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IN VITRO WOUND HEALING ACTIVITY OF ALPHA-LIPOIC ACID

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ABSTRACT

Chronic wounds represent a worldwide healthcare and socio-economic problem which significantly affect the patients' quality of life. Healing of chronic wounds is very complex process and requires constant wound management and treatment with agents that should stimulate wound healing. Alpha-lipoic acid (ALA) is a naturally occurring organosulfur compound with two thiol groups in its structure. It is a very potent antioxidant with other beneficial activities such as anti-inflammatory, anti-aging and neuroprotective. The aim of this study was to investigate *in vitro* wound healing activity of ALA and effect on proliferation of L929 fibroblasts. Wound healing activity was examined using *in vitro* 'scratch' assay while effect on cell proliferation was assessed using MTT test. Concentration-dependent effect of ALA on fibroblasts' proliferation was observed. ALA stimulated the wound closure and migration of fibroblasts in used *in vitro* wound healing model which suggests that ALA can be used as a potent agent in various pharmaceutical formulations for wound management and wound healing.

Key words: wound healing, chronic wounds, alpha-lipoic acid, cell proliferation, fibroblasts

INTRODUCTION

Wound healing (WH) represents highly dynamic biological process in the human body [1]. Interruptions, aberrancies, or prolongation in the programmed phases of WH such as hemostasis, inflammation, proliferation, and tissue remodeling, can lead to delayed WH or a non-healing chronic wound [2]. The complex and long-term process of chronic wounds healing starts with wound formation, can take months or year [3] and can result in significant negative impact on healthcare systems with serious impacts on the life quality of patients [4]. Recent estimations show that non-healing chronic wounds represent a huge socio-economic problem, and represent a silent epidemic that affects a 1-2% of the world population [5].

Despite numerous researches in the field of WH and chronic wounds, critical gaps of impaired healing process still remain [6]. Therefore, there is an immediate need for further analysis of factors associated with delayed healing, as well as identification of new candidates that can stimulate the proliferation of fibroblasts which are the cells that play a crucial role in all phases of WH [7]. Chronic inflammation and oxidative stress are distinguished as important factors of healing dysfunctions [8]. Therefore, regulation of redox balance through the modulation of reactive oxygen species (ROS) and antioxidant levels may improve the WH process. The interest in using antioxidants for wound treatment is growing, and numerous studies have been performed with aim to develop and examine different compounds with antioxidant properties which have the ability to enhance the healing process [3]. Among a large number of natural antioxidant compounds, alpha-lipoic acid (ALA) is standing out due to its noteworthy effects, and considered today as "the universal antioxidant" [9]. The molecular nature of ALA puts this unique dithiol compound in special focus, because ALA acts as an antioxidant and can exert immunomodulatory properties [10, 11]. Therefore, our aim was to examine the effect of ALA on proliferation of fibroblasts and WH activity *in vitro*.

MATERIALS AND METHODS

Cell line and culturing

In vitro WH activity testing of alpha lipoic acid ((±)-α-Lipoic acid, purity ≥98.0%, Cat. No. 62320, Sigma Aldrich, Germany) was performed on L929 fibroblast cell line (mouse skin fibroblasts). L929 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM stable glutamine, and antibiotic-antimycotic solution (which makes complete DMEM), at 37 °C in incubator with humidified atmosphere and 5% CO_2. Cell culture media and reagents used in the study were purchased from Capricorn Scientific GmbH, Germany.

