prevented the pain and bleeding typically caused by the use of gauze bandages. J. Liu and H. Shen investigated the efficacy of a chitosan-based hydrocolloid dressing to treat refractory wounds [21]. They found that such bandages helped both to relieve pain and itching and accelerated wound healing. Thus, many hydrogel wound dressings made of natural or artificial polymeric materials are being used for medical purposes and are proving to be efficient for healing skin wound defects of various etiologies [11, 22].

The application of dressings is not only limited to their primary purpose. The use of dressings in combination with cell products can significantly increase the effectiveness of wound treatment. Here, as well as being used simply as surface dressings to provide protection for the cells applied to the wound, the dressings themselves can act as carriers providing for the transfer of cells to the wound area (**Figure 1**). For example, there is a technique that involves the growth of fibroblast cultures on various carriers, culture transfer, and application of the cell layer directly to the wound bed, followed by removal of the carrier at an appropriate time after transplantation, depending on the condition of the wound.

The combined application of wound dressings with cell therapy brings additional requirements. It is known that wound dressings must be tested for safety and biocompatibility before any clinical application. ISO 10993-5:2009 [23] regulates preclinical studies for *in vitro* cytotoxicity. However, in addition to lack of cytotoxicity, a successful regenerative process requires the wound dressings not to interfere with cell recruitment to the wound area and for them to support the proliferative and functional activity of the cells. To use wound dressings as carriers requires that the cells can also adhere to their surfaces. The combination of such properties in dressings should ensure the efficiency of cell transfer to the wound area and the subsequent successful restoration of the damaged tissues. However, there are currently no specific recommendations or general approaches to assess the potential of using the approved dressings in combination with cell therapy.

The aim of the study was to investigate the interaction of 4 hydrogel dressings used in clinical practice with human cells in an in vitro

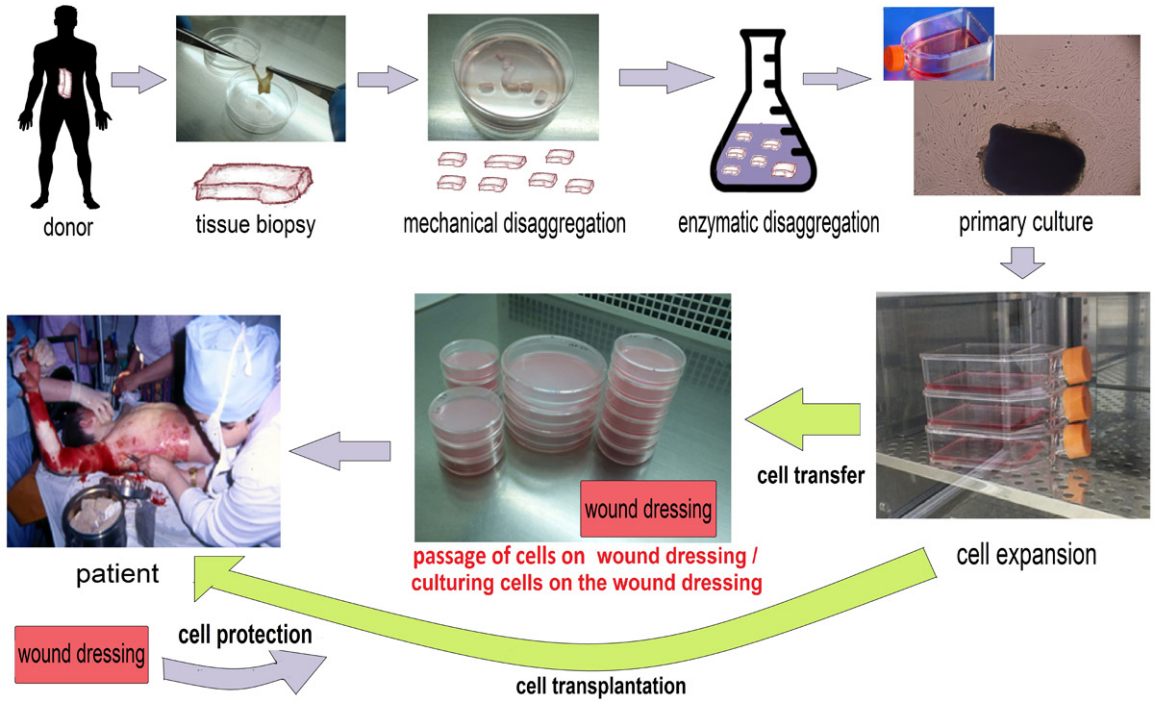

Figure 1. Principles of the application of wound dressings in combination with cell therapy.

model to determine if their use in combination with cell therapy is possible.

Materials and methods

Wound dressings

The authors studied the cytotoxicity and biocompatibility of 4 types of commercially available wound dressings:

Type 1: a wound dressing consisting of a polymer film (composition: hydrophilic vinyl acetate copolymers, dioxidine solution).

Type 2: a wound dressing consisting of an inner hydrocolloid layer in contact with the wound (composition: gelatin, pectin, sodium carboxymethylcellulose), located on an adhesive polymer base, with an outer layer of polyurethane film.

Type 3: a wound dressing consisting of a hydrocolloid matrix in contact with the wound (composition: highly elastic viscous polymer saturated with mineral oils, vegetable-based adhesive, E466 sodium carboxymethylcellulose supplement media), located on a polyurethane base.

Type 4: a wound dressing consisting of hydrogel polymer (composition: polyurethane and polyuria hybrid, propylene glycol), the reverse side of which (not in contact with the wound) was covered with a protective layer of polyurethane.

Cell culture

Four to six passage cultures of human dermal fibroblasts (hDFs) were used for the tests. Primary cultures of dermal fibroblasts were isolated from biopsy specimens of waste dermal skin obtained during cosmetic surgeries at the Department of Reconstructive and Plastic Surgery, University Hospital Federal State Budgetary Educational Institution of Higher Education «Privolzhsky Research Medical University» of the Ministry of Health of the Russian Federation (FSBEI HE PRMU MOH). Each patient participating in the study had provided his/her voluntary informed consent to the use of such material. The study protocol was approved by the local ethics committee of the FSBEI HE PRMU MOH (Minutes No. 11 of 09.06.2021). The tissue explants method was used to obtain the primary culture.

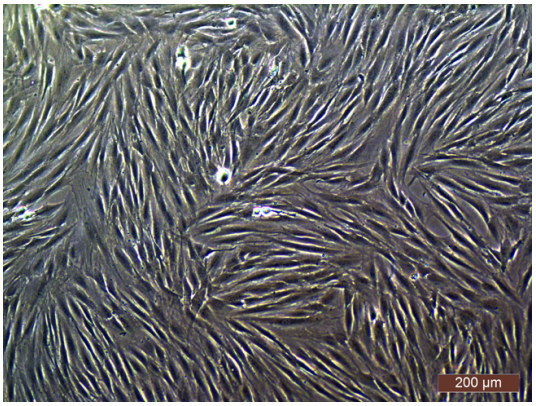

Figure 2. The hDF test culture (phase contrast).

All work with cells was conducted in a sterile box in line with aseptic regulations. The hDFs were cultured on complete growth medium: DMEMF/12 (Gibco™, Thermo Fisher, Waltham, MA, USA), 10% fetal bovine serum (FBS) (Gibco™, Thermo Fisher, Waltham, MA, USA), 2% glutamine, antibiotics (penicillin/streptomycin) (LLC PanEco, Moscow, Russia), using standard culture plastics (Corning, Arizona, USA). During growth, the growth medium was changed twice a week. After a subconfluent monolayer formation (70-80%), the cultures were transplanted. A 0.25% solution of trypsin in Versene (Gibco™, Thermo Fisher, Waltham, MA, USA) was used for reseeding. The cells were cultured in a CO_2 incubator under standard conditions (temperature 37°C, 5% CO_2, absolute humidity). Passaging was continued sequentially each time the subconfluent monolayer was reached until 4-6 passages had been reached. Cells from passages 4 to 6 were characterized and used for the study.

