$$w_1 = \frac{2}{1+3} = \frac{1}{2},\ w_2 = (3-1)\frac{2}{3(3+1)} = \frac{1}{3},\ w_3 = \frac{2}{3(3+1)} = \frac{1}{6}.$$

The respondent spent 2 actions to the first scenario[1]. Second and third scenarios required 4 actions including one incorrect. Thus,

$$S = \frac{1}{2}\cdot 2 + \frac{1}{3}\cdot 4 + \frac{1}{6}\cdot 4 = 3,$$

i.e. the respondent spent on average 3 actions to this scenario[2]. Second numeric characteristic of the interface, degree of falsity is calculated as ratio of wrong actions number to all actions number:

$$P = \frac{7}{19} = 0{,}37\ .$$

Statistical analysis

Thus, playing experiment gives researcher two-dimensional massive of numeric data S_i, P_i, for which a whole spectrum of known statistical methods is applicable: estimation distribution parameters, comparing means of two samples with Rosenbaum Q-test or Mann-Whitney U-test [2].

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[1] We consider as one action a cycle of actions that does not require a choice.
[2] If we use an arithmetic mean, we would get larger value S=3,33.

Materials of Conference

SYNTHESIS OF NATURAL-SCIENTIFIC AND HUMANITARIAN APPROACHES IN CREATIVE PROBLEMS OF DESIGNING

Fot Zh.A.
Omsk State Institute of Service
Omsk, Russia

Nowadays the topicality of carrying out the investigations, which would allow creating basic foundations of the universal design object form geometric theory, is evident. Such a theory should be based on mathematical instruments of space division investigation. Up to now the major attention is paid to artistic characteristics of the designed object (color, texture), material properties, technological effectiveness of constructions and the process of manufacturing; the form and its divisions depending traditionally on the designer's taste. A well thought-out form supposes a clearly organized arrangement of the parts, and that is expressed in its harmonic proportional structure. The development of such a problem solving approach, which would allow finding out the principles and laws managing the processes of form making in the outer world, is necessary. The unity of all outdoor and its various manifestations should condition the drawing together of the natural-scientific and humanitarian approaches while solving creative problems. The synthesis of these methods determines the content of the developed heuristic model of the form's proportional structure design and analysis. The innovative technologies of the computer algorithmic form making, by the virtue of their cheapness and universality combined with the historically accumulated experience of the form elements proportioning, give a new impetus to the design art development and, as a necessary condition, the integration of design solutions into engineering developments. The purpose: is not only to simplify the process of sketch design having shuffled off the routine on the computing device, but also to preserve the proportioning experience accumulated for centuries, to find the ways for its introduction into a new engineering activity. It will allow avoiding the formalism of combinatorial connection of the elements with each other excluding creativity, on the one hand; and giving an opportunity to acquire a new quality of integrity and harmony, on the other hand. The implementation problem of the new design methods based on digital technologies is of current interest in the most diverse applied spheres: professional equipment, interior and costume objects' design, automobile production, architectural engineering, etc.

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ANTIOXIDANT COMPLEX SELMEVIT IN HEMOSTASIS CORRECTION AT SOME UTERINE SURGERIES (report III)

Byshevsky A.Sh., Galyan S.L., Galushko M.G., Zabara Ye.S., Zvereva I.V., Karpova I.A., Nedorizanyuk M.A., Polyakova V.A., Rudzevich A.Yu., Shapovalov P.Ya., Shapovalova Ye.M.
Tyumen State Medical Academy, Tyumen, Russia

Overseeing 486 productive age women subjected to uterine cavity abrasion for the diagnostic purpose or for the purpose of legal abortion, hysterectomy by laparoscopic or laparotomic methods, cesarean section it is proved that a surgical intervention intensifies the registered in them lipid peroxidation acceleration, general blood clotting and the acceleration of thrombin-fibrinogen interaction in blood flow. The inclusion of the complex antioxidant selmevit into a usual therapy course (in pre- and post-surgical periods) restricts these shifts and accelerates their disappearance, somehow restricts intraoperative blood losses, that mitigates the risk of thromboembolic episodes.

Thromboses and embolias – are very dangerous complications of operative interventions on the uterine, the tissues of which – are the sources of fibrillation factors (FF). The uterine surgeries result in the outlet of the tissue factor, the connections like plasmatic FF V, VII, X, XIII and proaggregants [1, 2] into the blood flow. The most serious and frequent intraoperative complications – floods, thromboembolias – are connected with hemocoagulation changes [3]. Like other operative interventions, the uterine surgeries are attended by the lipid peroxidation (LPO) acceleration. The use of antioxidants limits the hemocoagulation shifts [4] like in other surgical interferences [5, 6]. The information about positive effects of antioxidants in the thromboembolias prophylaxis, their relations at obstetric-gynecologic pathology with hemocoagulation shifts, the absence of contraindications to vitamins-antioxidants application, the possibility of outpatient use substantiate their corrective effect [7 – 10].

The **purpose** of the work is the estimation of hemocoagulation changes, morphofunctional properties of thrombocytes (TC), LPO and antioxidant potential (AOP) in women after uterine surgeries, the advisability of selmevit antioxidant complex use for the hemostatic shifts prophylaxis.

Objects and methods of research

The women were examined at gynecological and maternal obstetric services of the Tyumen Regional Center of Maternity and Child Welfare Service and maternity hospital N3. The clinical and laboratory examination was carried out the day before and in 1, 3-4, 5-7 days after the operation (complaints and anamnesis study, general visual inspection, special obstetric and gynecological examination, bacterioscopy of cervical channel fluid, sheath and urethra, USI of true pelvis organs). The surgery duration, arterial tension, pulse, breathing rate, somatic temperature, anaesthesia form, intraoperative blood loss were taken into account. In the postsurgical period the thrombo-hemorrhagic complications frequency, hospitalization time, bed days were estimated. The throbmocytic hemostasis was estimated defining the thrombocytes (TC) number [11], their morphologic forms, the number and size of aggregates per 100 free cells, the quantity of aggregate-forming TC per 500 free cells; small (2-3 TC) and big aggregates (4 and more TC) per 100 free cells [12]. The hemocoagulation was estimated defining the ATR, APTT [13], prothrombin ratio (PR) [14]; for the thromboplastin international sensitivity index (ISI) account the PR was raised to the ISI power pointed at the marking and the international normalized ratio (INR) was calculated; the fibrinogen (FG) concentration was defined gravimetrically; the solvable fibrin-monomeric complexes (SFMC) [14], fibrin split products (FSP), antithrombin III activity (AT-III) [15], plasminogen reserve index

(PRI) (the reagents of the "Technology-Standard" firm, Barnaul) and f P_3 [16]. The LPO was estimated on the level of diene conjugates (DC) and malondialdehyde (MDA) [17]. The AOP was judged on the level of vitamin E in erythrocytes [18]. The mathematical treatment was performed by the variance analysis method for small series of observations calculating the arithmetic mean (M), its mean error (m), the mean-square deviation (σ), confidence coefficient of Student (t) and the degree of possibility (p).

The most abundant uterine surgeries (Table 1) were subject to observation. For this purpose two uniform groups were formed: the *comparison group* (usual preoperative preparation and postoperative treatment) and the *main group* (women received selmevit in addition to the usual therapy). The age, social status, clinical-anamnestic characteristics, occurrence of somatic and gynecological diseases, surgical indications and their scope were the same in both groups.

Table 1. Distribution of examined women in groups

№ group	Characteristics	n
1	Control – childbearing age women (II phase of menstrual cycle)	20
2	Women subjected to uterine cavity abrasion: - for purpose of legal abortion carrying out - for diagnostic purpose	140 90 50
3	Women subjected to conservative myomectomy: - didn't receive selmevit - received selmevit	79 49 30
4	Women after major uterine surgeries: - hysterectomy by laparotomy method - didn't receive selmevit - received selmevit - hysterectomy by laparoscopy method - didn't receive selmevit - received selmevit	155 136 76 60 19 10 9
5	Women subjected to cesarean section: - didn't receive selmevit - received selmevit	92 47 45
	Total:	486

Research results

At hysterectomy the operation time in the main group reduced by 3,0% (p >0,05), the intraoperatinal blood loss reduced by 15,2% (p >0,05), there were no complications registered. In the comparison group women undergone the *laparotomic hysterectomy* the surgery duration was reduced by 8,9% (p >0,05), the blood loss – by 15,7% (p< 0,05). The interconnection of hypercoagulation shifts and thrombembolias [20] allows speaking on the positive role of selmevit. In the main group women subjected to the conservative myomectomy the tendency to the surgery duration decrease (by 9,2%), the intraoperational blood loss decrease by 7,9%, the absence of thrombohemorrhagic complications (in the comparison group – 4,1%). In the main group women delivered with the help of *caesarian section* the intraoperational blood losses were lower by 13,8%, the operation time – by 21,9%, there were no postoperational thrombohemorrhagic complica-

tions registered (in the comparison group – 2,1%, i.e. I case).

Because of the limited article volume we document only a small part of the examined states – the conservative myomectomy. From the data of the Table 2 one can see that before the operation the APTT and TT are prolonged, the PR and INR are increased, the AT-III and PRI are reduced in the *comparison group*. In the *main group* the ATR was prolonged, the PR and INR factors became lower than in the comparison group, the AT-III and activity and PRI increased. In the comparison group women subjected to the conservative myomectomy the ATR was prolonged, the FG, SFMS and FSP levels were increased, the AT-III and PRI were decreased in 24 hours after the operation. The FG level decrease remained for 3-4 days, the ATR, AT-III, SFMC, FSP, PRI – for 7 days, the APTT, TT, PR, INR didn't differ from the initial ones, but didn't reach the control values. In the main group the TT, SFMC and FSP level increased, the AT-III and PRI level fell on the first day after the operation. On the 3-4th day the PRI and AT-III differences retained (compared to the pre-surgical values).

In the pre-surgical period in the comparison group there were differences from the control: the TC, S, SE, SAN, BAN, AN and f P_3 were increased, the DE number was decreased. The differences from the control were also registered in the main group women: the S, SAN and AN quantity were increased. Together with that the number of thrombocytes, SE, BAN and AN were authentically less (relative to the comparison group).

In the comparison group women in 24 hours after the operation the number of DE, SE, AFN, SAN, AN, f P_3 was increased and the number of D was decreased. The D, SE, NAF, SAN, AN and P_3 factors' differences from the pre-surgical ones retained for 3-4 days. Up to 7 days after the operation the S, SE, NAF, SAN, BAN and AN factors were decreasing and the D number was increasing. However, on the 5-7th day after the operation the SE, SAN, BAN and AN factors remained higher than those of the control.

In the main group patients the thrombocytic hemostasis changes after the operation were less manifested: on the first day the number of D, BAN, f P_3 were decreased, beginning with the 3-4th day the factors didn't differ from the pre-surgical ones, on the 5-7th day only SAN and AN are higher than in the control.

Table 2. Hemocoagulation, thrombocytic hemostasis and LPO before and after the laparoscopic conservative myomectomy in the comparison (1 line) and main (2 line) groups.

Factors	Control group, n = 20	Before operation	1 day	3-4 day	5-7 day
		without selmevit, n = 20, with selmevit n = 15	without selmevit, n = 20 with selmevit, n = 12	without selmevit, n = 20, with selmevit n = 12	without selmevit, n = 20, with selmevit, n = 12
ATR, sec	59,0±2,6	60,4±2,5 66,5±2,3*	70,1±5,7' 69±1,6	75,4±4,6' 60,6±2,6	75±3,1' 62,4±3,6
APTT, sec	40,6±1,3	46,2±1,5* 45,1±4,1	46,6±1,3 40,3±3,1	45,8±1,5 39,9±1,2	44,7±1,5 41,5±1,3
TT, sec	18,5±0,6	24,3±1,6* 19,2±1,6	24,5±1,5 23,7±0,9'	24,0±1,5 22,4±1,2	22±1,3 19,2±0,4
PR	1,4±0,1	2,1±0,3* 1,6±0,2"	1,7±0,2 1,9±0,2	2,6±0,3 1,6±0,1	2,2±0,3 1,5±0,1
INR	1,6±0,2	2,4±0,4* 1,9±0,2"	1,9±0,2 2,3±0,3	3,0±0,5 1,9±0,2	2,5±0,4 1,6±0,1#
FG, g/l	2,5±0,2	2,8±0,2 3,0±0,2	3,6±0,3' 2,8±0,3	3,4±0,2' 3,3±0,4	3,1±0,2 3,1±0,2

SFMC, mg/100 ml	3,5±0,1	3,8±0,4 3,6±0,2	6,7±0,6' 4,7±0,4'	5,2±0,5' 4,1±0,4	4,9±0,3' 3,8±0,3
FSP, mg %	0,547±0,02	0,562±0,03 0,554±0,02	0,640±0,02' 0,622±0,02'	0,632±0,02' 0,568±0,02	0,624±0,02' 0,562±0,02
AT III, %	95,1±2,7	80,4±3,2* 95,5±3,7"	66,8±4,6' 80,6±1,9'	70,6±3,8' 83,3±3,8'	71,2±3,1' 88,0±3,7
PRI, %	109,4±3,7	86,2±3,0* 99,1±2,1*"	68,6±3,0' 67,4±3,5'	73,1±2,8' 77,8±2,6'	77±3,2' 93,7±4,1
TC ($x10^9$)	248,5±5,4	376,4±22,6* 317,6±16,0 '	392,8±23,3 290,7±11,3	368,5±24,1 288,5±15,1	325,8±17,1 301,4±16,2
D, %	46,5±0,9	43,5±1,4 45,1±0,8	36,9±1,0" 48±0,8"	35,7±1,3" 47,2±0,7	43,1±1,7# 40,7±0,9#
DE,%	24,9±0,6	22,6±0,9* 23,7±0,6	28,7±1,2" 23,3±0,5	26,5±0,9" 24,5±0,5	26,9±1,4" 25,3±0,7#
S, %	18,0±0,5	23,4±0,9* 22,1±0,8*	21,3±0,8 21,7±0,5	24,3±1,3 20,4±0,6	17,9±0,9" # 18,5±0,6 #
SE, %	9,4±0,3	10,9±0,4* 9,0±0,3 '	13,6±0,6" 9,2±0,5	13,4±0,5" 9,4±0,4	11,5±0,4# 9,3±0,2
NAF (per 500 cells)	53,5±0,9	56,5±1,1 56,4±1,8	63,8±1,3" 53,3±1,6	63,1±1,1" 52,3±1,9	56,9±1,7# 53,3±1,7
SAN (per 100 cells)	7,7±0,7	12,3±1,9* 10,8±0,7*	22±2,0" 11,3±0,9	20,9±1,8" 11,6±1,1	14,8±1,8# 10,4±0,7
BAN (per 100 cells)	1,0±0,1	4,3±0,4* 1,1±0,3 '	4,7±0,6 2,0±0,3"	4,4±0,6 1,8±0,4	2,5±0,3# 1,4±0,3
AN (per 100 cells)	8,7±0,8	16,6±1,3* 11,9±1,0* '	26,7±2,6" 13,3±1,3	25,3±2,5" 13,4±1,5	17,3±2,1# 11,8±1,1
P_3, %	31,5±2,8	41,2±3,5* 36,6±3,7	60,3±4,1" 51,8±3,6"	53,4±3,9" 41,8±3,3	48,3±3,4# 39,7±4,2#
DC, nmol/ml	110,3±3,4	122,7±3,6* 121,2±3,5*	132,9±3,1" 132,6±3,4"	131,7±3,8" 126,5±3,6	129,8±4,1" 118,4±3,8
MDA, mol/ml	10,4±0,5	11,7±0,3* 10,9±0,4	14,1±0,4" 12,3±0,5"	13,6±0,5" 11,7±0,3	12,8±0,4" 11,2±0,4
Vitamin E, mol/ml	4,6±0,3	3,8±0,2* 4,1±0,2	3,2±0,2" 3,4±0,2"	3,3±0,1" 3,8±0,3	3,5±0,2 4,2±0,4

Designations here and in the text: ATR – activated time of recalcification, APTT – activated partial thromboplastin time, TT – thrombin time, PR – prothrombin ratio, INR – international normalized ratio, FG – fibrinogen, SFMC – soluble fibrin-monomeric complexes, FSP – Fibrin split products, AT III – antithrombin III, PRI – plasminogen reserve index; TC – thrombocytes, D – discocytes, DE – discoechinocytes, S – spherocytes, SE – spheroechinocytes, NAF – the number of aggregate-forming TC, SAN – small aggregates number with 2-3 TC per 100 free cells, BAN - big aggregates number with 4 and more TC per 100 free cells, AN – aggregates number – the total number of SAN and BAN per 100 free cells, DC – diene conjugates, MDA – malondialdehyde; * - authentically significant differences ($p \leq 0,05$) in healthy women, # - with values before the operation.