Cell Proliferation Assay

L929 cells were seeded in standard 96-well plates (purchased from Greiner Bio-One, Germany) at density of 5,000 cells per each well of 96-well plate. Twenty-four hours after cultivation of cells, ALA was added in various concentrations (ranging from 0 to 1,000 μM). Concentrations of ALA for testing were prepared by diluting the stock solution of ALA with complete DMEM. Stock solution of ALA was prepared according to the manufacturer's instruction, by dissolving ALA in methanol (purity p.a.) in concentration 100 mg/mL. The cells incubated only with complete DMEM, without ALA, were used as a control (untreated cells). Each tested concentration of ALA was examined in four to six replicates, and complete DMEM as a control as well. The cells were incubated with different concentrations of ALA or control medium for the next 72 hours. After the incubation period ended, MTT test was performed. The experiment was performed twice under the same conditions. The MTT test is widely used for assessment of cell proliferation and is based on the reduction of tetrazolium salt MTT (purchased from Carl Roth, Germany) by mitochondrial dehydrogenases of living cells, resulting in formazan crystals formation that corresponds to the number of viable cells. Prior to addition of 100 μL of MTT solution at concentration of 1 mg/mL,

media with ALA was discarded and cells were washed with phosphate buffer saline (DPBS, Capricorn, Germany). MTT substance was purchased from Carl Roth, Germany. The cells were incubated with MTT solution for the next three hours followed by dissolution of formed formazan crystals with 2-propanol (purchased from Thermo Fisher Scientific, USA). The absorbance of dissolved formazan was measured on a Multiskan Ascent Photometric plate reader (Thermo Labsystems, Finland) at a wavelength of 540 nm with reference wavelength of 650 nm for correction. The mean absorbance values were calculated for each tested sample, as well as for the control. The cell proliferation rate was calculated as: (absorbance value of cells treated with fibers/absorbance value of untreated cells) × 100.

In Vitro Wound Healing Assay

A "scratch" assay was performed to examine the *in vitro* WH effect of ALA, according to our previously published protocol [12-14]. Briefly, L929 fibroblasts were seeded in 48 well plates and incubated under the standard cell culture conditions previously described in the section "Cell line and culturing". After reaching the complete confluence, a wound ('scratch') was created by pipette tip in a cell monolayer, in the middle of each well. The cells were then washed with DPBS and 100 μM of ALA, as well as complete DMEM (control), were added. ALA and complete DMEM were tested in three replicates and the experiment was performed twice under the same conditions. Created "wounds" were incubated with ALA and complete DMEM for the next three days. A microscopic analysis of wounds' closure was then performed using inverted light microscope Observer. Z1 (Carl Zeiss, Germany), and morphometric measurements were made in ZEN 2 (blue edition) software (Carl Zeiss, Germany) after imaging the "wounds". To assess the wound closure, we measured the width of the remaining wounded area after three days of incubation with the ALA as well as with complete medium (control), and compared to the width of the area of initial wounds, before incubation with ALA. The WH activity is expressed as a percentage of wound closure. Additionally, cell migration zone was measured starting from the initial edge of the wound.

Statistical Analysis

The results of MTT test and morphometric measurements were analyzed using one-way analysis of variance (ANOVA). Results are expressed as a percentage of cell proliferation regarding the control culture which represented cells incubated in complete DMEM (untreated cells) under the same conditions and for which we considered cell proliferation rate to be 100%. Wound closure is expressed as percentage with relative standard deviation. As statistically significant, we considered those values for which $p < 0.05$.

RESULTS

The effect of ALA on proliferation of fibroblasts

The effect of various concentrations of ALA is shown in Figure 1. Concentration-dependent effect of ALA on L929 fibroblasts proliferation was observed and was mostly pronounced in concentrations from 50 to 1,000 μM. Slight anti-proliferative effect at concentrations 250 μM and above was observed, with the most pronounced effect of the highest examined concentration, while lower concentrations did not exhibit an anti-proliferative effect, they even had a mild stimulatory effect.

