



Anti-cytokine therapy for prevention of atherosclerosis



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ABSTRACT

Background: Currently a chronic inflammation is considered to be the one of the most important reasons of the atherosclerosis progression. A huge amount of researches over the past few decades are devoted to study the various mechanisms of inflammation in the development of atherosclerotic lesions.

Purpose: To review current capabilities of anti-inflammatory therapy for the prevention and treatment of atherosclerosis and its clinical manifestations.

Methods: Appropriate articles on inflammatory cytokines in atherosclerosis and anti-inflammatory prevention of atherosclerosis were searched in PubMed Database from their respective inception until October 2015.

Sections: "The role of inflammatory cytokines in the development of atherosclerotic lesions" describes available data on the possible inflammatory mechanisms of the atherogenesis with a special attention to the role of cytokines. "Modern experience of anti-inflammatory therapy for the treatment of atherosclerosis" describes modern anti-inflammatory preparations with anti-atherosclerotic effect including natural preparations. In "the development of anti-inflammatory herbal preparation for atherosclerosis prevention" an algorithm is demonstrated that includes screening of anti-cytokine activity of different natural products, the development of the most effective combination and estimation of its effect in cell culture model, in animal model of the acute aseptic inflammation and in a pilot clinical trial. A natural preparation "Inflaminat" based on black elder berries (*Sambucus nigra* L.), violet tricolor herb (*Viola tricolor* L.) and calendula flowers (*Calendula officinalis* L.) possessing anti-cytokine activity was developed using the designed algorithm. The results of the following 2-year double blind placebo-controlled clinical study show that "Inflaminat" reduces carotid IMT progression, i.e. has anti-atherosclerotic effect.

Conclusion: Anti-cytokine therapy may be a promising direction in moderation of atherogenesis, especially when it begins on the early stages of subclinical atherosclerosis. The use of herbal preparations with anti-cytokine mechanism of action is the most perspective for timely prevention of atherosclerosis, as they have no significant side effects and can be prescribed for long-term administration.

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Introduction

Atherosclerosis is a multifactorial disease, and it is the basis for stroke, coronary heart disease and myocardial infarction,

which remain the leading cause of mortality in Western countries.

A large number of studies on the role of inflammation in atherogenesis have been held since eighties of last century (Ammirati et al. 2015; Falk 2006; Hansson 2009; Tousoulis et al. 2015; Wolf et al. 2014). Currently there is a strong perception that local aseptic inflammation plays an important role in the progression of atherosclerosis (Aidinian et al. 2006; Libby 2006). It is known that the primary act of atherogenesis at the arterial wall is an accumulation of intracellular lipids, which is accompanied by other manifestations of atherosclerosis at the cellular level, such as stimulation of proliferation and increased synthesis and secretion

Abbreviations: BMI, body mass index; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule; IL-1, interleukin 1; IL-6, interleukin 6; cIMT, carotid intima-media thickness; LDL, low-density lipoproteins; M-CSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein 1; NSAID, nonsteroidal anti-inflammatory drug; TNF- α , tumor necrosis factor α ; VSMCs, vascular smooth muscle cells.

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of extracellular matrix and induction of synthesis and secretion of inflammatory cytokines (Orekhov et al. 1990). Under unfavorable circumstances the formation of foci of chronic inflammation leads to development of morphologically unstable plaques prone to rupture, which cause the clinical manifestations of atherosclerosis. It is necessary to conduct timely prevention in people with subclinical atherosclerosis to prevent the development of serious complications such as stroke, acute coronary syndrome and other life-threatening atherosclerotic diseases. Since atherosclerosis develops over many years, the atherosclerosis prevention should be lifelong. Taking into account the need for long-term antiatherosclerotic therapy our research team considers effective and safe natural products as promising candidates for the antiatherosclerotic agents that do not cause side effects and phenomena.

About 30 years ago, it was discovered the phenomenon of serum atherogenicity—its ability to induce lipid accumulation in cultured cells of the arterial intima (Chazov et al. 1986). It has been found that the atherogenic blood components are modified low-density lipoproteins (LDL) that are present in large amounts in the blood of patients with atherosclerosis (Tertov et al. 1989). Previously it has been established in a clinical trial using the ultrasound monitoring of the carotid atherosclerosis progression that a steady decrease of patients' serum atherogenicity leads to regression of atherosclerosis in the carotid arteries (Orekhov et al. 1995). Thus, the pathogenetic approach to the prevention of arteriosclerosis in its early stages was developed, which includes the suppression of the cholesterol accumulation in arterial wall cells. Currently, local inflammatory process is considered to be the most likely cause of the progressive development of atherosclerotic lesions in the arterial wall. The important role of inflammatory cytokines at all stages of the formation of atherosclerotic lesions and clinical manifestations of atherosclerosis is described in numerous studies (Daugherty et al. 2005; Gopal et al. 2014; Hansson GK et al. 2006; Ikonomidis et al. 2012; Ramji and Davies 2015; Von der Thusen et al. 2003; Young et al. 2002).

This review describes the symptoms of inflammation in atherosclerosis, that allow to consider atherosclerosis as a chronic inflammatory process. Modern experience of anti-inflammatory therapy in atherosclerosis treatment is presented. We have designed an algorithm of the development of anti-cytokine therapy for prevention and treatment of atherosclerosis that include screening of anti-cytokine activity of different natural substances, the development of the most effective combination and estimation of its effect in *in vitro*, *ex vivo* and *in vivo* models and clinical trial of the anti-atherosclerotic action.

Methods

Appropriate articles on inflammation in atherosclerosis and anti-inflammatory prevention of atherosclerosis were searched in PubMed Database from their respective inceptions until October 2015. More 50 publications describing the inflammatory mechanisms of atherogenesis, in particular, the role of inflammatory cytokines in the development of atherosclerotic lesions were examined in this review. About 20 publications describing the contemporary experience of anti-inflammatory therapy in the prevention and treatment of atherosclerosis, including natural preparations, were considered.

The role of inflammatory cytokines in the development of atherosclerotic lesions

Basic researches in the field of atherosclerosis reveal a lot of data about the role of inflammation in the cellular and molecular

mechanisms of atherogenesis in all its stages from the initial signs of the process until the destabilization of atherosclerotic plaques and thrombotic events, with special attention paid to the participation of inflammatory cytokines in the development of atherosclerotic lesions (Daugherty et al. 2005; Gopal et al. 2014; Hansson GK et al. 2006; Ikonomidis et al. 2012; Ramji and Davies 2015; Von der Thusen et al. 2003; Young et al. 2002).

Several types of immune cells, primarily monocytes, T- and B-lymphocytes and perhaps mast cells are involved in the inflammatory process in atherosclerosis. In the process of atherosclerotic inflammation the key role belongs to monocytes/macrophages (Chavez-Sanchez et al. 2014; Legein et al. 2013; Tuttolomondo et al. 2012). Apparently, the overexpression of inflammatory cytokines by dysfunctional endothelial and blood cells due to influence of modified LDL should be considered as the first step of inflammation in atherosclerosis (Nikoforov et al. 2013). Inflammatory cytokines, basically tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1) promote adhesion of monocytes to activated endothelial cells due to excessive expression of vascular cell adhesion molecules, intercellular adhesion molecules (ICAM-1), endothelial adhesion molecules for leukocytes and E-selectin are expressed by endothelial cells, vascular smooth muscle cells (VSMCs), tissue macrophages (Blake and Ridker 2001). Adhesion molecules cause rolling of blood cells, monocytes and lymphocytes, their binding and transendothelial migration. Endothelial adhesion molecules specifically and strongly bind to monocytes and lymphocytes of the blood, that is the basis for subsequent differential migration of these cells in the subendothelial space of the vessel, induced by specific factors (TNF- α , monocyte chemoattractant protein (MCP-1)). Soluble forms of the adhesion molecules can be determined in plasma and are an indicator of adhesion molecules expression on the cell membrane (Lind 2003). The next stage is the differentiation of monocytes into macrophages. A part of monocytes influenced by macrophage colony-stimulating (M-CSF), granulocyte-macrophage colony-stimulating and other factors secreted by endothelial cells penetrate in the intima and are exposed differentiation and proliferation, express scavenger receptors than transforming into macrophages (Rosenfeld et al. 1992). With the assistance of M-CSF the macrophage phenotype occurs that is not transformed into foam cells but subsequently secrete inflammatory cytokines (IL-1, TNF- α). Chemoattractants secreted by these macrophages, such as osteonin, mitogens and platelet derived growth factor activate VSMCs, causing their migration from media into intima of the arterial wall. Macrophages of the other phenotype uptake excess of modified LDL, are transformed into foam cells. Macrophages and mast cells secrete a growth factor which causes proliferation of VSMCs and regulates the production of the extracellular matrix, as well as metalloproteinases that cause the degradation of the extracellular matrix. Inflammatory cytokines may also have procoagulant effects, directly or via endothelial dysfunction. Thus, macrophages and mast cells regulate the growth of atherosclerotic plaque and contributing to its further destabilization with thrombosis (Biasucci et al. 1998; Galis et al. 1995).

