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Macrophage activity modulation via synergistic effect of a porous substrate and low-field laser therapy

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Purpose: The aim of this study was to investigate the effect of substrate – polycaprolactone (PCL)-based porous membrane modified with rosmarinic acid (RA), (PCL-RA) and to determine the optimal values of low field laser irradiation (LLLT) as stimulators of biological response of RAW 264.7 macrophages. *Methods*: The porous polymer membrane was obtained by the phase inversion method, the addition of rosmarinic acid was 1%wt. The reference material was pure polymer membrane. RAW 264.7 were deposited on the material and then irradiated with a laser with a wavelength of 808 nm, a power of 100 mW, an irradiation dose of 2 J/cm^2/cell well, applied continuously (C), (100/2/C) or pulsed (I), (100/2/I). *Results*: Macrophage irradiation resulted in an increase in their adhesion. Modifying the PCL membranes with rosmarinic acid had no effect on cell viability on day 3 of the cell culture. Irradiation of macrophages cultured on PCL-RA material increased their viability. Irradiation of macrophages cultured on PCL-RA material decreased macrophage secretion of NO and protein and the increase in TNF and MCP-1 secretion was only transient on day 3 of culture. *Conclusions*: Macrophage irradiation had a positive effect on macrophage attachment. Modification of PCL membranes with rosmarinic acid influenced the biological activity of macrophages. Culture of macrophages on rosmarinic acid-modified PCL membranes and simultaneous irradiation of LLLT cells resulted in anti-inflammatory effects.

Key words: *cytokines, macrophages, nitric oxide, rosmarinic acid, policaprolactone PCL*

1. Introduction

Macrophages are cells that play a key role in inflammation process. The main function of macrophages is to regulate the inflammatory response and maintain immune balance, which is related to their phenotypic plasticity/polarization. Macrophage polarization is triggered by signals from the surrounding tissue and results from a change in the expression of proteins on their surface, which determines the characteristics and activity of a given cell and affects other cells of the immune system and cells of the surrounding tissue. Macrophages are divided into: classically polarized with an M1 phenotype (pro-inflammatory) and alternatively polarized with an M2 phenotype (anti-inflammatory, reperative), which promote tissue repair and remodeling. The M1 phenotype is induced by interferon-γ (IFN-γ) and the presence of bacterial lipopolysaccharide (LPS). The M1 phenotype is characterized by an increase in inducible nitric oxide synthase (iNOS, inducible nitric oxide synthase) activity, production of reactive oxygen species (ROS), pro-inflammatory cytokines, including interleukin IL-1 and tumor necrosis factor α (TNF-α). In contrast, the M2 phenotype is induced by IL-4 or IL-13 and produces large amounts of anti-inflammatory cytokines min IL-10 and TGF-β [8], [21]. Data obtained from the literature show that in

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damaged tissue (*in vivo*) we have a spectrum of macrophage phenotypes that includes M1 and M2 phenotypes, but also intermediate phenotypes combining their features. The occurrence of the latter is most likely related to the simultaneous presence of different signals in the tissue [8], [21].

The low-field laser therapy (LLLT) is assigned a special role in accelerating tissue repair processes, especially in regenerative medicine [3]. The research results prove that LLLT affects cells in various aspects, depending on the beam application method (continuous or pulsed mode), its wavelength, power and a radiation dose. It has been shown that low-energy laser radiation increases cell proliferation, protein synthesis and secretion, production of ATP (adenosine triphosphate) and free oxygen radicals (reactive oxygen species – ROS). On the other hand, the LLLT application may lead to cell damage, activation of immune system cells (including macrophages) and pro-inflammatory cytokines secretion, initiating tissue inflammation [9], [17]. Work published by Fernandes' team LPS-stimulated macrophages J774 irradiated with two wavelengths: 780 nm (with a power of 70 mW and a dose of 2.6 J/cm^2) and 660 nm, (with a power of 15 mW and a dose of 7.5 J/cm^2). These studies showed a decrease in TNF-α and IL-6 secretion by cells irradiated with the 780 nm laser, and a decrease in TNF-α secretion and an increase in IL-6 secretion by macrophages irradiated with the 660 nm laser, compared to those not irradiated [10]. On the other hand, Leden et al. [19] stimulated LPS-stimulated BV2 macrophages by irradiating them with an 808 nm laser, with a power of 50 mW and doses of 0.2, 4 or 30 J/cm^2. In this case, it was shown that applying a dose of 0.2 J/cm^2 resulted in an increase in MCP-1 secretion by the cells while it had no effect on their NO secretion. In contrast, higher doses of 4 and 30 J/cm^2 had no effect on MCP-1 cell secretion, but resulted in an increase in macrophage NO secretion, compared to unexposed cells [10]. Also, Souza et al. [31] studied LPS-stimulated J774 macrophages, measuring their mitochondrial activity after irradiation with two laser wavelengths: 780 nm, with a power of 70 mW and a dose of 3 J/cm^2, and 660 nm, with a power of 15 mW and a dose of 7.5 J/cm^2. In this case, it was shown that the mitochondrial activity of the cells was modulated, depending on the irradiation wavelength used as well as the duration of culture. The first day of culture showed a decrease in the mitochondrial activity of the cells due to irradiation at 660 nm wavelength. On day 3, both laser irradiation wavelengths tested resulted in an increase in the mitochondrial activity of the cells, while on day 5 of culture, the mitochondrial activity of irradiated cells did not differ from that of non-irradiated cells [31].

In tissue repair processes, combination therapies are particularly beneficial for inflammatory responses. The simultaneous use of LLLT and the design of polymer materials for the controlled release of active polyphenols seems to be an innovative approach that can support tissue regeneration processes. Polyphenols are natural compounds of plant species (Lamiaceae) endowed with antioxidant properties. They also show unique affinity for functional molecules, such as receptors, enzymes or transcription factors. That is why, intensive research is being conducted on incorporating polyphenols into polymer dressing materials aimed to support regenerative processes by controlling the damaged tissue microenvironment. One of the substances classified as polyphenols is rosmarinic acid (RA) whose antioxidant activity is rated higher than the one of vitamin E [1], [2], [5], [7]. Additionally, RA is able to stabilize cell membranes and displays antimicrobial and antibacterial properties that promote the healing/regeneration of tissue [18]. There have only been few studies describing the comprehensive impact of low-energy laser and antioxidants on the biological response of cells, especially of macrophages developing inflammatory reactions.

