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*Original article*

# ***In vivo* inhibitory effect of *Aloe vera* gel on the ability of mouse parental splenic lymphocytes to induce cutaneous angiogenesis in recipient F1 mice**

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## **Abstract**

Lymphocyte-induced angiogenesis test (LIA) is a model of local graft-versus-host (GVH) reaction, marker of the earliest events resulting from activation of donor lymphocytes after contact with host semi-allogeneic histocompatibility antigens. The effect of *in vivo* oral administration of *Aloe vera* gel for 21 days to maternal strain (Balb/c) donor mice on the ability of their splenic lymphocytes to induce cutaneous angiogenesis (LIA test) in F1 Balb/c x C3H recipients, was studied.

Results: Neovascular reaction evaluated 72 hours after cells grafting was significantly lower in F1 mice grafted with lymphocytes collected from *Aloe*- fed donors, than in recipients of lymphocytes collected from respective controls.

Conclusions: This observation opens the promise of safe and ethically acceptable possibility of use of *Aloe vera* gel in human donors in prevention of GVHD in recipients of bone marrow grafts.

**Key words:** *Aloe vera*, mice, local GVH reaction, LIA test

## Introduction

Graft-versus-host disease is a common complication after allogeneic bone marrow transplantation. T lymphocytes present in a donor graft or derived from stem cells activated by recipient's antigens secrete various cytokines, some of them with angiogenic activity. Sidky and Auerbach (1975) elaborated and described lymphocyte-induced angiogenesis test (LIA) as a quantitative and sensitive local assay of graft-versus-host reaction in mice. In the case when host is semi-allogenic, (F1 hybrid of two various inbred strains), donor parental lymphocytes recognize alien histocompatibility antigens of the second parent and initiate the GVH reaction. Previously, we used LIA test for evaluating of pro- or anti-angiogenic activity of various herbal extracts (from plants *Echinacea purpurea*, *Echinacea pallida*, *Rhodiola rosea*, *Rhodiola quadrifida*, *Rhodiola kirilowii*) and remedies (Echinasal and Bioaron C), usually obtaining stimulatory effect (Skopińska-Różewska et al. 2002, 2008, 2011a,b, Siwicki et al. 2007, Wójcik et al. 2009). No effect was observed after feeding donor mice with extract from *Centella asiatica*, or with multi-component herbal remedy PERVIVO (Skopińska-Różewska et al. 2002, Bałan et al. 2013). Inhibitory effect was observed when donor mice were fed with FIBS – an aqueous solution of biogenic stimulators elaborated by prof. V.P.Filatov team (Skopiński et al. 2001).

The aim of this study was to evaluate the effect of feeding donor mice with *Aloe vera* gel, for 21 days before grafting their splenocytes to F1 recipients, on the ability of these cells to induce neovascularization in local cutaneous LIA test. In the available literature we have not found any similar investigation.

## Materials and Methods

### Drug

TRU-ALO 99% *Aloe vera* DRINKING GEL (*Aloe barbadensis* Miller folium succus), aloin content <40 ppm, produced by HI TECH ALOE VERA PTY LTD, Bundaberg, Australia.

### Animals

The study was performed on 54 male inbred Balb/c mice, 6- 8 weeks old, weighing about 20 g, and on 64 male F1 hybrids (Balb/c x C3H), 6 weeks old, delivered from the Polish Academy of Sciences breeding colony. For all performed experiments animals

were handled according to the Polish law on the protection of animals and NIH (National Institute of Health) standards. All experiments were accepted and conducted according to ethical guidance of Local Bioethical Committee (UWM42/N/19.11.2004). Mice were housed in a number of 4-6 per cage and maintained under conventional conditions (room temperature 22.5-23.0°C, relative humidity 50-70%, 12 h day/night cycle) with free access to standard rodent diet and water.

### Lymphocyte-induced angiogenesis test (LIA)

Balb/c mice were supplemented for 21 days with *Aloe vera*, in daily doses of 50 µl or 150 µl, served in drinking water, or water (controls), then bled under anaesthesia (ketamine 100 mg/kg. Ketamina 10%, Biowet Pulawy, Poland and xylazine 10 mg/kg; Sedazin, BIOWET, Pulawy, Poland) and sacrificed by cervical dislocation. Splenocytes were isolated from spleens under sterile conditions by pressing through stainless sieve and cotton gauze and centrifugation on Histopaque 1077 (Sigma-Aldrich, USA) for 8 min at 400g in order to remove erythrocytes. Isolated splenocytes were resuspended in Parker culture medium (TC 199, BIOMED, Lublin, Poland) and pooled within the groups of 6 mice. A local GVH reaction (lymphocyte-induced angiogenesis, LIA test) was performed according to Sidky and Auerbach (1975), and Auerbach et al. (1976) with own modifications (Skopińska-Różewska et al. 2011a). Shortly, spleen cells suspensions were grafted intradermally (1 million cells in 0.05 ml of Parker medium per graft) into F1 (Balb/c x C3H) recipients. Before performing injections mice were anaesthetized intraperitoneally with 3.6% chloral hydrate (Sigma Aldrich, USA; 0.1 ml per 10 g of body mass). Both flanks of each mouse were carefully shaved with a razor blade, each flank was injected with cells 2-3 times. Cell suspensions were supplemented with 0.05 mL/1 mL of 0.01% trypan blue solution in order to facilitate recognition of injection sites later. Grafted Balb/c splenic lymphocytes recognized C3H antigens and produced many immunological mediators including pro-angiogenic factors (immunological angiogenesis). In this test the number of newly-formed blood vessels was the measure of T cell reactivity. After 72 hours the mice were treated with a lethal dose of pentobarbital (Morbital, Biowet Pulawy, Poland).