IL-1 and interleukin-6 (IL-6) also have a great importance in the development of atherosclerosis as mediators of interactions between the leukocytes. IL-1 can induce a large part of local and systemic manifestations of the inflammatory response in atherosclerosis. This is achieved by improving the adhesion of the blood cells to the vascular endothelial cells and increasing blood procoagulant activity. IL-1 is a chemoattractant for a variety of cells, it increases the mobility of neutrophils, promotes cell activation in the locus of inflammation, enhances the production of other cytokines and prostaglandin as well as synthesis of collagen and fibronectin, stimulates phagocytosis, and production of superoxide radicals, cause degranulation of mast cells. IL-1 binds to a receptor on the endothelial surface and stimulates a whole cascade of

inflammatory cytokines, promotes the sticking of leukocytes to the endothelium with further penetrating into the subendothelial layers of the arterial wall. These features contribute to the development of exudative and proliferative components of the inflammatory response (Dinarello 2000; Moyer et al. 1991).

TNF- α is produced predominantly by monocytes/macrophages, endothelial cells and mast cells. TNF- α acts similarly to IL-1 and IL-6 as regards the spectrum of the target cells and biological effects. TNF- α affects the endothelium, increasing expression of adhesion molecules on it, activates macrophages, neutrophils, increases the secretion of prostaglandins, it also has chemotactic effects on various cells and causes the synthesis of acute-phase proteins. TNF- α is able to induce apoptosis leading to production of reactive oxygen species, superoxide radicals and nitric oxide (Parameswaran and Patial 2010; Yoshizumi et al. 1993). TNF- α is an important factor of atherosclerotic plaque destabilization, which leads to the development of acute coronary syndrome (Aukrust et al. 2011). It was also shown that post-ischemic reperfusion is accompanied by the release of inflammatory cytokines such as TNF- α , IL-1, IL-6 (Entman and Smith 1994).

IL-6 has a value in the development of atherosclerosis as a proinflammatory, hepatocyte-activating factor produced by monocytes, macrophages, lymphocytes, fibroblasts and endothelial cells. IL-6 is a central mediator of the acute phase response and one of the primary determinants of production of C-reactive protein (CRP). IL-6 has an important role in the development of atherosclerotic lesions from the early stages by regulation of endothelial cell adhesion molecule expression (Zhang et al. 2011). The main biological effect of IL-6 is participation in immune inflammatory response. Evidently, IL-6 affects the synthesis of acute phase proteins by hepatocytes (CRP, serum amyloid A, haptoglobin A, proteinase inhibitor, and fibrinogen) more than other inflammatory cytokines. Its effect on the local manifestations of inflammation is similar to the effect of IL-1. It is known that IL-6 promotes exacerbation of chronic inflammatory processes and the transformation of acute inflammation into chronic. IL-6 is released later than IL-1 and TNF- α and inhibits their production (and conversely they stimulate its release) and therefore IL-6 belongs to the cytokines that complete the development of inflammatory response (Kishimoto et al. 1995; Woods et al. 2000).

Investigations in animal models of atherosclerosis are widely used in the studies of inflammatory mechanisms of atherogenesis, in particular using knockout mice on various genes associated with atherosclerosis (Beaulieu et al. 2014; Ley et al. 2011; Lichtman 2013; Lind 2003). Furthermore, the presence of pro-inflammatory cytokines in atheromatous human tissue was demonstrated, and it was shown that increased levels of certain cytokines in serum correlated positively with the incidence and severity of atherosclerotic complications, including acute coronary syndrome (Mangge et al. 2004; Ridker et al. 2000). It is shown in studies in various animal models of atherosclerosis that the signs of inflammation detected in humans, can be observed at the initial stage of lipid infiltration of the vascular wall (Kleemann et al. 2008). Normal non-activated endothelium has no ability to bind leukocytes, but in animal models cell adhesion molecules are expressed on endothelium straight after the start of atherogenic diet, inducing recruitment of monocytes and T-lymphocytes (Cybulsky et al. 2001). There is a high ICAM-1 expression in atherosclerotic lesions in ApoE-deficient mice with hypercholesterolemia as compared to control mice (Nakashima et al. 1998). Blood leukocytes are located in early atherosclerotic lesions, not only in experimental models, but also in humans. It was investigated in the study on the model of atherosclerosis in rabbits that the increase of cell number in the atherosclerotic lesions is not only due to the proliferation of VSMCs and migration of blood cells, but also due to the proliferation of macrophages (Rosenfeld and Ross 1990). In several studies

the presence of TNF- α was demonstrated in atherosclerotic lesions (Barath et al. 1990; Rus et al. 1991). In the investigation of human atherosclerotic plaques obtained by endarterectomy the high levels of inflammatory T-cell cytokines such as IL-1, IL-8 and colony-stimulating factors for granulocytes and monocytes were detected (Frostegard et al. 1999).

CRP, a marker of inflammation, is considered as one of the most informative prognostic risk factors for cardiovascular complications of atherosclerosis (Poredos et al. 2015; Ridker 1998). It's worth noting that elevated level of CRP is a consequence of the cells' stimulation caused by overproduction of inflammatory cytokines. Thus, human smooth muscle cells of coronary arteries in cellular model produce significantly more CRP after incubation with IL-1, IL-6, TNF- α as compared with the control (Calabro et al. 2003).

Do anti-inflammatory cytokines have the anti-atherosclerotic effect? During the development of atherosclerotic lesions inflammatory cytokines IL-4 and IL-10, produced by activated lymphocytes, macrophages and mast cells, are the main inhibitors of inflammatory cytokine synthesis, they also decreases the activity of macrophages. IL-10 also reduces the stimulation of endothelial by modified LDL and production of metalloproteinases by macrophages, stimulates the synthesis of tissue inhibitor of metalloproteinase-1 by monocytes (Mallat et al. 1999). However, these cytokines are present in atherosclerotic lesions fewer than inflammatory cytokines at the stage of atherosclerotic plaque and are not detected in early atherosclerotic lesions (Frostegard et al. 1999).

Modern experience of anti-inflammatory therapy for the treatment of atherosclerosis

Currently it's no doubt that atherosclerosis is a pathological process with elements of local aseptic inflammation, and inflammatory cytokines play a role at every stage of the development of atherosclerotic lesions in the arterial wall. In this regard, preparations with systemic anti-inflammatory action may be effective for the prevention of atherosclerosis.

Previously, lipid-lowering drugs, such as statins, were the only drugs used for the atherosclerosis prevention. It was shown in numerous clinical studies that statins prevent the increasing of thickness of the intima-medial layer of carotid arteries (cIMT)—a surrogate marker of atherosclerosis, which is widely used at present as a key endpoint in studies of anti-atherosclerotic efficacy of various preparations, as well as a quantitative measure of atherosclerosis in epidemiological studies devoted to the study of atherosclerosis (Amarenco et al. 2004; Huang et al., 2013). Statins are preparations with pleiotropic mechanism of action, and their antiatherosclerotic effect is caused not only by lipid-lowering activity, but also by anti-inflammatory, antioxidant and anti-platelet properties, immunomodulation (Moyer et al. 1991; Profumo et al. 2104; Violi et al. 2014). Lipid-lowering drugs from other groups (fibrates, ezetimibe, etc.) have similar pleiotropic properties and are effective as anti-atherosclerotic agents (Crea and Niccoli 2015; Elisaf 2002).