Studies on RA proved its anti-inflammatory effectiveness achieved through inhibiting the expression of various pro-inflammatory factors, such as IL-6, IL-1β interleukins, defined as TNF-α (Tumor Necrosis Factor) [30]. As shown on RAW 264.7 cells in a mouse model, RA supported a decrease in NF-κB activity and prostaglandin E2 (PGE2), nitric oxide (NO) and cyclooxygenase-2 (COX-2) production. This mechanism helped prevent inflammation and oxidative stress [12]. RA also revealed anticancer activity by inhibiting healthy cells cytotoxicity, maintaining the constant mitochondrial membrane potential [16]. The RA use in the U937 cells showed anticancer effects registered as the decreased TNF-α production. In yet another research RA inhibited the reactive oxygen species (ROS) generation, apoptosis and NF-κB activation [23].

In the literature, also difficulties to properly dose and deliver RA to target sites in demand of anti-inflammatory, anti-cancer or anti-aging activities were indicates. Capsules, oils or micelles used as carriers neither cross the cell membrane nor integrate with body fluids easily.

The aim of this study was to investigate the effect of substrate – polycaprolactone (PCL)-based porous membrane modified with rosmarinic acid (RA), (PCL-RA), and to determine the optimal values of low field laser

irradiation (LLLT) as stimulators of biological response of RAW 264.7 macrophages.

2. Materials and methods

2.1. Substrates preparation

The research employed porous membranes based on the commercial polymer PCL (polycaprolactone 80 kDa, Fluka) obtained via non-inductive phase separation (NIPS). Aa a solvent, we used dichloromethane (DCM, chemical reagent, Avantor SA), dimethyl sulfoxide (DMSO, chemical reagent, Avantor SA) and dimethylformamide (chemical reagent, Avantor SA). The process of manufacturing membranes was analogous to that in the work [33]. In the final stage of polymer homogenization with the solvent mixture, 0.5 ml of DMSO mixed with 1% v/wt. rosmarinic acid (RA, No. S3612 Selleck Chemical's) was added. The membranes were air-dried and then washed in a sequence of diluted alcohols (EtOH 60, 70, 80 and 96%) both to remove DMSO and maintain the original pore morphology. The reference material was a membrane free of rosmarinic acid and DMSO. The obtained membranes were characterized in terms of microstructure (scanning electron microscope, NovaNano SEM) as well as pore size distribution and total porosity (ICP mercury porosimeter, Quantochrome). The RA influence on the physicochemical properties of the membrane, including wettability and antioxidant properties, was determined via the DPPH test (according to the procedure described in Food Chemistry 112 (2009) 654–658).

The following reagents were used for the test: synthetic 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma), methanol (MtOH, AR, Avantor SA). The solutions absorbance was measured at the 517 nm wavelength in the Shimadzu spectrophotometer 2001. A methyl alcohol solution served as the reference. The test material with an area of 24 cm^2 was placed in 15 ml of methyl alcohol (MtOH), from which 3 ml was collected at three subsequent time points. Then, we measured the absorbance (A_0) of the DPPH radical solution diluted with MtOH in the 1:3 ratio. Each solution was tested three times and the average absorbance value of the tested solution containing antioxidants was calculated (AAve). The ability to deactivate the DPPH radical of a given substance was calculated according to Eq. (1).

$$DPPH = \frac{(A_0 - A\text{Ave})}{A_0} \cdot 100\% . \tag{1}$$

The materials for biological tests were sterilized in ethyl alcohol and exposed to UV radiation for 10 minutes.

2.2. Macrophage culture conditions

The study was based on the RAW 264.7 macrophage cell line (ATCC, USA). The cells were cultured in 75 ml plastic bottles (Nest SB, USA), in the RPMI 1640 culture medium (Lonza, USA) with the addition of 10% FBS calf serum (Gibco, USA) and a 5% solution of two antibiotics: (penicillin and streptomycin from Sigma, USA). The atmosphere was set at 5% CO_2 and the temperature at 37 °C. Then, 1 ml of cell suspension with a concentration of 0.015 million cells/ml was placed in each well of a 24-well culture plate (Nest SB, USA). Prior to this, in each well we placed round culture slides – CTR group and a control (Menzel Glaser, Germany), reference PCL membranes (PCL group) or PCL membranes modified with rosmarinic acid (PCL-RA group).

2.3. Irradiation with low-energy laser radiation

The PhysioGo 400C device (ASTAR, Poland) was used to irradiate the macrophages with a laser. The low-energy laser generated electromagnetic infrared radiation with the 808 nm wavelength, 100 mW power. The radiation dose was 2 J/cm^2/well containing the cells. The laser beam was applied either continuously (C), (100/2/C) or in a pulsed manner (I) (100 Hz frequency with 50% duty cycle), (100/2/I). The irradiation was performed using a non-contact method at a 1 cm minimum distance from the cells (i.e., the plate well height), at a right angle to the irradiated surface.

The laser radiation was applied every 24 hours 2, 4, 6 times. On the next days of the experiment it was: 3 (2 applications of the laser beam), 5 (4 applications of the laser beam), 7 (6 applications of the laser beam). The macrophage culture was finished and the cells and the supernatant collected from above the culture were intended for further biological determinations.

2.4. Cell adhesion/proliferation (CV test)

The cells adhesion ability was examined using the crystal violet uptake test. The cells attached to the sub-

strate were fixed with 2% paraformaldehyde – PFA (Sigma, USA) for 5 minutes, stained for 5 minutes with the 0.5% crystal violet solution (Sigma, USA) and rinsed with water. Then, the dye absorbed by the macrophages was extracted by adding 0.5 ml of 100% methanol (Linegal Chemicals, Poland) to each well. The optical density (OD) of the fluid was read, using a FLUOstar Omega reader (BMG Labtech, Germany) at the 570 nm wavelength.