Before their introduction into the trial, the cultures were a uniform subconfluent monolayer formed of morphologically homogeneous cells with a predominance of spindle-shaped cells with clear contours, pronounced processes, and dense nuclei (**Figure 2**). The cultures used were sterile; there were no mycoplasmas or viruses found. Cell viability was 96-99%. The phenotype corresponded to that of typical mesenchymal cells: CD 90+, CD 105+, CD 73+, CD 44+, CD 10+, CD 45-, CD 14-, HLA DR-, CD 34-, CD 31-.

Study of wound dressing cytotoxicity using the MTT assay

For the MTT assay, 0.5 g samples of the wound dressings were placed in sterile test tubes to which were added 5 ml of DMEMF/12 culture medium. Specimens were incubated in a CO_2 incubator. Two series of specimens were prepared for each type of wound dressing under investigation. One series of dressing specimens was incubated in culture medium for 1 day and the second series incubated for 7 days to obtain the extracts. For further studies, the extracts obtained (the culture medium in which the specimens had been incubated) were collected in sterile tubes. 5 ml automatic single-channel mechanical pipettes were used to sample the extracts (Sartorius, Germany). Sterile conditions of a laminar flow box and sampling using aseptic techniques were observed. The resulting extracts were filtered through filters with 0.22 µm diameter pores. The wound dressing specimens were disposed of after extract sampling. A range of dilutions of the extracts were made up as follows: control-extract: growth medium, 0:1; dilutions-extract: growth medium, 1:0, 1:1, 1:2, 1:4 and 1:8, with mixing of the extract dilutions being ensured by rolling.

The hDF test culture had previously been seeded into 96-well plates in volumes of 100 µl of medium (DMEMF/12 medium with 2% FBS) per well with a cell concentration of 50,000 cells/ml and cultured in a CO_2 incubator for 24 hours. After this time, the growth medium was removed from the wells, and the various concentrations of extracts of the wound dressings to be tested were added to different cell culture wells in amounts of 200 µl per well. Each concentration was tested in 8 replicates. The plates were again placed in the CO_2 incubator for 72 hours. After that, 20 µl of a previously prepared MTT solution (Sigma-Aldrich, Taufkirchen, Germany) (5 mg/ml) was added to each well. The plates were replaced in the CO_2 incubator for a further 3 hours. After 3 hours of incubation with MTT, all liquid was removed from the wells and 200 µl of DMSO solution (Sigma-Aldrich, Taufkirchen, Germany) was added to each well. The resulting optical density (OD) was determined for each well at a wavelength of 540 nm using a TECAN plate reader and Magellan software (Tecan Group Ltd., Männedorf, Switzerland). To assess the cytotoxicity, the relative growth intensity (RGI) of each culture was calculated in line with the following formula: RGI (%) = mean OD in the test culture/mean OD in the control × 100. The

Table 1. Cytotoxicity rating scale

Relative growth intensity (RGI)	Cytotoxicity level (grade)
100	0
75-99	1
50-74	2
25-49	3
1-24	4
0	5

Note: Grade 0-1 - no cytotoxicity, 2-3 - moderate cytotoxicity, 4-5 - significant cytotoxicity.

results were assessed on the basis of a cytotoxicity rating scale (**Table 1**).

Investigation of the pH of wound dressing extracts

Isolation of the dressing extracts was conducted in line with the previously described method (Ref. clause 2.3 Materials and methods). The pH of each was measured using an HI 98103 Checker pH meter (HANNA Deutschland, Germany).

Determination of the viscosity of wound dressing extracts

Isolation and dilution of the wound dressing extracts were conducted in line with the previously described method (Ref. section 2.3 of Materials and methods). The viscosity of all extracts and their dilutions was determined using a Brookfield DV - II+ Pro (Brookfield, Middleboro, USA) viscometer. The studies were conducted at a sample temperature of 37°C.

Study of wound dressing cytotoxicity by direct contact method

In addition to the extraction method described above, the wound dressing cytotoxicity was assessed using a direct contact method. For this, hDF test cultures with a density of 15,000 cells/cm^2 were inoculated into 72 Petri dishes (each with an area of 20 cm^2). After 24 hours, provided that the cells had spread on the plastic and a subconfluent monolayer (80% of the area) had formed, the culture was considered ready for testing. The growth medium was removed, and the test samples were carefully placed on the surface of the culture in each dish. The area of the samples under study was 10% of the surface area of the Petri dishes. After the samples had been placed on the culture, fresh growth medium was poured into each Petri dish (Ref. clause 2.2 of Materials and methods). A total of 8 series were formed in triplicate: culture without samples (4 control series); Type 1 wound dressing (trial series 1); Type 2 wound dressing (trial series 2); Type 3 wound dressing (trial series 3); Type 4 wound dressing (trial series 4).

To assess the impact of the samples on the cells, the condition of each culture was recorded after 24, 48, and 72 hours with the following visual characteristics being noted: the nature of the monolayer and the cell morphology. Then the hDFs were detached using a mixture of trypsin/Versen and counted so that the concentration of cells and their density per unit area of the Petri dish could be calculated. Cell viability was assessed using *in vivo* trypan blue stain (Sigma-Aldrich, Taufkirchen, Germany). With this staining, dead cells become blue, while living cells, due to the selective permeability of their membranes, remain transparent. Each series was measured with a Countess counter (Thermo Scientific, California, USA) and the results were duplicated by recording the concentration using a Goryaev camera in two fields of view. The concentration, density and viability in the control series (cultures without samples) were evaluated simultaneously with the data from the trial series. With the direct contact method, samples of the growth medium were additionally taken to assess the secretory activity of the cells in terms of the VEGF-A (vascular endothelial growth factor-A) level (section 2.7. of Materials and Methods).

Assessment of the surface adhesive properties and of the wound dressing biocompatibility during cell cultivation

In order to assess the surface adhesive properties of the wound dressings, 1 cm^2 samples of each were placed in the wells of a 24-well plate. Then, the surface intended for contact with a wound was transfected with hDFs at a density of 20,000 cells/cm^2. The plates containing the samples were then placed in a CO_2 incubator under standard conditions. After 24 hours, the dressing samples were stained with fluorochromes and transferred to a new plate, so that any cells adhering to the wound dressings and

to the bottom of the culture wells could be visualized.

To characterize the biocompatibility of each dressing, the proliferative and secretory activity of the cells and their viability were assessed for cells cultivated on the surfaces of the dressings. The test periods were set as Day 1, Day 3, and Day 7. At the end of each test period, the condition of the cells was assessed for 3 samples of each type of wound dressing, with samples of the medium also being taken to determine the concentration of VEGF-A (section 2.8. of Materials and Methods). To assess cell proliferative activity and viability, the cells were stained with fluorochromes: Hoechst 3334 (BD, Franklin Lakes, NJ, USA), to estimate the total number of living cells, and TO-PRO™3 Ready Flow™ Reagent Invitrogen™ (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), to provide a discrete assessment of the proportion of dead cells. The number of cells was counted according to the previously described method [24] using a Cytation 5 imager (BioTek, Winooski, VE, USA). For statistical analysis, results obtained from 12 fields of view from each of the 3 specimens of each wound dressing type, and of the plastic surface under the wound dressing specimen were used.

Assessment of VEGF-A levels to determine the secretory activity of cells cohering to dressing samples

The concentration of vascular endothelial growth factor (VEGF-A) was determined in the culture medium samples that had been obtained during the studies described in sections 2.5-2.6 Materials and Methods. This was performed by enzymatic immunoassay using Invitrogen™ reagents (Thermo Fisher, Waltham, MA, USA), a TECAN plate reader and Magellan software (Tecan Group Ltd., Männedorf, Switzerland).

Statistical analysis

Statistical analysis was performed using the STATISTICA 6.0 software package. The research results were processed with nonparametric statistical methods, using the Wilcoxon paired comparison test. The results were presented as the mean (M) ± m. The level of significance was set as follow: $P<0.05$; $P<0.01$; $P<0.001$. Statistical significance of the results was judged at $P<0.05$. The result was judged a higher or extremely significant difference at $P<0.01$, $P<0.001$.