Currently, anti-inflammatory approach to the prevention and treatment of atherosclerosis is widely investigated (Berman et al. 2013; Foks et al. 2015; Ridker and Luscher 2014). Anti-inflammatory mechanisms of statins' anti-atherosclerotic action are the most studied. It was demonstrated that statins cause CRP reduction by modulation of inflammatory cytokines' action (Owens 2012). Among potential anti-atherosclerotic drugs novel preparations with anti-cytokine mechanism of action, such as canakinumab (a human monoclonal anti-human IL-1 β antibody) and anakinra (IL-1receptor antagonist), tocilizumab (IL-6 inhibitor), etanercept (TNF- α inhibitor) and darapladib (direct inhibitor of lipoprotein-associated phospholipase A2) and classic

anti-inflammatory drugs such as colchicine and methotrexate are the most promising agents for atherosclerosis medication that are being studied (Back et al. 2015; Owens 2012). Preparations that cause the suppression of IL-1 are the most highlighted and promising because this cytokine is the central mediator of inflammatory process in atherogenesis (Foks et al. 2015; Qamar and Rader 2012).

Atherosclerosis develops over many years, so anti-atherosclerotic therapy should be long-term or even lifelong. Only the timely start of the pathogenetic prevention of atherosclerosis may inhibit the development of such serious clinical complications of atherosclerosis as ischemic stroke, acute myocardial infarction, critical limb ischemia and gangrene. The progress in clinical diagnostic of atherosclerosis makes the timely start of the treatment possible. However, atherosclerosis can be subclinical at the early stages, and there is no need of urgent administration of aggressive synthetic preparations that have a spectrum of serious adverse effects. Patients could stabilize the atherosclerotic process by correcting the diet and using non-pharmaceutical products with beneficial properties. Modern experience of using preparations with anti-inflammatory activity in the treatment of atherosclerosis suggests that it may also be a promising direction for the prevention of subclinical atherosclerosis. The use of natural preparations is suitable for the early prevention of atherosclerosis because they have almost no side effects and exert regulatory effects at physiological limits, allowing longer, almost lifelong, medication.

Natural preparations with anti-inflammatory properties are also the subjects of researches as potential anti-atherosclerotic agents. Iridoids, a large group of cyclopentapyran monoterpenoids, that are present in most plants of Dicotyledonous plant families, are widely investigated in several fundamental researches. Different anti-inflammatory properties of herbs containing iridoids especially in species belonging to the Apocynaceae, Lamiaceae, Loganiaceae, Rubiaceae, Scrophulariaceae and Verbenaceae families were demonstrated in a huge amount of studies in *in vitro* and *in vivo* models (Bas et al. 2007; Viljoen et al. 2012). In *in vitro* and *in vivo* studies of Tripathi et al. the pleiotropic anti-inflammatory effects of a polyherbal formulation consisting of water-soluble fractions of five medicinal plants (*Commiphora mukul*, *Terminalia arjuna*, *Boswellia serrata*, *Semecarpus anacardium* and *Strychnos nuxvomica*) were demonstrated, i.e. a reduction of key inflammatory mediators of arachidonic acid cascade and inhibition of lipid peroxidation (Tripathi et al. 2004).

It was shown in the study of Narasimhulu et al. that administration of sesame oil reduces plasma level of inflammatory cytokines in mice model (Narasimhulu et al. 2015). Omega-3 fatty acids, that have a very beneficial effect on the lipid parameters by increasing of high-density lipoprotein and decreasing of triglycerides and low-density lipoprotein levels, have also been shown to provide anti-inflammatory properties by reducing IL-6, TNF- α , CRP (Ellulu et al. 2015). Bioactive peptides derived from food proteins have been evaluated for various beneficial effects, including anti-inflammatory and antioxidant properties and have been suggested to be effective for anti-atherosclerotic medication (Chakrabarti et al. 2014).

We can say that the use of natural preparations for the atherosclerosis prevention is only a matter of time. Anti-inflammatory properties of natural substances are studied in a number of basic researches. However, currently there is no clear standard for evaluating the effectiveness of natural products with anti-inflammatory properties as anti-atherosclerotic agents, especially in clinical trials. Despite the fact that the study of the influence of natural products on the natural history of atherosclerosis in humans is a popular direction of current research, anti-atherosclerotic efficiency of natural preparations with anti-inflammatory activity in clinical studies is little studied.

The development of anti-inflammatory herbal preparation for atherosclerosis prevention

Since it is believed that natural preparations can be promising agents for anti-atherosclerotic therapy, we have designed an algorithm of the development of anti-cytokine therapy for atherosclerosis prevention, that includes screening of anti-cytokine activity of different natural substances, the development of the most effective combination and estimation of its effect in *in vitro*, *ex vivo* and *in vivo* models and in clinical trials. Below the developed algorithm is described in detail.

Screening of natural products in *in vitro* model

The main aim of the study was to develop a natural preparation that possesses anti-cytokine effect and to study its effect on natural history of subclinical atherosclerosis. At the first stage of the study the anti-cytokine activity of following 31 natural products was investigated in *in vitro* model: black chokeberry (*Aronia melanocarpa* (Michx.) Elliott), amur barberry (*Berberis amurensis* Rupr.), hawthorn (*Crataegus ambigua* C.A.Mey ex A.K.Becker), black elderberry (*Sambucus nigra* L.), ling (*Calluna vulgaris* (L.) Hull), common gypsyweed (*Veronica officinalis* L.), pedunculate oak (*Quercus robur* L.), ginseng (*Panax ginseng* C.A.Mey), St. John's wort (*Hypericum perforatum* L.), white willow (*Salix alba* L.), kalanchoe (*Kalanchoe pinnata* (Lam.) Pers.), calendula (*Calendula officinalis* L.), red clover (*Trifolium pratense* L.), willow-herb (*Chamerion angustifolium* (L.) Holub), cranberry (*Oxycoccus palustris* Pers.), great nettle (*Urtica dioica* L.), silverweed cinquefoil (*Potentilla anserina* L.), common burdock (*Arctium lappa* L.), coltsfoot (*Tussilago farfara* L.), lungwort (*Pulmonaria officinalis* L.), common juniper (*Juniperus communis* L.), peppermint (*Mentha piperita* L.), plantain (*Plantago major* L.), couch grass (*Elytrigia repens* (L.) Nevsky), chamomile (*Matricaria chamomilla* L.), licorice (*Glycyrrhiza glabra* L.), bearberry (*Arctostaphylos uva-ursi* (L.) Spreng.), common yarrow (*Achillea millefolium* L.), viola tricolor (*Viola tricolor* L.), tripartite bur-marigold (*Bidens tripartita* L.), clary sage (*Salvia sclarea* L.). Plant names were checked with <http://www.theplantlist.org>. Cell culture model of human blood-derived monocytes was used to test the effect of these products on IL-1 expression. Venous blood for isolation of monocytes was taken from apparently healthy donors at an amount of 50 ml in a sterile tube and supplied with sodium citrate as an anticoagulant. Monocytes were isolated by gradient low-speed centrifugation with LSM 1077 lymphocyte separation medium under sterile conditions. Isolated cells were seeded into 48-well plastic clusters for cell cultures under sterile conditions. The obtained cells were incubated overnight with DMEM medium containing 10% fetal calf serum for cell adhesion and growth. On the second day in culture, the cells were washed with sterile phosphate buffered saline and supplied with DMEM medium containing aqueous extract of studied herbs. Bacterial lipopolysaccharide was added to stimulate the expression of inflammatory cytokines by cultured cells. After 24 h of incubation the cultural medium was removed, the cells were washed with phosphate buffered saline and then fixed with hexane-isopropanol mixture. The expression of inflammatory cytokine IL-1 was estimated by the enzyme-linked immune-sorbent assay using monoclonal antibodies to IL-1 («Biogenesis», UK). Expression of IL-1 was assessed in terms of optical density.