2.5. Cell viability (ViaLight test)

The ViaLight test (Lozna, Switzerland) was used to test the macrophages viability in culture. 200 µl of Cell Lysis Reagent was added to the wells containing cells and 600 µl of the supernatant. After a 10-minute incubation, 200 µl of the supernatant and the lysis reagent mixture was transferred to a white 96-well plate (Nest SB, USA) and 200 µl of the AMR PLUS reagent was added. Two minutes later, the emitted radiation was determined, using a FLUOstar Omega reader (BMG Labtech, Germany).

2.6. Level of released adenylate kinase AK (ToxiLight test)

The AK level was determined via quantification, using the bioluminescence method (Toxilight, Lonza, Switzerland).

The supernatant from the cell culture (20 µl) was collected and transferred to a white 96-well plate (Nest SB, USA). 100 µl of the AK Detection Reagent solution was added to each well, according to manufacturer's protocol. After 5 minutes of incubation, the luminescence value was read, using a FLUOstar Omega reader (BMG Labtech, Germany).

2.7. Level of secreted protein (BCA test)

10 µl of each tested sample was transferred individually to the wells of a 96-well plate (Nest SB, USA). Then, 200 µl of the 1:50 mixture consisting of CS (II), i.e., copper sulphate II (Sigma-Aldrich, Germany) and BCA, i.e., bicinchoninic acid (Sigma-Aldrich, Germany) were added. The plates were incubated for 30 minutes in the dark. After the designated time, the optical density (OD) of the liquid was read at the 570 nm wavelength, using a FLUOstar Omega reader (BMG Labtech, Germany).

2.8. Level of NO secretion (Griess test)

The secreted nitrite ion amount was determined according to the following procedure: 100 µl of the cells supernatant was transferred to each well of a 96-well plate (Nest SB, USA). Then we added 100 µl of a 1:1 reagents mixture (Sigma-Aldrich, Germany) – Griess A (1% sulphanilamide in 5% phosphate acid) and B (0.1% (1-naphthyl) ethylenediamine in H_2O). After 5 minutes, the optical density (OD) of the liquid was read at the 540 nm wavelength, using a FLUOstar Omega reader (BMG Labtech, Germany).

2.9. Level of secreted cytokines

Cytokine levels in the cell culture supernatants were measured via flow cytometry using Flex Set kits (CBA, BD Biosciences). The entire assay procedure, all measurements and analyses were performed in accordance with the manufacturer's instructions, using a Beckman Coulter flow cytometer (Life Science, USA). Thanks to the Mouse Inflammation Kit (BD Biosciences, USA) the simultaneous determination of 6 cytokines was possible: interleukin (MCP-1), tumour necrosis factor (TNF-α), interferon gamma (IFN-γ, interleukin 12p70 (IL-12p70), interleukin 6 (IL-6) and interleukin 10 (IL-10). The data analyses and determination of cytokine concentrations were performed in Microsoft Excel, using standard curves based on the subsequent dilutions of the standard.

2.10. Statistical analysis

The data are presented as mean values and standard error. The differences between the control group and the study group were obtained with the use of the Student's *T*-test if the assumptions for parametric tests were met. If the assumptions were not met, the non-parametric Mann–Whitney *U*-test was applied. The differences between the control group and the study groups were established using one-way analysis of variance (ANOVA), and then, the Tukey's test was used for post-hoc evaluation. The significance level of $p \leq 0.05$ was adopted. Microsoft Excel 2007 and GraphPad 7 (GraphPad Software, San Diego, CA, USA)

were used for the statistical and graphical analysis of the results.

3. Results

3.1. Characteristics of substrates modified with rosmarinic acid

All the obtained membranes were similar in thickness – approximately 120 µm, surface porosity (approx. 68%) and volume porosity (above 60%) (Fig. 1f). The pore size distribution in the PCL and PCL-RA membranes was unimodal. The PCL membrane had round and larger pores (average pore size of approx. 60 µm), while the PCL-RA membrane had the pores with sharp boundaries and the size of approx. 40 µm. (Figs. 1a, b, d, e). The introduction of rosmarinic acid into the polymer matrix affected the pore formation process; their shape and size (Fig. 1c). However, the RA presence did not affect the membrane porosity (Table 1). The smaller pores in the PCL-RA membrane and the RA presence in the matrix did not alter the surface wettability values. The presence of the acid was confirmed by antioxidant tests which showed a visible reduction of the DPPH free radicals already after the 15-minute incubation of PCL-RA (Fig. 2). Moreover, the material was still able to reduce free radicals after 60 minutes of incubation. Such changes were not observed for the reference material without RA (PCL).