Results and discussion

One of the key aspects in planning investigations of the properties of wound dressings is the choice of the test culture. ISO 10993-5:2009 [23] provides fairly general recommendations about this. In this work, the authors used cultures of human dermal fibroblasts for this purpose. Fibroblasts are surface-dependent cells of the mesenchymal series, being the main cells of the dermal layer of the skin and, as such, having both a pronounced proliferative potential and a marked level of secretory activity [25]. These cells secrete a large number of cytokines and growth factors, including insulin-like growth factor (IGF), vasculoendothelial growth factor (VEGF), which stimulates neoangiogenesis, collagenase and protease remodeling enzymes, extracellular matrix proteins, and, in particular, epidermal growth factor, which regulates the proliferation and growth of the epidermis [26, 27]. Effective regeneration of skin wounds is not possible without a complete restoration of the dermis; therefore, fibroblast cultures are often used for cell therapy in the treatment of skin defects [28, 29]. Based on the above, the authors considered it reasonable to be guided by the conditions of the clinical use of the tested wound dressings when choosing the test culture. To obtain a sufficiently complete range of data on the potential application of the dressings in association with relevant cell therapy, it is important to take into account the characteristics of the cell culture-its required growth conditions, proliferation activity, secretory capacity, sensitivity to changes in physical and chemical environmental factors, etc.

Assessment of the cytotoxic effects of wound dressings

The most common methods used to determine cytotoxicity are quantitative, involving assessment of the number and viability of cells in absolute units. In turn, these quantitative methods are divided into direct contact methods (based on the direct cohesion of the test material with the cell culture) and extraction or indirect contact methods (based on assessments of the effects on the extract cells). The

MTT assay is a traditional extraction method. It uses a colorimetric method based on the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase into purple-blue intracellular crystals of MTT-formazan that are soluble in DMSO. Since the reduction process is catalyzed by NADP-H-dependent cell oxidoreductase enzymes, it can only occur in living cells. Thus, the method provides for assessment of the cytotoxic properties of the studied samples by comparative appraisal of the proportions of cells that remain alive. This method is widely accepted and recommended in the ISO standards.

The MTT assay: The authors conducted a study to assess the cytotoxicity of extracts isolated following 1 day (hereinafter, one-day-old extract) or 7 days (hereinafter, seven-day-old extract) of incubation of the different wound dressings in growth medium. The results of the MTT assay demonstrated that the one-day-old extracts of the Type 1 samples did not show cytotoxicity (**Table 2**), the extract taken from the samples after one day of incubation having a cytotoxicity grade of 1, however the relative growth intensity (RGI) of the cells in this treatment was slightly lower than in the control. When the extract incubated with Type 1 samples was diluted, the cytotoxicity grade ranged from 0 to 1. Whereas our aim was to investigate cytotoxicity, it should be noted that the RGI of the cells, in fact, exceeded the control values in the case of the 1:2 and 1:4 dilutions of the extract, which may indicate that these lower concentrations of the extract actually create favorable conditions for the cells' vital activities. However, at a dilution of 1:8, the cells' relative growth intensity decreased, which could be explained by the loss of that positive effect on cell growth in the case of greater dilution of the extract. Determining the cytotoxicity of the extract isolated after 7 days of incubation with the Type 1 samples showed that this extract was cytotoxic. The RGI of the cells in the presence of the undiluted extract was only 22.6%, indicating a high level of sample cytotoxicity. When the extract was diluted, a decrease in the cytotoxic effect was observed, however, even at a dilution of 1:8 the cytotoxicity grade was greater than 1. Thus, the substances released from the Type 1 wound dressing over 7 days had a pronounced cytotoxic effect. This effect was probably cumulative, as the one-day-old extract had not shown such a cytotoxic effect and for the 7-day extract it decreased with dilution.

When studying extract samples incubated with the Type 2 dressings, an inverse relationship was found in comparison with the results for Type 1. Here, the neat one-day-old extract of the Type 2 samples, as well as its 1:1 and 1:2 dilutions, did not have a cytotoxic effect, and the cells' relative growth intensity decreased with each dilution. For example, when studying the undiluted extract (1:0), the RGI was 99.4%, but when it was diluted 1:2, this value fell to 74.8%. With further extract dilution (1:4, 1:8), the cytotoxicity grade increased to 2, and the RGI went down to 50.5-67.9%. This effect of cytotoxicity increase with dilution of the extract may be associated with the so-called "paradoxical" and the "small doses" effects found in some biological systems. The small doses effect provides that very low concentrations of an active substance have an opposite effect on biological subjects compared to its higher concentrations. For example, when high concentrations of the active substance have a negative impact on biological subjects, a decrease in the concentration of the active substance results in the negative impact being reduced or completely leveled. However, a further decrease in the concentration of the active substance, in particular, to 10^{-4} and below compared to the initial level, reveals a paradoxical effect, where the negative effect on biological subjects is restored and may even exceed the initial effect observed at high concentrations of the active substance. The reasons for this phenomenon are still unknown to scientists. Most often, the effects are explained by the ligand-receptor interaction of molecules or a change in cell membrane structure, which entails a change in the cells' functional activity and responses to external factors [30, 31].

During the assessment of the effect of the seven-day-old extract of the Type 2 dressing samples, a pronounced cytotoxic effect (RGI = 23.9%) was found. This effect decreased by 3.7 times when diluted 1:1, with a corresponding decrease in the cytotoxicity from Grade 4 to Grade 1. These data also indicated the existence of the "paradoxical effect". It is likely that the pronounced cytotoxicity of the seven-day-

Table 2. Assessment of wound dressing cytotoxicity - MTT assay

Sample		Type 1		Type 2		Type 3		Type 4	
		1 day	7 days	1 day	7 days	1 day	7 days	1 day	7 days
Control (n=8) 0:1	OD (M ± m)	0.381±0.012	0.456±0.010	0.473±0.017	0.352±0.03	0.409±0.040	0.461±0.041	0.335±0.019	0.345±0.025
	RGI (%)	100	100	100	100	100	100	100	100
	Cytotoxicity grade	0	0	0	0	0	0	0	0
Extract (n=8) 1:0	OD (M ± m)	0.308±0.010	0.103±0.005	0.470±0.021	0.084±0.003	0.391±0.027	0.113±0.010	0.348±0.011	0.082±0.004
	RGI (%)	81	23	99	24	96	25	104	24
	Cytotoxicity grade	1	4	1	4	1	3	0	4
Extract 1:1 (n=8)	OD (M ± m)	0.378±0.015	0.269±0.007	0.426±0.023	0.309±0.072	0.368±0.020	0.248±0.046	0.363±0.025	0.590±0.038
	RGI (%)	99	59	90	88	90	54	108	171
	Cytotoxicity grade	1	2	1	1	1	2	0	0
Extract 1:2 (n=8)	OD (M ± m)	0.439±0.013	0.330±0.010	0.354±0.01	0.495±0.025	0.343±0.019	0.401±0.036	0.313±0.021	0.494±0.031
	RGI (%)	115	72	75	141	84	87	93	143
	Cytotoxicity grade	0	2	1	0	1	1	1	0
Extract 1:4 (n=8)	OD (M ± m)	0.478±0.024	0.337±0.026	0.239±0.006	0.373±0.024	0.355±0.013	0.391±0.027	0.297±0.022	0.385±0.045
	RGI (%)	126	74	51	106	87	85	89	112
	Cytotoxicity grade	0	2	2	0	1	1	1	0
Extract 1:8 (n=8)	OD (M ± m)	0.343±0.021	0.312±0.045	0.321±0.004	0.438±0.033	0.332±0.025	0.393±0.030	0.422±0.014	0.411±0.017
	RGI (%)	90	68	68	124	81	85	126	119
	Cytotoxicity grade	1	2	2	0	1	1	0	0

old extract (1:0) is due to a cumulative effect. Whereas the lack of cytotoxicity of the one-day-old extract (1:0) was comparable to the effect of the seven-day-old extract in a 1:1 dilution, it should be noted that in the case of further dilutions (1:2, 1:4, 1:8) of the seven-day-old extract, the cells' relative growth intensity exceeded the control values, indicating that these concentrations of the extract stimulated cell growth.