Significant decrease of LPS-induced expression of IL-1 (no less than 15%) in cultured monocyte-macrophages after 24-h incubation with aqueous herbal extract (tincture) at a concentration of 1 mg/ml was a criterion of anti-inflammatory efficacy. A criterion for the safety assessment was the lack of cytotoxicity of aqueous extract. As a result of screening the following herbs were selected:

Table 1
Inflammatory cytokines expression after single dose of diclofenac administration.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	80.0 \pm 10.0	88.0 \pm 7.5	61.3 \pm 6.6*
IL-1 expression, % from baseline	78.0 \pm 5.5*	73.3 \pm 7.9*	51.0 \pm 21.8*
HLA-DR expression, % from baseline	86.7 \pm 6.9	83.3 \pm 9.0	71.7 \pm 7.9
ICAM-1 expression, % from baseline	84.7 \pm 8.1	71.3 \pm 6.4*	67.7 \pm 13.3*

* Significant result, $p < 0.05$.

Table 2
Serum atherogenicity after single dose of allicor administration.

Time after single dose oral intake	2 h	4 h	8 h	12 h
Serum atherogenicity, % from baseline	34.2 \pm 7.8*	31.6 \pm 4.6*	53.0 \pm 5.0*	67.3 \pm 5.4*

* Significant result, $p < 0.05$.

Table 3
Effect of black elder berries intake on inflammatory cytokines expression and serum atherogenicity.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	80.3 \pm 2.0*	98.0 \pm 14.1	121.7 \pm 21.6
IL-1 expression, % from baseline	68.0 \pm 2.6*	70.7 \pm 2.4*	88.3 \pm 2.0*
HLA-DR expression, % from baseline	93.7 \pm 4.1	70.3 \pm 9.8*	111.7 \pm 28.8
ICAM-1 expression, % from baseline	85.7 \pm 1.2*	82.7 \pm 2.2*	86.7 \pm 9.6
Serum atherogenicity, % from baseline	58.0 \pm 16.5	14.0 \pm 14.0*	44.7 \pm 8.3*

* Significant result, $p < 0.05$.

hawthorn, black elderberry, St. John's wort, calendula, viola tricolor.

The investigation of anti-inflammatory and anti-atherogenic effects of selected natural products and their combinations in ex vivo model

The development of the standard for assessment of anti-inflammatory efficiency

Nonsteroidal anti-inflammatory drug (NSAID) diclofenac was used as a standard that has a pronounced anti-inflammatory activity. The study was conducted at three volunteers who previously had the increased pro-inflammatory potential of blood serum, *i.e.* the ability to induce the expression of inflammatory cytokines in cell culture. Primary culture of monocytes/macrophages was prepared for the experiment as described above. On the second day in culture, the cells were supplied with DMEM medium containing 10% of tested serum sample. Serum samples were taken from volunteers before diclofenac intake and after 2, 4 and 8 h after single dose 100 mg of diclofenac oral administration.

Results were expressed in terms of means and S.E.M. Significance of differences was evaluated using SPSS 10.1.7 statistical program package (SPSS Inc., USA). Significance was defined at the 0.05 level of confidence.

Baseline cytokine expression was taken for 100%. Results are presented in percentage from baseline. It was shown that diclofenac's anti-inflammatory effect developed within 4 h after administration with a peak after 8 h (Table 1). The most sensitive marker was IL-1.

The development of the standard for assessment of anti-atherogenic efficiency

Allicor, natural preparation based on garlic was used as a standard that has a pronounced anti-atherogenic activity. The study was conducted in 20 volunteers who had high serum atherogenicity, *i.e.* ability of blood serum to induce cholesterol accumulation in cultured monocytes/macrophages. We estimated anti-atherogenic effect of Allicor using serum samples taken from volunteers after 2, 4 and 8 h after single dose (150 mg) of preparation adminis-

tration in described *ex vivo* model. Atherogenic potential of blood serum was significantly decreased during 2 h after a single dose of Allicor intake. Anti-atherogenic effect lasts 8–12 h after Allicor administration (Table 2).

The assessment of anti-inflammatory and anti-atherogenic effects of selected natural products

The effects of the natural products on the expression of inflammatory markers, as well as serum atherogenicity were studied in *ex vivo* model. The study of each product was carried out at 3 volunteers. Inflammatory cytokines expression and serum atherogenicity were evaluated before and at 2, 4 and 8 h after natural products' intake in the described above *ex vivo* model. Results are presented in Tables 3–7.

The tincture of 2.5 g of black elder berries had strong anti-inflammatory effect, comparable to that of diclofenac, the maximum effect was observed after 2–4 h. Anti-atherogenic effect of black elder was comparable to that of Allicor in 4–8 h after ingestion (Table 3).

The tincture of 3 g St. John's wort herb had anti-inflammatory effect in 2–4 h after administration, comparable to that of diclofenac, but less in duration. In addition, St. John's wort had unstable anti-atherogenic effect that made impossible to compare the anti-atherogenic activity of Allicor and St. John's wort (Table 4).

The tincture of 2.5 g calendula flowers had a pronounced anti-inflammatory action comparable with diclofenac effect. Furthermore, calendula had anti-atherogenic effect comparable to Allicor but with less duration of effect (Table 5).

The tincture of 1.5 g of viola tricolor herb had anti-inflammatory effect in 2–4 h after administration, comparable to that of diclofenac, but less in duration. In addition, viola herb had strong anti-atherogenic effect comparable to that of Allicor (Table 6).

The tincture of 8 g hawthorn fruit had anti-inflammatory effect that was much smaller than diclofenac effect in expression and duration. Anti-atherogenic effect of hawthorn fruit intake was comparable with effect of Allicor in terms of expression, but less in duration (Table 7).

Table 4

Effect of St. John's wort intake on inflammatory cytokines expression and serum atherogenicity.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	84.0 \pm 1.7*	87.3 \pm 1.8*	97.3 \pm 3.3
IL-1 expression, % from baseline	90.3 \pm 4.7	83.0 \pm 2.1*	95.7 \pm 5.4
HLA-DR expression, % from baseline	76.0 \pm 8.0*	79.3 \pm 7.1*	91.3 \pm 3.8
ICAM-1 expression, % from baseline	86.7 \pm 5.0*	83.7 \pm 6.2*	89.3 \pm 9.0
Serum atherogenicity, % from baseline	37.0 \pm 37.0	36.7 \pm 29.0	56.0 \pm 29.5

* Significant result, $p < 0.05$.**Table 5**

Effect of calendula flowers intake on inflammatory cytokines expression and serum atherogenicity.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	72.7 \pm 9.0*	71.0 \pm 11.5*	70.3 \pm 21.2
IL-1 expression, % from baseline	78.3 \pm 9.2*	77.3 \pm 13.2*	81.7 \pm 11.4
HLA-DR expression, % from baseline	86.7 \pm 6.4	87.3 \pm 9.3	73.7 \pm 0.7*
ICAM-1 expression, % from baseline	75.3 \pm 7.0*	90.3 \pm 2.8*	75.0 \pm 5.1*
Serum atherogenicity, % from baseline	54.7 \pm 20.6*	26.0 \pm 26.0*	73.3 \pm 36.7

* Significant result, $p < 0.05$.**Table 6**

Effect of viola herb intake on inflammatory cytokines expression and serum atherogenicity.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	85.3 \pm 10.3	83.3 \pm 14.6	80.3 \pm 4.1*
IL-1 expression, % from baseline	68.7 \pm 3.8*	84.7 \pm 5.2*	85.3 \pm 9.4
HLA-DR expression, % from baseline	79.3 \pm 3.7*	73.0 \pm 5.0*	102.7 \pm 8.3
ICAM-1 expression, % from baseline	65.7 \pm 11.5*	85.7 \pm 17.3	83.7 \pm 14.2
Serum atherogenicity, % from baseline	49.3 \pm 12.2*	44.8 \pm 14.6*	62.0 \pm 9.3*

* Significant result, $p < 0.05$.**Table 7**

Effect of hawthorn fruit intake on inflammatory cytokines expression and serum atherogenicity.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	94.3 \pm 2.7*	96.3 \pm 5.8	113.3 \pm 6.7
IL-1 expression, % from baseline	87.3 \pm 3.4*	84.0 \pm 2.1*	88.7 \pm 9.4
HLA-DR expression, % from baseline	92.3 \pm 3.9*	81.3 \pm 6.2*	105.7 \pm 6.7
ICAM-1 expression, % from baseline	86.0 \pm 2.6*	85.3 \pm 2.4*	102.3 \pm 11.3
Serum atherogenicity, % from baseline	26.7 \pm 12.1*	16.7 \pm 16.7*	35.3 \pm 24.3

* Significant result, $p < 0.05$.