The LPO speed in the pre-surgical period in the comparison group was higher than in the control one: the DC and MDA level was increased; the vitamin E level was decreased. In the main group women the DC increase was less high; the rest LPO factors didn't differ from the control ones.

On the first day after the operation the DC and MDA level increased and vitamin E level – decreased in the comparison group; that retained up to 3-4, and DC and MDA – up to 5-7 days.

In the main group on the first day of the operation the content of DC and MDA increased, vitamin E – decreased. On the 3-4th day and on the 5-7 day there were no authentic differences compared to the pre-surgical ones found.

So, the prescription of selmevit to the women subjected to conservative myomectomy by laparoscopic method restricts the LPO shifts, coagulative and thrombocytic hemistasis mitigating the risk of thrombo-hemorrhagic complications.

After other operations (uterine cavity abrasion for diagnostic purpose, for legal abortion performance, hysterectomy by laparoscopic or laparotomic method, cesarean section) the changes of coagulative and thrombocytic components of hemostasis, the LPO speed changes were the same in directivity differing in intensity. As a whole, the intensity of shifts is consistent with the latitude of the operative intervention: the more significant the tissue traumatism is – the more evidently the LPO is promoted, the AOP is decreased and the TC coagulative activity is increased. At all kinds of the studied surgical aggressions the shifts degree and their duration were lower in the patients of main groups, i.e. the groups having received selmevit. In all the observations the signs of hypofibrinogenemia of consumption expressed in various measures to the power proportional to the LPO shifts intensity and the degree of TC activation became apparent.

Thus, various traumatism degree uterine interventions promote the activation of coagulative hemostasis to the power depending on the extent of the operation. The hemostasis activation reaches the degree resulting in the development of secondary hypocoagulemia – the hypocoagulemia of consumption. The TFI products level reflecting the acceleration of continuous intravascular clotting (CIC) in the blood flow testifies to the FG consumption. Considering the fact that the CIC acceleration attends obstetric-gynecologic operative interventions, one can assume that in these situations the disseminated intravascular clotting is initiated [15].

At the same time, after surgical interventions, thrombocytes activate – their ability to aggregate formation and release reaction increases. On the basis of hemocoagulation and TC changes time sequence data at extreme conditions or factors [5] the TC activation has been reputed to be the initiator of hemocoagulative shifts in our observations.

The fact that together with the activation of hemostasis the LPO accelerates and the AOP dies out [4] testifies that the activation of the hemostasis coagulative component after the operative intervention into the uterine cavity is conditioned by the LPO acceleration, as it is shown in other pathological states determined by various extreme conditions or factors [8, 10]. The abovementioned has been acknowledged by the fact that the introduction of selmevit increasing the AOP and inhibiting LPO diminishes the hemostatic shifts caused by the operation upon the uterine and the hemostatic profiles are normalized quicker (it is common knowledge that vitamins composing selmevit do not influence hemostasis essentially in conditions of health [8], but normalize hemostasis if its disorders are connected with the LPO activation [8, 10]. It testifies that the LPO shifts initiate hemostatic shifts as it was shown in the experiment [5, 6]. The abovementioned is also confirmed by the fact that the antioxidant effect is manifested, although to a not large extent, by an intraoperative blood loss decrease – the factor depending on the hemostasis state.

So, our findings confirm the idea about the connection between hemostasis and free-radical oxidation at the LPO level, and it proves the applicability of selmevit in pre-surgical preparation and post-surgical treatment as means of non-specific correction of hemostatic shifts appearing at the states attended with an oxidative stress, at gynecological diseases requiring surgical aggression, in particular.

Conclusions

1. In women with uterine surgical indications the LPO speed, the TC aggregate-forming ability, general blood coagulability and TFI markers content are increased. In 24 hours after uterus lining biopsy or abortion by conservative myomectomy the shifts are redoubled without reaching the critical extent.

2. In women subjected to laparotomic or laparoscopic hysterectomy, cesarean section the hemostatic shifts reach the degree of the blood DIC initial stage (the shifts do not disappear by the 5-7th day after the operation).

3. The supplement of usual therapy with selmevit (before and after the uterine surgeries) reduces the hemocoagulation shifts degree in the pre-surgical period, and especially the shifts caused by an operative intervention.

4. At all the examined states the blood clotting activation is connected with the LPO acceleration and AOP decrease. The prescription of selmevit restricts both of them that allows associating the selmevit effect with its antioxidant properties.

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REGULATION OF CELL CYCLE TRANSITION AND INDUCTION OF APOPTOSIS IN HL-60 LEUKEMIA CELLS BY LIPOIC ACID: ROLE IN CANCER PREVENTION AND THERAPY

Elangovan Selvakumar, Tze-chen Hsieh
Department of Biochemistry and Molecular Biology,
New York Medical College, Valhalla, New York 10595, USA

Background: Lipoic acid (LA), a potent antioxidant, has been used as a dietary supplement to prevent and treat many diseases, including stroke, diabetes, neurodegenerative and hepatic disorders. Recently, potent anti-tumorigenic effects induced by LA were also reported and evident as assayed by suppression of cell proliferation and induction of apoptosis in malignant cells. However, the mechanism by which LA elicits its chemopreventive effects remains unclear.
Methods and Results: Herein, we investigated whether LA elicits its anti-tumor effects by inducing cell cycle arrest and cell death in human promyelocytic HL-60 cells. The results showed that LA inhibits both cell growth and viability in a time- and dose-dependent manner. Disruption of the G_1/S and G_2/M phases of cell cycle progression accompanied by the induction of apoptosis was also observed following LA treatment. Cell cycle arrest by LA was correlated with dose-dependent down regulation of Rb phosphorylation, likely via suppression of E2F-dependent cell cycle progression with an accompanying inhibition of cyclin E/cdk2 and cyclin B1/cdk1 levels. Evidence supporting the induction of apoptosis by LA was based on the appearance of sub-G_1 peak in flow cytometry analysis and the cleavage of poly(ADP-ribose) polymerase (PARP) from its native 112- kDa form to the 89-kDa truncated product in immunoblot assays. Apoptosis elicited by LA was preceded by diminution in the expression of anti-apoptotic protein bcl-2 and increased expression of apoptogenic protein bax, and also the release and translocation of apoptosis inducing factor AIF and cytochrome c from the mitochondria to the nucleus, without altering the subcellular distribution of the caspases.
Conclusion: This study provides evidence that LA induces multiple cell cycle checkpoint arrest and caspase-independent cell death in HL-60 cells, in support of its efficacious potential as a chemopreventive agent.

Background

α-Lipoic acid (LA), also known as thioctic acid, occurs naturally as a prosthetic group in various mitochondrial enzymatic complexes and plays a fundamental role in metabolism. It is involved in different multienzyme complexes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-keto acid dehydrogenase, and glycine decarboxylase complex [1]. The two sulfur molecules in LA undergo cycles of oxidation and reduction, enabling it to function as a potent antioxidant that is capable of directly terminating potentially damaging free radicals. Several features have been described for LA such as (a) specificity of free radical scavenging in both oxidized and reduced forms, (b) interaction with other antioxidants, (c) metal-chelating activity, (d) effects on gene expression, (e) bioavailability, (f) location (in aqueous or membrane domains, or both), and (g) ability to repair oxidative damage, which make it an outstanding antioxidant [2-4]. Added to cell culture medium *in vitro*, LA readily enters cells and is reduced by mitochondrial and cytosolic enzymes to dihydrolipoic acid, most of which is rapidly effluxed from the cell to the culture medium [5]. Experimental and clinical studies have indicated the potential usefulness of exogenous LA as a therapeutic agent for the prevention and treatment of various pathologies including diabetes [6], atherosclerosis [7], ischemia-reperfusion injury [8], degenerative processes in neurons [9], diseases of joints [10], radiation injury [11], heavy metal poisoning [12] and HIV activation [13]. LA is readily absorbed from the diet, and to date, only mild side effects have been detected following LA administration; supports the overall feasibility of using LA as a dietary supplement [3].

In recent years, LA has gained considerable attention in the cancer field as an anticancer agent [14,15]. Results from antiproliferation studies on cancerous cell-based models have suggested that the tumor-suppressive effect of LA corresponds with apoptosis induction, a critical parameter impaired in cancer cells, and this induction is

selectively exerted in cancer and transformed cell lines, while being less active toward normal nontransformed cells [16-18]. Thus, LA was shown to induce apoptosis in tumor Jurkat, FaDu, Ki-v-Ras-transformed mesenchymal cells and human lung epithelial cancer H460 cells [19,20]. In human leukemic T cells, LA also potentiated Fas-mediated apoptosis through redox regulation without affecting peripheral blood monocytes from healthy humans [21]. In experiments using antioxidant response element (ARE) reporter assays, LA has also been shown to induce phase II protective genes which are involved in the prevention of carcinogenesis, in non-cancerous animal- and cell-based studies [22-24]. These studies support the potential utility of LA as an anticancer agent and the importance of the elucidation of the detailed mechanism of its antitumor activity. Because of its widespread use and therapeutic potential of LA, however, the mechanism by which LA elicits its chemopreventive effects remains largely unknown.

We sought to determine the LA-induced apoptosis and cell cycle arrest and the underlying mechanisms of action. Our study shows for the first time that LA is capable to block multiple cell cycle checkpoints including G_1/S and G_2/M and induce caspase-independent cell death via AIF/ cytochrome c translocation from the mitochondria to the nucleus. Our findings provide mechanistic support to the potential utility of LA as an agent for the treatment of leukemia.

Materials and methods

Reagents

DL-α-Lipoic acid was purchased from LKT laboratories (St Paul, MN). Primary antibodies like anti-Rb, anti-E2F, anti-cyclin B1, anti-cyclin D, anti-cyclin E, anti-cdk1, anti- cdk2, anti-AIF, anti-cytochrome c, anti-bcl-2, anti-bax, anti-actin, anti-histone H1, and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibodies like anti-pRb (ser 780) and anti-pRb (ser 807/811) were purchased from Biosource International, Inc. (Camarillo, CA). Anti-PARP was purchased from Biomol International, L.P. (Ply- mouth Meeting, PA). Fetal calf serum, RPMI 1640, penicillin and streptomycin were purchased from Cellgro, Inc (Herndon, VA). All other chemicals and solvents used were of analytical grade.

Cell culture and growth inhibition assay

Human HL-60 cells were obtained from American Tissue Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with penicillin, streptomycin and 10% heat inactivated fetal calf serum as previously described [25-27]. For treatment, cells were seeded at a density of 1 Ч 10^5 cells/ml. LA dissolved in 1 N NaOH solution and neutralized with HCl, was added to the culture media to the final concentration specified in the text. At the specified times, control and treated cells were harvested. Cell count was performed using a hemocytometer and cell viability was determined by trypan blue exclusion [25-27]. Harvested cells were washed twice with PBS, and pellets were stored at -80°C for additional biochemical and molecular analyses.

Cell cycle analysis

Cell cycle phase distribution was assayed by flow cytometry. Following 24 and 48 h treatment of HL-60 cells with different concentrations of LA (0, 2.5, and 5 mM), cells were washed with PBS and stained with 1.0 μg/ml DAPI containing 100 mM NaCl, 2 mM $MgCl_2$ and 0.1% Triton X-100 (Sigma) at pH 6.8, as described [26,28,29]. The DNA-specific DAPI fluorescence was excited with UV light emitting laser (Ni-Cad), and collected with appropriate filters in an ICP-22 (Ortho Diagnostic, Westwood, MA) flow cytometer. MultiCycle software from Phoenix Flow Systems (San Diego, CA) was used to deconvolute the cellular DNA content histograms to obtain quantitation of the percentage of cells in the respective phases (G_1, S and G_2/M) of the cell cycle. Flow cytometry was also used to show cells undergoing apoptosis, evident by ance of the sub-G_1 peak [26,28,29].

Preparation of whole cell extracts and subcell fractionation

For immunoblotting experiments, cells were collected by centrifugation and were lysed in ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1 % SDS, 1 mM dithiothreitol and 10 µl/ml protease inhibitor cocktail). The extracts were centrifuged and the clear supernatants were stored in aliquots at -70°C for further analysis. Subcellular fractionation was performed using mitochondria isolation kit obtained from Sigma (Sigma Chemicals, St Louis, MO) and different compartmental proteins were used to study the translocation of AIF and cytochrome c. Protein content of cell lysates and subcellular fractions was determined by coomassie protein assay kit (Pierce, Rockford, IL) with BSA as standard.

Immunoblotting

The aliquots of lysates (20 µg of protein) were boiled with sample buffer for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blocked in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl and 0.05% Tween 20) containing 3% non-fat dried milk overnight at 4°C. The blots were incubated with various primary antibodies, followed by incubation for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase in TBST. Actin and histone expression was used as loading control. Fractionation of the mitochondrial and nuclear proteins was confirmed by probing the membrane for mitochondrial specific cytochrome c oxidase antibody or nuclear specific histone H1 using their specific antibodies. The intensity of the specific immunoreactive bands were detected by enhanced chemiluminescence (ECL), using the manufacturer's protocol (Kirkegared & Perry Laboratories) and quantified by densitometry and expressed as a ratio to actin or histone, as previously described [27].

Results

Inhibition of HL-60 cell growth by LA is both time and dose dependent

Initially, we investigated the effect of LA on cell growth inhibition. Exponentially growing HL-60 cells were treated with increasing doses and exposure times of LA, and subjected to trypan blue exclusion assay to measure the cell growth and viability. LA treatment resulted in dose- and time-dependent inhibition of cell growth, compared with controls, and the magnitude of cell growth suppression was seen as early as 24 h exposure to 5 mM LA (89%; Fig. 1A). By 48 h there was a ~8%, ~64% and 86% diminution of cell growth by 1, 2.5 and 5 mM LA, respectively, which was accompanied by ~1, ~3% and 36% temporal, dose-dependent decrease in cell viability (Fig. 1B).