The study of Type 3 samples demonstrated that the one-day-old extract and its dilutions did not have a cytotoxic effect (their cytotoxicity grade was 1). However, with each subsequent dilution of the extract, there was a trend towards reduction in the relative intensity of cell growth. Furthermore, the seven-day-old extract demonstrated pronounced cytotoxicity (**Table 2**). For instance, the RGI impacted by the seven-day-old extract was more than 4 times lower than the control values, with a cytotoxicity of Grade 3. Such values are most likely associated with the cumulative effect of the active substance during the dressing's prolonged incubation in the growth medium, as with the Type 1 and Type 2 wound dressings. However, the RGI increased for subsequent dilutions, and even at a 1:2 dilution of the seven-day-old extract there was a virtual absence of cytotoxicity. The cytotoxicity grade in this and all greater dilutions was 1. Nevertheless, despite a clear decrease in cytotoxicity with dilution of the extract, the control values were not achieved even for an extract dilution of 1:8. It should be noted that no stimulating effect was seen in any of the dilutions of either the one-day-old or seven-day-old extracts of the Type 3 dressing.

For the Type 4 wound dressing samples, the one-day-old extract had no cytotoxic effect. However, for neat extract and a 1:1 dilution there were increases in the relative growth intensities compared to the control values. This may indicate a beneficial effect of the extract on the vital activity of the cells. However, in the cases of further dilution (1:2, 1:4), a negative trend was noted-the cytotoxicity grade was 1, and the RGI ranged from 88.7 to 93.4%. When one-day-old extract was diluted 1:8, the RGI increased and exceeded the values observed at for neat extract and 1:1 dilutions. Thus, overall, the one-day-old extract of the Type 4 wound dressings had no cytotoxic effect, and at certain concentrations it had a stimulating effect on the cells. When studying the seven-day-old extract, it was found that the undiluted extract (1:0) showed significant cytotoxicity (Grade 4). Such results may indicate that the concentration of toxic substances in the extract was high because of the cumulative effect during the prolonged incubation. However, when the extract was diluted with growth medium 1:1, the toxic effect was completely leveled. In this dilution, there was a marked difference in the cells' relative growth intensity, which exceeded the control values by 1.7 times! Thus, a two-fold decrease in the concentration of the seven-day-old extract of the Type 4 sample had a pronounced stimulating effect on the proliferative activity of the cells. With further dilution of the samples this positive effect decreased, but was not completely lost even at a dilution of 1:8 (**Table 2**).

Change in viscosity of the medium under the impact of wound dressings: Obviously, all the substances released by wound dressings end up in the medium with which they are in contact, and can change its physical and chemical properties. Thus, any substances released by dressings can violate normal functioning of cells not only when they directly affect cell metabolism, but also by changing the properties of the growth medium. It is known that cell metabolism is affected by many factors, such as the temperature of the medium and the presence of antibiotics, amino acids, growth factors, as well as by its viscosity. There is a high probability of a decrease in the cells' proliferative and functional activity in a medium that is too viscous, causing reduced cohesion between the cells, difficulties in obtaining nutrients, impaired gas diffusion, etc. [32-34]. In the case of dressing applications in combination with cells, one must take into account that many wound dressings can absorb or coagulate fluids. When such dressings are applied to a wound, they can absorb or thicken the exudate secreted by the damaged tissues [2, 35-37]. Since the dressings investigated here are of hydrogel nature, and hydrogels can retain up to 90% fluid or even more [38-40], there is no doubt that they can enter into various interactions with a liquid growth medium, including changing its viscosity.

Table 3. Measurement of viscosity of culture media following their incubation with different wound dressings

Sample, Type	time	dilutions				
		Extract (1:0)	1:1	1:2	1:4	1:8
1	1 day	1.09	1.03	1.11	1.08	1.06
	7 day	1.13	1.10	1.02	1.02	1.05
2	1 day	6.51	2.62	1.60	1.18	1.06
	7 day	11.40	3.57	1.95	1.37	1.15
3	1 day	23.40	6.39	2.88	1.62	1.11
	7 day	26.40	6.80	2.90	1.82	1.28
4	1 day	1.02	1.03	1.00	1.02	1.05
	7 day	1.02	1.02	1.02	1.03	1.09

Note: viscosity of the medium - centipoise units (cP), control (growth medium) =1 cP.

The authors studied the viscosity of the medium in extracts collected after either one day or seven days in contact with the different wound dressings. It was found that the Type 1 and Type 4 dressings did not critically change the viscosity of the medium (**Table 3**). The maximum increase was seen for a seven-day-old extract of the Type 1 dressing, but even this did not exceed 13% greater than the control values. A totally different result was observed for extracts of the Type 2 and Type 3 dressings. These significantly increased the viscosity of the growth medium. For instance, the viscosity of the one-day-old extract of the Type 2 dressing was 6.5 times higher than the viscosity of the original growth medium, and the viscosity of the seven-day-old extract was more than 11 times higher. When diluting the extract, a decrease in viscosity was observed, and it was almost completely leveled when the extract was diluted 1:8 (**Table 3**). Changes in the viscosity of the medium impacted by the Type 3 dressing were the most pronounced in comparison with the other samples. Here, the viscosity of the one-day-old extract exceeded the control values by more than 23 times, and for the seven-day-old extract it was more than 26 times higher. When these extracts were diluted, the viscosity decreased, and when diluted 1:8, it approached the control values.

The revealed differences can be explained by the structure of the wound dressings. It is known that hydrogels can vary greatly in their characteristics. They can have the form of a colloidal gel, in which the liquid is the dispersion medium, or the form of three-dimensional structures developed as a result of the hydrophilic polymer chains being held together by cross-links [41, 42]. According to the manufacturers' own descriptions of the various dressings, Types 1 and 4 involve cross-linked polymers, while the Type 2 and Type 3 dressings are made on the basis of hydrocolloid matrices. These properties of hydrogels explain the changes in the viscosity of the medium in which they were incubated. Thus, the hydrocolloid matrices contain unbound chains that can be released into the medium, expand in it, and increase its viscosity. Unlike hydrocolloid matrices, the structure of the cross-linked hydrogels prevents detachment of any of the components; such hydrogels can expand during hydration but do so without changing the viscosity of the medium.

When comparing the data from the study of the viscosity of the growth medium in which samples of the wound dressings had been incubated with the results of the MTT assay, it would be logical to predict that a significant change in the viscosity of the medium could be involved in the cytotoxic effect seen in the seven-day-old extracts of the Type 2 and Type 3 dressings. However, our analysis of the data excludes this hypothesis. For instance, one-day-old extracts of the Type 2 and Type 3 dressings had a high viscosity, yet they did not show any cytotoxic effect. It should be noted that the viscosity of the one-day-old extract of the Type 3 dressing even exceeded the viscosity of the seven-day-old extract of the Type 2 dressing by more than 2 times. Thus, the cytotoxic effect of these dressings cannot be associated with the observed change in the viscosity of the medium.