As a result of the total rank evaluation of the anti-inflammatory and anti-atherogenic efficiency of natural products, hawthorn was excluded from further study as the least effective.

The development of the most effective combination of natural products

Anti-inflammatory effects of various combinations of natural products have been studied in *ex vivo* model. The flowers of calendula (2.5 g), berries of black elder (2.5 g), herb of St. John's wort (3 g) and herb of violet tricolor (1.5 g) were used for the preparation of mixtures. The effect of mixtures intake on the expression of inflammatory cytokines TNF- α and IL-1 was studied in three volunteers for each mixture. Baseline expression is taken for 100%. The results of natural products' combinations effect on inflammatory cytokines expression are presented as percentage from baseline (Table 8).

After evaluation of the anti-inflammatory activity of the natural products combinations, mean levels of anti-cytokine effects were estimated in comparison with diclofenac, which allowed the ranking of the mixtures effectiveness. The combination of viola, calendula and black elder had the most pronounced anti-cytokine activity and its integral effect was 87.7% of the effect of diclofenac. Study results allowed to design natural preparation inflaminat con-

taining 165 mg of calendula flowers, 165 mg of black elder berries and 165 mg of viola tricolor herb.

Anti-cytokine and anti-atherogenic effects of inflaminat in *ex vivo* model

Anti-cytokine effect of inflaminat

The effect of a single dose of inflaminat (1 tablet 500 mg) administration on the expression of inflammatory cytokines was studied in 4 volunteers. The results of evaluation of inflaminat anti-cytokine effect are presented in Table 9.

When comparing the effect of inflaminat with diclofenac (Table 1), a standard for anti-cytokine activity, there were no significant difference.

As follows from the data presented in Table 9, a single dose of inflaminat intake led to significant reduction in pro-inflammatory activity of blood serum, but the effect duration didn't exceed 8 h, which resulted in the need to develop the optimal mode of the preparation administration to achieve a stable reduction of serum pro-inflammatory potential.

The following reception modes of natural preparation inflaminat were studied for optimization of dosing:

- A single dose of 3 tablets in the morning (mode A)

Table 8
Effect of natural products combinations' intake on inflammatory cytokines expression.

Combination	Inflammatory cytokine	Time after intake, h		
		2	4	8
Calendula + Black elder	IL-1	90.0 ± 6.9	84.3 ± 6.7*	82.2 ± 7.1*
	TNF- α	89.3 ± 4.2*	82.8 ± 7.6*	94.5 ± 5.5
St. John's wort + Black elder	IL-1	88.3 ± 2.2*	87.2 ± 3.4*	93.8 ± 6.7
	TNF- α	91.3 ± 9.2	83.1 ± 9.7*	89.5 ± 12.4
Calendula + St. John's wort	IL-1	86.4 ± 4.5*	82.5 ± 7.6*	98.2 ± 5.8
	TNF- α	94.2 ± 6.4	87.3 ± 4.2*	96.5 ± 12.1
Viola tricolor + Black elder	IL-1	77.3 ± 12.7*	80.7 ± 13.8	97.7 ± 16.0
	TNF- α	68.0 ± 8.5*	82.7 ± 6.1*	77.0 ± 7.0*
Calendula + Viola tricolor	IL-1	73.7 ± 2.8*	85.0 ± 7.9*	89.7 ± 10.1
	TNF- α	88.7 ± 4.4*	87.0 ± 5.5*	122.3 ± 12.8
Viola tricolor + St. John's wort	IL-1	85.0 ± 5.0*	96.3 ± 1.9	91.0 ± 5.6
	TNF- α	90.0 ± 4.0*	88.7 ± 3.0*	84.0 ± 9.0
Calendula + Viola tricolor + Black elder	IL-1	69.3 ± 2.7*	77.3 ± 0.3*	83.0 ± 3.6*
	TNF- α	74.3 ± 2.7*	83.7 ± 0.9*	91.7 ± 1.8*
Viola tricolor + Calendula + St. John's wort	IL-1	79.0 ± 1.7*	88.0 ± 2.5*	90.3 ± 7.5
	TNF- α	86.7 ± 1.2*	87.3 ± 1.8*	96.0 ± 2.5
Calendula + Black elder + St. John's wort	IL-1	91.3 ± 4.3	90.7 ± 4.1*	82.0 ± 4.6*
	TNF- α	95.0 ± 3.1	85.7 ± 4.4*	83.7 ± 9.2*
Viola tricolor + Black elder + St. John's wort	IL-1	81.7 ± 6.0*	87.3 ± 6.3	93.7 ± 3.3
	TNF- α	82.3 ± 1.5*	86.7 ± 4.9*	92.7 ± 3.5
Viola tricolor + Black elder + Calendula + St. John's wort	IL-1	84.3 ± 5.2*	86.0 ± 5.7*	85.3 ± 3.5*
	TNF- α	92.7 ± 3.0*	83.7 ± 1.9*	96.3 ± 2.6

* Significant result, $p < 0.05$.

Table 9
Inflammatory cytokines expression after single dose of inflaminat administration.

Time after single dose oral intake	2 h	4 h	8 h
TNF- α expression, % from baseline	68.1 ± 4.1*	70.4 ± 6.0*	82.0 ± 9.0
IL-1 expression, % from baseline	70.3 ± 6.2*	66.1 ± 1.1*	75.5 ± 6.4*
HLA-DR expression, % from baseline	84.2 ± 6.0*	84.0 ± 5.5*	94.1 ± 5.0
ICAM-1 expression, % from baseline	73.1 ± 6.2*	71.4 ± 5.3*	87.2 ± 6.4

* Significant result, $p < 0.05$.

Table 10
IL-1 expression at different modes of inflaminat administration.

Time, h	The average inflammatory cytokines expression, % from baseline		
	Mode A	Mode B	Mode C
0	100	100	100
4	59.3 ± 6.1*	64.0 ± 5.3*	66.7 ± 7.1*
8	66.0 ± 7.4*	68.9 ± 6.0*	70.1 ± 7.3*
12	88.2 ± 7.1	63.2 ± 8.0*	62.3 ± 9.3*
24	102.2 ± 7.2	78.1 ± 7.4*	74.1 ± 5.0*

* Significant result, $p < 0.05$.

- 2 tablets in the morning and 1 tablet in the evening (mode B)
- 1 tablet 3 times a day every 8 h (mode C).

The expression of IL-1 induced by blood serum taken at different periods of time during the day was measured in *ex vivo* model at 4 volunteers for each mode of inflaminat administration. The expression and duration of the anti-cytokine effect at the various modes of reception were compared. The results are presented in percentage from baseline in Table 10.

It was found that a single dose of 3 tablets of inflaminat (mode A) increased the anti-cytokine effect of the preparation, but didn't increase the duration of its effect. Modes B and C didn't differ significantly in the expression and duration of the anti-cytokine effect. Both modes reduced serum pro-inflammatory potential at 22–38% from baseline, and this effect persisted for 24 h. Mode C (receiving of 1 tablet 3 times a day with an interval between doses of about 8 h) is more preferred for use in clinical practice as the

Table 11
Serum atherogenicity after single dose of inflaminat administration.