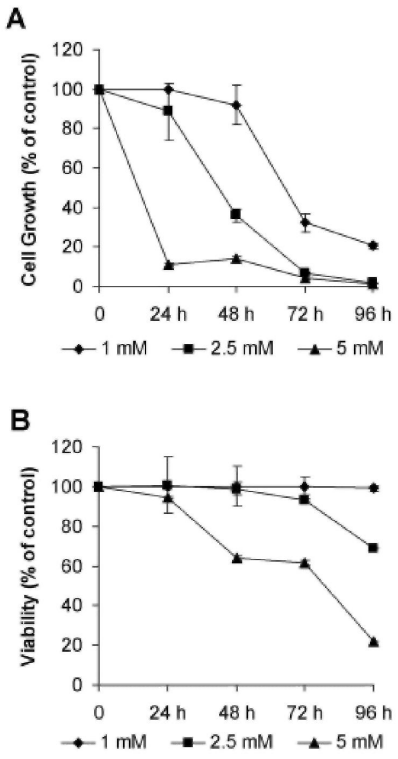

Figure 1. Control of cell growth viability of HL-60 cells by LA. (A) Cells were treated with 0, 1, 2.5 and 5 mM LA and the cell numbers were determined at 24, 48, 72 and 96 h. (B) Cell viability was measured trypan blue dye exclusion assay. Effects of LA were presented as a percentage of control, and values are expressed as mean ±SD for three experiments.

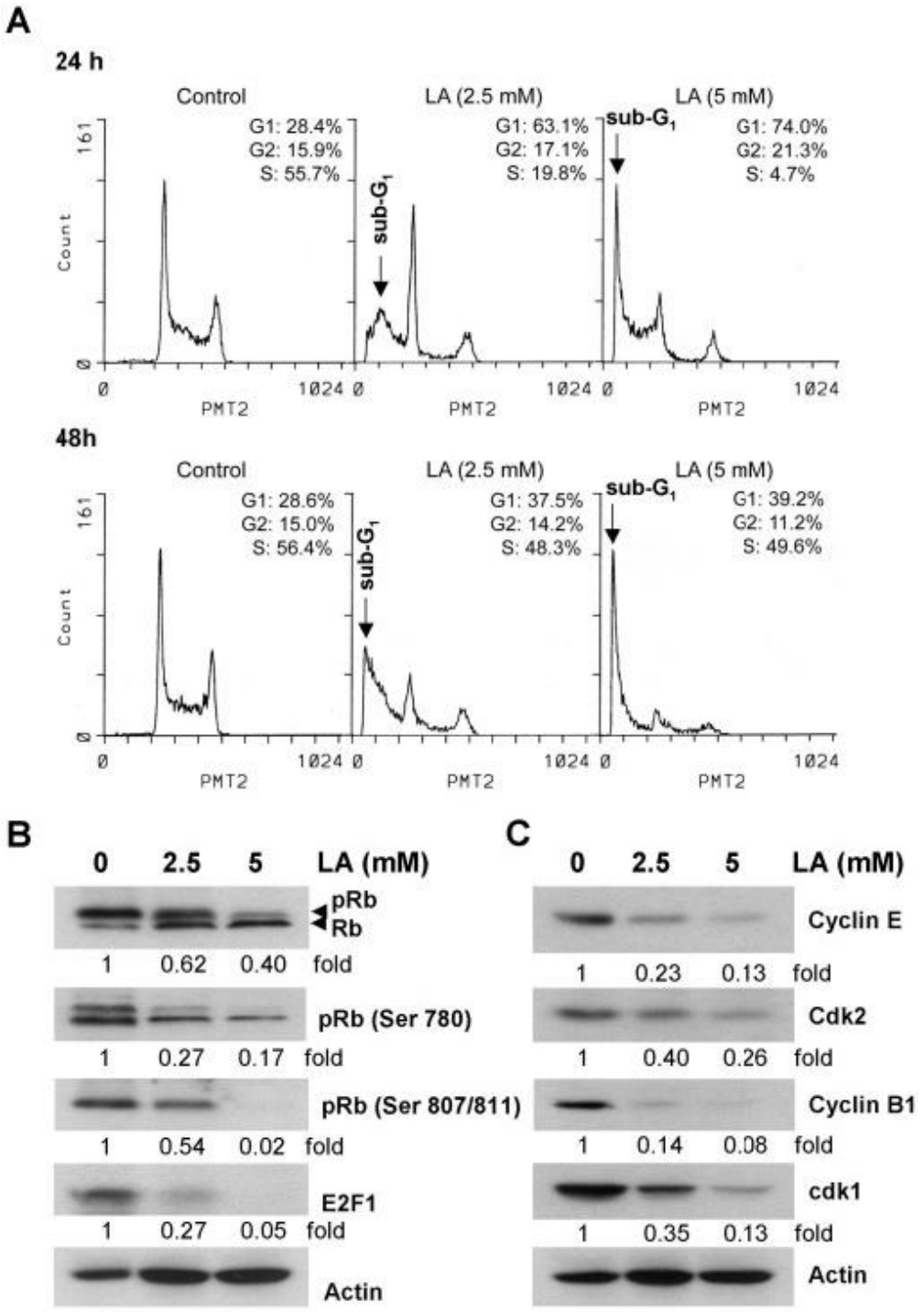

Figure 2. Effects of LA on cell cycle phase distribution and the expression of various cell cycle regulatory proteins in HL-60 cells. (A) Cells were treated with 0, 2.5 and 5 mM LA for 24 and 48 h and analyzed by flow cytometry. Cells with hypodiploid DNA content (sub-G1) represent apoptotic cell fractions. (B) Western blot analysis of total Rb, pRB (ser780), pRB (ser 807/811) and E2F expression in cell lysate treated with LA for 48 h. (C) The level of immunoreactive cyclins B1, E, cdk1 and cdk2 in LA-treated HL-60. The intensity of the specific immunoreactive bands were quantified by densitometry and expressed as a fold difference against actin.

LA induces HL-60 cell cycle arrest by altering the expressions of specific signaling proteins

To assess LA-induced cell growth suppression is mediated via alterations in cell cycle, we evaluated the cell cycle dis- tribution by flow cytometry. Since 48 h treatment with 1 mM LA showed minimum affects on cell growth and via- bility, only cells exposed to 2.5 and 5 mM LA for 24 and 48 h were analyzed. The percentage of cells in G_1, S, and G_2 phases were calculated and presented as histograms in Fig. 2A. LA caused a significant decrease in S-phase cell population (55.6% in control vs. 19.8% and 4.7% in cells treated with 2.5 and 5 mM LA, respectively), accompanied by a concomitant accumulation in the G_1 phase cell population (28.4% in control vs. 63.1% and 74% in 2.5 and 5 mM LA treated cells). To further explore the cell cycle arrest by LA in HL-60 cells, specific cell cycle regulatory proteins required for G_1, G_1/S and S phase transition were measured by Westerm blot analysis. First, we measured the expressions of cyclins D, E and cdk2, as they play a pivotal role in controlling the phosphorylation status of Rb, which in turn activate transcription factor E2F to induce cell entry into the S-phase. Results in Fig. 2B show that LA treatment caused a dose-dependent reduction in cyclin E/ cdk2 expression without affecting cyclin D1 (data not shown), and at the same time LA treatment also resulted in ~38 to 60% suppression of the phosphorylated Rb (pRb). Moreover, LA caused a significant reduction in the phosphrylation of Rb at two specific sites, Ser-780 and Ser-807/811, was also observed (Fig. 2B). In addition, a more pronounced decrease in the expression of E2F was also detected in the treated cells (Fig. 2B), suggesting that these changes collectively contributed to the decrease in S phase cell population by LA (Fig. 2A).

Since LA-treated cells also show alterations in G_2/M progression, we also assayed the expression of cyclins A, B and cdk1 expression and observed a dose-dependent down regulation of cyclin B1/cdk2 (Fig. 2C) without a corresponding alteration in the expression of cyclin A (data not shown).

LA induces apoptosis by increasing bax/bcl2 ratio and by causing poly(ADP-ribose) polymerase (PARP) cleavage

Cell cycle analysis revealed that LA apparently induced apoptosis as evident by the appearance of sub-G1 fraction (Fig. 2A); notably, the percentage of apoptotic cells increased from 1.4% in control cells to 59.6% and 72.9% in 24 and 48 h, 2.5 and 5 mM LA-treated cells, which might contribute to the growth inhibitory effects of LA (Fig. 3A). Corroborative evidence of induction of apoptosis was obtained by biochemical analysis showing that PARP cleavage was substantially increased in cells treated for 48 h with increasing doses of LA (Fig. 3B). As additional support, other apoptosis markers including AIF, cytochrome c and bax/bcl-2 ratio were also examined to further ascertain the response of cells to LA treatment, by western blot analysis. Treatment of HL-60 cells with 2.5 mM LA for 24 h resulted in a 1.5 fold increase in total cytochrome c, while the total AIF levels remained unchanged (Fig. 3C). As bcl-2 plays an integral role in the release of cytochrome c during cell death, we determined its expression and correspondingly, also bax, an apoptosis agonist, in control and LA-treated whole cell extracts. Western blot analysis clearly showed a dose-dependent suppression of bcl-2 expression, accompanied by concomitant increases in bax, in LA-treated cells, compared to control cells (Fig. 3D), which was most vividly illustrated as a marked increase in bax-to-bcl-2 expression ratio (Fig. 3D). These results further support the ability of LA to acti- vate the mitochondria-dependent apoptotic cascade.

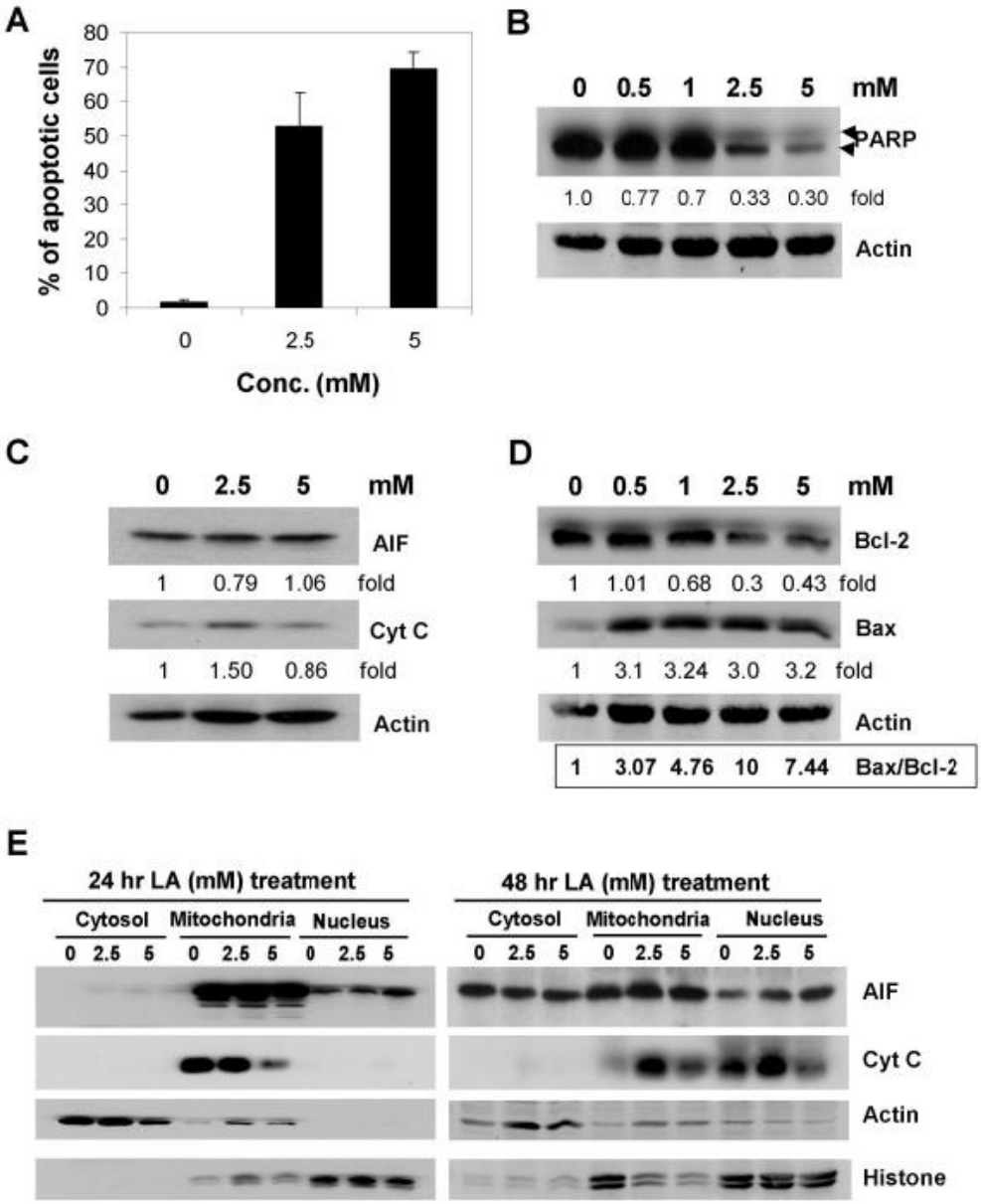

Figure 3. Induction of apoptosis by LA and analysis on poly(ADP-ribose) polymerase (PARP) cleavage, AIF/cytochrome c expression, and bax/bcl-2 ratio and subcellular distribution of AIF/cytochrome c by LA. (A) HL-60 cells were treated with 0, 2.5 and 5 mM LA for 24 to 48 h; LA induced cell death, evident by the flow cytometric measured sub-G1 fraction was calculated and shown as % of total cell population. (B) Western blot analysis revealed down regulation of PARP expression at accompanied by appearance of 89 kDa cleaved PARP fragment in > 2.5 mM, 48 h LA treated cells. (C) AIF and cytochrome c (Cyt C) expression in 48 h LA treated cells. (D) The actin-adjusted level of bax and bcl-2 and changes in the ratio of bax to bcl-2 in HL-60 cells treated for 48 h with increasing dose of LA. (E) Subcellular distribution of immunoreactive AIF and Cyt C in the cytosol, mitochondria and nucleus in control and 24 and 48 h LA-treated HL-60 cells. Actin and histone was used as loading control for cytosol and nucleus fractions, respectively. For mitochondria fraction verification was performed as detailed in Methods.

LA induces translocation of cytochrome c and AIF

Induction of apoptosis by LA conceivably may involve the translocation of cytochrome c and AIF. This possibility was tested by biochemically fractionating different subcellular compartments and quantifying the appearance of cytochrome c and AIF by western blot analysis, following treatment with LA. Typical results in cells treated with 2.5 and 5 mM LA for 24 and 48 h showed a spatiotemporal release of AIF from mitochondria into the nucleus (Fig. 3E). Similarly, cytochrome c was also apparently released from the mitochondria, and unexpectedly, was not accompanied by a concomitant cytoplasmic increase (Fig. 3E). These results suggest that LA-elicited cell death may not occur via a classical cytochrome c mitochondriacytosol translocation mechanism but rather, a caspase-independent mode of cell death via the nucleus directed shuttling of AIF and cytochrome c.

Discussion

LA has pleiotropic pharmacologic effects. The therapeutic potential of LA in cancer treatment has been shown in several studies [14,17,20], however, the mechanisms by which LA elicits its chemopreventive properties remain largely unknown. Using HL-60 cells, we have confirmed the cancer cell growth suppressive effects of LA. Further, we now provide evidence for two novel LA-elicited changes that possibly contribute to its chemopreventive potentials: (i) LA induces blockade at both well established cell cycle checkpoint, respectively, G_1/S and G_2/M, (ii) LA promotes the demise of treated HL-60 cells, possibly by a combination of mechanisms that includes the mitochondria-dependent apoptotic cascade encompassing a caspase-independent mode of cell death mediated via the translocation of AIF/cytochrome c. The proposed mechanism of LA is depicted in Figure 4.