Impact of wound dressings on pH of the growth medium: It is known that the optimal pH of growth media for the active growth and functional activity of cells ranges between 7.2 and 7.4 [43, 44]. Any deviation has a negative effect on the cells and may result in changes in their metabolism or even in their death [45, 46]. During the MTT assay, the authors noted that during the incubation of some samples of the dressings used to obtain extracts, the color of the growth medium changed. The authors therefore tried a growth medium containing phenol red as a pH indicator: pink-red at pH

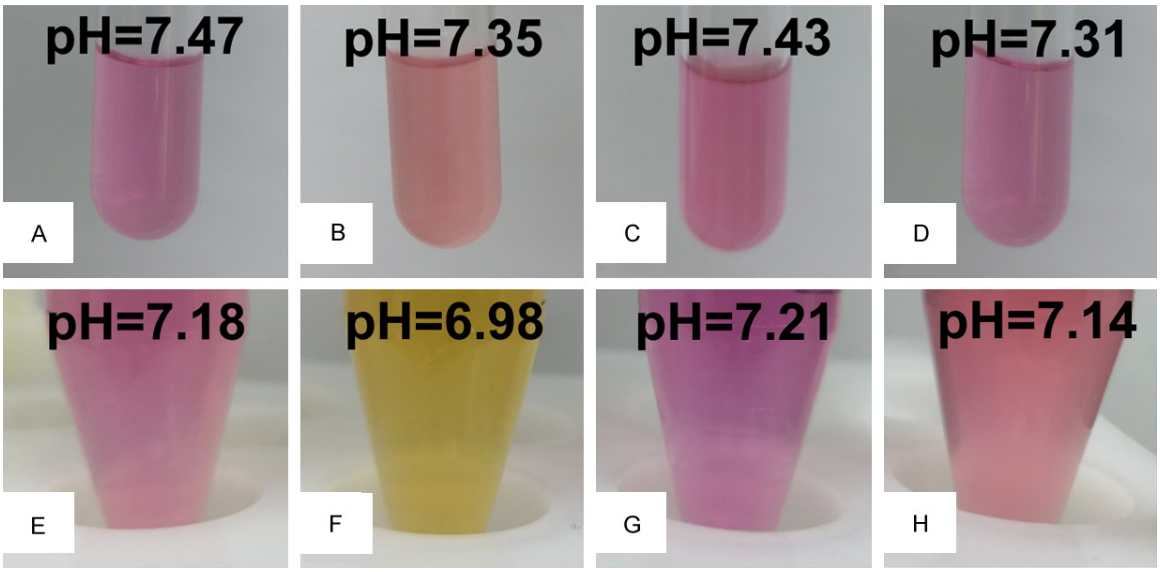

Figure 3. Changes in the culture medium pH impacted by incubation with the wound dressings. A-D. 1 day incubation. E-H. 7 days incubation. A, E. Type 1; B, F. Type 2; C, G. Type 3; D, H. Type 4.

7.2-7.4, changing its color to orange or yellow when the pH value decreases (acidification of the medium) and to bright purple when the pH value increases (alkalization of the medium). Thus it was possible to assess the level of acidity of the wound dressing extracts used in the MTT assay.

The results of the study showed that no significant pH fluctuations occurred for the one-day-old extracts. Although extracts from the Type 1 and Type 3 samples had a slightly alkaline reaction these changes did not have a significant effect on the cell culture during the MTT assay, their cytotoxicity being Grade 1, while their RGIs were over 80%. Meanwhile, the extracts from the Type 2 and Type 4 samples retained pH values within the optimal range of 7.2-7.4 (**Figure 3**).

A totally different result was seen for the pH levels in the seven-day-old extracts. It was found that while the pH of the extracts of the Type 3 dressing samples remained within the optimal range, the pH values of the Type 1 and Type 4 samples were slightly acidified. However, the seven-day-old extract of the Type 2 wound dressing demonstrated a sharp acidification (**Figure 3**). Comparing the data of the pH studies of the extracts with the results of the MTT assay, the authors conclude that the cytotoxic effect of the seven-day-old extract of the Type 2 wound dressing is highly likely to be due to this marked acidification. This is supported by the leveling of the cytotoxic effect when the extract was diluted with a growth medium that contained a buffer solution able to restore the original pH level.

Thus, almost all samples influenced the acidity of the medium in which they were incubated in some manner. This demonstrates the importance of analyzing the pH of the medium as well as conducting regular MTT assays if assessing the properties of different dressings. Doing so provides the opportunity to identify, and perhaps exclude one of the possible reasons for the cytotoxic effects seen in the test samples. It should also be noted that a change in the pH of the medium in the wound caused by the wound dressing can adversely affect the regeneration process and lead to undesirable effects, such as slower tissue repair, impaired angiogenesis, and even the wound defect turning into a chronic wound [47-49].

Direct contact method

The direct contact method, in contrast to the MTT assay, provides for the assessment of changes in the concentration of cells and their viability in the case of direct contact of the samples under study with the cell culture. The direct contact method is considered more sensitive

and more easily extrapolated to *in vivo* processes. According to the results of our study of the dressing samples using this method, the cell density under the impact of the Type 1 dressing did not differ from that of the control (cell culture cultivated under regular conditions) (**Table 4**). The cells maintained a high viability of over 95% throughout the experiment. Microscopic assessments of the condition of the cell cultures in the experiment and in the control were identical. The cells in each formed a subconfluent monolayer, the majority of the cells having the spindle-like shape typical of hDFs (**Figure 4**). In the same study, samples were also taken to assess the secretory activity of the cells by determining the concentration in the conditioned medium of one of the key factors secreted by fibroblasts, VEGF-A (vascular endothelial growth factor) [50, 51]. It was found that the concentration of VEGF-A in samples from hDFs cultured for 24 hours with Type 1 dressings was 31% lower than in the control. However, one day later (48 hours from the start of the trial), the concentration of VEGF-A in the trial series exceeded the control values at the corresponding cultivation period by 2.2 times, and the daily values of the trial by 3 times (**Table 5**). The extent of this difference decreased after 72 hours and the concentration of the growth factor in the trial samples exceeded that of the control by only 36.2%. The concentration of VEGF-A in the trial samples after 3 days was 1.8 times higher than in the 48-hour samples and 5.7 times higher than in the 24-hour samples. Thus, the Type 1 wound dressing samples on Day 1 depressed the secretory activity of the cells, but it was restored with longer cultivation. Taking into account the subsequent significant differences in the values of the control and the trial samples (at 48 hours and 72 hours), overall, the wound dressing samples had a stimulating effect on the secretory function of the hDFs. While the Type 1 wound dressing did affect the cell culture, influencing the cells' secretory activity, it still allowed them to maintain their proliferative activity and high viability.

The effects of the Type 2 dressings on the hDF cultures were more pronounced compared with those of Type 1. After 24 hours, the cell density impacted by the samples was reduced by 27%, and after 72 hours by 36.5% compared to the control values. The authors saw no growth of the cell culture; under the impact of the dressing sample, the culture appeared "paralyzed" (**Table 4**). By contrast, the cell density in the control had increased over time. However, the viability of the hDFs throughout the study stayed at a level of over 95%. Microscopic assessment of the state of the cell culture after 72 hours of cohesion with samples of the Type 2 dressing showed that although the cells had retained their characteristic spindle-like shape and formed a monolayer, in the trial series this monolayer was less dense compared to that in the control series (**Figure 4**). Multiple inclusions were visualized on the cell surfaces of the cultures after cohesion with the dressing samples. It is assumed that these were hydrocolloid hydrogel particles adsorbed onto the cell membranes. On assessment of the levels of VEGF-A, it was shown that the cells had maintained their secretory activity at a fairly high level under the impact of the dressing samples (**Table 5**). For instance, the concentration of VEGF-A increased dynamically over time, and in the samples taken after 72 hours of cohesion it exceeded the values in the samples taken after 24 hours by more than 4 times. Nevertheless, it should be noted that the VEGF-A level was lower in the samples taken 24 hours and 48 hours after the cohesion of the samples with the cell culture than in the samples from the control culture. However, by 72 hours the concentration of VEGF-A in the trial samples exceeded that in the controls, as noted above. Thus, despite the inhibition of proliferative activity, the Type 2 wound dressing samples did not significantly affect the viability of the hDF cultures and ultimately allowed the cells to maintain their high secretory activity.