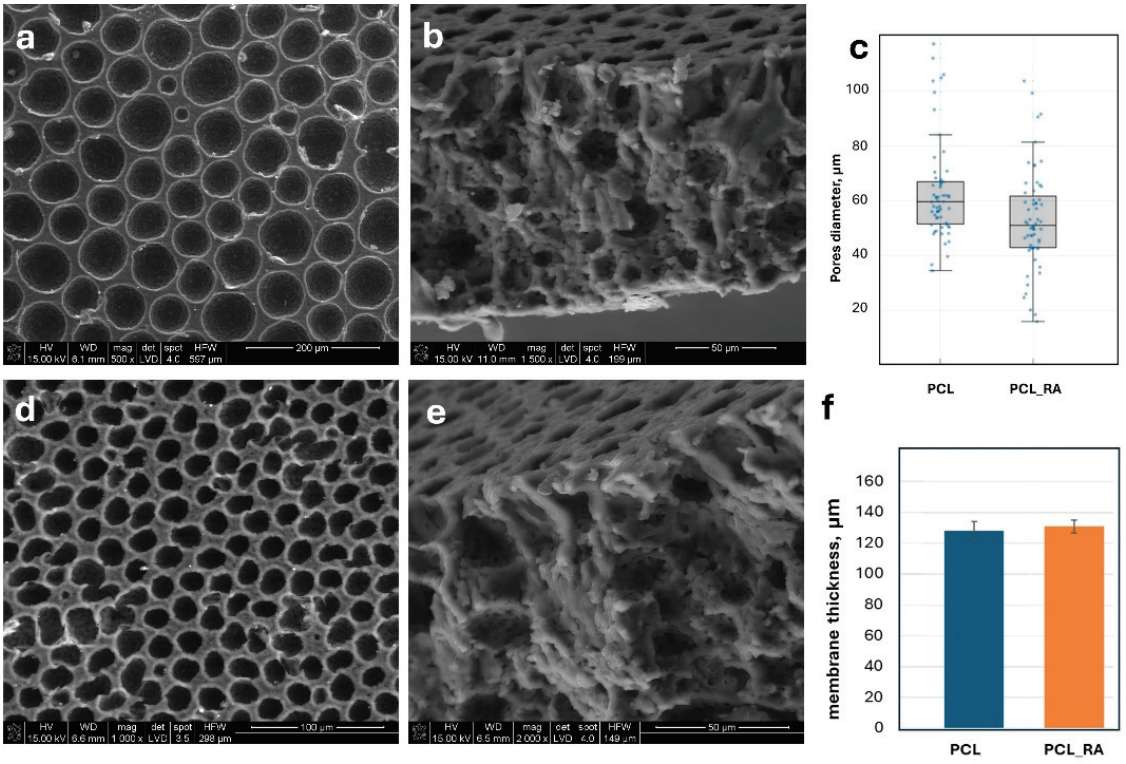

Fig. 1. Microstructures of membrane materials: PCL reference sample, PCL membrane area (a), PCL membrane cross-section (b), and PCL-RA rosmarinic acid modified membrane: PCL-RA membrane area (d), PCL-RA membrane cross-section (e). Average membrane thickness (f), membrane pore size distribution (c)

Table 1. Summary data characterizing the PCL reference membrane and rosmarinic acid modified membrane (PCL-RA)

Substrate	Total porosity [%]	Surface porosity [%]	Diameter of pores [µm]	Wettability, contact angle
PCL	62 ± 2.5	68 ± 1.2	63 ± 4.6	67 ± 2.8
PCL-RA	64 ± 1.8	67 ± 2.1	41 ± 5.2	65 ± 4.3

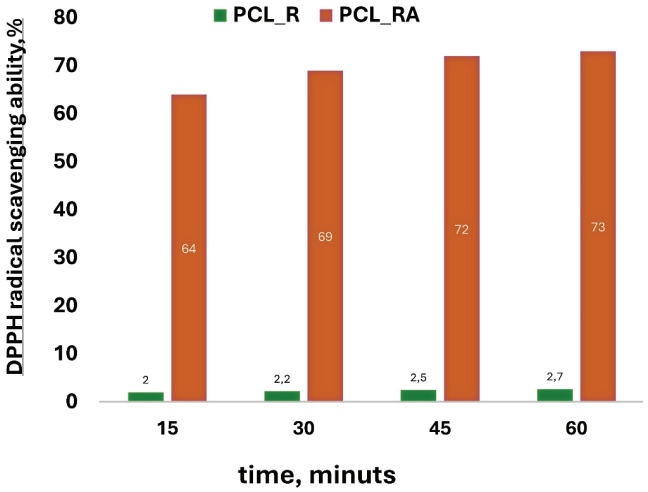

Fig. 2. DPPH radical deactivation scavenging ability in contact with the tested PCL and PCL-RA membrane material at several time points (15–60 min)

3.2. Adhesion/proliferation of macrophages irradiated with low-energy laser

In the course of the experiment, the non-irradiated macrophages from the control group (CTR) proliferated, which increased the adherent cells number on days 5 and 7 in comparison to day 3 of the culture. The 2-, 4- and 6-fold irradiation of the macrophages with both a continuous (100/2/C) and pulsed (100/2/I) laser beam increased the macrophage adhesion/proliferation on all days of the experiment (days 3, 5 and 7), (Fig. 3a, b).

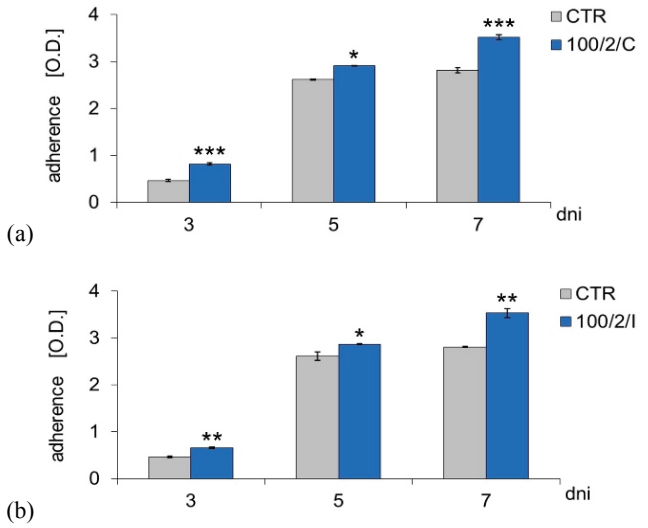

Fig. 3. Effect of continuous laser beam (C, a) and pulsed laser beam (I, b) on macrophage adhesion of the RAW 264.7 cell line. Cells were cultured on slides for a specified number of days and irradiated with a 100 mW laser, an irradiation dose of 2 J/cm^2/cell well. On subsequent days of the experiment (3, 5, 7), cells were stained with crystal fillet. O.D. – optical density was measured at 570 nm. Mean values ± standard deviation. *, **, *** – differences between cells irradiated with laser of different parameters and cells not irradiated (CTR) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.3. Viability and secretory activity of macrophages cultured on porous PCL membranes

Due to the PCL membrane structure, it was impossible to perform a cell adhesion test using crystal violet on this substrate, as the membrane itself absorbed CV. For this reason, we evaluated the macrophages viability, adenylate kinase and NO release, and the protein and cytokine secretion in the next stage of the study. There were no statistically significant differences between the cell viability, AK release and NO secretion observed for the macrophages growing on PCL and the control macrophages grown on culture slides (Figs. 4–c). At the same time, a transient increase in the secretion of protein and cytokines TNF-α and MCP-1 was demonstrated by the PCL macrophages on day 5 of the culture, compared to the CTR control group (Figs. 4d–f).