Targeting dual checkpoints of the cell cycle by LA is particularly noteworthy as it effectively, as a single agent, accomplishes the same cellular endpoint as what has been eloquently proposed by Li et al [30] of inducing malignant cell demise through the deliberate bi-checkpoint blockade-mediated induction of apoptosis, as exemplified by the combined administration of β-lapachone and taxol to deliver a one-two punch for tumor cell killing and eradication. The mechanism by which LA acts in dual cell cycle checkpoint control may be complex and appears to involve at least the down regulation of cyclin E/cdk2 and cyclin B1/cdk1 in a manner that effects synergistic cell cycle arrest and induction of apoptosis [30,31]. It is notable that earlier studies have also demonstrated the post- translational elevation of p27Kip1 and p21Cip1 as specific LA elicited effects [14,19]. Taken together, these results not only reinforce the essential role of LA in cell cycle control but are likely to be directly involved in contributing to its therapeutic potential in cancer treatment.

Results of flow cytometry analysis assessing the presence of cells with fractional DNA content (evident as the sub- G_1 peak), in combination with the appearance of specifically processed 89-kD PARP product as demonstrated by immunoblot analysis (Figures 2 and 3), showed clearly the restoration/activation of programmed cell death in HL-60 cells treated with LA. Since the flow cytometric data appeared to show a more pronounced effect of prolonged treatment by LA, especially at the higher concentrations it is possible that more than one mode of cell death is triggered by LA. Equally likely is the possibility that these two assays alone are not sufficiently definitive to establish the mode of cell death in the treated cells. Experiments exploring TUNEL and agarose gel electrophoresis for detecting appearance of DNA ladders, and the use of caspase inhibitors are contemplated to address these and other possibilities. Despite the limitations mentioned above, it is important to point out a significant finding in this study, i.e., the demonstration of translocation of two proteins, respectively, AIF and cytochrome c

from mitochondria to the nucleus after LA treatment. A dose- dependent increase of AIF appearing in the nuclear fraction was observed as early as 24 h, whereas cytochrome c release and nuclear accumulation occurred at 48 h. Recent studies have demonstrated that AIF plays a critical role in caspase-independent induction of apoptosis [32,33]. Our studies also showed that LA down regulated bcl-2 expres- sion, which in turn may aid the release of AIF by altering mitochondrial permeability and contributing to its relocalization to the nucleus, and thereby promoting the induction of caspase-independent apoptosis. A companion and equally important change in this regard may be the cellular fate of cytochrome c, which, in our studies of the effects of LA, became nuclear bound. It is notable that previous studies have demonstrated a novel role of cytochrome c in the activation caspase-independent apoptosis, as involving the nucleus accumulation of cytochrome c instead of a more generally accepted classical mechanism in which the cytoplasmic translocation of cytochrome c from mitochondria provides a key trigger for caspase-dependent apoptosis [34]. Indeed, there is increasing awareness and acceptance regarding the co-existence of caspase-dependent and caspase-independent apoptotic and other modes of cell death for a given cell type [35]. Such as notion is consistent with and supported by our observation of the re-localization of mitochondrial proteins, AIF and cytochrome c into nucleus by LA treatment, suggesting that LA signals cell death in responsive cells by a caspase-independent, nuclear activated other apoptotic and perhaps other cell death mechanism.

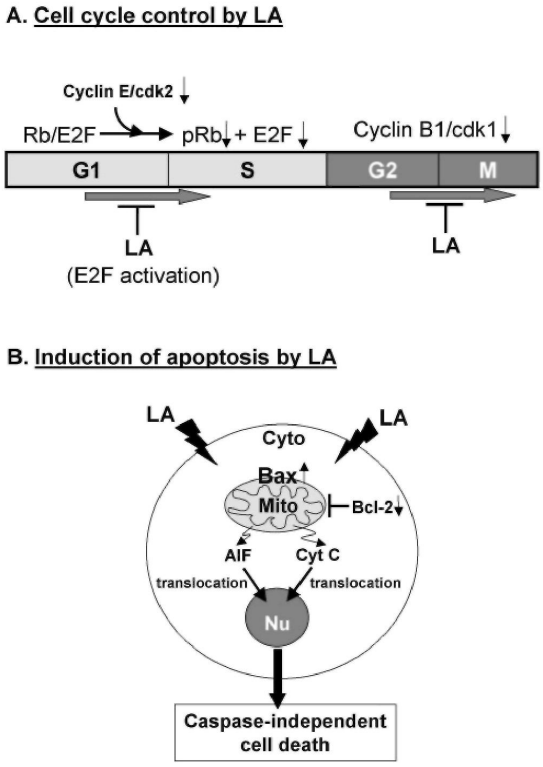

Figure 4. Proposed mechanism of action of LA. In this model, the ability of LA to suppress cell proliferation and induce apoptosis in HL- 60 cells is hypothesized to involve (A) disruption of cell cycle control, (B) perturbation in apoptogenic/anti-apoptotic (bax/bcl- 2) regulatory protein expression and translocation of mitochondrial AIF and cytochrome c (Cyt C) from mitochondria to nucleus and promoting the caspase-independent induction of apoptosis.

Importantly, the concentrations of LA used in this study are similar to those used in other *in vitro* studies reporting the cell cycle arrest and apoptosis inducing properties of LA [19]. Notably also, mM LA concentrations have been reported in the plasma after oral dosing in pharmacokinetic studies and are considered non-toxic. Moreover, the half-life of LA in plasma is short (30 min), suggesting that it is rapidly taken up into tissues or further metabolized [36]. Therefore, it is plausible that high concentrations, in the mM range, may accumulate in target tissues.

Conclusion

The results of this study demonstrate conclusively that LA treatment causes cell cycle arrest and alterations in the expression/translocation of mitochondrial apoptogenic/ anti-apoptotic proteins including AIF and cytochrome c, and the net result being a reduction in cell proliferation concomitant with cell cycle arrest and induction of apoptosis. These findings may be part of the mechanisms that underlie or contribute to the beneficial effects of this readily available dietary supplement in cancer prevention.

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EFFECTIVENESS OF ACUPUNCTURE, SPECIAL DRESSINGS AND SIMPLE, LOW-ADHERENCE DRESSINGS FOR HEALING VENOUS LEG ULCERS IN PRIMARY HEALTHCARE: STUDY PROTOCOL FOR A CLUSTER-RANDOMIZED OPEN-LABELED TRIAL

Jorge Vas*[1], Manuela Modesto[1], Camila Mendez[2], Emilio Perea-Milla[3], Inmaculada Aguilar[1], Jesus Manuel Carrasco-Lozano[4], Vicente Faus[4], Francisco Martos[5]

[1]Pain Treatment Unit, Primary Healthcare Centre, Dos Hermanas, Spain, [2]Andalusian Public Health System, Sevilla, Spain, [3]Support Research Unit (Network and Cooperative Research Centres of Epidemiology. CIBERESP), Costa del Sol Hospital, Marbella, Spain, [4]Costa del Sol Hospital, Marbella, Spain and [5]Department of Pharmacology, Malaga University, Spain

Background: Venous leg ulcers constitute a chronic recurring complaint that affects 1.0-1.3% of the adult population at some time in life, and which corresponds to approximately 75% of all chronic ulcers of the leg. Multilayer compression bandaging is, at present, the only treatment that has been proved to be effective in treating this type of ulcer. There is no consensus, however, about the dressings that may be applied, beneath the compression, to promote the healing of this type of ulcer, as there does not seem to be any added benefit from using special dressings rather than simple, low-adherence ones. As well as analgesia, acupuncture provokes peripheral vasodilation, in skin and muscles - which has been demonstrated both experimentally and in clinical practice - probably due to the axon reflex, among other mechanisms. The aim of the present study is to measure the effectiveness and cost of compression treatment for venous leg ulcers combined with special dressings, in comparison with low- adherence ones and acupuncture.

Methods/design: Cluster-randomized open-labeled trial, at 15 primary healthcare clinics in the Sevilla-Sur Healthcare District, with a control group treated with compression bandaging and low-adherence dressings; the experiment will consist, on the one hand, of the compression treatment applied in combination with special dressings (Treatment 1), and on the other, the compression treatment applied in association with low-adherence dressings, together with acupuncture (Treatment 2).

Discussion: The results will be measured and recorded in terms of the median time elapsed until complete healing of the ulcer, and the rate of complete healing at 3 months after beginning the treatment. An economic analysis will also be made.

This study, carried out in the context of real clinical practice, will provide information for decision-taking concerning the effectiveness of special dressings. Moreover, for the first time a high-quality study will evaluate the effectiveness of acupuncture in the process of healing venous leg ulcers.

Trial registration: Current Controlled Trials ISRCTN26438275.

Background

Venous leg ulcers (VLU) constitute a chronic recurring complaint that affects 1.0–1.3% of the adult population at some time in life, and which corresponds to approximately 75% of all chronic ulcers of the leg [1]. They are caused by sustained venous hypertension, which is the result, in almost 50% of cases, from superficial venous insufficiency or from malfunctioning valves in perforating veins, with a normally-functioning deep venous system. Other factors that may result in sustained venous hyper- tension are those that impede the pumping function of the leg muscles, such as conditions that reduce spontaneous movements (Parkinson's disease, stroke, spinal cord injuries or excessive sedation). This type of chronic venous insufficiency is characterized by oedema, venous dilation, painful legs and stasis dermatitis. VLU patients have a worse quality of life than non-affected persons of the same age due to the pain, malodour and loss of independence that it causes [2]. The precise procedure by which hypertension leads to ulceration has yet to be clarified, but various mechanisms are known to be capable of intervening in its development and maintenance, such as plasma extravasation, pericapillary deposits of fibrin [3], disorders of the fibrinolytic system [4], the fixing or retention of growth factors by macromolecules

in the dermis, or leukocytes in the veins of the legs [5-7].

In the USA, VLUs have been estimated to cost a billion dollars a year, representing an average cost of over 40,000 dollars during the lifetime of each patient [1], without taking into account the financial effects of impaired quality of life, sick leave and frequent hospitalization. In the United Kingdom, the average cost of treating venous ulcers varied between 814 to 1,994 euros per year, of which a large proportion is devoted to nursing expenses [8,9]. In Europe, the total cost of VLU treatment amounts to 1% of the annual healthcare budget [10], with an estimated mean cost of 250 euros in Spain per patient, per year of treatment [11].

Developing a healthcare plan for VLU patients includes curing active ulcers and preventing relapses. To achieve these goals, such a plan must address the treatment of infections associated with these ulcers, preventing the development of infection, stimulating granulation and epithelial tissues, compression, raising the leg position, improving mobility, reducing obesity, improving nutrition and, in some cases, performing surgery [12,13].

Perhaps the most controversial aspect of the local treatment of VLUs concerns the type of dressings to use [14]. The use of wet dressings has been shown to favour granulation and epithelialization processes and, therefore, the healing of the ulcer, but no evidence has been provided to assist in choosing between special dressings and simple, low-adherence ones [15]. The application of external compression causes various complex physiological and biochemical effects that affect the venous, arterial and lymphatic systems. Provided the degree of compression does not adversely affect arterial blood flow, and that the correct techniques and materials are applied, the effects of compression may be very positive, reducing oedema and pain, whilst at the same time favouring the healing of ulcers caused by venous insufficiency. However, there are many ways in which this compression may be applied, and there is no consensus as to which is best [16]. Depending on the size of the ulcer, healing rates with compression treatment range from 40–70% at three months to 50–80% at 6 months [17,18]. The latest systematic review carried out by the Cochrane Collaboration recommends compression therapy and low-cost, low- adherence dressings as a first-line treatment for healing VLUs [15].

In addition to analgesia, acupuncture provokes peripheral vasodilation in the skin and in muscles, probably due to the axon reflex [19-21]. Stimulation of Aδ and C fibres releases vasoactive neuropeptides, as well as proinflammatory agents like the calcitonin gene related peptide (CGRP), the P substance (SP), neurokinin A (NKA), opioids, galanin, somatostatin and vasoactive intestinal pep- tide (VIP) [22,23]. Deep, prolonged vasodilation may also be mediated by CGRP [24-26]. Some studies have suggested that the neuropeptides released by sensory nerve stimulation are beneficial in maintaining skin integrity [23,27,28], for healing ulcers [29,30] and for peripheral vascular disorders [31,32]. Another key element in regulating the vascular tone and the blood flow is the endothelial synthase of nitric oxide (NO), which is responsible for catalyzing the NO [33], which is active in arteriolar vasodilation and reduces peripheral resistance, facilitating normal blood flow to the tissues [34]. It has been shown that the expression of NO-synthase (NOS) is greater in skin regions where acupuncture points and channels are located. In these areas, moreover, there are raised levels of NO in the blood after acupuncture [35,36], which suggests that the acupunctural stimulation of the sensorial nerves may act as an *in vivo* modulator of NO levels [32]. Although studies have been carried out to examine the effectiveness of acupuncture in treating VLUs, these have been of poor quality and have reported contradictory results [37-39].

In this paper, we propose a multicentre cluster-randomized study, with a predetermined sample size, and appropriate follow

up, assessing the patients' quality of life and employing an objective results measure that will enable us to accurately evaluate the length of time needed for patients' ulcers to heal, thus meeting the recommendations of the Cochrane Collaboration's review body [15,16].

Methods/Design ***Research aims and questions***

The aim of this study is to determine whether the combination of compression bandaging with simple, low- adherence dressings and acupuncture is more effective than when the same bandaging is employed in combination with special dressings or with simple, low-adherence dressings, but no sensory stimulation (acupuncture), with respect to the complete healing of venous leg ulcers.

The following research questions are proposed:

▪ In terms of the healings of venous leg ulcers, is it better to employ compression bandaging associated with sensory stimulation (acupuncture) and simple dressings, than the same bandaging without sensory stimulation, with special dressings, or than the compression bandaging without sensory stimulation and with simple dressings?

▪ What are the differences between healing treatments with special dressings and those with simple, low-adherence ones, in terms of the complete healing of venous leg ulcers?

▪ How long does it take for venous leg ulcers to recur?

▪ How effective is the treatment in terms of reducing pain intensity?

▪ How effective is the treatment in terms of reducing the amount of analgesic and anti-inflammatory medicines consumed?

▪ Are there any quality of life changes related to the health of patients with venous leg ulcers?

▪ What is the total financial cost of each of the treatments studied?

▪ What is the cost-effectiveness ratio of the treatments studied, for patients with venous leg ulcers?

▪ What is the opportunity cost of treatments not implemented?

Design

Open-labeled, controlled multicentre prospective study, with random cluster allocation to treatment groups (Primary Healthcare Clinics) in the Sevilla-Sur Health District (Andalusian Public Health System).

Study duration

April 2008-December 2010

Subjects

Target population

patients with venous leg ulcers who request treatment at the Primary Healthcare Clinic.

Study population

patients with venous leg ulcers who request treatment by a healthcare professional (doctor or nurse) at one of the Primary Healthcare Clinics in the Sevilla-Sur Health District taking part in the study, with at least one active venous leg ulcer (open, CEAP C6 [40] with a diameter exceeding 1 cm.

Patients with one or more of the following will be **excluded** from the study:

▪ Arterial pathology (systolic pressure in the ankle of less than 80 mm Hg or ankle/arm systolic index less than 0.8).

▪ Diabetic ulcer of the foot, rheumatoid arthritis or systemic vasculitis.