Assessment of the impact of direct contact of the Type 3 dressing samples with the hDF cultures showed that the cell density was significantly lower than that in the controls throughout the trial. For instance, 24 hours after the start of the trial, the cell density impacted by the samples was 37% lower than that in the control culture. Over time, this difference increased to 55% after 48 hours and to 71% after 72 hours. Analysis of the cell density changes under the impact of the dressing samples over time showed that Type 3 dressings affected the hDFs in a similar manner to the Type 2 dressing-the culture was "paralyzed". However, microscopic assessment revealed

Table 4. Comparative study of the impact of wound dressing samples on hDF cultures (direct contact method)

t	Type 1				Type 2				Type 3				Type 4			
	control		test		control		test		control		test		control		test	
	Cd	V	Cd	V	Cd	V	Cd	V	Cd	V	Cd	V	Cd	V	Cd	V
24	28.70±0.70	99	28.28±2.24	96	35.09±2.48	96	25.51±3.40	96	21.66±0.99	97	13.75±1.17	97	19.26±1.08	98	19.16±1.57	99
48	33.97±0.70	98	33.40±0.19	98	39.39±2.13	99	29.35±1.71	98	37.91±3.18	99	16.85±0.60	98	26.48±1.59	99	23.47±1.08	99
72	37.00±0.74	99	35.28±1.04	98	40.83±3.30	99.5	25.88±2.21	96	50.32±1.48	99	17.50±2.01	87	27.50±0.78	98	20.69±0.79	99

Note: Cd - cell density, thousand/cm^2; V - cell viability, %; t - time, h.

Table 5. Change in VEGF-A concentration (pg/ml) during cultivation of hDF cultures with wound dressings samples (direct contact method)

Time, h	Type 1		Type 2		Type 3		Type 4	
	control	test	control	test	control	test	control	test
24	200.30±39.97	138.26±19.07	477.60±154.84	427.19±33.16	441.41±83.98	289.76±58.67	338.86±96.03	730.79±132.96
48	193.34±54.88	427.33±56.40	1285.37±98.63	978.47±120.86	974.44±33.01	944.30±110.95	1483.79±78.26	1511.78±130.23
72	574.48±106.92	782.59±130.93	1204.81±159.54	1853.75±34.27	1503.59±8.61	1696.58±176.15	3136.93±52.55	2853.33±150.50

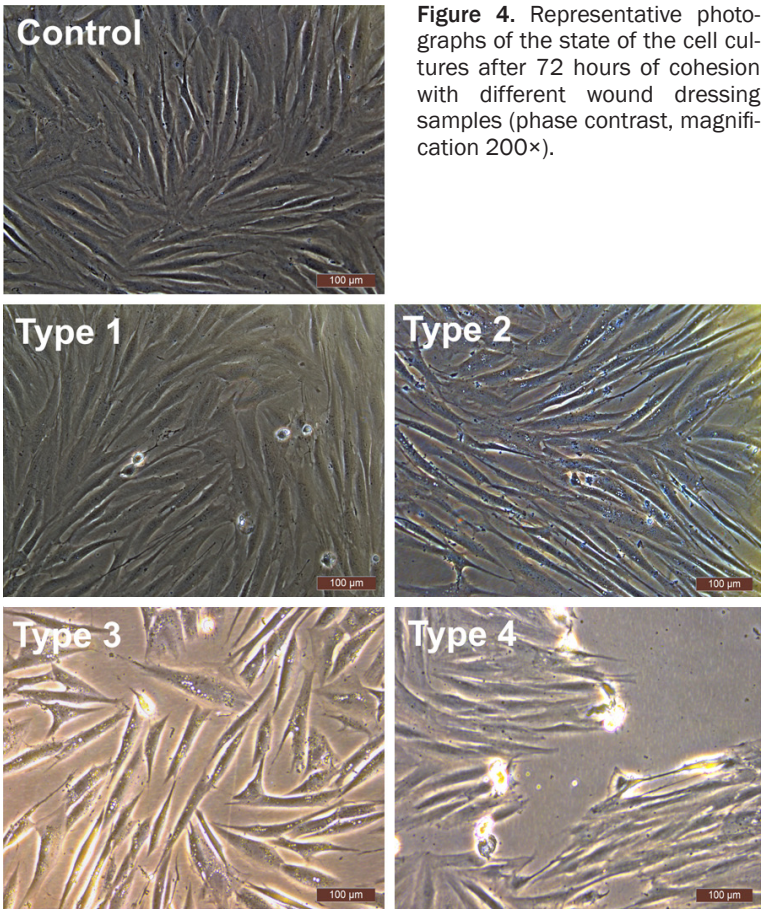

Figure 4. Representative photographs of the state of the cell cultures after 72 hours of cohesion with different wound dressing samples (phase contrast, magnification 200×).

that the cells interacting with the Type 3 samples did not form a subconfluent monolayer like that seen in the control. The state of the culture can best be described as a very thin monolayer, formed by predominantly spindle-shaped cells. As with the impact of the Type 2 dressing, multiple inclusions were well visualized on the cell surfaces exposed to the Type 3 samples (**Figure 4**). Taking into account that both the Type 2 and Type 3 dressings are hydrocolloid hydrogels, it is no surprise that similar results were found in the study. It is likely that the "paralysis" of culture growth in both cases was due to the impact of the hydrocolloid particles that became fixed to the cell membranes. That the inhibition was more pronounced with the Type 3 dressings correlates with the data from the study of the viscosities of the medium extracts taken during the MTT assay (**Table 3**). There, the values of the medium viscosity were significantly higher for the Type 3 dressing extracts than for Type 2. Cell viability under the impact of the Type 3 dressing samples remained at a high level over the first 48 hours (97-98%) although it had decreased to 87% by 72 hours after the start of the trial. The dynamics of the changes in the VEGF-A level in the medium during cell cultivation in the presence of the Type 3 dressings was similar to that for cultivation in the presence of the Type 2 samples. For instance, the concentrations of VEGF-A in samples taken after 24 hours and 48 hours were lower than in the control at these times, whereas after 72 hours it exceeded the control value. In fact, the VEGF-A concentration had increased so markedly over that 24 hours that, by 72 hours, it exceeded the values in the samples taken after one day by almost 6 times (**Table 5**). Thus, the Type 3 wound dressings had a pronounced negative effect on the hDF cultures, significantly suppressing their proliferative activity, reducing cell viability in the late stages of the study, and reducing secretory activity in its early stages.

Analysis of the data from the study of direct contact with Type 4 samples showed that after 24 hours the cell density in the cultures of the trial and control series did not differ. After further cultivation, a trend towards decreasing cell density in the hDF culture cultivated with the dressing samples was noted, and by 72 hours the cell density in the trial series was 25% lower than the values in the controls. The dynamics of cell culture growth impacted by the Type 4 samples was characterized by an increase in cell density up to 48 hours of cultivation, followed by growth termination (72 hours). However, the Type 4-exposed hDF culture was characterized by high viability-99% (**Table 4**). Microscopic examination of the state of the culture after 72 hours of cohesion with the Type 4 samples showed that the cells had a morphology typical of fibroblasts and had formed a subconfluent monolayer comparable in density to that in the control. However, the areas of the monolayer, where the dressings were directly located, had multiple "tearings" i.e., areas without cells. Furthermore, no foreign inclusions