3.4. Viability and secretory activity of macrophages cultured on porous PCL membranes modified with rosmarinic acid (PCL-RA)

On day 3 of the culture, no differences in cell viability were found between the cells cultured on the PCL material modified with rosmarinic acid (PCL-RA group) and the macrophages cultured on the reference PCL material (PCL group). Further culture of macrophages (day 5 and 7) on PCL-RA displayed the cell viability lower than the one of PCL cells (Fig. 5a). Regardless of the irradiation mode (continuous or pulsed beam), the viability of macrophages cultured on the PCL-RA material was lower than the cells viability on PCL (Figs. 4b, c).

On all days of the experiment, culturing cells on PCL-RA material resulted in a decrease in the release of the adenylyl kinase, AK, from dead cells (Fig. 5b). Modification of the membranes with rosmarinic acid results in an increase in NO and protein secretion (on days 3 and 7 of culture) by macrophages cultured on this material compared to macrophages cultured on PCL membranes (Figs. 5c, d).

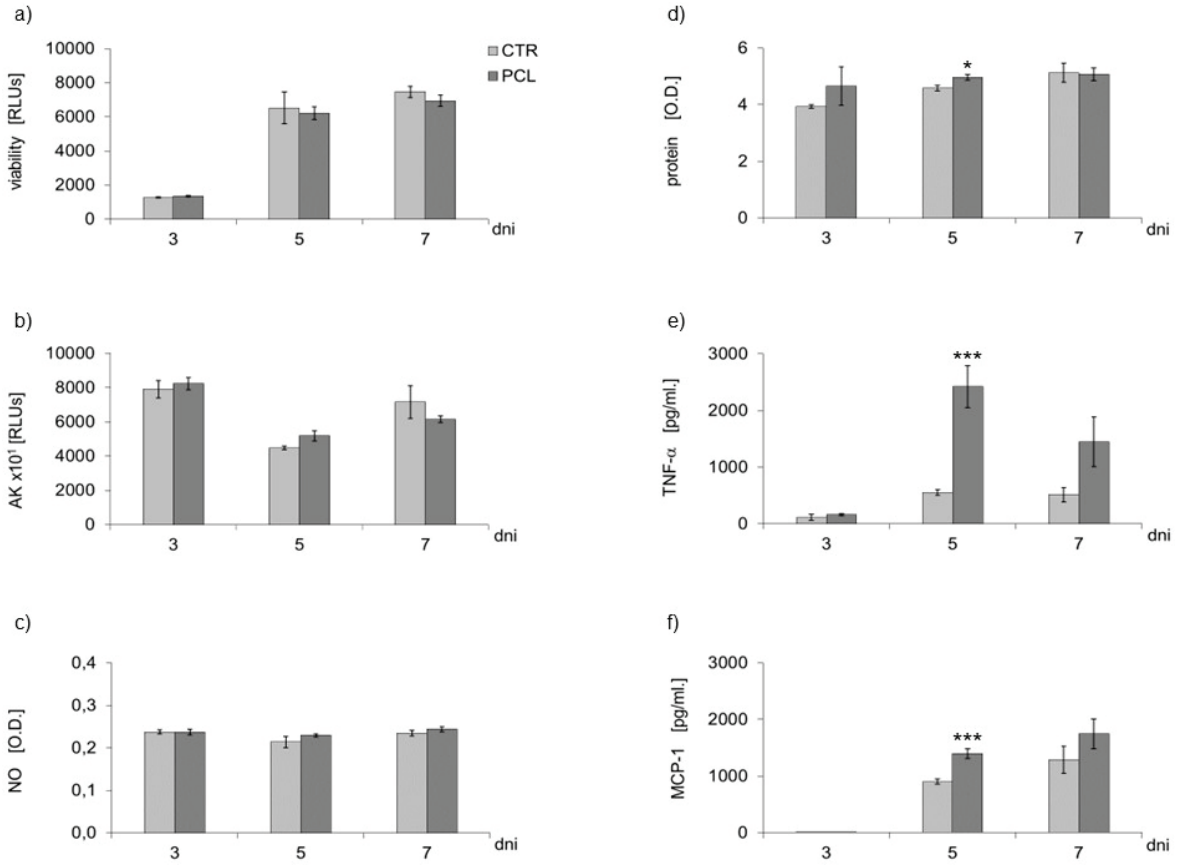

Fig. 4. Effect of porous polycaprolactone membrane (PCL group) on the viability (a) of RAW 264.7 cell line macrophages and the levels of released adenylyl kinase (AK, b), secreted nitric oxide (NO, c), protein (d), TNF-α (e), MCP-1 (f). Cells were cultured on slides (CTR group) or PCL membranes for a defined number of days. Cell viability was measured on the next 3, 5 and 7 days of the experiment. RLUs – luminometer flux unit, O.D. – optical density was measured at 540 nm. Mean values ± standard deviation. *, *** – differences between PCL group and CTR group (* $p < 0.05$, *** $p < 0.001$)

It was shown that the level of secreted TNF-α and MCP-1 by macrophages cultured on PCL-RA material did not differ from the level of secretion of these cytokines by macrophages cultured on PCL reference material (Figs. 5e, f). At the same time, the cytokines interferon gamma (IFN-γ) interleukin-12p70 (IL-12p70), interleukin-6 (IL-6) and interleukin-10 (IL-10) were not secreted in any of the cell groups studied.