All newly-formed blood vessels were identified and counted in dissection microscope on the inner skin surface, using criteria suggested by the authors of the method, at magnification of 6 x, in 1/3 central area of microscopic field. Identification was based on the



Fig. 1. Typical picture of neovascular reaction 3 days after intradermal injection of Balb/c splenocytes to F1 (Balb/c x C3H) hybrids.

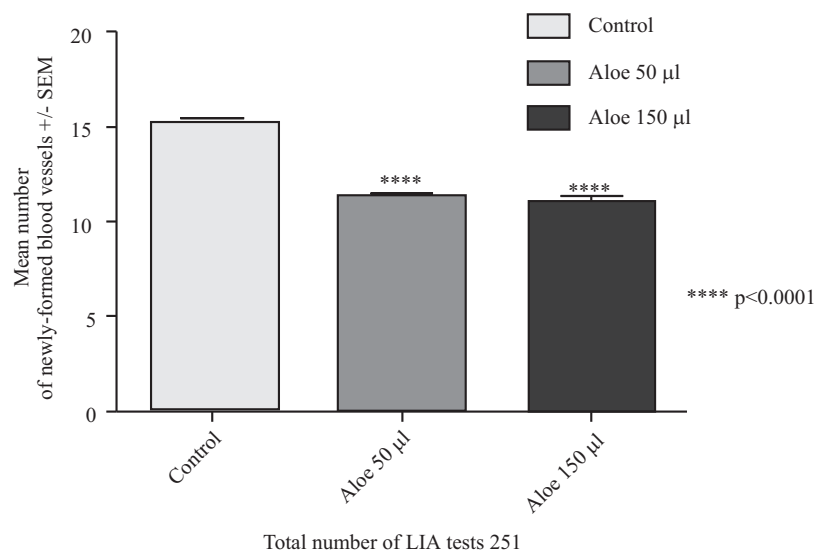


Fig. 2. Inhibitory effect of feeding Balb/c mice with *Aloe vera* gel on the ability of their splenocytes to induce neovascular reaction in skin of F1 (Balb/c x C3H) hybrids.

Table 1. Statistical analysis of the results presented in Fig. 2. One-way ANOVA, Bonferroni's Multiple Comparison Test.

	Mean Diff.	t	p<0.05	Summary
Control vs Aloe 50	3.60	10.08	Yes	****
Control vs Aloe 150	4.10	11.86	Yes	****
Aloe 50 vs Aloe 150	0.50	1.389	No	ns

fact that new blood vessels are thin, directed to the point of cells injection, with ramifications, and some of them are tortuous (Fig. 1)

### Statistical analysis

Statistical evaluation of the results was performed by one-way ANOVA with Bartlett's test for equal variances, and the significance of differences between the groups was verified with a Bonferroni Multiple Comparison Post Test (Graph Pad Prism).

### Results

The inhibitory effect of feeding Balb/c mice with *Aloe vera* gel (50  $\mu$ l and 150  $\mu$ l) on the ability of their splenocytes to induce neovascular reaction in skin of F1 (Balb/c x C3H) hybrids was evaluated. The results are shown in Fig. 2. We have found an inhibitory effect in both groups fed with *Aloe* while comparing to the control group. There are no statistical differences between the number of newly-formed blood vessels induced by splenocytes collected from both experimental groups of mice fed with *Aloe* gel (Table 1).

### Discussion

In this study we have shown for the first time that the *in vivo* administration of *Aloe vera* succus to Balb/c mice, donors of spleen cells, results in a diminished neovascular response induced by these cells in the skin of F1 Balb/c x C3H hybrids, a model of local graft-versus-host (g-v-h) reaction. Graft-versus-host disease is a common serious complication of allogeneic bone marrow transplantation, rich in stem and lymphoid cells. There were some attempts before, to ameliorate and reduce severity of experimental g-v-h disease by pretreatment of donors of graft, mice or rats, with granulocyte colony-stimulating factor, what polarized donor T-lymphocytes toward type 2 cytokine production and reduced severity of experimental graft-versus-host disease (Pan et al. 1995); by pretreatment with progenipoiectin-1 (Mac Donald et al. 2003), or with various immunosuppressive drugs (Jacobs 1985, Cooper et al. 1991, Ambrus et al. 1996). However, these drugs can have significant side effects and ethical questions arise regarding the administration of these drugs to human transplant donors (Volk et al. 1999). Regarding other aspects of graft function, Bernhardt et al. (2009) examined the effect of donor treatment with a specific prolyl-hydroxylase (PHD)

