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Polidocanol (Lauromacrogol 400) has anti-angiogenic effects *in vitro* and *in vivo*

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Abstract. – OBJECTIVE: Polidocanol is the most frequently used sclerosant for sclerotherapy all around the world. Our experimental research aims to find out the angiogenic effects of Polidocanol.

MATERIALS AND METHODS: Angiogenic activity of polidocanol was examined *in vivo* in the chick chorioallantoic membrane (CAM) model, cell viability assay (human umbilical vein endothelial cells – HUVECs) and *in vitro* tube formation assay of HUVECs.

RESULTS: In CAM assay, a significant decrease on CAM vessel growth was observed after the application of polidocanol solutions. Vessel growth inhibition was strongly dose-dependent. There was a cytotoxic effect on HUVECs in the presence of polidocanol observed with MTT assay ($p < 0.05$). In the tube formation assay, statistically significant decrease in tube formation was observed in polidocanol group. It was found that polidocanol had an anti-angiogenic effect ($p < 0.05$). The results provide evidence that polidocanol decreases angiogenesis and has a cytotoxic effect on ECs.

CONCLUSIONS: These results provide evidence that Polidocanol (lauromacrogol 400) have strong anti-angiogenic effects *in vitro* and *in vivo*. Further researches needed to reveal early and long-term effects of polidocanol in the human vascular system and new treatment approach as an anti-angiogenic therapy.

Key Words:
Polidocanol, Anti-angiogenesis, CAM assay, Cell tube formation, *In vitro*, *In vivo*.

Introduction

Angiogenesis is called as the formation of new capillaries from an existing vascular structure[1]. This is a complex process that has the main steps of activation, migration, and proliferation of endothelial cells (ECs)[2]. Angiogenic and angiostatic factors should be kept in balance during whole this process. Dysregulation of this balance causes many diseases such as cancers, psoriasis, arthritis, diabetes, obesity, asthma, and atherosclerosis. Moreover, a defect in angiogenesis can cause heart and brain ischemia, neurodegeneration, hypertension, osteoporosis, respiratory distress syndrome, preeclampsia, endometriosis, postpartum cardiomyopathy, ovarian hyperstimulation syndrome and pulmonary hypertension[3,4].

Polidocanol (lauromacrogol 400) (POL) is the most frequently used sclerosant for sclerotherapy. The target of POL as a sclerosant is endothelial cells (ECs). Endothelial cells have an important role in the coordination of many physiologic functions, such as angiogenesis and vessel repair, immune responses, tissue permeability, coagulation, and vascular tone[5].

Sclerosants, are anionic surfactants and cause damage to endothelial cell membrane phospholipid bilayer in concentration dependent manner[6]. Higher concentrations cause lysis, while lower sclerosant concentrations potentially injure endothelial cells exposed for a prolonged period. Sclerosants at much lower concentrations cause cell activation and release procoagulant microparticles[7]. The process is mediated by the interaction of surfactant molecule with phospholipid content of cell membranes. Sclerosants have also the ability to activate intracellular signaling pathways which in turn cause intracellular calcium release or alterations in nitric oxide production heralding cellular injury or death. Cell death may result from activation of apoptotic pathways, induction of necrosis, or direct chemical toxicity effects on the cell membrane[8]. Additionally, depending on the ionic nature of the surfactant molecule, sclerosants may interfere with plasma proteins and the protein contents of cell membranes[9].

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Although today POL has been mainly used for sclerotherapy, there is no information about the angiogenic effect of POL in the current literature. The aim of this study was to evaluate the angiogenic effects of POL *in vivo* and *in vitro*.

Materials and Methods

Cell viability assay, endothelial cell tube formation assay and chorioallantoic membrane (CAM) assay protocols were performed according to our previous works[4,10,11].

Polidocanol (lauromacrogol 400) Solution

POL solution was prepared as a stock solution of 20 mM. Required concentrations of POL solutions were prepared with serial dilutions from this stock solution.