3.5. The viability and secretory activity of macrophages cultured on porous membranes modified with rosmarinic acid PCL-RA and irradiated with low-energy laser radiation

On all experimental days studied, regardless of the type of continuous beam (100/2/C) or pulse beam (100/2/I) laser irradiation used, the viability of macrophages cultured on PCL-RA rosmarinic acid-modified material was higher than that of cells cultured on PCL--RA and non-laser irradiated material (Fig. 6a).

Continuous-beam irradiation of macrophages (100/2/C) cultured on PCL-RA material resulted in an increase in AK release on days 3 and 7 of culture, while pulsed-beam irradiation of cells (100/2/I) resulted in an increase in AK release only on day 7 of culture compared to cells cultured on PCL-RA and non-laser--exposed material (Fig. 6b). Irradiation with continuous (100/2/C) and pulsed (100/2/I) laser beams of macrophages cultured on PCL-RA material resulted in a decrease in NO secretion by the cells on all experimental days tested, as well as a decrease in protein secretion on days 3 and 7 of culture, compared to macrophages cultured on PCL-RA material and unexposed macrophages (Figs. 6c, d). Treatment of cells growing on PCL-RA material with both a continuous (100/2/C) and pulsed (100/2/I) laser beam resulted in a transient (on day 3 of culture) increase in TNF-α and MCP-1 secretion by the cells compared to a group of unexposed cells growing on PCL-RA material (Fig. 6e, f). Further irradiation of the cells with a continuous

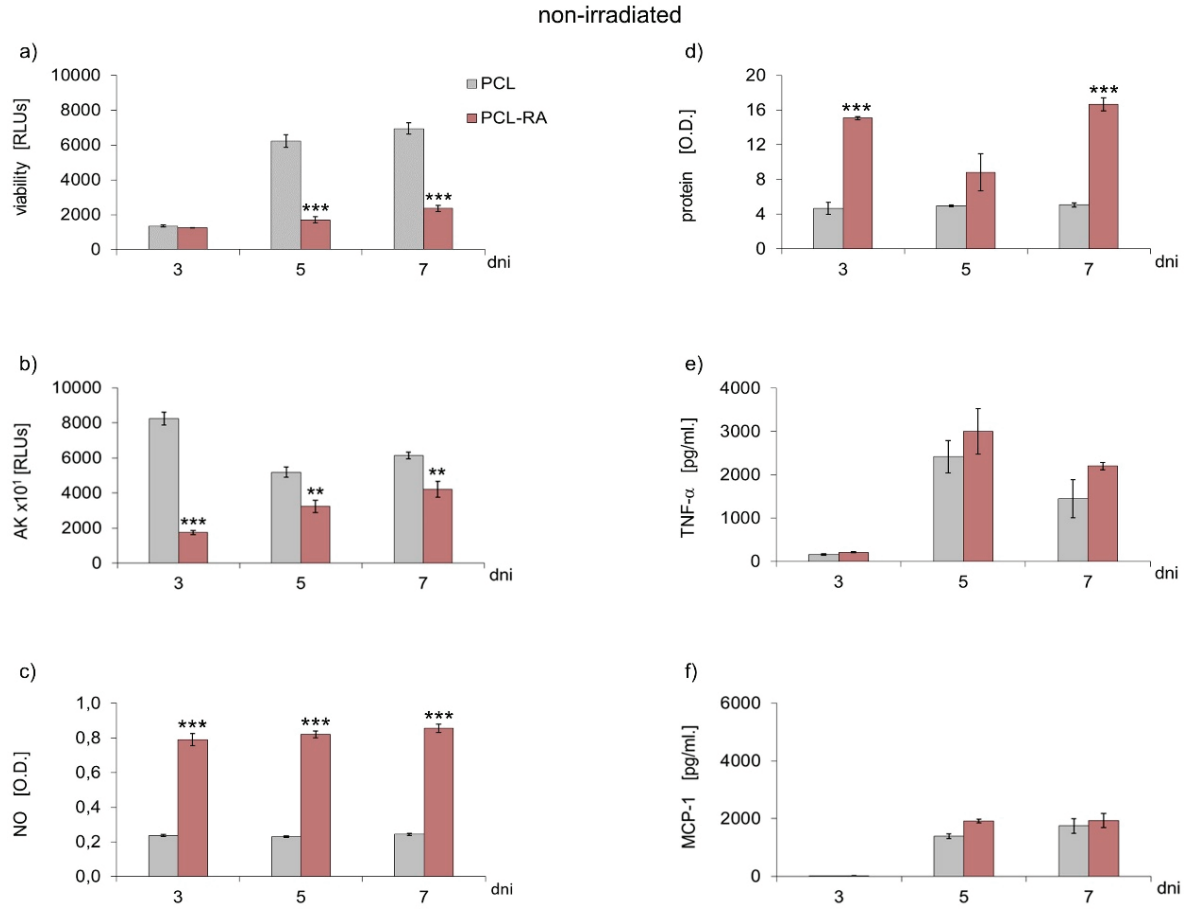

Fig. 5. Effect of polycaprolactone membranes modified with RA rosmarinic acid (PCL-RA group) on the viability (a) of RAW 264.7 cell line macrophages and the levels of released adenylyl kinase (AK, b), secreted nitric oxide (NO, c), protein (d), TNF-α (e), MCP-1 (f). Cells were cultured for a predetermined number of days (3, 5 and 7) with polycaprolactone membranes (PCL group) or membranes modified with RA rosmarinic acid (PCL-RA group). RLUs – luminometer flux unit, O.D. – optical density was measured at 540 nm. Mean values ± standard deviation. **, *** – differences between PCL-RA and reference PCL group (** $p < 0.01$, *** $p < 0.001$)

beam (100/2/C) laser caused a decrease in TNF-α secretion on day 5 of culture and a decrease in MCP-1 secretion on day 7 of culture. In contrast, further irradiation of cells with a pulsed laser beam (100/2/I) had no effect on their secretion of TNF-α and MCP-1 compared to the levels of cytokines secreted by unexposed macrophages growing on PCL-RA material (Figs. 6e, f).