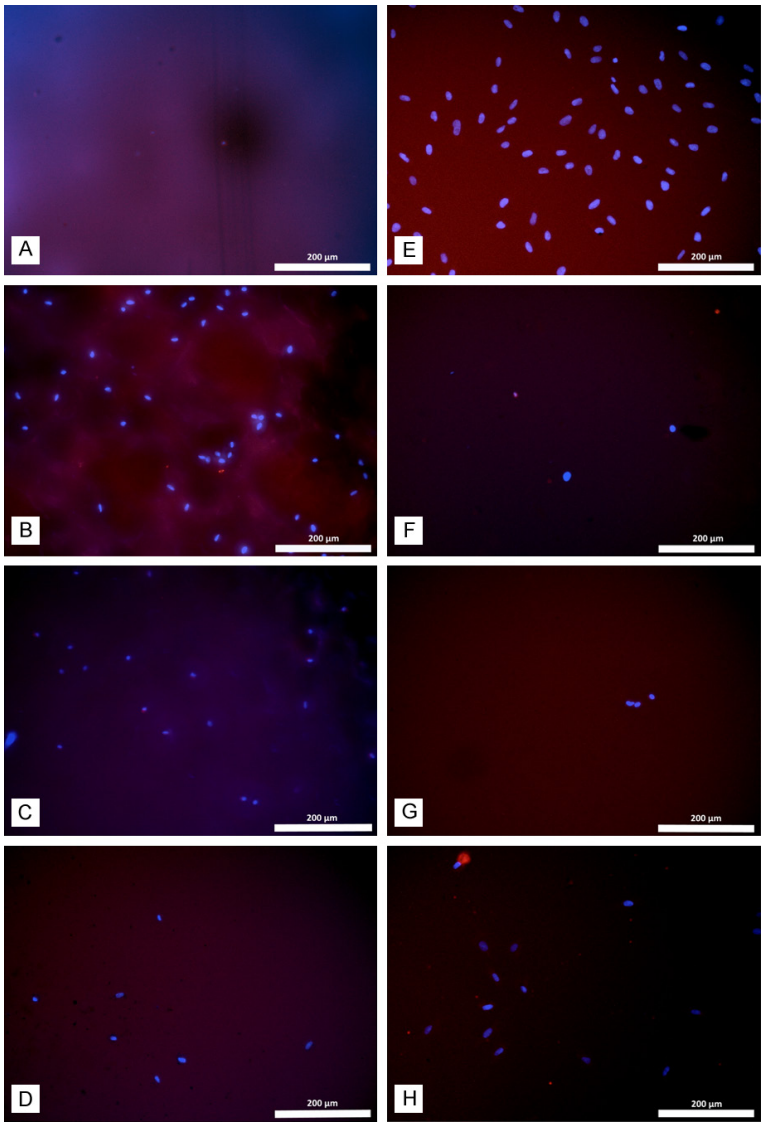

Figure 5. Representative photographs from the assessment of hDF adhesion to the surfaces of the different wound dressings. A-D. Cell nuclei on the surface of wound dressings. E-H. Cell nuclei on the surface of the plastic plates under the wound dressings. A, E. Type 1; B, F. Type 2; C, G. Type 3; D, H. Type 4 (intravital fluorescent staining: blue-nuclei of living cells stained by Hoechst 3334; red-nuclei of dead cells as revealed by TO-PRO 3 Ready Flow).

were seen either on the cells or in the empty areas (**Figure 4**). Data from the VEGF-A concentration analysis showed that the dressing had had a stimulatory effect on the secretory function of the cells during the early stages. For instance, 24 hours after the start of the trial, the VEGF-A level exceeded the control value by more than 2 times (**Table 5**). However, the further dynamics changed: after 48 hours the values in the trial and control samples had become almost equal, and after 72 hours the concentration of VEGF-A in the trial samples was 9% lower than the control value. Despite this, it can be stated that the Type 4 wound dressings had had no significant negative effect on the secretory activity of the cells. In other words, the VEGF-A concentration in the trial samples had risen throughout the experiment and increased almost 4 times (from 24 hours to 72 hours). Thus, although the Type 4 dressing did affect the proliferative activity of the cells during prolonged exposure (72 hours), it still allowed the cells to maintain high viability and secretory activity throughout the trial.

Assessment of the properties of wound dressings with hDFs seeded on their surfaces

Adhesive properties of the wound dressing surface: When such material is to be analyzed for its value in regenerative treatment, the studies cannot be limited just to assessment of the severity of its potential cytotoxicity. The material must also promote adhesion and allow the cells to maintain high viability, proliferative and secretory activity. If the dressing is able to provide appropriate conditions for cell adhesion it may therefore additionally be considered as a potential carrier for cell transfer to the wound area.

The authors assessed the adhesive properties of the surfaces of the different types of wound dressing. For this purpose, hDFs were seeded onto the dressing surfaces, and after 24 hours, the cell nuclei (live/dead) were differentially colored using specific fluorochromes; here, the presence of cells on both the upper surface of each sample and below the sample was assessed. It was found that the cells did not adhere to the surface of the Type 1 wound dressing. All cells applied to its surface

Table 6. Assessment of proliferative activity and viability of cells during their prolonged cultivation on wound dressings

Sample (type)	Day	On the wound dressing surface		On the surface of plastic plate under wound dressing	
		Total number of cells (pcs./mm^2)	% of dead cells	Total number of cells (pcs./mm^2)	% of dead cells
1	Day 1	*	*	111.66±18.61	*
	Day 3	*	*	287.08±52.64	*
	Day 7	*	*	508.93±40.71•,■	*
2	Day 1	100.78±16.80	*	*	*
	Day 3	129.16±9.43•	*	*	*
	Day 7	150.44±14.54•	*	*	*
3	Day 1	23.89±3.98	*	*	*
	Day 3	95.36±12.38•	*	10.81±2.97	27.55
	Day 7	362.69±20.11•,■	*	*	*
4	Day 1	20.24±3.37	*	61.39±10.39	*
	Day 3	55.37±12.99•	5.68	23.46±5.98•	*
	Day 7	-	-	13.49±3.03•	6.69

Note: * - cells were sporadic or absent in the field of view for dead cells - up to 5%; • - P<0.001 - comparison with day one, ■ - comparison with day three.

"slipped" off the samples and spread out onto the surface of the culture plastic (**Figure 5**). Adherent hDFs could be observed on the surface of the Type 2 and Type 3 dressings 24 hours after seeding, furthermore, the nuclei of separated cells could be visualized beneath the dressing. A somewhat different result was observed for the study of cell adhesion to the surface of the Type 4 samples. Some cells adhered to the surface of the dressing, but at the same time, a significant proportion of the cells were beneath it and had become fixed to the plastic (**Figure 5**).

Assessment of the proliferative activity and viability of hDFs when seeded onto dressing samples: After the cells were seeded onto the samples, they were cultured to assess their viability, proliferative and secretory activity changes over time. As, shown in the previous section, the cells did not adhere to the Type 1 wound dressing, but adhered well to the surface of the plastic beneath it. It was found that when cultivated under the wound dressing, the hDFs retained high viability and maintained a pronounced proliferative activity (**Table 6**; **Figure 6**). For instance, the total number of cells increased by 4.6 times from Day 1 to Day 7, and the percentage of dead cells did not exceed 5% throughout the study. Samples of the culture medium were also taken to gauge the secretory activity of the cells by assessing the levels of VEGF-A in the medium. It was found that during cultivation, cells under the Type 1 wound dressing maintained high secretory activity, with the VEGF-A levels increasing dynamically over time (**Figure 7**).

For the cells cultivated on the Type 2 dressing, it was found that, although they maintained their proliferative activity, the rate of culture growth on the dressing was not very pronounced, the total number of cells increasing only by a maximum of 1.5 times from Day 1 to Day 7. However, the cells did retain high viability and some secretory activity, although the latter was fairly limited, with the VEGF-A concentration increasing by less than 20% from Day 1 to Day 7.

When cultivating hDFs on the Type 3 dressing the cells maintained their proliferative activity and high viability. Indeed, the number of cells increased by almost 4 times from Day 1 to Day 3, 3.8 times from Day 3 to Day 7, and, from days 1 to 7, by a total of 15 times! Assessment of the secretory activity of hDFs cultured on the Type 3 dressings revealed a negative trend (**Figure 7**). Thus, despite the high proliferative activity of the cells, their secretory activity was significantly reduced. It should also be noted that on Day 1, although single cells could be observed beneath the Type 3 dressing, and that by Day 3 their number had increased, almost a third of those present were not viable. By Day 7, in the field of view, there were, again, only single, separate cells visible under the dressing, as was the case on Day 1. This can be explained by cell death and destruction.