Cell Viability Assay

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method to evaluate the cell viability assay. We use the same MTT method protocol as our previous studies[3,9,10]. Briefly, human umbilical vein endothelial cells (HUVECs) (4×10^4 cells/well) were incubated in a 96-well plate in the presence of various concentrations of POL (10, 20, 40 and 80 μM) for 24-48 hours to determine the effect on endothelial cell proliferation.

In vitro Endothelial Cell Tube Formation Assay

The *in vitro* endothelial cell tube formation assay was used to determine whether POL had an effect on tube formation of endothelial cells. 20 μL of 20 μM POL solutions were placed on Matrigel. This assay was performed according to the steps that described previously [4,10,11].

CAM Assay

CAM assay was used for *in vivo* evaluation of angiogenesis. POL with concentrations of 100, 150, 200 μM were prepared, and 50 μL of each solution was used on CAMs. Several control CAMs received phosphate-buffered saline (PBS). A standardized procedure including the same steps with our published studies[4,10,11] was followed to perform CAM assay, and the effect on CAM vascular area was scored.

Statistical Analysis

Chi-square test was used for the non-parametric tests. In addition, Yates correction analysis was performed for the statistically significant difference. Statistical analysis was carried out using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). We used in the same data situations as a Pearson's correlation. The statistical analysis was carried out using Statistical Package for the Social Sciences software for Windows, Version 15.00 (SPSS Inc., Chicago, IL, USA). The value of $p < 0.05$ was considered statistically significant.

Results

Cell Viability Assay of Polidocanol (lauromacrogol 400)

HUVECs were incubated with increasing doses of POL for 24 – 48 h, and cell viability was observed with the MTT assay. There was a cytotoxic effect on HUVECs in the presence of POL for 24 and 48 h, and this was statistically significant ($p < 0.05$) (Figure 1). And LD50 value was determined as 80 μM on 48th h.

Effect of Polidocanol (lauromacrogol 400) on Tube Formation

In the tube formation assay, on the 18th hour of incubation, the results were assessed. Comparing the tube length/area ratio values, there was statistically significant decrease in POL group with respect to the control group ($p < 0.05$). So, it was found that POL had an antiangiogenic effect (Figures 2 and 3).

Polidocanol (lauromacrogol 400) Inhibits Angiogenesis on CAM

To determine the mode of action and dose dependency of POL during days 6 and 8 on CAMs, we applied various concentrations of POL solution to CAMs on day 6 and evaluated their effects on CAM vessels growth, angiogenesis on day 8. On day 8, the treatment of CAMs with POL solutions caused a significant dose-dependent decrease in CAM vascular area. The vessel formation in CAM vascular area was scored[3]. While physiological angiogenesis was observed in the form of some allantoic vessels in the control group (score 0), a significant decrease (slim and fading vessels, necrotic areas) in CAM area of eggs treated with POL solution was appreciable macroscopically (score +2). Significant decrease on CAM vascu-