▪ Use of anticoagulants

▪ Pregnancy

Sampling units

the Primary Healthcare Clinics participating in the study.

Analysis units

per Healthcare Clinic (according to the group assigned) and per patient.

Randomization and blinding procedure

a list of random numbers will be created, using statistical software, to which the researchers involved in the study will not have access. This list will be applied to the list of participating Clinics until the clusters for each group are obtained. Three types of cluster will be used, according to the treatment for the venous leg ulcer provided at the Clinic (**A:** compression therapy plus simple,

low-adherence dressings and acupuncture; **B:** compression therapy plus special dressings; **C:** compression therapy plus simple, low-adherence dressings). Before beginning, the list of Clinics will be randomly ordered, by drawing lots blindly, with no repetitions, from a bag containing the names of all the Clinics taking part. None of those participating in this process will belong to the research group, and none of them will play any further part in the study.

Ethical criteria

The ethical validity of this study has been analyzed and approved by the Andalusian Government Committee for Clinical Trials, following the approval of the corresponding Research Commission at each of the participating Clinics. The study design takes into account the fundamental principles set out in the Helsinki Declaration, and those of the Council of Europe Convention concerning human rights and biomedicine, as well as the requirements under Spanish law in the field of biomedical research, the protection of personal data, and bioethics. All the patients involved must sign their informed consent to the proposed clinical research procedures. During the course of the study, audits will be performed as required by the relevant Research and Ethics Committee, as well as those of each Clinic's Quality Committee, independently of any external audits (such as that of the research financing body) that may be necessary.

Sample size

The sample size has been calculated following the parameters of Campbell et al. [41], on the basis of historical data [18]. The success rate for complete healing of venous leg ulcers at 3 months, with compression bandaging plus special dressings, is 44%. With an alpha (significance) level of 0.05 and a power of detection of differences of 80%, assuming a two-tailed approach, the total sample size needed will be 375 patients, in order to detect differences of 15% between the groups, as regards the rate of complete healing of venous leg ulcers at 3 months. This calculation assumes a possible dropout rate of 5% and an intra- cluster coefficient of correlation of 0.05 (according to the expected variance of the response within a single cluster). There will be 15 clusters (Primary Healthcare Clinics) and so each Clinic will need to recruit 25 subjects. Therefore, 3 treatment arms will be formed, with 125 patients in each (with 5 Clinics per arm).

Interventions

The compression treatment and application of dressings will be performed in accordance with the standard procedure and the criteria of the nursing staff responsible. A priori, one treatment session per week is planned, but this may be modified in the light of the patient's response. Follow up of the patients recruited into this study will be continued for one year after the randomization process. The acupuncture sessions in Group A will be given at the same time as the cleansing of the ulcer and the changing of the compression bandage, and will continue for 3 months, or until the ulcer has healed completely (whichever occurs first). If, after these 3 months, the ulcer has not healed, the sessions will be continued (as before, together with the cleansing of the ulcer) for another 3 months. The acupuncture treatment will not be continued for longer than 6 months (figure 1).

The patients taking part in the study will be given recommendations about suitable hygiene-posture routines (resting with the legs raised, not standing for long periods, taking regular walks, keeping the skin well hydrated) and nutritional advice, if necessary. Both the compression treatment and the application of dressings will continue until the ulcer has completely healed. If a recurrence occurs before the one-year follow-up period has ended, the patient will be entered into a sub-cohort and given a new treatment plan.

Compression therapy (Groups A, B and C in the study) Compression therapy will be given to all the participants in the study. Once the patient has been examined and the ankle/arm index calculated by means of a Doppler scan, an evaluation will be

made of the state of the skin and of the shape of the leg, after confirming the absence of neuropathology, arterial pathology and cardiac insufficiency. The bandaging will be of a Class 3, high-compression multilayer type [42], following cleansing and application of the dressing, as follows: 1) With a 50% overlap, apply a cotton bandage in a spiral pattern from the base of the toes to just below the knee, without imposing any tension; 2) Apply an elastic bandage in a spiral pattern, with 50% overlap and stretched to 50% of its extension, from the base of the toes to just below the knee; 3) Apply a cohesive elastic bandage, with 50% overlap and stretched to 50% of its extension, from the base of the toes to just below the knee, and apply light pressure to ensure the adherence of the bandage. The oedema of the leg should be reduced by raising the limb for 15 minutes before applying compression.

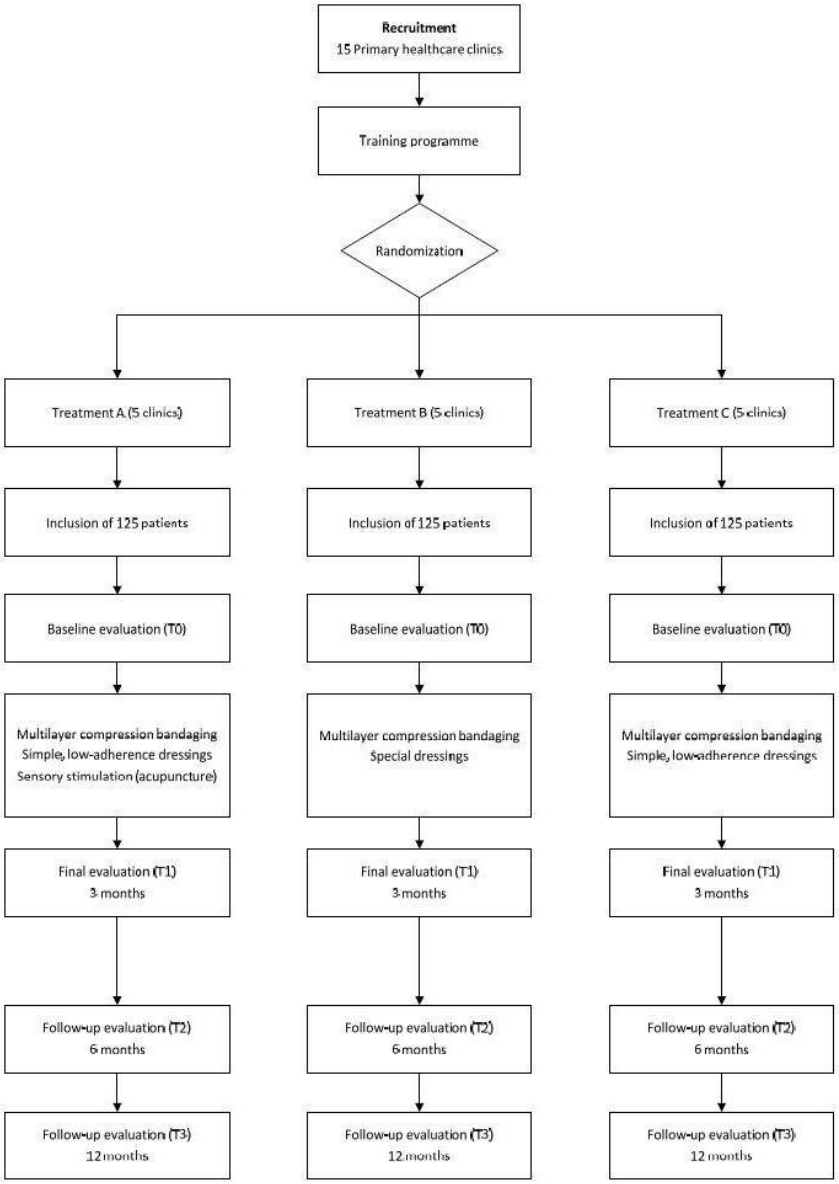

Figure 1. Flow diagram for the study. Flow diagram for the diverse phases of the study

Treatment of the ulcer for the patients given low-adherence, simple dressings (Study groups A and C)

▪ Cleaning: with tap water and mild soap [43-45], followed by careful drying

▪ Debridement: if necrotic tissue or sloughing are observed, perform enzymatic debridement, which may be combined with excision debridement, if necessary

▪ Managing infection: if infection is suspected, take a sample and begin treatment with silver sulfadiazine for a maximum of 2 weeks. If a culture study confirms the infection, treat using oral antibiotics

▪ Dressings: simple, low-adherence type (paraffin-impregnated tulle gras)

Any adverse reaction or side effect that may occur should be recorded in the Data Record (DR), with a detailed description and the date of occurrence.

Treatment of the ulcer for the patients given special dressings (Study group B)

▪ Cleaning: with tap water and mild soap, followed by careful drying

▪ Debridement: if necrotic tissue or sloughing are observed, perform enzymatic debridement, which may be combined with hydrogel to favour autolysis. If necessary, excision debridement should be applied

▪ Managing infection: if infection is suspected, take a sample and begin treatment with silver dressings (hydrofibre or hydropolymer) for a maximum of 2 weeks. If a culture study confirms the infection, treat using oral antibiotics

▪ Dressings: Cover with hydropolymers. Use alginate or hydrofibre dressings if exudate must be controlled

Any adverse reaction or side effect that may occur should be recorded in the DR, with a detailed description and the date of occurrence.

Acupuncture (Study Group A)

The nursing staff will be trained in the specific techniques of localization, puncture and manipulation in order to apply acupuncture at the perilesional zone of the ulcer(s), in healthy skin, using 4–8 needles, and at 2 points located on either side of the tibial crest of the affected limb (*Yinlingquan* SP9 and *Zusanli* ST36). The puncture will be carried out after cleansing of the ulcer. The needle to be inserted is a sterile, single-use filiform acupuncture needle, 30 mm long and 0.30 mm in diameter, fitted with a guide tube (S-J3030, manufactured by Seirin Corporation). Application will be made at each of the above-mentioned points, after sterilizing the skin, and with the patient lying face up or sideways. The puncture will be made vertically, unless otherwise specified, to a depth of 10–28 mm. The insertion will be followed by broad bidirectional rotational movements of the needle handle in order to produce the sensation of *Deqi*, commonly described as an irradiated feeling. The needle will be held in place for 20 minutes, and moved for 10 seconds every 5 minutes (4 manipulations per session). The acupuncture sessions will be applied for the first 3 months of this treatment programme or until the ulcer has completely healed. If complete healing is not achieved in this period, the sessions may be prolonged for another 3 months, after which the acupuncture treatment will be discontinued.

For each session, the number of needles used and the exact location of their application will be recorded. Any adverse reaction or side effect arising from this treatment should be recorded in the DR, with a detailed description and the date of occurrence.

A priori, the ulcer should be cleansed once weekly, the same frequency as the sensory stimulation with acupuncture. Nevertheless, this may be increased or decreased, at the discretion of the nursing staff.

All treatment will be given by the professional healthcare staff (nurses) employed at the Primary Healthcare Clinics, coordinated by the treatment programme head in each case. In order to standardize criteria and training procedures in the use of compression bandages, the data record system and the application of the study treatments, a **specific ad-hoc training course** will be given to all the healthcare staff involved in

the study. This training will be structured as follows:

▪ Aims: acquisition of skills in applying acupuncture protocols for venous ulcers, in applying treatment techniques using the different kinds of dressing and in using the data record system. Standardization of criteria for applying compression bandages.

▪ Content: differential diagnosis of leg ulcers, calculation of the ankle/arm index, selection and localization of acupuncture points, the insertion of needles, the preparation and application of ulcer cleansing treatment, the reaction to possible infection, training in techniques with compression bandages, giving instructions to patients, implementing follow up, and giving all the study participants hygiene, posture and nutrition recommendations

▪ Instruction method: workshop

▪ Duration: 10 teaching hours

Outcome measures

Primary outcome

Time until complete healing is achieved

Complete healing is defined as the complete epithelialization of all the leg venous ulcers, not just the reference one.

Secondary outcome variables

Complete healing at 3 months after beginning treatment Complete healing is defined as the complete epithelialization of all the leg venous ulcers, not just the reference one (table 1).

Changes in the size of the surface area of the ulcer (in cm^2) When the baseline evaluation is made, the ulcer will be measured; if there are more than one, the largest ("the main ulcer") will be assessed. Nevertheless, the outcome variable of complete healing will be determined on the basis of all the ulcers observed on the patient when the baseline assessment is made. The area of the ulcer will be calculated using a compact digital tablet (Visitrak), with which the area can be assessed quickly, conveniently and accurately. The outline of the ulcer is drawn on a tracing sheet used as the digital screen; this has three layers to minimize the risk of cross contamination or secondary infection, and the layer in contact with the patient is sterile. The surface on which the data are recorded is kept clean and may be included in the patient's case history.

Pain intensity measured on a visual analogue scale There exists sufficient evidence to corroborate the validity of the visual analogue scale of pain intensity. Many studies have demonstrated the validity of the concept [46] and its reliability [47,48]. With this method, the subjective intensity of pain may be measured quickly and straight- forwardly. The patient is asked to mark the degree of pain intensity on a millimetric scale, from 0 (no pain) to 100 (the worst pain imaginable).

Changes in the health-related quality of life at 3, 6 and 12 months (12-item Short Form health survey, version 2) [49].

Adverse events – Adverse effects

A record will be kept of any adverse events or adverse effect observed by healthcare staff or reported by the patient or by his/her carer.

Cost-utility

Health utility will be measured in terms of the quality-adjusted life years (QALY) gained for each patient, calculated from the area beneath the SF-12 curve at 3 months from the start of treatment.

Opportunity cost

This will include the sum of the outputs from the activities potentially left undone as a result of providing a given treatment, rather than a possible alternative, together with the potential financial cost of the resources employed. Using the ABC method, the resources imputed to each patient will be distributed in a multidisciplinary table based on a modular "counter"-type framework for each case, in order to apply the analytical model and to obtain the SF-12 curve showing the variation in health-related quality of life and the potential cost savings for the Public Healthcare System.

Table 1. Outcome measures. Work scheme with description of assessment visits and times

	Baseline (T0)	**Weekly treatment**	**Evaluation at 3 months (T1)**	**Weekly treatment (if necessary)**	**Evaluation at 6 months (T2)**	**Evaluation at 12 months (T3)**
Sociodemographic data	X					
Ankle/arm index	X					
Nutritional study	X					
Localization of ulcer(s)	X		X		X	X
Area of main ulcer	X	X	X	X	X	X
Duration of the main ulcer	X					
Exudate culture (if necessary)	X	X	X	X	X	X
Changes in the size of the main ulcer		X	X	X	X	X
Characteristics of the main ulcer	X	X	X	X	X	X
Complete healing (date)		X	X	X	X	X
Pain intensity		X	X	X	X	X
SF-12	X		X		X	X
Adverse events/effects		X	X	X	X	X
Compliance with hygiene-posture recommendations	X		X		X	X
Location of treatment (clinic/home)		X		X		
Time employed in treatment		X		X		
Materials employed in treatment		X		X		
Number of needles used (Group A)		X		X		
Obtention of Deqi (Group A)		X		X		

Covariables

The covariables recorded will be the patient's age, sex, educational level, occupation, income, weight (kg), height (cm), mobility, systolic arterial pressure, diastolic arterial pressure, arm/ankle index and ankle diameter (cm), the duration of the active ulcer (weeks), the location of the ulcer (if more than one ulcer is present, stating the location of the largest one, which is taken as the reference ulcer), whether the ulcer is unilateral or bilateral, the number of ulcers on the leg(s), whether the ulcer is new or recurrent (in the latter case, recording the time since

the first appearance, in years and months), the signs of granulation at the base of the ulcer, the signs of epithelialization at the edges of the ulcer, the presence of fibrinous and/or necrotic tissue, the presence of lipodermatosclerosis, and the presence of infection. Other variables recorded will include dependence on tobacco, alcohol or other psychoactive substances, the presence of diabetes mellitus, the results of a nutritional study (haemogram and biochemical tests: albumin, prealbumin, transferrin and proteinogram), the time elapsed until recurrence occurs (weeks), compliance with hygiene-posture recommendations, self care, and the assistance or otherwise of non-professional carers. In addition, a record will be kept of all medication taken (corticoids, immunosuppressors, anticoagulants, etc.) and information about possible cultures of the ulcer exudate.