4. Discussion

Numerous research works have shown that irradiation of tissues with low-energy laser is a promising therapeutic method to modulate biochemical and molecular processes occurring in living cells [28], [29], [37]. However, these studies focused mainly on examining the effect of laser irradiation on connective tissue cells [6], [15], [25]. Little is still known about the effect of low-energy laser on the response of immune system cells, such as macrophages, whose activation is crucial for the inflammation process of tissues [22], [26], [35]. In the present research, we made the first attempts to determine how the 2-, 4- and 6-fold exposure to a continuous or pulsed laser beam of the 200 mW power and the 2/J/cm^2/well dose affected the macrophages adhesion/proliferation. Macrophages, playing a key role in the immune response, require adherence to the substrate for their proper growth and proliferation. In our tests, regardless of the applied laser beam mode, there was a rise in the macrophages adhesion/proliferation, as compared to the control non-irradiated cells. Golovynska et al. [11] also proved a 2.5-fold increase in macrophage adhesion in the first hours after exposure to laser light. Additionally, they showed that the macrophages viability was higher after irradiation with lower doses (0.3–1 J/cm^2) and decreased with higher doses (18–30 J/cm^2). Song et al. [28] examined the effect of a continuous laser beam of the 810 nm

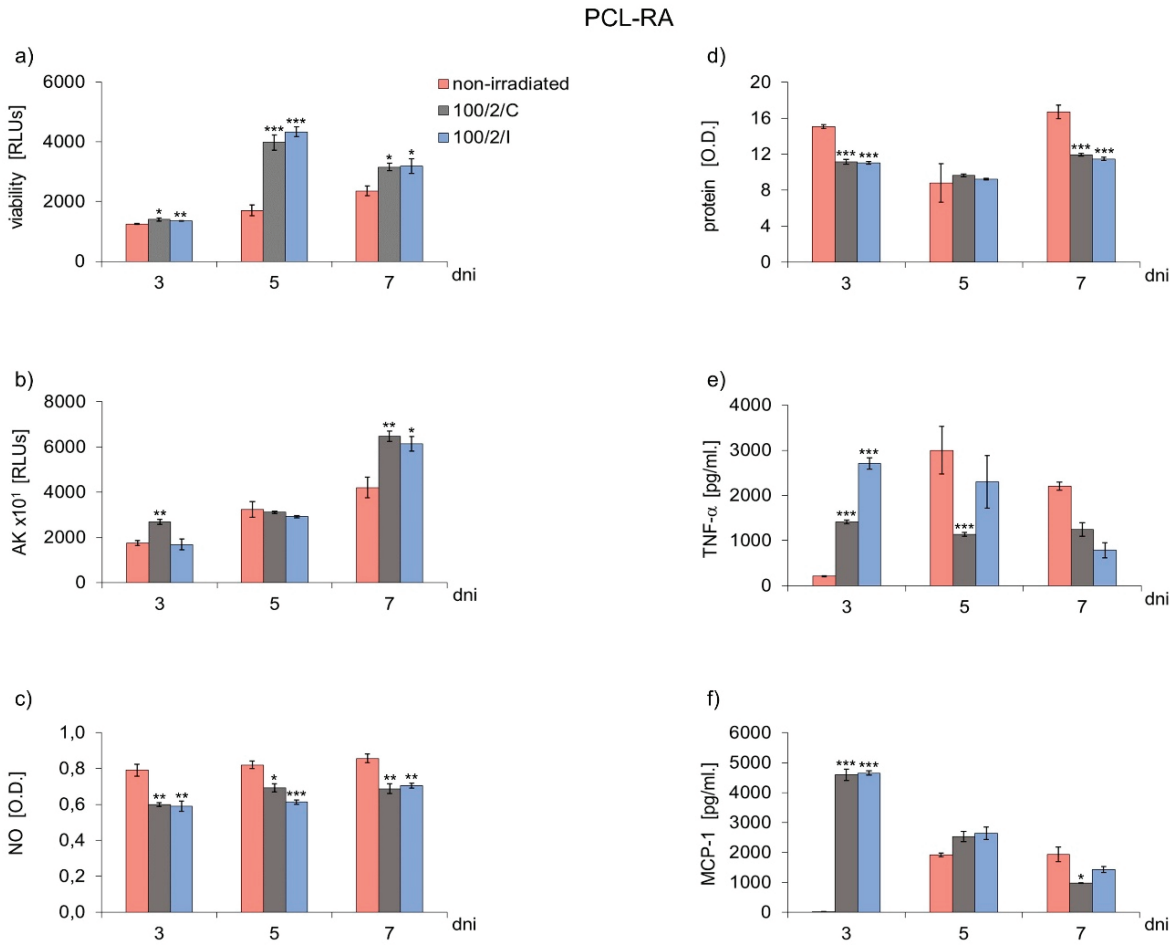

Fig. 6. Effect of continuous (C) and pulsed (I) laser beam irradiation on the on the viability (a) of RAW 264.7 cell line macrophages and the levels of released adenylyl kinase (AK, b), secreted nitric oxide (NO, c), protein (d), TNF-α (e), MCP-1 (f) levels of the cytokines secreted by macrophages of the RAW 264.7 cell line. Cells were cultured for a predetermined number of days (3, 5 and 7) on polycaprolactone membranes modified with rosmarinic acid (PCL-RA) and irradiated with a 100 mW laser with an irradiation dose of 2 J/cm^2/cell well. RLUs – luminometer flux unit, O.D. – optical density was measured at 540 nm. Mean values ± standard deviation. *, **, *** – differences between cells irradiated with laser of different parameters (100/2/C group or 100/2/I group) and cells non-irradiated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

wavelength and 80 mW power. They irradiated RAW 264.7 macrophages with doses of 0.4 J/cm^2, 1.2 J/cm^2 and 2.4 J/cm^2. The 2.4 J/cm^2 dose significantly increased cell viability 24 hours after irradiation. In turn, Alves et al. [4] described the effect of 50 and 100 mW low-energy laser therapy at a dose of 4 J/cm^2 on macrophages present in the synovial fluid from the inflamed joint capsule. It was revealed that only the 100 mW laser beam caused a remarkable rise in the macrophages number in the culture.