Totally different results were found when the cells were seeded onto samples of the Type 4 dressings. Some of the cells adhered to the surface of the dressing, however, a significant proportion of them were under the dressing by 24

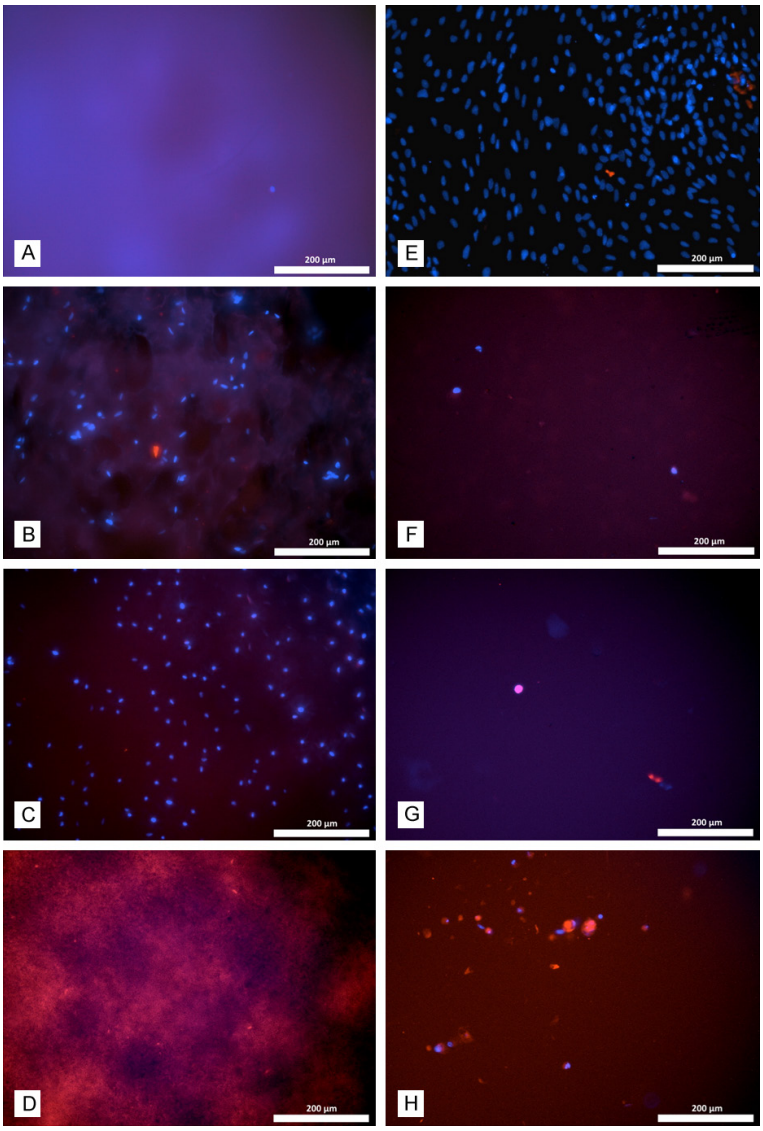

Figure 6. hDFs on the surface and beneath wound dressings samples 7 days after seeding. A-D. Cell nuclei on the surface of wound dressings. E-H, cell nuclei on the surface of the plastic plates under the wound dressings. A, E. Type 1; B, F. Type 2; C, G. Type 3; D, H. Type 4 (representative photographs; *in vivo* fluorescent staining: blue-nuclei of live cells, stained with Hoechst 3334; red-nuclei of dead cells stained with TO-PRO 3 Ready Flow).

hours after seeding (**Table 6**). Over the cultivation period, the total number of cells beneath the dressing decreased sharply. In particular, from Day 1 to Day 7 the number of cells decreased by 4.5 times. One should note that the proportion of visualized dead cell nuclei was less than 5%, whereas by Day 7 it was about 7%. At the same time, most of the visualized nuclei were “expanded” and had indistinct shapes indicating they were undergoing destruction. This explains the decrease in total cell count over time. An even more interesting result was noted when studying the state of the cells cultured on the surface of the dressing. Here, the number of cells increased by more than 2.5 times between Day 1 and Day 3. However, in parallel with the increase in the number of cells, a negative trend was seen in relation to their viability, although concurrently, the dynamics of the VEGF-A changes were positive. With reference to the above sections, in order to assess the secretory activity of the cells, the authors sampled the medium in which the cells had been cultivated both on and under the dressings. From an analysis of these results we concluded that the observed dynamics of the increase in the VEGF-A concentration in the growth medium shows little association with the true secretory activity of the cells. It is most likely that the growth factors detected were actually released into the culture medium as a result of cell destruction rather than by secretion, thereby explaining the increase in VEGF-A concentration.

One of the key conclusions of the work was the need for a comprehensive study of different wound dressings, using various methodological approaches at an *in vitro* phase in order to determine the possibilities for their application in combination with cell therapy. The limitation of *in vitro* studies is that they may provide false positive results, which, if taken into account during the choice of dressing could lead to erroneous assessments of their potential for cell therapy, and, worse still, may lead to a negative result of treatment and to a deterioration in the patient. It should, however, be noted that a comprehensive, initial, *in vitro* approach to the study of wound dressings is still economically preferable, even

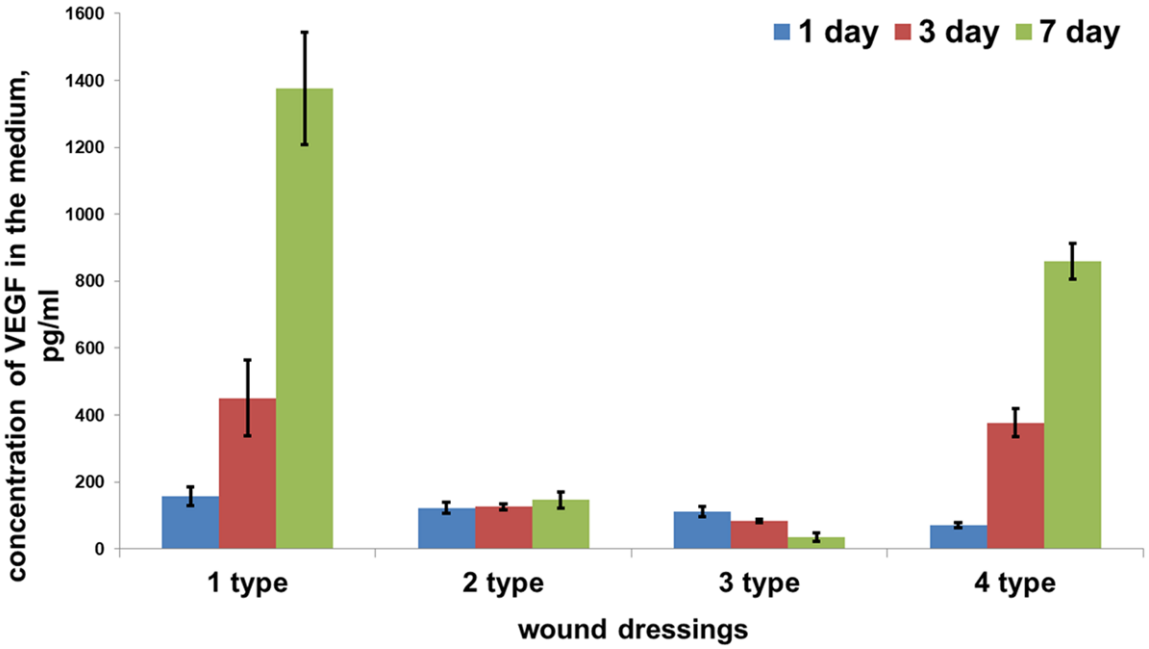

Figure 7. Dynamics of changes in VEGF-A levels in the growth medium around seeded dressings.

though it involves quite expensive trials, as it ultimately allows greater subsequent savings at the stage of *in vivo* and clinical studies. The authors hope that the methodological base and research results demonstrated herein will allow specialists working in the field of regenerative treatment and cell technologies to optimize their criteria for choosing the most appropriate wound dressings to be applied in combination with cell therapies.

Conclusions

The studies we conducted showed that each of the tested hydrogel wound dressings interacted with the cells but affected the test cultures in different ways. Taking into account all of our results, it is only reasonable to recommend the Type 1 dressing for applications involving cell therapy where it can function as a protective wound dressing after cell transplantation into the wound area. Based on the results of our work, none of the wound dressings investigated can be recommended as a carrier for cell transfer to the wound.

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Disclosure of conflict of interest

None.

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