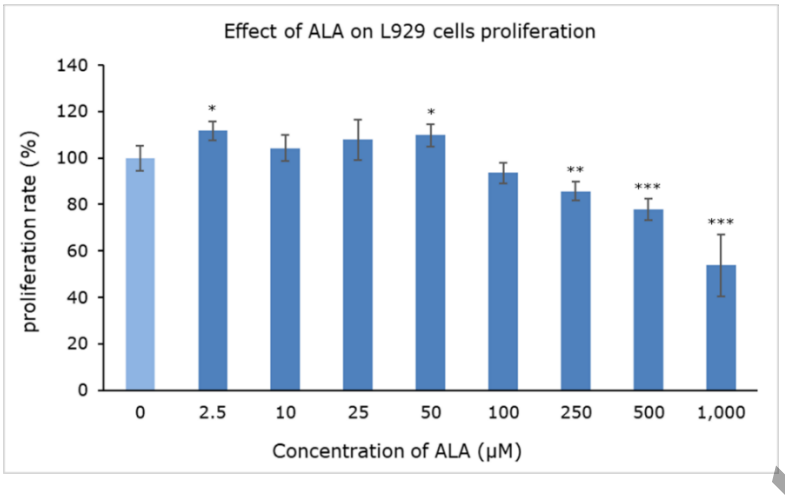

Figure 1. The effect of various ALA concentrations on L929 cells proliferation, measured by MTT test. Results presented as mean % of cell proliferation compared to the control cell culture with relative standard deviation; (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

In vitro wound healing activity of ALA

Wound healing effect of ALA, determined by *in vitro* 'scratch' assay, is shown in Figure 2. The effect of ALA was compared with the control which represented complete DMEM without ALA. Three parameters were measured after three days of incubation: cell migration zone (the extent of cell growth and migration starting from the initial edge of the wound), wound area after the treatment (width of remained wound area) and the percentage of wound closure (calculated by the formula: 100% - (width of remained wound area – width of the initial wound area) x 100%). For WH activity testing, we chose a concentration which did not alter fibroblasts proliferation.

The stimulating WH effect of ALA was noticed, determined by *in vitro* 'scratch' assay. Statistically significant greater migration zone, smaller width of the remained wound area and higher percentage of wound closure in the presence of ALA was observed, when we compare ALA treatment with the control.