Time after single dose oral intake	2 h	4 h	8 h
Serum atherogenicity, % from baseline	38.2 ± 7.3*	28.6 ± 7.6*	42.0 ± 9.1*

* Significant result, $p < 0.05$.

most convenient, safe and the most optimized to provide sustained biological effect.

Anti-atherogenic effect of inflaminat

In the study of inflaminat anti-atherogenic effect (Table 11) the average reduction of serum pro-atherogenic potential by $63.9 \pm 5.1\%$ ($p < 0.001$) during 8 h after single dose of inflaminat intake was demonstrated. When comparing the anti-atherogenic effect of inflaminat with Allicor (Table 2) no significant difference was revealed.

So, it was shown that natural preparation inflaminat based on black elder berries, calendula flowers and violet tricolor herb caused the reduction of the serum-induced expression of inflammatory cytokines in primary culture of macrophages and anti-cytokine effect of inflaminat was comparable with the effect of NSAID diclofenac. It was also shown that inflaminat possessed anti-atherogenic activity along with anti-inflammatory action. Efficiency of inflaminat in suppressing the ability of blood serum to induce the cholesterol accumulation in cell culture was comparable with the effect of Allicor, natural preparation with strong anti-atherogenic activity. Thus, a natural preparation with a double mechanism of action was developed and studied in cell culture.

Table 12

Effects of inflaminat and diclofenac on the skin thickness at different times after cryodamage.

Group	Day after cryodamage					
	Before cryodamage	0	1	2	5	7
Intact rats	68 ± 3					
1 gr. (Diclofenac)		265 ± 9*	96 ± 12*. [†]	76 ± 17 [#]	72 ± 9 [#]	66 ± 2 [#]
2 gr. (Inflaminat)		265 ± 10*	84 ± 17*. [†]	77 ± 15 [#]	63 ± 6 [#]	65 ± 4 [#]
3 gr. (control)		265 ± 8*	144 ± 39*. [#]	94 ± 10*. [#]	74 ± 9 [#]	65 ± 8 [#]

* Significant difference from intact rats, $p < 0.05$;[#] Significant change as compared with the state after the operation, $p < 0.05$;[†] Significant difference from control group, $p < 0.05$.**Table 13**

Effects of inflaminat and diclofenac on the thickness of the reticular layer of skin at different times after cryodamage.

Group	Day after cryodamage					
	Before cryodamage	0	1	2	5	7
Intact rats	15 ± 4					
1 gr. (Diclofenac)		190 ± 10*	44 ± 5*. [†]	23 ± 9*. [#]	21 ± 5*. [#]	14 ± 3 [#]
2 gr. (Inflaminat)		190 ± 10*	35 ± 10*. [†]	25 ± 6*. [#]	15 ± 2*. [†]	16 ± 3 [#]
3 gr. (control)		190 ± 10*	89 ± 37*. [#]	38 ± 9*. [#]	27 ± 7*. [#]	19 ± 6 [#]

* Significant difference from intact rats, $p < 0.05$;[#] Significant change as compared with the state after the operation, $p < 0.05$;[†] Significant difference from control group, $p < 0.05$.

Anti-inflammatory effect of inflaminat in animal model of acute aseptic inflammation

The aim of this investigation was to study the anti-inflammatory activity of the natural preparation inflaminat in animal model of aseptic inflammation in the loose connective tissue of skin of rats induced by cryodamage. The research was conducted in accordance with internationally accepted principles for laboratory animal use and care (e.g. European community guidelines).

All experiments were performed on white gay linear Wistar rats (females) weighing 150–200 g aged 2–4 weeks, contained on a standard diet. Aseptic inflammation was caused by cryodamage of 6 cm² rats' skin using liquid nitrogen. All rats were divided into 4 groups. Rats of 1st experimental group received diclofenac 50 mg once daily for 8 days. Rats of 2nd experimental group received inflaminat 250 mg once daily for 8 days. Preparations were given at the rate of 1/6 of the single human dose. Rats of 3rd (control) group didn't receive any preparations. The last group of intact rats with intact skin was used for comparison. In total, 65 rats, 20 rats in the 1st, 2nd and 3rd groups, and 5 rats in the 4th group were used in the study. Material for the preparation of histological samples (the skin samples of 1 × 2 cm of the damaged section of the experimental rats or similar area of skin of intact rats) was taken on the day of experiment and at 1, 2, 5 and 7 days thereafter.

Histological specimens were prepared from obtained tissue samples using reagents Sigma Aldrich (USA). Semifine sections with thickness of 10 micron were prepared on a microtome (Carl Zeiss, Jena, Germany). Coloring of specimens for hematogenous cells was performed using monoclonal antibodies to CDLC T- and B-lymphocytes (Dako, USA). Coloring of specimens for mast cells was performed using a 0.05% solution of the toluidine blue dye in 0.5 N HCl, pH = 0.5. Coloring of sections for collagen was performed using picrofuchsin. The thickness of skin and reticular layer of skin was measured using the ocular micrometer on a light microscope "Jenaval" (Carl Zeiss, Germany). Computer processing of histological specimens was performed to determine the amount of collagen in the skin using the program «NIH Image» (National Institutes of Health, USA). The amount of collagen was evaluated in

arbitrary units on the degree of picrofuchsin coloring. The number of positively stained mast and hematogenous cells was determined in sections on the light microscope "Jenaval" (Carl Zeiss, Germany). Study statistics was performed using software package SPSS (SPSS Corporation, version 10.0, USA).

It was demonstrated that skin thickness increased an average of 3.9-fold ($p < 0.001$) immediately after cryodamage in all groups. The thickness of rat's skin decreased significantly in two experimental groups receiving inflaminat and diclofenac in the first day after the cryodamage. In the control group skin thickness decreased to the initial value only after 5 day cryodamage (Table 12).

The thickness of the skin increased mainly due to the reticular layer. Immediately after cryodamage the thickness of the reticular layer increases significantly with an average 12-fold ($p < 0.001$) in all groups. On the first day after cryodamage reticular layer thickness was significantly lower in the two experimental groups receiving inflaminat and diclofenac than in the control group (Table 13).

The content of collagen after the cryodamage didn't change significantly in control and experimental groups (data are not shown).

It was found that during the first day after cryodamage number of positively stained mast cells significantly decreased in all groups. Seven days after the cryodamage number of positively stained mast cells in two experimental groups reached the initial values, and was significantly different from the control group, $p < 0.05$ (Table 14).

On the first day after the cryodamage the number of hematogenous cells increased dramatically in all groups. After 2 days after cryodamage number of hematogenous cells reached the initial values in rats of two experimental groups receiving inflaminat and diclofenac, while in the control group it remained significantly elevated for up to 5 days (Table 15).

Thus, inflaminat administration on the first day after cryodamage resulted in a significant reduction of edema, and in the next few days—led to the complete elimination of edema. Diclofenac also led to a reduction of edema from the first day after cryodamage. No significant differences in anti-edema effect of diclofenac and inflaminat were detected ($p = 0.869$). Cryodamage

Table 14

Effects of inflaminat and diclofenac on the number of positively stained mast cells at different times after cryodamage.

Group	Day after cryodamage					
	Before cryodamage	0	1	2	5	7
Intact rats	11.0 ± 0.7					
1 gr. (Diclofenac)	10.3 ± 0.5	2.1 ± 0.8*†	4.6 ± 0.5*	7.3 ± 1.6*#	10.5 ± 1.6#	
2 gr. (Inflaminat)	10.3 ± 0.5	5.0 ± 0.2*	2.4 ± 0.8*†	4.7 ± 1.1*	11.3 ± 1.8#	
3 gr. (control)	10.3 ± 0.5	6.4 ± 0.4*	3.8 ± 0.3*	4.9 ± 0.1*	7.6 ± 0.5	

* Significant difference from intact rats, $p < 0.05$;# Significant increase of mast cells number as compared with control group, $p < 0.05$;† Significant reduction of mast cells number as compared with control group, $p < 0.05$.**Table 15**

Effects of inflaminat and diclofenac on the number of hematogenous cells at different times after cryodamage.

