Calophyllum inophyllum in vaginitis treatment: Stimulated by electroporation with an in vitro approach

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online)

Adv Clin Exp Med. 2019;28(2):223–228

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Funding sources

This work was supported with the Wrocław Medical University Statutory Funds ST.E130.16.060 (Prof. J. Zalewski).

Conflict of interest

None declared

Acknowledgements

We owe special thanks to Thomas A. Burley for critical review of the manuscript. Authors would like to express their gratitude to Professor Małgorzata Kotulska from the Wrocław University of Science and Technology for the possibility of performing electroporation tests on ECM 830 Square Wave Electroporation System (BTX, Syngen Biotech, Poland).

Received on August 9, 2017

Reviewed on October 25, 2017

Accepted on March 19, 2018

Published online on November 22, 2018

Cite as

Zalewski J, Mączyńska J, Biezuńska-Kusiak K, et al. *Calophyllum inophyllum* in vaginitis treatment: Stimulated by electroporation with an in vitro approach. Adv Clin Exp Med. 2019;28(2):223–228. doi:10.17219/acem/87045

DOI

10.17219/acem/87045

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Abstract

Background. Vaginitis is one of the most common problems in clinical medicine and is cited most often during visits to obstetricians and gynecologists. Most of the inflammation cases are caused by candidiasis trichomoniasis and bacterial vaginosis. Therefore, treatment of vaginal infections must use antibiotic or antifungal drugs, which often provide quick relief to the patient. The real cause of the problem – disrupting the ecosystem of the vagina – remains unchanged. Thus, new therapeutic compounds are being explored.

Objectives. The aim of our study was to evaluate the effect of a natural substance: tamanu oil, an extract from the plant *Calophyllum inophyllum*, applied to the human fibroblast cell line (normal human dermal fibroblasts – NHDFs) and to the isolated human fibroblasts from the vagina (human vaginal fibroblasts – HVFs) in vitro.

Material and methods. We evaluated the viability of cells with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay after incubation only with tamanu oil and with electroporation (EP). We also examined the immunocytochemical reaction of collagen type III and mitochondrial superoxide dismutase (MnSOD) under established conditions.

Results. Tamanu oil increased the proliferation of cells and the amount of collagen III. It has been shown that the *C. inophyllum* extract stimulates the proliferation of commercial fibroblasts. For direct application in patients, one should use *C. inophyllum* extract in the range of 1:10–1:100 (saline dilution).

Conclusions. The use of this extract (at concentrations indicated by the studies presented here) stimulates the healing processes (increased expression of collagen type III), and has anti-inflammatory, analgesic and antiseptic qualities.

Key words: electroporation, vaginitis inflammation, primary fibroblasts culture, tamanu oil

Introduction

Currently, obstetricians and gynecologists often struggle to treat vaginitis, which is one of the most common problems in clinical medicine. This problem affects women of various ages during their reproductive stage and menopause. Commonly, it is defined as a change in the normal vaginal bacterial flora, in which the normal *Lactobacilli* production is disturbed and, consequently, leads to the overgrowth of a predominant anaerobic bacteria species, such as *Gardnerella vaginalis*, *Protea* spp., *Bacteroides* spp., *Mobiluncus* spp., Gram-positive bacteria, and *Mycoplasma*, which can lead to serious complications.¹ Untreated vaginal infection can result in chronic infection of the vagina, and when the infection reaches the fallopian tubes and cervix, infertility can occur. Maintaining a healthy vaginal ecosystem remains an issue underestimated by both patients and the doctors treating them. Most of the cases of vaginitis are caused by candidiasis, trichomoniasis and bacterial vaginosis.^{2,3} The pathomechanisms responsible for the bacterial and fungal infections remain unknown. The most significant role in the elimination of vaginal inflammation (VI) is played by non-specific immunity: macrophages, natural killer (NK) cells and neutrophils. Eosinophils indifference and other humoral factors (lysozyme, low pH) constitute a barrier and play an important role in long-term humoral immune protection. However, specific immunity based on the production of antibodies does not serve an important function in VI. Bacterial vaginosis is caused by an imbalance in bacteria colonizing the vagina. A characteristic feature of bacterial vaginosis is the lack of leukocyte infiltration. It is thought that succinic acid and acetic acid are secreted by the bacteria in order to suppress the local immune response. However, in recurrent infections of the vagina and cervix, immune disorders are diagnosed by increased levels of heat shock proteins. This indicates that the normal immune status of women may be imperative in preventing the development of vaginal infections. However, this problem is more complicated, particularly when the immunological system is impaired through disease, immunosuppressants or cancer.⁴ Some authors have highlighted the role of cytokines (e.g., recombinant proinflammatory cytokines) in the immune response to fungal pathogens and bacterial infections, and their potential use for prevention or treatment of fungal infections.⁵