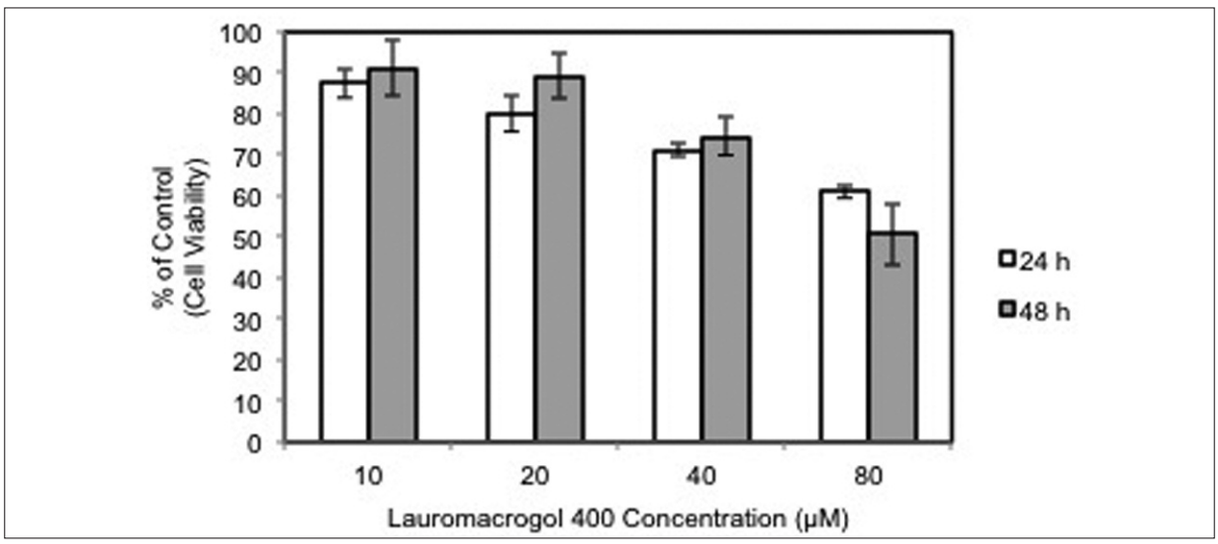

Figure 1. Cell proliferation of HUVECs under Polidocanol (lauromacrogol 400) incubation (cell viability was indicated as percentage of control). HUVECs: human umbilical vein endothelial cells.

larity was seen for 80 μM POL solution. Especially the obliteration of the affected vessels and the necrotic area can be clearly seen in Figure 4. The efficacies at different doses were compared using χ^2 test, and there was a statistically significant difference (Yates correction $\chi^2 = 13.307$, $p < 0.05$). The efficacy of increasing doses was determined with Spearman's correlation test and a strong correlation was detected ($r = 0.76$, $p < 0.001$). Macroscopically evaluated results of CAMs were shown in Table I.

Discussion

POL is a synthetic fatty alcohol, alkyl polyglycol ether of lauryl alcohol. It is a nonionic surfactant whose chemical structure forms an alkyl chain of 12 carbon atoms and an ethylene oxide chain of 9 ethylene oxide units ($C_{30}H_{62}O_{10}$)[12]. The molecular weight is approximately 600[9].

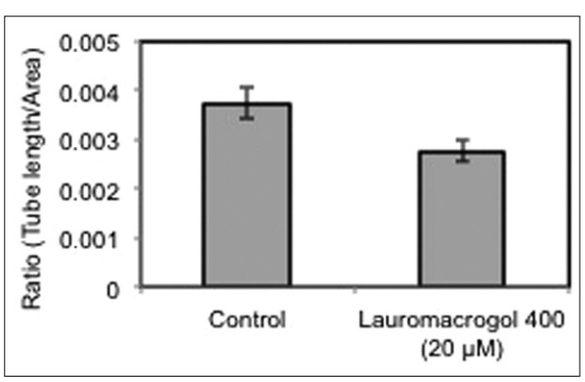

Figure 2. Ratio of tube length to area under Polidocanol (lauromacrogol 400) incubation and control.

POL was first developed in 1931 for personal care products as a detergent such as facial cream, body lotions, shampoo, and hair-conditioner[12]. As a topical and local anesthetic agent, it was introduced in Germany in 1936[13].

Injection of POL causes protein theft denaturation in which an aggregation of detergent molecules forms a lipid bilayer that induces disruption of cell surface membrane in a concentration dependent manner, activates cellular calcium signaling and nitric oxide pathways and produces endothelial cell death[14]. During sclerotherapy, concentration ranges between 0.5% and 4% is used nearly without reported side effects or allergic reactions[15]. POL is available in different concentrations, for example 0.25, 0.5, 1, 2 and 3%. Exceeding a daily dose of 2 mg polidocanol per kg body weight is not recommended[16].