Data handling

The data on the variables of interest will be recorded on a purpose-designed form to be completed by each researcher at each clinic. This information will be entered into a database for subsequent statistical analysis.

Data collection

The routine follow-up evaluation, to be made following cleansing session, will be performed by the nursing staff responsible. The baseline evaluation (T0), that of the results at 3 months (T1), and the follow-up evaluations at 6 and 12 months (T2 and T3) will be carried out by the healthcare personnel responsible for the treatment programme (and who will be blinded with respect to the group to which each patient is assigned).

Measurements (measurement times)

Baseline evaluation basal (T0)

▪ The data obtained in the examination made prior to beginning treatment will include personal and sociodemographic characteristics, background and case history, ankle/arm index, nutritional status, previous treatment received, results of the ulcer exudate culture, the dimensions of the ulcer and observations regarding its condition, location and duration in an active state, as well as about the patient's health-related quality of life.

▪ Follow up. At each visit, the nurse applying the treatment will record the macroscopic status of the ulcer, any adverse events that may have occurred, the time employed in providing the treatment, the location of the treatment (clinic/ home) and the name of the person performing it. If total healing of the ulcer is detected during a routine examination, the treatment programme official at the relevant Clinic should be informed. For the patients in Group A, a record should also be kept of the data concerning the acupuncture session (the number and location of local points).

Follow-up evaluation at 3 months after starting treatment (T1) At three months after starting the treatment, a fresh assessment will be made of the patient's condition, quality of life and compliance with hygiene-posture recommenda- tions, as well as of the physical status of the ulcer (appearance and size).

Follow-up evaluation at 6 months after starting treatment (T2) With the same content as the previous evaluation.

Follow-up evaluation at 12 months after starting treatment (T3) With the same content as the previous evaluation.

Data analysis

The data analysis will be carried out by personnel blinded to the treatment groups, and for two types of populations: (1) intention to treat (ITT), with all the patients randomized; (2) per protocol (PP), including only the patients with no more than slight variances from the protocol.

The baseline variables for the different groups will be compared, to test the homogeneity produced by random allocation, in terms of differences of the means and of proportions, and the only differences taken into consideration in this comparison will be those that are clinically relevant for subsequent analysis. The magnitude of the difference in any imbalance produced by the random allocation to groups will be evaluated

by using ratios of the means, or medians, or proportions, bearing in mind whether the level of analysis is per cluster or per individual.

The final analysis will be carried out as a univariate survival analysis (using the Kaplan-Meyer method and the log-rank-test for inter-group comparison). The hazard ratios for each treatment will be calculated, together with the corresponding NNTs, calculating a 95% confidence interval, using Cox's proportional risk regression model. The assumption of proportionality will be assessed from the interaction between the time elapsed until success (total healing) and treatment group, and will be tested graphically by observing the parallelism in the log-log curves. A multilevel analysis will be carried out to adjust for possible unbalanced variables in the baseline analysis, taking the patients as level 1 and the Primary Healthcare Clinics as level 2. The interactions between clinic and treatment group will be examined, as will the possible variability in the intercept. The level of significance will be set at $p < 0.05$, for all the tests made. Cost-effectiveness, cost- utility and opportunity cost analyses will be made.

Discussion

We have identified three shortcomings in the present study design: the first arises from its conceptualization, in attempting to measure the effectiveness of procedures (acupuncture) whose efficacy has yet to be proven. It might be argued that a previous step should be to design a double-blinded study of effectiveness, comparing the above methods with a placebo procedure. However, apart from the impossibility of implementing a double-blind test for acupuncture, our viewpoint is to seek to determine the validity of clinical practices that are feasible in pragmatic contexts. The second limitation concerns the variability of the effects produced by nursing care; nevertheless, the cluster design employed is aimed at minimizing the risk of habit-formed contamination in the treatments applied and that of contamination between patients, in the sense of their sharing information about the results. These problems are addressed by standardizing the proto- cols/procedures and by organizing meetings to standardize the criteria applied, as well as by performing an a posteriori statistical analysis of the influence of the "therapist" factor. Finally, the fact that the outcome variable assessed is that of the complete healing of all leg venous ulcers suffered by the patient complicates analysis, but we believe a more limited study would have less probability of being of interest to patients concerned with the time elapsed until the complete healing of all their ulcers.

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INDUCTION OF MATURATION AND ACTIVATION OF HUMAN DENDRITIC CELLS: A MECHANISM UNDERLYING THE BENEFICIAL EFFECT OF *VISCUM ALBUM* AS COMPLIMENTARY THERAPY IN CANCER

Sri Ramulu Elluru[1,2,3,4], Jean-Paul Duong van Huyen[1,2,3,5], Sandrine Delignat[1,2,3], Michel D Kazatchkine[1,2,3], Alain Friboulet[4], Srini V Kaveri*[1,2,3] and Jagadeesh Bayry[1,2,3]

[1]Centre de Recherche des Cordeliers, Universite Pierre et Marie Curie – Paris6, UMR S 872, Paris, 75006, France, [2]Universite Paris Descartes, UMR S 872, Paris, F-75006, France, [3]INSERM, U872, Paris, F-75006, France, [4]CNRS UMR6022, Universite de Technologie, Compiugne, France and [5]Laboratoire d'Anatomie Pathologique, Hopital Europeen Georges Pompidou, 20-40 rue Leblanc, Paris, France

Background: *Viscum album* (VA) preparations have been used as a complimentary therapy in cancer. In addition to their cytotoxic properties, they have also been shown to have immunostimulatory properties. In the present study, we examine the hypothesis that the VA preparations induce activation of human DC that facilitates effective tumor regression.

Methods: Four day old monocyte-derived immature DCs were treated with VA Qu Spez at 5, 10 and 15 |ig/ml for 48 hrs. The expression of surface molecules was analyzed by flow cytometry. The ability of Qu Spez-educated DC to stimulate T cells was analyzed by allogeneic mixed lymphocyte reaction and activation of Melan-A/MART-1-specific M77-80 CD8+T cells. Cytokines in cell free culture supernatant was analyzed by cytokine bead array assay.

Results: VA Qu Spez stimulated DCs presented with increased expression of antigen presenting molecule HLA-DR and of co-stimulatory molecules CD40, CD80 and CD86. The VA Qu Spez also induced the secretion of inflammatory cytokines IL-6 and IL-8. Further, Qu Spez-educated DC stimulated CD4+T cells in a allogeneic mixed lymphocyte reaction and activated melanoma antigen Melan-A/MART-1-specific M77-80 CD8+T cells as evidenced by increased secretion of TNF-a and IFNy.

Conclusion: The VA preparations stimulate the maturation and activation of human DCs, which may facilitate anti-tumoral immune responses. These results should assist in understanding the immunostimulatory properties of VA preparations and improving the therapeutic strategies.

Background

VA preparations are aqueous extracts from *Viscum album* (also known as European mistletoe) consisting of different types of lectins [1-3]. In addition to mistletoe lectins (ML), biologically active components of VA preparations include viscotoxins, several enzymes, peptides (such as viscumamide), amino acids, thiols, amines, polysaccharides, cyclitoles, lipids, phytosterols, triterpines, flavonoids, phenylpropanes and minerals [3,4]. VA preparations have been used as a complimentary therapy in cancer. Several studies have reported the clinical benefits of VA preparations in cancer patients [5, 6]. Treatment with VA preparations or purified ML has also been shown to be associated with tumor regression in several experimental models [7, 8]. The mechanisms underlying the anti-tumoral activity of VA preparations are complex and not completely understood. The proposed mechanisms include induction of apoptosis of tumor cells and lymphocytes, inhibition of angiogenesis and stimulation of the cellular compartment of the immune system [9-14].

During the course of tumor development, the tumor evades the immune system through the secretion of various factors such as VEGF, IL-10 and PGE_2 that have been shown to inactivate the immune system [15]. The different pathways of immune evasion by tumors involve: induction of immune tolerance, resistance to killing by immune effector cells, and imparting functional paralysis of professional antigen presenting cells (APCs) such as dendritic cells (DCs) [15].

DCs are the professional APCs that are specialized in the uptake of antigens and their transport from peripheral tissues to the lymphoid organs [16,17]. Because of their capacity to stimulate naive T cells, DCs have a central role in the initiation of primary immune responses [18]. DCs reside in periphery as immature cells with a high ability to endocytose target antigens [19]. Upon receiving appropriate stimuli and in the context of inflammation, DCs undergo maturation process characterized by increased surface expression of antigen presenting HLA molecules and co-stimulatory molecules such as CD80 and CD86 and secrete several pro-inflammatory cytokines [20].

Tumor cells suppress the maturation and activation process of DCs [21]. Thus, several studies have demonstrated that DCs that reside in the tumor site or in the vicinity of tumor are immature with a decreased ability to stimulate T cells [22, 23]. In addition, tumor cells secrete several anti-inflammatory cytokines such as IL-10 and TGF, which can suppress the functions of DCs [24-26]. In view of the anti-tumoral and immunostimulatory properties of VA preparations, and the central role of DCs in anti-tumoral immune response, we examined the hypothesis that VA preparations stimulate the DCs, which in part explains the mechanisms underlying the beneficial effect of VA preparations in cancer therapy.

Table 1. Concentrations of Mistletoe Lectins and Viscotoxins in VA Preparation Concentration used (μg/ml)

Preparation	Concentration used (μg/ml)	Lectin (ng/ml)	Viscotoxin (ng/ml)
VA Qu Spez	5	0.375	0.012
VA Qu Spez	10	0.750	0.024
VA Qu Spez	15	1.125	0.036

Methods

Antibodies and reagents

Recombinant human (rh) interleukin-4 (IL-4) was obtained from R&D Systems (Lille, France), and rh granu- locyte macrophage-colony-stimulating factor (rh GM-CSF), rhIL-2 and rhTNFα were obtained from Immunotools (Friesoythe, Germany). FITC-conjugated monoclonal antibodies (mAb) to HLA-DR and CD80, PE-conjugated mAbs to CD86, CD40 and CD83 and APC-conjugated mAbs to CD11c were obtained from BD biosciences (France).

VA preparations

VA Qu Spez, was a kind gift from Weleda AG (Arlesheim, Switzerland). VA Qu Spez is the extract of *Viscum album* growing on oak trees. The VA preparations are therapeutic preparations that are free from endotoxins. VA prepara- tions are formulated in sodium chloride (NaCl 0.9%) isotonic solution as 5 mg/ml vials. During the manufacturing process, VA preparations are prepared by standardizing the levels of Mistletoe lectins and Viscotoxins. The concentrations of the lectins and viscotoxins of the different preparations used in the study are summarized in Table 1.

Generation and culture of monocyte-derived human dendritic cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors purchased from Hopital Hotel Dieu, Etablissement Fran3ais du Sang (06/ EFS/029, dated 29.05.2006), upon ethical approval for the use of such materials. The percentage of monocytes in the PBMC preparations was in the range of 9 to 14%. Monocytes were positively isolated using CD14 beads (Miltenyi Biotec, France). The purity of the monocytes after purification is > 98%. Immature DCs were generated by culturing monocytes for 4 days in RPMI 1640 containing 10% FCS, 50 U/ml penicillin, 50 ng/ml streptomycin, rhIL-4 (500 IU/10^6 cells), and rhGM-CSF (1000 IU/10^6 cells). Half of the medium, including all supplements, was replaced on second day.

Analysis of the expression of surface molecules by flow cytometry

To investigate the effect of VA Qu Spez on DCs, 0.5 Ч 10^6 immature four-day old DCs were either untreated or treated with TNFa (15 ng) or VA preparations (5, 10 and 15 |ig) for 48 h. On day 6, cell surface staining was per- formed with specifically labeled mAbs and proceeded for flow-cytometry (LSR II, BD Biosciences, France). Ten thousand events were recorded and analyzed for each sample. Data were analyzed by BD FACSDIVA software (BD Biosciences, France).

Mixed lymphocyte reaction (MLR) with allogenic CD4+ T cells

Responder CD4+ T cells used for allogeneic MLR assays were isolated from PBMC of healthy donors using a negative isolation kit (Dynal biotech-Invitrogen, France). DCs following 48 hr treatment with VA Qu Spez were washed extensively and were seeded with 1 Ч 10^5 responder allogeneic T cells at DC:T cell ratios of 1:10, 1:20 and 1:40. After 4 days, the cells were pulsed for 16 h with 0.5 |iCi (0.037 MBq) of (^{3}H)thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as counts per minute (mean ± SEM of triplicate values) after subtracting values of responder T cell cultures alone.

Anergy assay to determine the activation status of the CD4 T cells in the co-culture with VA-treated DCs

The anergy assay was performed according to a modified protocol originally described by Steinbrink et al [27]. Briefly, four-day old DCs were treated for 48 hrs with VA Qu Spez (15 ng/ml) or untreated or TNFa (15 ng/ml). Responder CD4+ T cells were then co-cultured during the first incubation at a density of 10^5 cells with 10^4 DC for 72 hrs. Then, T cells from the co-cultures were isolated by using CD4+ beads (Miltenyl Biotech) and rested for 24 hrs in the culture medium containing 2 U/ml IL-2. Subsequently, CD4+ T cells were re-stimulated with DCs generated from the same donor as that used for the first stimulation and have undergone similar VA Qu Spez treatment. After 48 hrs, the cells were pulsed for 16 h with 0.5 |iCi (0.037 MBq) of (^{3}H)thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as counts per minute (mean ± SEM of triplicate values). Tests were conducted in triplicates. Additionally, the levels of cytokines TNF-a and IFNy in the co-culture were analysed.

Activation of melanoma specific cytotoxic T cell (CTL) clones by VA Qu Spez-treated DCs

The melan-A-specific CTL clone M77-80 that was derived from tumor infiltrating lymphocytes of melanoma patient M77 is a kind gift from Dr. Nathalie Labarriere and Dr. Francine Jotereau [28,29]. The VA Qu Spez-treated DCs from HLA matched donor (HLA-A2, 10^4/well/200 |il RPMI 1640 medium supplemented with 10% AB serum) were cultured overnight with M77 CTLs (10^5) in 96 well round-bottomed plates along with the MART-1 peptide (1 |iM) and 25 IU/mL rh IL-2. The activation of M77-80 was analyzed by measuring IFNy and TNFa in the cell free- supernatants.

Analysis of cytokines

Cytokines in the cell-free culture supernatant were quantified using BD CBA Human Inflammation kit and Human Th1/Th2 kits (BD Biosciences, France).

Statistical analysis

Statistical significance was determined using the Mann- Whitney U test.

Results

VA Qu Spez enhances the expression of antigen presenting and co-stimulatory molecules on human DCs

We initially characterized the effect of VA Qu Spez on the phenotype of human DCs. Four-day old immature DCs were treated with VA preparations for 48 hrs and cells were analyzed for the expression of various surface molecules (Figure 1). We have used DCs treated with 15 ng/ml of TNF-a as control in addition to the DCs that

were left untreated. VA Qu Spez enhanced the expression by DCs of co-stimulatory molecules CD80 and CD86 (Figure 1) in dose-dependent manner. The expression of CD80 on DCs by 15 ng/ml concentration of VA Qu Spez (81.74 ± 2.3% population and 1632.8 ± 152 mean fluorescence intensity, MFI) was comparable to the DCs treated with TNF-a (84.93 ± 1.1% and 1644.25 ± 195.4 MFI). In addition, Qu Spez also significantly enhanced the percentage expression CD86 in a dose-dependent manner (Figure 1). How- ever, the expression of HLA-DR, CD40 and CD83 were either unaltered or marginally increased (data not shown).