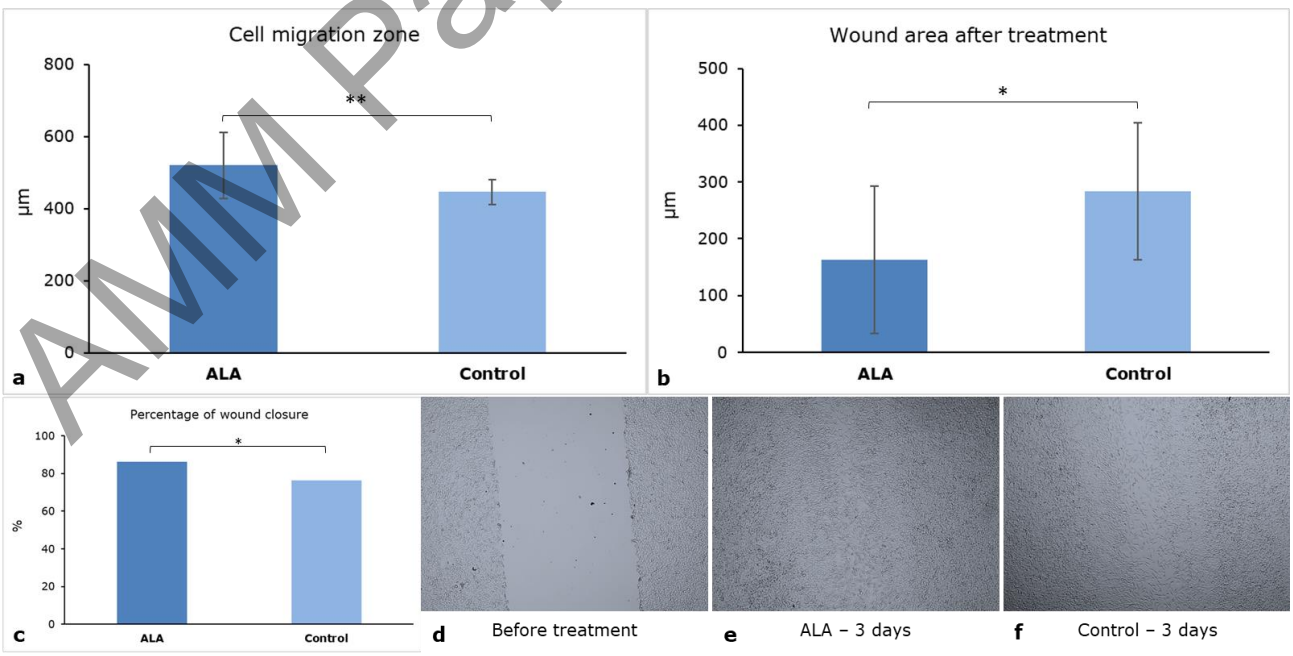

Figure 2. The wound healing effect of ALA, determined by 'scratch' assay. Results presented as mean width (μm) ± SD of cell migration zone (a) and remained wound area after treatment(b) and

% of wound closure after treatment (c); microscopical image of the wound before treatment (d), after three days of incubation with ALA (e) and control medium (f); (*) $p<0.05$, (**) $p<0.01$.

DISSCUSSION

A meticulous and very complex process of WH is achieved through several phases and requires the interaction and communication of different cell types present in the wound [15]. Fibroblasts are distinguished as key effector cells in all phases of WH process, with the note that accumulation and activation of the fibroblasts is closely related to and is responsible for the formation and deposition of granulation tissue and finally lead to the wound contraction [16]. Therefore, quick WH might be due to stimulation of proliferation and migration of fibroblasts, but it is also necessary to take into account a level of reactive oxygen species (ROS) playing a crucial role in the WH process [17]. Growing evidence supports the fact that oxidative stress plays a significant role in WH phases and processes such as regulation of inflammation, angiogenesis, cell proliferation, formation of granulation tissue and the formation of extracellular matrix (ECM) [18]. Because of the complexity of WH process and participation of oxidative stress in that process, various antioxidant compounds have become the focus of research [17].

Alpha-lipoic acid is a naturally derived organosulfur compound with two thiol groups in its structure, which may participate in redox reactions [19]. It exists in oxidized and reduced forms and is characterized by unique antioxidant potential, since both endogenously and exogenously synthesized form is actively involved in ROS neutralization, restores the intrinsic antioxidant systems and supports their production [10,19]. ALA occurs usually in mitochondria, acting as a coenzyme for some enzyme complexes, and plays major role in protein-, carbohydrate and fatty acids metabolism, and manages gene activation [11]. Its amphiphilic characteristic sets ALA apart from other antioxidants, and can elicit antioxidant and anti-inflammatory actions in both the cytosol and plasma membrane [20]. Currently, it is attracting attention because of its distinctive antioxidant properties and influence on various cellular functions leading to the beneficial effects on human health [21].

In this study, we analized WH activity of ALA using a well-established *in vitro* cell 'scratch' assay. Before performing WH assay, we investigated the effect of ALA on proliferation of fibroblasts. We observed concentration-dependent effect of ALA on the proliferation of L929 fibroblasts, with slight anti-proliferative effect in concentrations above 250 μM. Lower concentrations of ALA did not exhibit an anti-proliferative effect and they even had a mild stimulatory effect on fibroblasts' proliferation which makes ALA for a good candidate for topical applications (Figure 1.). The cell scratch assay using skin cells such as fibroblasts is a widely used method as an *in vitro* WH model that provides information about the activity of different compounds and natural products [22]. In the present study, we used this assay with focus on three parameters: cell migration zone, resting wound area and percentage of wound closure after three days of incubation with ALA. Our results showed that ALA. Representative images in Figure 2, statistically significant larger migration zone, smaller width of the remained wound area and higher percentage of wound closure comparing to the control, clearly show that ALA significantly stimulated WH in an *in vitro* cell model and may be used as a powerful WH agent in numerous topical pharmaceutical formulations intended for wound treatment and healing. The obtained results might be due to the strong and already confirmed antioxidative activity of ALA [22, 23], however, we assume that ALA exerts other activities that are involved in stimulation of fibroblasts' proliferation and migration.