The most common cause of vaginal infection is due to a chronic disorder of the vaginal ecosystem. Normal microflora of the vagina, which consists of naturally occurring bacteria in the reproductive tract – primarily of the *Lactobacillus* genus – is essential for the protection of these areas. These bacteria maintain an acidic environment (pH 3.8–4.5) in the vagina and produce substances that prevent the growth of pathogenic bacteria and fungi. Disturbance of the vaginal ecosystem and the reduction in the number of these bacteria contributes

to the development of vaginal infections.^{1,3} Therefore, treatment of vaginal infections with antibiotic or antifungal drugs is often successful only for a limited time, as the root cause of the problem, the disrupted ecosystem of the vagina, remains unaddressed. For this reason, it is important to discover and develop new therapeutic compounds to improve the treatment of vaginal infections. In recent years, interest in the use of plant or animal extracts for the production of pharmacological compounds has increased. Additionally, numerous studies have shown that natural components can prevent different diseases.^{6–9} The introduction of new natural substances with medicinal properties can increase the effectiveness and quality of treatment. Therefore, we decided to evaluate another natural substance for vaginitis treatment: an extract from the plant *Calophyllum inophyllum*, tamanu oil. It contains a lot of unsaturated acids, which are necessary, i.e., to keep the skin healthy and properly hydrated. We hypothesize that this examined extract can be used as a potential therapeutic factor for healing wounds in gynecological diseases as well as protecting mucous membrane integrity. Additionally, we used the electroporation (EP) process to improve therapeutic effects of the tested compound. Electroporation has been widely utilized in recent years as a safe and effective technique to successfully deliver drugs into target cells for both experimental and therapeutic approaches.¹⁰

Material and methods

Cell culture

The established fibroblast cell line (Normal Human Dermal Fibroblasts – NHDF; PromoCell, Biomedica Poland, Piaseczno, Poland) and normal human primary fibroblast isolated from the vaginal mucus fragment (human vaginal fibroblasts – HVFs) of a healthy patient were used. The primary human fibroblasts were used as a comparison to the established human fibroblast line. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, USA) containing 2mM glutamine, 50 µg/mL streptomycin and 10% fetal bovine serum. Cells were incubated at 37°C in 5% CO₂. The culture medium was replaced twice a week. Before every experiment, cells were detached by 0.25% trypsin with 0.02% ethylenedinitrilotetraacetic acid (EDTA) (Sigma-Aldrich). The appropriate dilutions (1:2–1:100) of tamanu oil, the pure extract from the plant *C. inophyllum*, were prepared in cell culture medium and examined.

Electroporation

Cells were grown as monolayers in 75 cm² flasks; then they were trypsinized and centrifuged (5 min, 1000 rpm). Next, cells were counted to obtain the volume of 3×10^6 /mL and resuspended in 200 µL of EP buffer

with a low electrical conductivity of 0.14 S/m (10mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 1mM MgCl_2 , and 250mM sucrose; pH 7.4). The cell suspension was pulsed in a cuvette with 2 aluminum plate electrodes (4 mm space between electrodes) with electrical field strength up to 3,000 V/cm using 8 pulses of 100 μs duration. Rectangular electrical pulses were delivered by an electroporator ECM 830 (BTX Harvard Apparatus; Syngen Biotech, Wrocław, Poland). In the case of the cells being treated with tamanu oil and undergoing EP, electrical fields with the intensity of 800 V/cm and 1,000 V/cm were used. Following pulsation, the cells were left for 10 min at 37°C, centrifuged, resuspended in fresh cell culture medium, and reseeded for a viability assay and immunocytochemistry (ABC method).

Cellular viability

Cellular viability was studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (In Vitro Toxicology Assay; Sigma-Aldrich), which assesses the mitochondrial redox activity as an indicator of the cellular proliferation potential. The MTT assay was performed 24 h and 72 h post-treatment, according to the manufacturer's protocol. The absorbance was measured at 570 nm using a multiwell plate reader (EnSpire Multimode Reader; Perkin Elmer Polska, Kraków, Poland). The assay was performed independently 3 times using 3 repetition samples, and the mean values and standard deviation (SD) of combined results were calculated.