VA Qu Spez induces the secretion of pro-inflammatory cytokines IL-6 and IL-8 by DCs

In addition to co-stimulatory molecules, DC-derived cytokines play a crucial role in priming T-cell response. We therefore analyzed whether the maturation process of DCs induced by VA Qu Spez is associated with the secretion of pro-inflammatory cytokines such as IL-6 and IL-8. The control DCs secreted 86.02 ± 23.3 pg/ml of IL-8 and 11.55 ± 6.28 pg/ml of IL-6 (n = 5 donors). However, as shown in Figure 2, VA Qu Spez significantly stimulated the secretion of IL-8 (308.052 ± 48.60 pg/ml) and IL-6 (54.97 ± 41.27 pg/ml) by DCs. Together these results indicate that in addition to stimulating the expression of co- stimulatory molecules on DCs, VA Qu Spez induce the secretion of pro-inflammatory cytokines.

VA Qu Spez-treated DCs stimulate T cell proliferation

A major function of DCs is their ability to trigger the activation and proliferation of T cells. We thus examined whether maturation of DCs induced by VA Qu Spez is reflected in their capacity to stimulate CD4+ T cells in an allogeneic MLR. As shown in Figure 3, VA Qu Spez-treated DCs, stimulated the proliferation of CD4+ T cells in a dose-dependent manner. The extent of CD4+ T cell proliferation induced by VA Qu Spez-treated DCs was significant at DC-T cell ratios of 1:10 and 1:20.

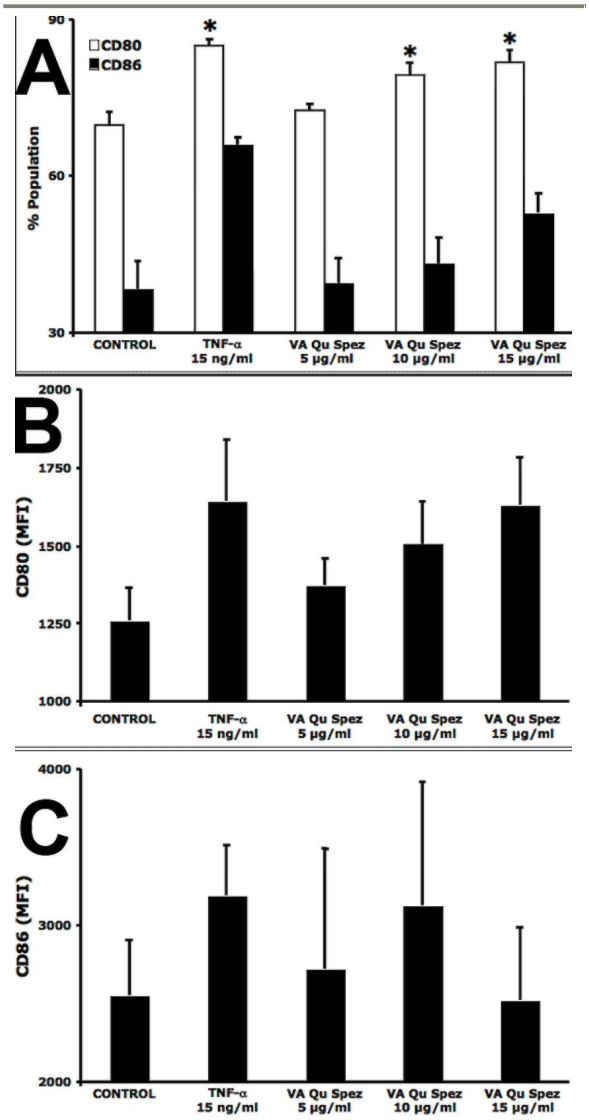

Figure 1. DCs treated with VA Qu Spez show an increased expression of co-stimulatory molecules CD80 and CD86. The 4-day-old DCs (0.5 Ч 10^6) were treated with medium alone (Control) or with 15 ng/ml TNF-α or with 5, 10 and 15 µg/ml of VA Qu Spez for 48 hours. The expression of CD80 (A and B) and CD86 (A and C) was analysed by flow cytometry (BD LSRII). Panel A shows the % of DCs pos- itive for CD80 (open bars) and CD86 (filled bars), while mean fluorescence intensities were presented in Panels B and C. Data are presented as mean ± SEM from from five to six independent donors. Statistical significance (*, $p<0.05$) as analysed by Mann-Whitney test is indicated

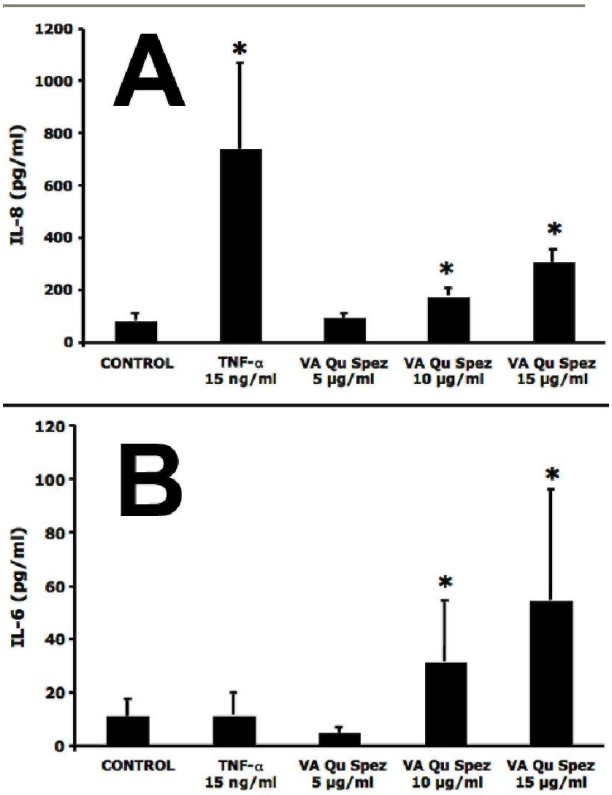

Figure 2. DCs treated with VA Qu Spez show an increased secretion of inflammatory cytokines IL-8 and IL-6. The 4-day-old DCs (0.5 Ч 10^6) were treated with medium alone (Control) or with 15 ng/ml TNF-α or with 5, 10 and 15 µg/ml of VA Qu Spez for 48 hours. The secretion (pg/ml) of IL-8 (Panel A) and IL-6 (Panel B) in cell free supernatants were analysed by cytokine bead array assay. Data are presented as mean ± SEM from five to six independent donors. Statistical significance (*, $p < 0.05$) as analysed by Mann-Whitney test is indicated

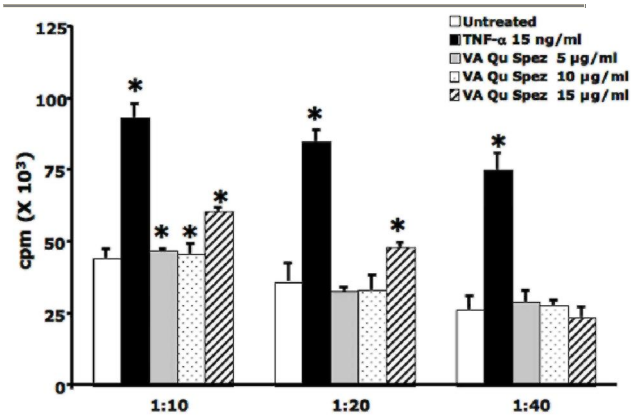

Figure 3. VA Qu Spez-treated DCs stimulate the proliferation of allogeneic CD4+ T cells. The 4-day-old DCs (0.5 * 10^6) were treated with medium alone (Control) or with 15 ng/ml TNF-α or with 5, 10 and 15 µg/ml of VA Qu Spez for 48 hours. After 48 hr treatments, DCs were co-cultured with the allogeneic CD4+ T cells at different ratios in a round bottom 96-welled plate. After 4 days of co-culture, the cells were pulsed overnight with 0.5 µCi (0.037 MBq) of (^{3}H)thymidine to quantify T-cell proliferation. Radioactive incorporation was measured by standard liquid scintillation counting, and the results were expressed as counts per minute (mean ± SEM of triplicate values). Statistical significance (*, $p < 0.05$) as analysed by Mann-Whitney test is indicated

VA Qu Spez-treated DCs do not induce anergy of CD4+ T cells

We performed anergy assay to determine the activation status of the CD4 T cells in the co-culture with VA Qu Spez-treated DCs. As shown in Figure 4a, during first cycle of CD4+ T cell stimulation in a MLR (day 0–day 7), VA Qu Spez-treated DCs (81920 ± 9070 cpm) show similar ability to stimulate the proliferation of CD4+ T cells as compared to TNF-α treated DCs (87439 ± 3910 cpm). Interestingly, CD4+ T cells that were re-stimulated / challenged during second cycle of stimulation with VA Qu Spez-treated DCs show increased proliferation (202276 ± 2052 cpm) as compared to the control DCs (76236 ± 4436 cpm) and TNF-α treated DCs (154341 ± 3224 cpm).

To further confirm that VA Qu Spez-treated DCs do not impart CD4+T cell anergy, we analysed for the secretion of T cell cytokines TNF-α and IFNγ in the DC-CD4+T cell co- cultures. As shown in Figure 4b, CD4+ T cells that were re- stimu-

lated/challenged during second cycle of stimulation with VA Qu Spez-treated DCs show increased secretion of above cytokines as compared to control DCs. These results suggest that maturation and activation of DCs induced by VA Qu Spez have functional repercussion on T cell activation and not T cell anergy.

VA Qu Spez-treated DCs stimulate melanoma specific M77-80 CTL clone

Since, VA preparations have been used as a complimentary therapy in cancer we examined whether VA Qu Spez- stimulated DCs facilitate anti-tumoral T cell responses. Therefore, HLA-matched VA Qu Spez-treated DCs were co-cultured with Melan-A/MART-1 specific M77-80 CTL clone (Figure 5). Strikingly, VA Qu Spez-treated DCs activated tumor antigen specific CTL clone as analyzed by the secretion of cytokines TNF-α and IFNγ.

Discussion

Although VA preparations are widely used in clinical practice and cancer therapy, their mechanisms of action are yet to be fully understood. In our previous studies, we have shown that in addition to cytotoxic properties, VA preparations have immunostimulatory effects that facilitate tumor regression in experimental models [13]. However, to mount an effective anti-tumoral immune response, an increased expression of co-stimulatory molecules on the DCs, the sentinels of the immune system, accompanied by an enhanced secretion of pro-inflammatory cytokines that culminates in T cell proliferation is necessary.

DCs found within the tumor microenvironment are found to have a relatively immature phenotype characterized by low levels CD86, and surface HLA-DR expression and inability to produce pro-inflammatory cytokine [30,31]. Clinical studies with mistletoe lectins have shown that VA preparations stimulate the cytokine secretion and function of monocytes, the precursors of DCs [32]. The previous studies by Stein et al demonstrated that mistletoe extract and their isolated components influences the maturation of DC with an increased expression of co-stimulatory and antigen presenting molecules [33,34]. Furthermore, we found that the up-regulation of these molecules is accompanied by the induction of inflammatory cytokines by the VA preparations and stimulation of tumor specific T cells. Together these results suggest that induction of maturation and activation of human DCs is one of the mechanisms underlying the beneficial effect of VA preparations as complimentary therapy in cancer.

Previously, it has been demonstrated that VA lectin induces the gene expression of IL-1 alpha, IL-1 beta, IL-6, TNF-α, IFN-γ and GM-CSF from PBMC [35]. A recent clinical study has shown that the CD14+ monocytes from multiple myeloma patients could be induced to differentiate into functional DCs by culturing them with the cytokine cocktail consisting of GM-CSF, IL-4, IL-6, TNF-α and IL-1β for use in cancer immunotherapy [36]. Our data demonstrates that VA Qu Spez-mediated maturation of DCs and secretion of pro-inflammatory cytokines (IL-6 and IL-8) has repercussion on the stimulation of CD4+ T cells and their cytokine secretion. It is interesting to note that VA Qu Spez-treated DCs do not induce anergy of T cells as shown by the induction of proliferation and the secretion of TNF-α and IFNγ by the CD4+ T cells. Thus, induction of DC-cytokines and T cell cytokines by VA Qu Spez represents a critical determinant in the development of effective innate immune responses against the tumor cells [37].

$CD8^+$ cytotoxic T lymphocytes (CTLs) are critical for the elimination of tumor. Thus, therapies aimed at expansion of CTLs and their functions holds the key in mounting an effective anti-tumor immune response. The ability of the CTLs to recognize the processed peptides derived from the cellular genes, such as those encoding MART-1 or tyrosinase in melanoma, led to the recognition that protective immune responses are often directed towards tumor-associated, rather than tumor-specific, antigens [28,29]. Using Melan-A/MART-1 specific M77-80

CTL clone, we have shown that DCs "educated" by VA Qu Spez can mount an anti-tumoral immune response as suggested by the increased levels of secretion of TNF-α and IFNγ by the CTLs in the co-culture. Further studies on the effect of the VA preparations on the DCs that have been subjected to inactivation by tumor factors, may provide strategies in dissecting the stimulatory effects of the VA preparations on the DCs.