inhibitor (FG-4497), activating hypoxia-inducible transcription factors (HIFs), on graft function in the rat model of allogeneic kidney transplantation (KTx). Authors concluded that pretreatment of organ donors with FG-4497 improves short and long-term outcomes after allogeneic KTx. They outlined that inhibition of PHDs appears to be an attractive strategy for organ preservation that deserves clinical investigation.

Experimental studies have shown the role of cytokines as mediators of acute GVHD (Krenger et al. 1995, Rus et al. 1995). CD4+ and CD8+ T cells produce two patterns of cytokines. The one (type 1) is pro-inflammatory, such as interferon  $\gamma$  (IFN  $\gamma$ ), interleukin 2 (IL-2) and the second one is anti-inflammatory (type 2), such as IL-4 and IL-10. Generally, T lymphocytes with type 2 cytokine responses can prevent acute GVHD. Pan et al. (1995) showed that pretreatment of donor mice with granulocyte colony-stimulating factor (G-CSF) can modulate immunological donor properties toward type 2 cytokine production and reduces severity of experimental GVHD.

Data on donor pretreatment in human treatment are almost lacking. There is interesting retrospective analysis of outcomes in 567 patients with hematologic malignancies who had hematopoietic cell transplantation from human leukocyte antigen-identical sibling donors. The aim of the study was to check a correlation between statin use (by the donor and not by the recipient) and risk of graft-versus-host disease. The results of these investigations suggest that donor statin treatment may be a promising strategy to prevent severe acute GVHD (Rotta et al. 2010).

*Aloe* gel is a clear, jelly-like substance, found in the inner part of the *aloe* plant leaf. The gel comprises more than seventy-five compounds, among them polysaccharides, amino acids, steroids, organic acids, enzymes and antibiotic agents. It is a traditional remedy, used for many purposes, without unwanted side effects. Recently, Sehgal et al. (2013) reported that a commercial stabilized *aloe* gel (but not *aloe* latex and its component *aloe* emodin), consumed as a beverage, was not genotoxic or toxic *in vivo* in mice.

*Aloe* gel is widely used to relieve thermal burn and sunburn. This remedy also promotes wounds and ulcers healing, exerts hematopoietic activity and possesses antimicrobial and anti-inflammatory properties (Talmadge et al. 2004). Thai authors reported the effects of *Aloe vera* in rat models of gastric ulcers induced with acetic acid or *Helicobacter pylori* infection (Eamlammam et al. 2006, Prabjone et al. 2006). Treatment with *Aloe vera* resulted in suppressing of serum TNF-alpha and enhancement of IL-10 levels and promoted gastric ulcer healing. It would partly explain our present results demonstrating lower g-v-h

activity of splenocytes collected from Aloe-fed Balb/c donors, because IL-10 is described as a key immune-regulatory cytokine also contributing to the regulation of experimental graft-versus-host disease. Recently, Tawara et al (2012) demonstrated that IL-10 from donor T regulatory cells (Tregs – CD4<sup>+</sup>, CD25<sup>+</sup>, Foxp3<sup>+</sup>) or donor bone marrow is important for optimal Treg – mediated suppression of GVHD.

Another possibility is direct inhibition of pro-inflammatory and pro-angiogenic cytokines release, or some enzymes activity, in donor splenic immune cells by feeding donor Balb/c mice for 21 days with Aloe gel. Studies performed *in vitro* with *Aloe vera* and human monocytic cell line, human blood mononuclear cells and isolated human and microbial metalloproteinases (MMP), described suppressing bacterial-induced pro-inflammatory cytokines, inhibition of collagenase and metalloproteinases, and down-regulation of MMP-9 in these cells (Barrantes and Guinea 2003, Habeeb et al. 2007, Vijayalakshmi et al. 2012). Elucidation of the mechanisms of Aloe gel action on donor lymphocytes will be the matter of our further study. This observation opens the promise of safe and ethically acceptable use of the studied drug in donors for prevention of GVHD in recipients of the graft that hampers the curative and life-saving effect of the graft after high-dose chemotherapy regimens, used in hematological malignances and solid tumors as replacement of diseased or otherwise normal bone marrow. However, in many instances graft-versus leukemia or graft-versus tumor effect is desirable for the success of the therapy, although it may break out of the control and eventually hamper the outcome of the therapy. A modulatory influence of donor pretreatment with *Aloe vera* extracts, or its compounds, on the graft-versus tumor reaction, especially in mini-allografts used to boost tumor immunological response in tumors sensitive to such therapy, would be of interest.

### Conflict of interests

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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