Immunocytochemical ABC staining of collagen III and mitochondrial superoxide dismutase

The expression of selected proteins was examined by the immunocytochemical Avidin-Biotin Complex (ABC) method. After fixation in 4% paraformaldehyde, the samples were permeabilized and blocked by incubation with 0.1% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline (PBS). The protein expression was visualized with a polyclonal antibody COL-III (1:100, anti-collagen III) and anti-SOD2 (Santa Cruz Biotechnology, Dallas, USA). Immunocytochemical staining was performed with ImmPRESS Universal Reagent (Vector Laboratories, Burlingame, USA).

Results

Cellular viability

The influence of tamanu oil, electric field intensity and the combination of both on cellular viability was assessed using MTT assay. The investigation indicated that using the tamanu oil increased viability in human fibroblast cell line, stimulating the proliferation of NHDF cells at every dilution, in contrast to HVF cells, in which higher viability

Fig. 1. The effect of *Calophyllum inophyllum* extract on the viability of normal human dermal fibroblast (NHDF) cells and cells from primary cell cultures (human vaginal fibroblasts – HVFs)

Fig. 2. The impact of pulsed electric field on human fibroblast (normal human dermal fibroblasts (NHDFs) and human vaginal fibroblasts (HVFs)) viability a) 24 h and b) 72 h after exposition

was observed only for further dilutions (1:20; 1:50; 1:100) (Fig. 1). From the experiment in which different currents were used, we selected the electric field of 800 V/cm intensity as the most beneficial for further experiments with the application of the oil extract from *C. inophyllum* on both cell lines (Fig. 2). Surprisingly, combining EP with tamanu oil resulted in a much greater increase in cell proliferation of HVF cells compared to NHDF cells. The highest proliferation was observed at 800 V/cm electrical field intensity and 1:10 dilution of tamanu oil (Fig. 3).

Immunocytochemical ABC reaction: collagen III and MnSOD

The results of immunostaining with anti-collagen III and anti-mitochondrial superoxide dismutase 2 (anti-MnSOD) are presented in Tables 1–4 and in Fig. 4 and 5. An increase in collagen III expression was noted only 24 h post-incubation with tamanu oil (Table 1, Fig. 4). The most intense immune reaction was observed after incubation with tamanu oil at a dilution of 1:10 with application of an electric field

of 800 V/cm intensity (c.a. 70% for NHDF and 30% for HVF) after 24 h of incubation. After 72-hour incubation with tamanu oil in combination with EP, the level of COL-III insignificantly decreased in NHDF cells and increased in primary HVF cells (Table 1, Fig. 4). Contrary to collagen III, the MnSOD expression indicated more intense staining reaction. The intensity of anti-MnSOD reaction increased proportionally with the increasing electric field intensity (Tables 1,2, Fig. 5) in NHDF cells. The highest expression was noted after a 24-hour incubation for cells treated only with tamanu oil and for cells treated with oil-EP combination (90% and 100%, respectively) (Table 1, Fig. 5). The expression of this antioxidant enzyme was lower after 72 h than 24 h post-treatment for cells treated only with tamanu oil and for the combined treatment (Table 2, Fig. 5). In HVF cells, the expression of MnSOD also increased at the higher EP parameters after 72 h in 100% of cells (Tables 3,4).

Discussion

Prognosis of vaginitis in many cases is promising, but most of the infections are not completely cured and, therefore, reoccur. The recurring vaginal infection might lead to chronic infections and scarring,¹⁰ but in most cases when suitably treated do not cause permanent problems. Conversely, untreated vaginal infections can spread to other pelvic structures and result in chronic diseases.¹¹ In such cases, another course of treatment is necessary. Natural compounds are commonly applied in many diseases of the human body, against both the precancerous state and cancer. However, it is commonly known that some of these compounds can be used in other non-cancer diseases. As an example can serve tropical tamanu oil, an extract from *C. inophyllum*. The properties of this oil have been known for a long time, especially for healing wounds. Women frequently use tamanu oil to achieve healthy, clear skin, as it helps to clear acne and scars. These compounds

Fig. 3. The influence of *Calophyllum inophyllum* extract combined with electroporation (EP) on human fibroblasts viability 24 h and 72 h post-treatment in: a) normal human dermal fibroblasts (NHDFs) and b) human vaginal fibroblasts (HVF)