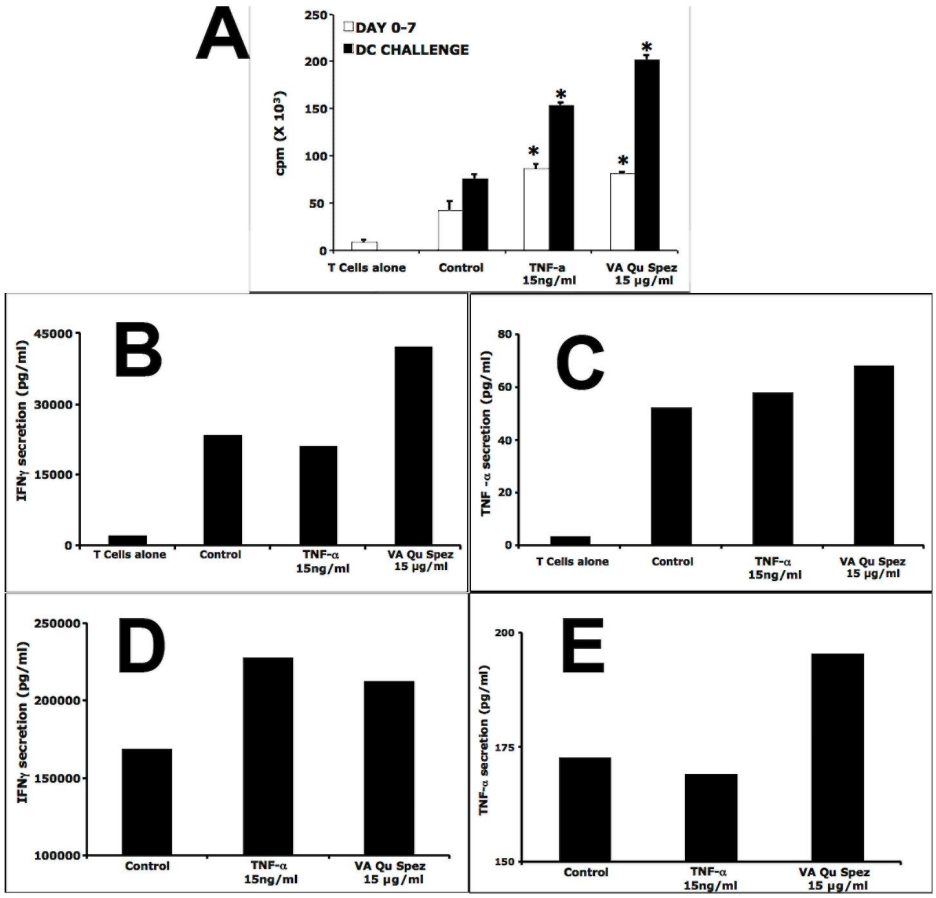

Figure 4. VA Qu Spez-treated DCs do not induce CD4+ T cell anergy. The 4-day-old DCs ($0.5*10^6$) were treated with medium alone (Control) or with 15 ng/ml TNF-oc or with 15 jug/ml of VA Qu Spez for 48 hours. The DCs were then co- cultured with the allogeneic CD4+ T cells at 1:10 ratio in a round bottom 96-welled plate for 72 hrs for the first cycle of stimulation. The T cells from in the co-cultures were then purified and were rested for 24 hrs in the presence of 2 IU/ ml of IL-2. These CD4+ T cells were then subjected for second cycle of stimulation with similiarly treated DCs from same donor. After 48 hrs of co-culture, the cells were pulsed overnight with 0.5 jiiCi (0.037 MBq) of (^{3}H)thymidine to quantify T-cell proliferation (Panel A, filled bars). Radioactive incorporation was measured by standard liquid scintillation counting, and the results were expressed as counts per minute (mean ± SEM of triplicate values). DC-T cell co-cultures of first cycle of stimulation that were maintained for 7 days were used for the comparison (Panel A, open bars). Statistical significance (*, $p < 0.05$) as analysed by Mann-Whitney test is indicated. The level of T cell cytokines IFNy (Panels B and D) and TNF-oc (Panels C and E) in the cell-free supernatants from above cultures were analysed by cytokine bead array. Panels B and C indicate the level of cytokines in DC-T cell co-cultures of first cycle of stimulation that were maintained for 7 days. Panels D and E present the level of cytokines in DC-T cell co-cultures of second cycle of stimulation

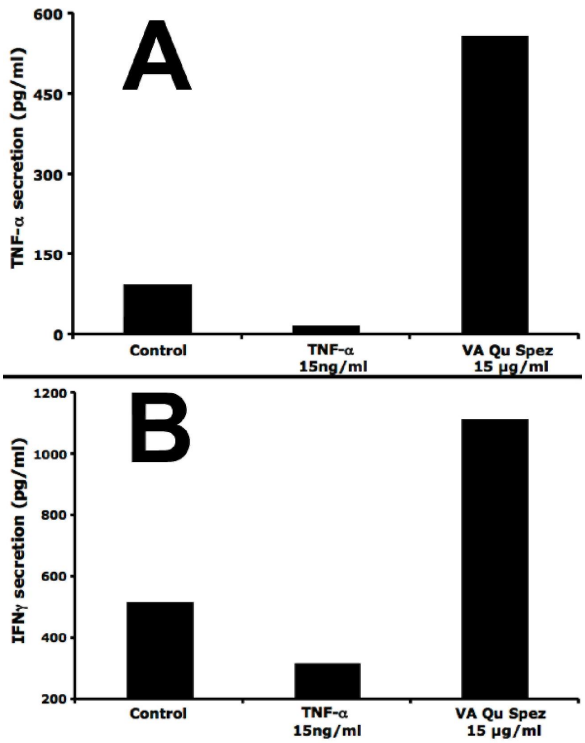

Figure 5. VA Qu Spez-treated DCs stimulate melanoma specific M77-80 CTL clone. The 4-day-old DCs (0.5 × 10^6) from HLA-A2 donor were treated with medium alone (Control) or with 15 ng/ml TNF-oc or with 15 jug/ml of VA Qu Spez for 48 hours. The DCs (10^4/well/200 JLIl medium) were then cultured overnight with M77-80 CTLs (10^5) in 96 well round-bottomed plates along with the MART-1 peptide (1 JLIM) and 25 IU/mL rh IL-2. The activation of M77-80 was analyzed by measuring TNFoc (Panel A) and IFNy (Panel B) in the cell free-supernatants by using cytokine bead array

Conclusion

VA preparations are known to have cytotoxic properties towards the tumor cells. They are also known to improve the quality of life in the cancer patients. However, the mechanisms by which VA preparations stimulate the immune system and exert beneficial effects in patients are not yet clear. We have demonstrated the role of the VA preparations in stimulating the DCs with implications in the induction of anti-tumor immunity. However, these *in vitro* results need to be validated further in the context of clinical studies. The elucidation of immunostimulatory mechanisms of VA preparations is critical in understanding their role as complimentary therapy in cancer.

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IMMUNE SYSTEM STATE IN CHRONIC CHOLECYSTITIS AND METABOLIC SYNDROME PATIENTS

Yurenko A.V., Antonyuk M.V., Khodosova K.K., Demyanenko N.B.
Research Institute of Climatology and medical rehabilitation, Vladivostok, Russia

On the ground of 127 chronic cholecystitis patients' examination results the immunological aspects of metabolic syndrome and chronic cholecystitis comorbid course were elucidated. The research results showed that in the patients with chronic cholecystitis the immune status changes take place together with the development of metabolic disorders (dyslipodemia, insulin-resistance) due to suppressing the cellular component of the immune system, nonspecific resistance factors' stimulation, cytokine level increase.
Key words: chronic cholecystitis, metabolic syndrome, immunity.

The development of chronic cholecystitis is conditioned, as a rule, by the bacterial flora, the penetration of which into the bile cyst happens by the enterogenous, hematogenous or lymphogenous ways [1]. The chronic inflammation at the chronic cholecystitis long course results in the metabolic and immune status disorders. In spite of the fact that cytokines are not specific factors of inflammation the determination of their concentration in blood gives the information about functional activity of various types of immunocompetent cells, the inflammatory process severity and disease prognostication [6].

The connection between the immune state and metabolic syndrome (MS), which often takes course against the background of chronic cholecystitis, is widely discussed in literature. At that, the data got by different investigators are rather contradictory [10].

Up to the present day the immunological aspects at the MS and chronic cholecystitis (CC) comorbid course remain one of the poorly studied problems.

The purpose of the research is to evaluate the immune system state in the patients with chronic cholecystitis at the MS formation.

127 persons aged from 20 to 55 years old, 39 men and 88 women, took part in the investigation. According to the research tasks three study groups were formed. The first group (control) was represented by 32 patients without chronic cholecystitis and MS; the second group – were 49 patients with chronic cholecystitis without MS manifestations and the third group – 49 patients with chronic cholecystitis and MS. Diabetes was diagnosed in 15%, the overweight – in 30%, adiposis – in 65% of the third group patients, in 82% of the patients all these changes were combined with hypertension.

The chronic cholecystitis diagnosis was made on the basis of clinical, laboratorial and functional research methods. The chronic non-calculous cholecystitis was detected in 75 patients, the chronic calculous cholecystitis – in 20 patients. The metabolic syndrome was diagnosed according to the USA National Committee criteria on cholesterol (ATR, 2001) [5].

In blood serum the level of total cholesterol, high-density lipoprotein cholesterol, triglycerides («OLVEX DIAGNOSTICUM» sets), apo-A1 and apo-B («DiaSys» sets) were determined. The apo-protein atherogenicity coefficient was calculated on the quotient of apo-proteins – apo-B to apo-A1 [7]. The carbohydrate metabolism investigation included the detection of glucose content in blood serum on the empty stomach, insulin – by the enzyme multiplied immunoassay method («DRG Diagnostics» sets). To determine the insulin resistance the NOMA index was used [9]. For the purpose of inflammation markers detecting in blood serum the tumor necrosis factor level – alpha (TNF- α) and receptor to TNF- α (TNF RI), were determined by the enzyme multiplied immunoassay method (the chemical reagents of the "BD Bioscience" firm). The peripheral

blood immunocompetent cells phenotyping was carried out using monoclonal antibodies to the cells CD3, CD4, CD8, CD 16, CD 22, CD 25, HLA-DR (Vitebsk) [3]. To determine the nonspecific resistance of the body the immune system monocytic macrophageal element cells' functional abilities were investigated. The neutrophils' metabolic activity study was carried out by means of the Nitro Blue Tetrazolium Reduction Test (NTR-test), the Nitro Blue Tetrazolium Reduction Test reserve (NTRR), the neutrophils' activation index (NAI) and the neutrophils' activation index reserve (NAIR) on the method of Park B.H. in the modification of Shmelev Ye.V. [12]. The A, M, G immunoglobulines concentration was determined in blood serum by the enzyme multiplied immunoassay ("Vector-Best" sets). The detection of the circulating immune complexes (CIC) of large (C3) and small (C4) sizes was carried out by the method of M. Digeon, M. H. Jover, J. Rizo in the modification of Struchkov P.V. [9]. As the pathogenicity factor their quotient (K = C4/C3) was used. At the static processing of the data the Student's t-test was determined; the differences were considered authentic at $p<0,05$.

In the patients with CC and MS the level of arterial blood tension, body-weight index, waist and thighs circuit, and also the studied factors of lipid and carbohydrate metabolism differed from the analogous factors in the first and second groups ($p<0,05$).

The immunological status analysis revealed a tendency to the immune system imbalance formation in chronic cholecystitis patients at metabolic disorders augmentation. As it is seen from table 1, in the second group patients the T suppressors' quantity reduction, tellingly for the chronic inflammatory process, takes place against the background of the T helpers' quantity reduction. In the third group patients there was a tendency to the "adult" T lymphocytes' (CD3), T helpers' (CD4) quantity reduction and cytotoxic T lymphocytes' (CD8) quantity increase compared to the control group marked. The CD4/CD8 index fell in the third group patients compared to the 1st and 2nd groups.

In the third group patients there was a modest rise of the B "adult" lymphocytes' level against Ig G level increase ($p<0,05$) registered compared to the 1st and 2nd groups.

The given changes testify to the immune resistance suppression in CC and MS patients. On the one hand, the humoral immunity activation happens in this group; on the other hand – the immune system cellular component's functional abilities decrease takes place.

In the patients of the 2nd and 3rd groups there was an increase of the level of the cells containing receptors to the interleukin-2 (IL-2) ($p<0,05$) compared to the control group registered, that reflects the production stimulation of IL-2 promoting, in its turn, the activation and maturation of different subpopulations of T-cells, B-cells, natural killers and macrophages. The decrease of the "adult" T lymphocytes', T helpers' CD4/CD8 index', natural killers' (CD 16) level in the third group patients testifies to a negative activation of lymphocytes at the MS formation against the background of chronic cholecystitis [4].

The results of antigens' state study are of concern. The HLA-DR increase in the second and third groups compared to the control was registered ($p<0,05$). There are no analogous investigations of the HLA antigens' state in the CC and MS patients described in the works of other authors. The findings, in the course of which the associative relations between the HLA antigens and various diseases were set, it letting high risk groups be emphasized, the preconditions for various pathological forms combination be found out and the course be prognosticated; are submitted in literature [4].

Table 1. Immunity factors in chronic cholecystitis patients

Factors	1 group, control, n=32	2 group, n=49	3group, n=46
Leukocytes, g/l	5,55±0,27	5,60±0,28	6,02±0,34
Lymphocytes, %	27,70±1,38	27,85±1,3	26,2±1,48
T – lymphocytes "adult" (CD3), %	33,39±2,19	30,24±1,55	29,97±1,55
T –helpers (CD4), %	33,30±1,85	29,42±1,66	27,83±1,57 $p_{3-1} < 0,05$
T –suppressors (CD8), %	21,35±0,91	19,42±0,93	23,53±1,47 $p_{2-3} < 0,05$
CD4/ CD8 index	1,50±0,06	1,55±0,08	1,24±0,07 $p_{3-1}, p_{2-3} < 0,05$
B – lymphocytes "adult" (CD22), %	24,26±1,9	24,18±1,33	26,03±1,79
Natural killers (CD16), %	19,74±1,43	20,33±1,13	19,8±1,34
IL-2-receptor bearing T- and B-lymphocytes (early stage of activation) (CD 25), %	11,26±0,86	18,09±1,39 $p_{2-1} < 0,05$	18,47±1,52 $p_{3-1} < 0,05$
Activated T- and B-lymphocytes (late activation stage), bearing receptors HLA-DR , %	13,61±0,81	18,55±1,44 $p_{2-1} < 0,05$	16,69±1,07 $p_{3-1} < 0,05$
Oxidative metabolism of neutrophils (NTR-test), %	10,14±0,8	12,94±1,55	16,55±1,48 $p_{3-1} < 0,05$
NTR reserve,c.u.	1,30±0,09	1,12±0,08	1,09±0,1
Neutrophils' activation index (NAI), %	0,17±0,05	0,24±0,06	0,19±0,02
NAI reserve, c.u.	1,32±0,09	1,13±0,08	1,07±0, 1
Ig A, mg/ml	1,63±0,1	1,88±0,07 $p_{2-1} < 0,05$	1,64±0,1 $p_{3-2} < 0,05$
Ig M, mg/ml	1,25±0,1	1,22±0,08	1,36±0,08
Ig G, mg/ml	9,70±0,2	9,84±0,18	10,41±0,2 $p_{3-1}, p_{2-3} < 0,05$
CIC: C3, c.u.	0,44±0,02	0,52±0,04	0,49±0,03
CIC: C4, c.u.	0,51±0,02	0,55±0,03	0,56±0,03
К, c.u.	1,17±0,04	1,12±0,03	1,15±0,04
TNF-α, pg/ml	4,88±0,17	5,68±0,38	6,62±0,35 $p_{3-1} < 0,05$
TNF RI pg/ml	1039,1±209,11	1926,35±132,7 $p_{2-1} < 0,05$	2520,2±123,36 $p_{3-1}, p_{3-2} < 0,05$

Note: p_{3-1}, p_{2-1} –reliability of distinctions between factors in comparison with the control; p_{2-3} – reliability of distinctions between the 2nd and 3rd group.

The state of non-specific resistance factors was characterized by the neutrophils' oxidative metabolism activity (NTR) increase with the decrease of neutrophilic granulocytes' reserve opportunities and increase of C3 levels by 11% and C4 CIC by 8% in the patients of the 2nd and 3rd groups compared to the control ($p<0,05$). An authentic increase of the TNF- α factors and TNF- α receptors is registered in the CC and MS patients compared to the control ($p<0,05$). The anti-inflammatory cytokines' level increase and neutrophils' oxidative metabolism activation is considered by many authors as the factor of vessels' endothelium injury through the intensification of oxidation processes resulting in nitrogen oxide inactivation, the release of the enzymes, which catalyze the formation of kinins, which promote vascular permeability and change the vascular wall structure [11, 2].

Thus, the research results testified that in CC patients together with the development of metabolic disorders (dyslipodemia, insulin-resistance) the immunological status changes take place due to the immunity due to suppressing the immune system cellular component, nonspecific resistance factors' stimulation, cytokine level increase. The revealed changes reflect systemic reactions of the body at chronic inflammation and are associated with metabolic disorders resulting in the development of MS clinical implications – hypertension, diabetes, in chronic cholecystitis patients.

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