Group	Day after cryodamage					
	Before cryodamage	0	1	2	5	7
Intact rats	13.8 ± 1.2					
1 gr. (Diclofenac)	14.3 ± 1.4	63.1 ± 3.4*	10.5 ± 6.9#	10.9 ± 7.6	10.4 ± 1.1	
2 gr. (Inflaminat)	14.3 ± 1.4	66.4 ± 5.7*	13.3 ± 1.3#	13.1 ± 1.0	14.3 ± 2.5	
3 gr. (control)	14.3 ± 1.4	64.6 ± 2.6*	30.7 ± 4.6*	16.3 ± 3.0	12.5 ± 1.1	

* Significant difference from intact rats, $p < 0.05$;# Significant reduction of mast cells number as compared with control group, $p < 0.05$.

didn't cause intense degranulation of mast cells, the number of positively stained mast cells decreased dramatically during the first day after surgery. Therefore, this indicator characterized the infiltrative phase of inflammation. It was shown that the anti-inflammatory effect of inflaminat was slower than diclofenac, and reached a maximum at the second rather than the first day. However, complete recovery of the pool of positively stained mast cells occurred in the same time in both groups. During a day after cryodamage number of hematogenous cells increased. Thus, this indicator also characterized the infiltrative phase of inflammation. On the first day after cryodamage nor inflaminat neither diclofenac prevented the infiltration of affected skin by hematogenous cells. Both preparations equally stopped infiltration on the second day after the cryodamage, ($p = 0.793$). Thus, it was shown that inflaminat possessed anti-inflammatory effect in animal model of acute aseptic inflammation compared to that of NSAID diclofenac.

Pilot clinical trial of anti-inflammatory activity of inflaminat

A pilot clinical trial was conducted to evaluate the safety and anti-inflammatory efficacy of natural preparation inflaminat in patients with reactive arthritis. Statistical evaluation of the results was carried out using a statistical software package SPSS (SPSS Corporation, version 10.0, USA). After assessing the nature of the distribution Mann–Whitney test or t -test were used for intergroup comparisons, Wilcoxon test was used to assess changes in indicators.

In total, 11 participants (10 women and 1 man) aged 44.1 ± 5.8 (30–62 years) were included in the study. All participants administered 1 capsule of inflaminat 3 times a day during one month. Characteristic of pain syndrome caused by reactive arthritis at baseline and after treatment is presented in Table 16.

In the analysis of the severity of pain syndrome positive dynamics was demonstrated. Initially, 3 patients characterized their pain as severe and 8 patients as moderate, while after treatment severe pain preserved only in 1 patient, 6 patients have noted pain of moderate intensity, 3 patients—mild pain and 2 patients—the lack of pain.

At baseline most patients complained of pain in several joints (2–3 joints in one patient), while after the therapy a reduction in

Table 16

Characteristics of pain syndrome.

Type of pain	The number of patients who complained of this type of pain	
	At baseline	After treatment
Nagging	8	5
Bursting	5	3
Sharp	1	0
Periodic	3	4
Constant	8	5
Mild	0	2
Moderate	8	6
Severe	3	1
Absent	0	2

Table 17

Subjective assessment of preparation efficacy.

Assessment of preparation efficacy	Number of participants	Z-statistics
Full effect	2	−3.69
Satisfactory effect, comparable with NSAIDs	1	−2.38
Satisfactory effect but lower than that of NSAIDs	6	−0.49*
Absence of effect	2	−3.67

the total number of affected joints was demonstrated (28 versus 17 joints in 11 participants, at baseline and after treatment, respectively). Subjective efficacy of the preparation was assessed using Z-test results are presented in Table 17.

The effect of inflaminat can be considered as satisfactory on the results of the subjective evaluation of preparation effectiveness using 0-hypothesis, since it was rated satisfactory by 6 of 11 participants.

Leukocyte counts were within normal limits before and after treatment. No significant deviations were observed. Humoral immunity (IgA, IgM IgG, and circulating immune complexes) was evaluated in all subjects before and after treatment. The level of these immunoglobulins was within the normal range, no significant deviations were observed. Biochemical parameters (alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase,

Table 18
The average level of cytokines at baseline and after treatment.

Cytokines, U/l	IL-1	IL-6	TNF- α
At baseline	349 \pm 121	106 \pm 36	78 \pm 48
After treatment	326 \pm 114	109 \pm 58	42 \pm 28

Note: Data are presented as mean and standard deviation due to the small sample size

total protein, bilirubin, urea) were within normal limits at baseline and after treatment. Analysis of inflammatory cytokines (IL-1, IL-6 and TNF- α) was performed before and after treatment, the results are presented in [Table 18](#).

Elevated levels of inflammatory cytokines were found in the majority of patients at the initial examination. After follow-up the effect of treatment on the level of IL-1 and IL-6 wasn't observed, but a tendency to reduce the level of TNF- α in plasma was detected, however these changes weren't statistically significant, $p = 0.521$.

Thus, the positive clinical dynamics expressed in reducing the number of affected joints, reducing pain intensity and changing of its character was observed during inflaminat treatment in patients with reactive arthritis. Natural preparation inflaminat didn't cause any adverse changes in biochemical and immunological parameters of blood plasma.

Double blind placebo-controlled clinical study of the effect of natural preparation inflaminat on carotid IMT progression

The aim of this study was to evaluate the effect of long-term anti-cytokine therapy on progression of atherosclerotic lesions in carotid arteries. Natural preparation inflaminat based on calendula flowers, black elder berries and viola herb that has a pronounced anti-inflammatory effect, which was confirmed in laboratory studies ([Gorchakova et al. 2007](#)), and in a pilot clinical trial in patients with reactive arthritis was used as an anti-cytokine agent.

The study followed the guidelines of the Declaration of Helsinki and Tokyo for humans (ClinicalTrials.gov Identifier: NCT01743404). In total, 85 men aged 40–74 years with asymptomatic atherosclerosis, which was detected as an increase of maximum cIMT in common carotid arteries more 1000 μm were included in the study. Other inclusion criteria were absence of chronic diseases requiring continuous medication except mild hypertension requiring continuous use of angiotensin-converting enzyme inhibitors and also obligatory condition was signing of informed consent form.

Clinical and laboratory examinations of study participants were performed at baseline, and then 1 time in 6 months during 2 years of follow-up. Examination of study participants included biochem-

ical analysis of serum lipids, identification of the main risk factors for cardiovascular disease (body mass index, blood pressure, smoking, left ventricular hypertrophy detected on ECG, family history of arterial hypertension, diabetes and coronary heart disease) the calculation of prognostic 10-year risk of coronary heart disease, and ultrasound of carotid arteries.

Prognostic risk of coronary heart disease during the next 10 years was calculated using the Weibull model, developed on the basis of the Framingham study ([Odell et al. 1994](#)). Prognostic risk of myocardial infarction within next 10 years was calculated using Cox proportional hazards model, developed on the basis of Munster study ([Assmann et al. 2002](#)).

The protocol of ultrasound examination involved the scanning of the right and left common carotid artery and the area of the carotid sinus (bulb) as high up as possible in B-mode ([Lonn 1999](#)). Ultrasound examinations were made with scanner SonoScape SSI-6000 (SonoScape, China). Three fixed angles of interrogation were used (anterolateral, lateral, and posterolateral). Images were focused on the posterior wall of the artery. Ultrasonographic examinations were performed with the subject in the supine position after a rest of 15 mins. All measurements were always done consecutively in the same session for each subject. The B-mode ultrasound system used a 7.5 MHz linear array probe. cIMT measurements were carried out with M'Ath software (M'Ath Std., France). The measurements were always performed at 10-mm section of common carotid artery adjacent to the carotid bulb. cIMT of the posterior wall was measured as the distance from the leading edge of the first echogenic (bright) line to the leading edge of the second echogenic line. The mean of three measurements (in anterolateral, lateral, and posterolateral positions) was considered to be the integral cIMT estimate.

Results were expressed in terms of means and S.E.M. Significance of differences was evaluated using SPSS 14.0 statistical program package (SPSS Inc., USA). Significance was defined at the 0.05 level of confidence. The ultrasound data and the changes from baseline to the mean of follow-up visits were analyzed by a two-way ANCOVA and paired two-tailed t -test.