Fig. 4. The immunocytochemical staining reaction of collagen III in normal human dermal fibroblasts (NHDFs) 24 h and 72 h post-treatment

also have anti-inflammatory properties reducing swelling of rashes, insect bites and sunburns. Tamanu oil additionally possesses significant antimicrobial, antibacterial and antifungal qualities.¹¹ In this investigation, we examined the effect of tamanu oil on the proliferation of human

established cell line fibroblast line (NHDF) and human primary fibroblast isolated from vagina (HVF). Additionally, we used EP in order to improve the transport of the examined compound. However, we observed an increase in proliferation in both human fibroblast cell lines

Table 1. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in established normal human dermal fibroblast (NHDF) cells 24 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	<5% +	55% +/++
Group I	1:10	0	50% +	90% ++
Group II	1:10	800	75% ++	100% ++
Group III	1:10	1,000	<5% +	100% ++

Table 2. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in established normal human dermal fibroblast (NHDF) cells 72 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	<5% +	70% +/++
Group I	1:10	0	<5% -/+	80% ++
Group II	1:10	800	35% +/++	85% ++
Group III	1:10	1,000	10% +	95% +++

Table 3. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in primary human vaginal fibroblast (HVF) cells 24 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	–	45% ++
Group I	1:10	0	10% ++	100% ++
Group II	1:10	800	30% ++	90% ++
Group III	1:10	1,000	35% ++	100% +++

Table 4. The immunocytochemical evaluation of collagen III and mitochondrial superoxide dismutase (MnSOD) proteins in primary human vaginal fibroblast (HVF) cells 72 h after incubation with tamanu oil and after electroporation (EP) combined with examined compound for 800 V/cm and 1,000 V/cm electric field intensity

Sample group	Tamanu oil concentration	EP (V/cm)	Intensity of immunoreaction	
			collagen III	MnSOD
Control group	no tamanu oil used	no EP applied	–	100% +++
Group I	1:10	0	15% ++	100% ++/+++
Group II	1:10	800	40% ++	100% +++
Group III	1:10	1,000	75% ++	100% +++

Fig. 5. The immunocytochemical staining reaction of superoxide dismutase (anti-MnSOD) in normal human dermal fibroblasts (NHDFs) 24 h and 72 h post-treatment

only for the electric field strength of 800 V/cm. A higher electric field intensity caused a similar effect in the control non-treated cells. Our results indicated that it stimulated cell proliferation, which suggests that tamanu oil could become a promising agent for human fibroblast re-growth. Therefore, it could help in developing a new, more effective method in vaginitis treatment. During the healing phase (so-called “proliferation”) collagen and elastin fibers are formed to build up a strong and flexible skeleton, which is then filled by proteoglycan molecules and glycoproteins that combine matrix and healing cells in the wound.^{12,13} In order to verify the healing properties of the chosen natural oil, the expression of selected extracellular matrix protein (collagen III) was examined. An increase of collagen III expression was noted 24 h and 72 h after incubation with the extract from *C. inophyllum* seeds. The highest expression was observed after incubation with tamanu oil at a dilution of 1:10, with a pulsed electric field (800 V/cm). Higher (1,000 V/cm) EP parameters induced decrease of immunoassayed reaction with anti-collagen III, suggesting that the higher EP parameters in combination with tamanu oil do not support collagen expression in NHDF and HVF cells. Initial wound healing involves the synthesis of type III collagen, which is characteristic for immature connective tissue. As wound healing progresses, type III collagen, which is the main component of the granulation tissue, is replaced by type I collagen. Type I collagen is the main and most common type present in dermal tissue and is responsible for the tensile strength of the tissue.¹⁴ Our results suggest that tamanu oil can aid in early stages of the healing process by increasing the expression of type III collagen by human fibroblasts. In wound healing and inflammatory processes, the development of scar remodeling is important in order to obtain a sufficiently strong substitute tissue that is resistant to mechanical stimuli.^{15,16} The synthesis of collagen proteins is a very complex process, which is dependent on the level of the natural antioxidant, vitamin C.^{17,18} Thus, the expression of main antioxidant enzyme MnSOD was evaluated. The MnSOD protein content was found to increase with increasing pulsed electric field intensity combined with tamanu oil use, in comparison to control non-treated cells.

Conclusions

It has been shown that the *C. inophyllum* extract stimulates the proliferation of established and primary fibroblasts to a different degree. The use of tamanu oil

at the concentrations indicated by this study suggests that the stimulated proliferation process could be utilized as an anti-inflammatory, analgesic and antiseptic agent in the healing process (increased expression of collagen type III) of vaginal infections. Moreover, tamanu oil is known for its strong antimicrobial properties. The obtained results might be the basis for the future development of protocols involving the application of the EP method combined with the oil extract from *C. inophyllum* in preclinical and clinical treatment.

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