Effects of higher sclerosant concentrations to achieve local therapeutic result risks systemic exposure of vascular endothelium in unaffected vessels upon sclerosant release into the circulation[17,18]. The lytic effect of POL is not limited to ECs. It also shows lytic effects on erythrocytes, leukocytes, and platelets at concentrations specific to cell types. Sclerosants are deactivated by plasma proteins and membrane lipids which result in a prominent drop in effective concentration and a reduced lytic effect on ECs. Detergent

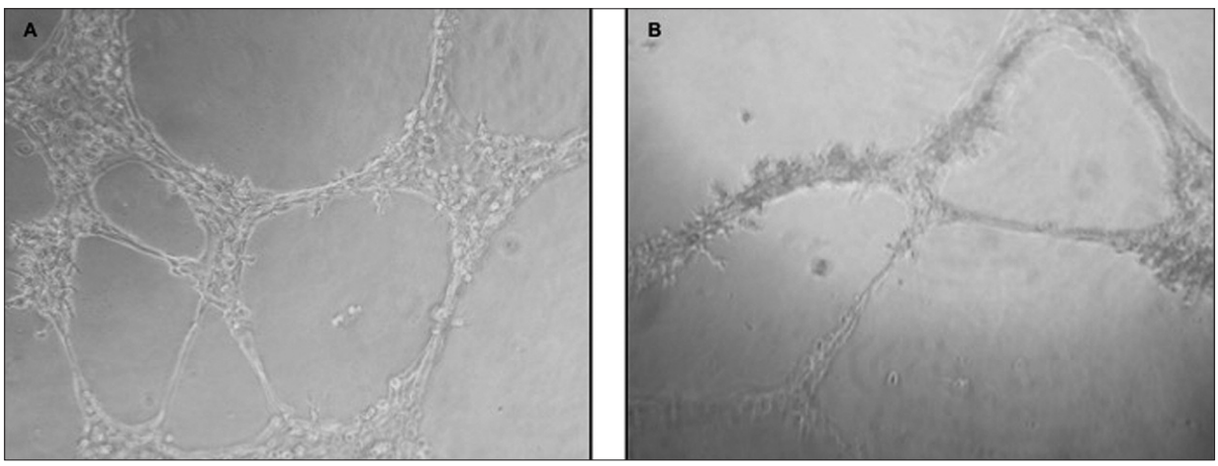

Figure 3. Tube formations of control group ***(A)*** and under Polidocanol (lauromacrogol 400) incubation ***(B)***.

sclerosants not only cause cell lysis but also cause platelet activation and release of platelet microparticles at sublytic concentrations[19].

By using a cell culture model, we have neglected *in vivo* constituents such as hormones, cytokines, and other molecules that may be crucial factors of endothelial cell and vessel wall responses to POL application. The exclusion of available proteins in blood also eliminates effects of protein binding, which may provide reduced POL toxicity. Sclerosants may also cause apoptosis to occur in some tissues by initially inducing sublethal cellular injury[20].

Angiogenesis is inevitable for reproduction, development, repair, as well as angiogenic and neoplastic diseases. During embryonic development, vasculogenesis, formation of endothelium-lined channels from precursor cells, precedes angiogenesis[1]. Early angiogenic researchers observed that the growth of human tumors was accompanied by increased vascularity in common. The existence of tumor-derived factors is responsible for promoting new vessel growth. Tumor growth and development of a neovascular supply is strongly correlated[21].

It was hypothesized that inhibition of angiogenesis (antiangiogenesis) would be an effective strategy to treat human cancer, and there are ongoing active searches for angiogenesis inducers and inhibitors[22]. In 1971, Folkman[23] proposed the angiogenesis-dependent correlation between tumor growth and metastasis. Therefore, blocking