Since the aim of our study was to investigate the combined effect of low-energy laser irradiation and rosmarinic acid on the macrophages, the next stage was to assess the influence of RA alone. The modification of PCL membranes with rosmarinic acid (PCL-RA) allowed its gradual release throughout the culture duration. First, we assessed the PCL membranes toxicity towards cells. The test results confirmed the polymer membranes biocompatibility with RAW 264.7 macrophages. Culturing the macrophages on PCL did not impair their viability and secretory activity, as measured by the level of secreted AK, NO in comparison to the control macrophages. The observations carried out on day 5 revealed the increased secretion of protein, MCP-1 and TNF-α, compared to the level of these cytokines secreted in the control culture. This result proved that culturing macrophages on the PCL membranes stimulated the pro-inflammatory activity of these cells. Yet, the stimulation was only transient. The PCL effect on EMF mouse fibroblasts was shown that there were no differences in the morphology, adhesion, viability, and mitotic activity between the cells cultured on PCL and the control cells [32]. In turn, Liu et al. [20] described a positive effect on the adhesion of human

melanocytes and keratinocytes to PCL fibers. Valente et al. [34] discovered that PCL inhibited the adhesion of HaCaT keratinocytes and MRC-5 and HGF fibroblasts, but had no effect on the RAW 264.7 macrophages adhesion to its surface.

In yet other studies, Nakkala et al. [24] examined the effect of PCL nanofibers on classically activated M1 macrophages and alternatively activated M2 macrophages. The authors confirmed that PCL, especially in contact with M2 macrophages, inhibited the secretion of pro-inflammatory cytokines, such as TNF-α and IL-1β, and stimulated the secretion of anti-inflammatory factors, such as Arg-1, IL-10 and TGF-β. In our research, we showed that modifying the PCL membranes with rosmarinic acid had no effect on cell viability on day 3 of the cell culture. However, extending the culture time to 5 and 7 days reduced the macrophages viability in comparison with the ones grown on the unmodified PCL. The lowered cell viability under the RA influence was accompanied by the decreased adenylate kinase release and increased secretion of NO and protein. It should be noted that, at the same time, no significant differences were observed in the levels of secreted TNF-α and MCP-1 cytokines between the PCL-RA macrophages and the PCL cells. Similar positive effects of rosmarinic acid on cells were showed that 50 and 100 ppm RA concentrations had no cytotoxic effect on RAW 264.7 microphages. Additionally, these cells stimulated with LPS and INF-γ and then RA treated proved the lower iNOS expression than the stimulated cells [27]. It was showed that an ethanol extract of rosemary leaves effectively inhibited the NO production by activated macrophages, which was related to the extract's influence on the expression of the gene encoding iNOS [18]. The study how the rosmarinic acid methyl ester influenced RAW 264.7 macrophages showed that it reduced the IL-1β, IL-6, IL-10, MCP-1 levels and suppressed the protein and NO synthesis, dependently on the dose [36]. Additionally, rosmarinic acid inhibited the production of inflammatory mediators, such as NO and PGE2 prostaglandins, in the LPS-stimulated RAW 264.7 macrophages [13]. A new results presented by Jiang et al. [14] again confirmed the anti-inflammatory effect of rosmarinic acid. In this case, RAW 264.7 macrophages stimulated for 24 hours with LPS and then treated with rosmarinic acid at a 80 μm concentration noted the lower expression of iNOS and COX-2, and consistently produced less NO and PGE2.

In our research, the discrepancy in the effect of rosmarinic acid on the RAW 264.7 macrophages may result from its various concentrations and the cells' different exposure time to RA. In most published studies, rosmarinic acid was applied to the cells previously stimulated towards inflammation in order to examine the RA effect on suppressing the inflammatory response. In our study, we used RA on the unstimulated cells (M0 resting macrophages) whose reaction to the modifier presence resulted from their first contact. The available literature data are mainly concerned with the effects of LLLT and various active agents (including rosmarinic acid) on macrophages previously stimulated with lipopolysaccharide (LPS). LPS-stimulated macrophages determine the polarization of M0 macrophages toward M1-type cells. However, there is no information on the directional effects of LLLT and rosmarinic acid on the biological activity of resting M0 macrophages, previously unstimulated. In our study, previously unstimulated (unpolarized) macrophages were used, hence, the results obtained differ from the results of other authors quoted in the paper, who conducted studies on macrophages polarized to the M1 pro-inflammatory phenotype. In the case of these studies, rosmarinic acid was applied to cells previously stimulated toward inflammation, in order to study its effect on suppressing the inflammatory response (min. antioxidant effect, reduction of the level of NO secreted by macrophages). In order to check whether low-energy laser irradiation would modify the RA influence on the macrophages, we exposed the cells growing on the PCL-RA membranes to a continuous and pulsed laser beam with the 100 mW power and the 2 J/cm^2 dose. Irradiation of macrophages cultured on PCL-RA material had a beneficial effect on the cells, causing an increase in their viability. The concomitant (at some time points) increase in AK release by LLLT-treated PCL-RA cells was transient or, at further culture time points (day 7) a result of the lack of free surface area for macrophage adhesion. Irradiation of macrophages cultured on PCL--RA material decreased macrophage secretion of NO and protein and on subsequent days resulted in a decrease in their secretion, or no effect on their secretion.

5. Conclusions

In this study, we examined the activity of macrophages (resting macrophages – M0) through a synergistic effect: rosmarinic acid-modified porous medium and low-field laser therapy. Based on the results obtained, the following can be concluded:

(a) macrophage irradiation had a positive effect on macrophage adhesion/proliferation;
(b) modification of PCL membranes with rosmarinic acid (PCL-RA) affected the biological activity of

macrophages causing a decrease in cell viability and an increase in secretory activity measured by NO and protein levels (BCA test);

(c) culture of macrophages on PCL and rosmarinic acid modified membranes (PCL-RA) and simultaneous exposure of LLLT cells to these media induces anti-inflammatory effects.

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