Baseline characteristics of study participants are presented in [Table 19](#).

The main characteristics of groups did not differ significantly at baseline. The dynamics of the main parameters between the first and last visits is presented in [Table 20](#).

It was shown after 2 years of follow-up that in inflaminat group there were multiple statistically significant favorable changes of clinical and laboratory parameters which are main cardiovascular risk factors. In placebo group there was only significant reduction in total cholesterol and LDL, probably related to the implementation of dietary recommendations. These changes caused significant

Table 19
Baseline clinical and laboratory characteristics of study participants.

Characteristic	Group		p^*
	Inflaminat	Placebo	
Number of participants	41	37	–
Age, years	62.8 \pm 6.9	63.9 \pm 6.9	0.910
Body mass index, kg/m ²	26.9 \pm 2.9	27.0 \pm 4.6	0.277
Systolic blood pressure, mmHg	150 \pm 21	151 \pm 19	0.509
Diastolic blood pressure, mmHg	92 \pm 12	89 \pm 8	0.549
Total cholesterol, mg/dl	262 \pm 48	275 \pm 65	0.434
HDL, mg/dl	63.5 \pm 2.3	59.5 \pm 2.6	0.239
LDL, mg/dl	153.6 \pm 6.4	148.9 \pm 6.8	0.579
Triglycerides, mg/dl	157 \pm 90	159 \pm 68	0.433
Atherogenic index	2.7 \pm 1.0	2.6 \pm 0.7	0.935
10-year risk of developing coronary heart disease, %	36.3 \pm 17.4	37.7 \pm 16.9	0.948
10-year risk of developing myocardial infarction, %	13.1 \pm 11.9	15.1 \pm 10.6	0.900
Mean cIMT, μm	946 \pm 121	910 \pm 139	0.846
Maximal cIMT, μm	1101 \pm 149	1048 \pm 162	0.810

Table 20
Dynamics of clinical and laboratory characteristics of study participants after 2 years of follow-up.

Characteristic	Group		p**
	Inflaminat	Placebo	
Number of participants	41	37	–
Body mass index, kg/m ²	0.2 ± 0.9	0.2 ± 1.0	0.776
Systolic blood pressure, mmHg	–19 ± 17*	–11 ± 20	0.284
Diastolic blood pressure, mmHg	–6 ± 7*	–5 ± 7	0.605
Total cholesterol, mg/dl	–49 ± 42*	–51 ± 49*	0.813
HDL, mg/dl	–4 ± 2*	–3 ± 4	0.824
LDL, mg/dl	–18 ± 7*	–19 ± 10*	0.929
Triglycerides, mg/dl	–8 ± 32	–35 ± 107	0.813
Atherogenic index	–0.7 ± 1.4	0.7 ± 1.7	0.133
10-year risk of developing coronary heart disease, %	–10.5 ± 12.9*	3.2 ± 22.1	0.193
10-year risk of developing myocardial infarction, %	–10.6 ± 13.1*	1.1 ± 16.4	0.230
Mean cIMT, μm	12 ± 8	44 ± 10*	0.045**
Maximal cIMT, μm	2 ± 9	52 ± 9*	0.036**

* Significant change after follow-up period, $p < 0.05$;

** Significant difference between groups.

decrease of 10-year prognostic risk of coronary heart disease and myocardial infarction by 10%.

The most important changes occurred in inflaminat group in ultrasonic parameters which are direct quantitative characteristics of atherosclerosis. The mean cIMT increased by 6 μm/year, the maximum cIMT by 1 μm/year. Thus, there were no statistically significant changes of cIMT in the group receiving inflaminat after 2 years of observation. In placebo group the dynamics of change in cIMT is significantly different from the dynamics in inflaminat group. During the observation period the mean cIMT increased by 22 μm/year, the maximum—26 μm/year, it was shown that cIMT after 2-year observation differs significantly from baseline in placebo group.

The results showed that the natural preparation inflaminat has direct anti-atherosclerotic effect, which comprises in slowing the progression of early atherosclerotic lesions, namely the cIMT of carotid arteries. Thus, there was a weak, not statistically significant increase of cIMT in the group of study participants receiving inflaminat during 2-year follow-up period. While the rate of cIMT progression in placebo group was 22 μm/year that cause significant changes of cIMT. These data are consistent with the results obtained in control groups in a number of different clinical trials of anti-atherosclerotic agents. Thus, in the study MARS (Monitored Atherosclerosis Regression Study) the average rate of cIMT progression was 20 μm/year (Blankenhorn et al. 1993), in the study KAPS (Kuopio Atherosclerosis Prevention Study)—29 μm/year (Salonen et al. 1995), in the study CLAS (Cholesterol Lowering Atherosclerosis Study)—23 μm/year (Azen et al. 1996).

Furthermore, it was shown that the natural preparation inflaminat has a favorable effect on main cardiovascular risk factors, which leads to decrease of prognostic risk of coronary heart disease and myocardial infarction as well as inflaminat has no side effects and phenomena, which allows using it for long-term treatment.

The designed algorithm allowed us to produce a natural preparation inflaminat that possesses anti-cytokine and anti-atherogenic activity in cell culture model, has anti-inflammatory effect in animal model of aseptic inflammation and in a pilot clinical trial, and reduce carotid IMT progression in double blind placebo-controlled clinical study, thus it can be considered as a natural preparation for primary prevention of atherosclerosis.

Conclusions

Different inflammatory mechanism of the development of atherosclerotic lesions is described in numerous studies. Special attention is given to studying the role of inflammatory cytokines in

the progression of atherosclerosis, as they play an important role at all stages of the atherosclerotic process. Anti-cytokine therapy may be a promising direction in moderation of atherogenesis, especially when it begins on the early stages of subclinical atherosclerosis. The use of herbal preparations with anti-cytokine mechanism of action is the most perspective for timely prevention of atherosclerosis, since they have no significant side effects and can be prescribed for long-term administration.

In this manuscript algorithm of the development and results of the clinical study of the effectiveness of herbal preparation inflaminat with anti-inflammatory mechanism of action is described. The developed unique standard of the anti-inflammatory effectiveness' evaluation using widely known drug diclofenac as a model for comparison has no analogues in the world. Anti-inflammatory activity of various components of medicinal plants was studied on *in vitro* and *ex vivo* models, that allowed to determine the best combination of natural ingredients to create a herbal preparation inflaminat. It was shown that this herbal preparation significantly inhibited inflammatory cytokines expression in cell culture models. Anti-inflammatory effect of inflaminat was confirmed on *in vivo* model of acute aseptic inflammation. The optimal mode of administration of inflaminat for achieving sustainable anti-inflammatory action was developed and a clinical study of the efficacy and safety of the preparation in patients with reactive arthritis was conducted. In depth statistical analysis of the study results it was found that the use of the preparation lead to positive clinical dynamics, resulting in lower amounts of the affected joints, reduction of pain intensity and change of its character. It was established that the preparation inflaminat caused clinical anti-inflammatory effect, comparable to that of the widely used in clinical practice NSAIDs—substances of synthetic origin. Finally, a clinical study of the anti-atherosclerotic effect of the herbal preparation inflaminat was conducted in patients with subclinical atherosclerosis, using cIMT as a key endpoint. It was shown that 2-year administration of inflaminat leads to significant reduction of cIMT progression.

Currently, a large number of studies of anti-atherosclerotic effect of various preparations with anti-inflammatory mechanism of action, including natural products are carried out. Exactly natural preparations with anti-cytokine mechanism of action are the most promising candidates for early prevention of subclinical atherosclerosis, as inflammatory cytokines are mediators of the inflammatory process at all stages of the formation of atherosclerotic lesions, from the very first stage. At the same time we have the opportunity to use natural preparations in people without clinical manifestations of atherosclerosis, with a high prognostic cardiovascular risk, because unlike synthetic drugs, they do not have

severe side effects and can be recommended practically for life-long treatment.

Conflict of interest

The authors confirm that there is no conflict of interest associated with this publication.

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