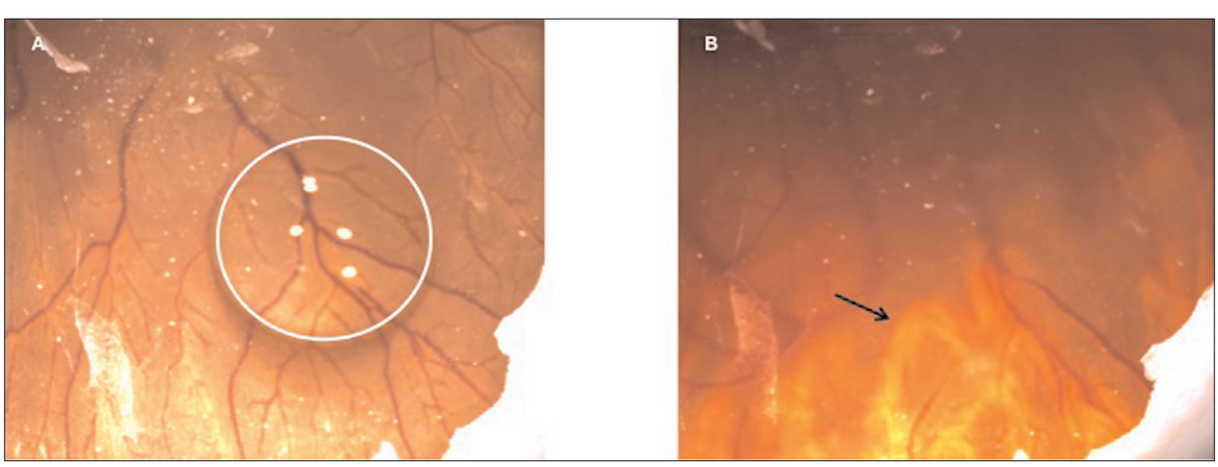

Figure 4. Effect of Polidocanol (lauromacrogol 400) solution on CAM before ***(A)*** and after 48 h ***(B)***. White circle shows the area where Polidocanol (lauromacrogol 400) was placed on first. Black arrow shows the affected vessels and the necrotic area. CAM: chorioallantoic membrane.

Table I. Macroscopic evaluation of the effect of Polidocanol (lauromacrogol 400) treatment on CAM.

Group		Efficacy			
		Ineffective	1	2	Total
Control	n	6	0	0	6
	%	100.0	0	0	100
100 μM	n	3	3	0	6
	%	50.0	50.0	0	100
150 μM	n	1	3	2	6
	%	16.67	50	33.33	100
200 μM	n	0	4	3	7
	%	0	57.14	42.86	100
Total	n	10	10	5	25
	%	40	40	20	100

angiogenesis could be a strategy to arrest tumor growth. Gullino et al[24] demonstrated that cells in pre-cancerous tissue acquire angiogenic capacity during a process becoming cancerous. Recent observations have provided further evidence for dysregulated angiogenesis. Dysregulated angiogenesis is one of the main responsible courses in the pathophysiology of pulmonary arterial hypertension[4]. According to the results of successful preclinical studies, several anti-angiogenic agents alone or in combination with conventional therapies are now in use for several studies. These trials are based on strategies that interfere with angiogenic ligands, their receptors or downstream signaling; upregulate or deliver endogenous inhibitors, or directly target tumor vasculature. These approaches offer new hope for the successful treatment of cancer[25].

Sclerosants have been used for the treatment of vascular lesions as vascular malformations[26]. Endothelial damage is the first impact of the treatment and anti-angiogenesis may be the second goal for vascular lesions and cancer, POL may be a good option for this purpose. Our study may provoke the usage of anti-angiogenic agents such as POL as an adjunctive therapy for the treatment of vascular lesions. Further researches will demonstrate the clinical importance of POL for the treatment of vascular lesions and other diseases with angiogenic activity.

Conclusions

Our study claims the importance of potential anti-angiogenic effects of commonly used sclerosant agent: Polidocanol (lauromacrogol 400). The current study evidences that POL has a potent anti-angiogenic effect *in vitro* and *in vivo*. Research of the angiogenic effects of such a commonly used agent polidocanol is crucial. Further researches need to reveal early and long-term effects of polidocanol in the human vascular system and new treatment approache as an anti-angiogenic therapy.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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