ALA has been in the research focus for several decades and literature data reveals the effectiveness of ALA in the prevention of many oxidative stress-mediated pathologic conditions such as obesity, cardiovascular disease, diabetes and related complications, osteoporosis, cognitive dysfunction, malignant diseases, glaucoma, and many others [24]. However, its potential application in WH has not yet been thoroughly investigated. There is limited number of studies with different experimental design makes it difficult to compare the results (*in vitro*, animal models or human clinical studies) [23, 25, 26]. Türkez et al. showed that ALA conjugated boron nanoparticles (concentrations of 50 ug/mL and lower) enhance WH and antimicrobial processes in human dermal fibroblasts cell culture [25]. The group of authors from China showed that ALA enhanced injury repair in a dose-dependent manner in human colon epithelial cells NCM460 [27]. Reported results of one study conducted by Külkamp-Guerreiro et al. confirmed that topical application of non-encapsulated ALA induces increased skin WH, which is shown using an *in vivo*

model of experimentally induced skin wounds [23]. Human clinical study conducted in Italy showed that supplementation with ALA, in combination with hyperbaric oxygen therapy, promotes progression of the healing process by down-regulating the growth factors and inflammatory cytokines production [26]. The results of our study presented here, together with the literature data, indicate that ALA shows a good potential to be used as WH agent in wound treatment, and provide a basis for further investigation to clarify therapeutic potential of ALA in WH. Further *in vivo* research on experimental animals is necessary to explain the precise role of ALA in complex and chronic WH process.

CONCLUSION

We can conclude that alpha-lipoic acid, besides many beneficial effects, show a great wound healing activity determined *in vitro* using "scratch" assay on fibroblasts. This indicates that ALA can be used as a potent agent in various pharmaceutical formulations for wound management and wound healing.

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IN VITRO EFEKAT ALFA-LIPOINSKE KISELINE NA ZARASTANJE RANA

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SAŽETAK

Hronične rane predstavljaju svetski zdravstveni i socio-ekonomski problem koji značajno utiče na kvalitet života pacijenata. Zarastanje hroničnih rana je veoma složen proces i zahteva stalnu obradu rana i tretiranje rana sredstvima koja treba da stimulišu zarastanje rana. Alfa-lipoinska kiselina je prirodno organsko sumporno jedinjenje sa dve tiolne grupe u svojoj strukturi. Veoma je moćan antioksidans sa drugim značajnim aktivnostima kao što su anti-inflamatorna, *anti-aging* i neuroprotektivna. Cilj ove studije je bio da se ispita efekat alfa-lipoinske kiseline na zarastanje rana *in vitro* i efekat na proliferaciju L929 fibroblasta. Efekat na zarastanje rana je ispitivan korišćenjem *in vitro* testa koji podrazumeva pravljenje „ogrebotine" u monosloju ćelija u kulturi, dok je efekat na proliferaciju ćelija ispitivan primenom MTT testa. Primećen je koncetracijski-zavisan efekat alfa-lipoinske kiseline na proliferaciju fibroblasta. Alfa-lipoinska kiselina je stimulisala zarastanje tj. zatvaranje napravljene rane (ogrebotine) i migraciju fibroblasta u korišćenom *in vitro* modelu zarastanja rana, što sugeriše da se alfa-lipoinska kiselina može koristiti kao potentan agens u različitim farmaceutskim formulacijama za lečenje i zarastanje rana.

Ključne reči: zarastanje rana, hronične rane, alfa-lipoinska kiselina, proliferacija ćelija, fibroblasti