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Effects of calcium dobesilate and diosmin-hesperidin on apoptosis of venous wall in primary varicose veins

E. Iriz[1], C. Vural[2], E. Ereren[1], A. Poyraz[2], D. Erer[2], L. Oktar[1], L. Gokgoz[1], V. Halit[1] and H. Soncul[1]

[1] Gazi University, School of Medicine, Department of Cardiovascular Surgery, Ankara, Turkey
[2] Gazi University, School of Medicine, Department of Pathology, Ankara, Turkey

Summary

Background: Evaluation of the therapeutic effects of calcium dobesilate and diosmin-hesperidin through regulation of apoptosis.
Patients and methods: 56 Patients were divided into four groups; Group 1 consisted of patients (n = 18) with the recent diagnosis of primary varicose disorder who have never used medications, Group 2 consisted of patients (n = 14) who have used diosmin-hesperidin for at least six weeks prior to the operation, Group 3 consisted of patients (n = 14) who have used calcium dobesilate for at least six weeks prior to the operation and finally Group 4 (Control group) consisted of normal saphenous vein biopsies (n = 10). All biopsies were stained with Hematoxylin and Eosin. Tissue samples from 56 patients were immunohistochemically stained with antibodies of anti-bcl-2, anti-bax and anti-p53. Apoptosis was evaluated by TUNEL method.
Results: There were no statistically significant differences among the groups in respect to gender distribution and smoking status. Immunohistochemical evaluation of apoptosis related proteins revealed a statistically significant difference between Group 4 and the other groups with respect to the apoptag staining on venous wall ($p = 0.026$). There were significant differences in the presence of bcl-2 protein expression between groups 4 and Group 1 ($p = 0.0002$) and between Group 1 and Group 3 ($p = 0.023$).
Conclusions: Our study highlights the significance of apoptosis in varicose disorders and suggests that calcium dobesilate, which is used in the treatment of varicose veins, could be of benefit by regulating apoptosis.

Key words: Apoptosis, varicose veins, calcium dobesilate, lipid peroxidation, venous wall

Zusammenfassung

Die Effekte von Calcium dobesilate und Diosmin-hesperidin auf die Apoptose der Venenwand in primär varikösen Venen
Hintergrund: Evaluation der therapeutischen Effekte von calcium dobesilate und Diosmin-hesperidin durch Regulation der Apoptose.
Patienten und Methoden: 56 Patienten wurden in vier Gruppen unterteilt. Gruppe 1 bestand aus Patienten (n = 18) mit der Diagnose einer primären Varikose ohne medikamentöse Therapie. Gruppe 2 enthielt 14 Patienten, die vor der Operation mindestens sechs Wochen lang Diosmin-hesperidin einnahmen. Die Patienten der Gruppe 3 (n = 14) nahmen präoperativ sechs Wochen lang Calcium dobesilate ein. Die letzte Gruppe stellte als Kontrollgruppe ein Kollektiv aus 10 Patienten mit normalen Biopsien der V.saphena dar. Alle Biopsien wurden mit Hematoxylin und Eosin entnommen. Die Proben von 56 Patienten wurden immunhistochemisch mit den Antikörpern anti-bcl-2, anti-bax und anti-p53 gefärbt. Die Apoptose wurde mit der TUNEL Methode ausgewertet.
Ergebnisse: Es zeigten sich keine statistisch signifikanten Unterschiede zwischen den Gruppen hinsichtlich der Geschlechterverteilung oder der Nikotinkonsums. Die immunhistochemische Auswertung der Apoptose assoziierten Proteine wies hinsichtlich der Apoptag Färbung an den Venenwänden eine statistisch signifikante Differenz zwischen Gruppe 4 und den übrigen Gruppen auf ($p = 0.026$). Es zeigten sich signifikante Unterschiede hinsichtlich bcl-2 Protein Expression zwischen den Gruppen 4 und 1 ($p = 0.0002$) und zwischen den Gruppen 1 und 3 ($p = 0.023$).
Schlussfolgerungen: Unsere Studie hebt die Signifikanz der Apoptose bei den varikösen Erkrankungen hervor und lässt darauf schließen, dass Calcium dobesilate, das zur Therapie der Varikosis genutzt wird, durch die Regulation der Apoptose von Vorteil sein kann.

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Introduction

The etiology of varicose veins remains unknown despite its widespread occurrence. Many factors are implicated in the etiology of varicose veins. These factors include pregnancy [15], obesity [33], some occupational diseases, alterations on vessel wall [4, 13, 38, 39] and genetic factors [11]. Recently, it has been indicated that apoptosis is effective in the vessel wall weakness and in the development of varicose veins [4, 13, 38, 39]. There exist two theories about the pathology of varicose veins [39]; one is the valve failure theory and the second is vessel wall weakness progressed by metabolic and physiologic alterations in vessel wall cells [39]. The more accepted second theory suggests that dysregulated apoptosis affects the natural process and causes structural and functional deterioration in vessel wall. Changes in elastin and collagen content of vessel wall [13, 39] or changes in concentrations of inhibitors and metalloproteinases in extracellular matrix [5] are the possible explanations for development of varicose veins.

Apoptosis or programmed cell death is regulated by intrinsic and extrinsic mechanisms involving many proteins. Bcl-2 family and specific caspases take place in intrinsic pathway (mitochondrial or type 1 pathway). Extrinsic pathway (transmembrane or type 2 pathway) involves proteins such as Fas and TNF-R [1, 13]. In recent years apoptosis has been implicated in some of the vascular pathologies such as atherosclerosis, inflammatory vessel diseases and aneurysms [7, 29]. p53 is an important regulator of the cell cycle as it triggers growth arrest or leads to apoptosis in response to cellular stress.

One of our study drugs calcium dobesilate (Doxium® 500 mg) has been previously shown to have an in vitro antioxidant effect [18]. This molecule is suggested to have anti-apoptotic effects through inhibition of membrane lipid peroxidation by free oxygen radicals in human erythrocytes and polymorphonuclear leucocytes and through clearance of superoxide anions [18].

Flavonoids (Diosmin+Hesperidin – Daflon® 500 mg) are known to protect tissues and cells against reactive oxygen radicals in literature [8]. In one study, this drug is proposed to exert a protective effect by the clearance of free radicals and other oxidant products from the tissue [12]. This drug is also protective for neutrophil-mediated venous wall injury [2]. Reports are also available about drug which suggests a regulatory effect for apoptosis in cancer patients [26].

In our study, we analysed the relation of apoptosis with the varicose veins. The main purpose of the study was to show the effects of calcium dobesilate and diosmin-hesperidin on apoptosis of venous wall.

Patients and methods

Patients

This study was supported by Scientific Research Projects of our University (Nr. 2005–61) and approved by the ethic committee. Informed consents were obtained from all patients. Total 46 patients, who underwent an operation between February 2005 and December 2006 for C2, 3 and 4 primary varicose veins according to CEAP classification were included in the study. In all patients actual diagnosis was primary varicose veins. Also in the same period, saphenous vein biopsies were obtained from 10 patients (control group) admitted for coronary bypass operation and who were found to have normal great saphenous vein in both preoperative Doppler ultrasonography (USG) and intraoperative examination. Forty-six patients with varicose veins and 10 patients undergoing coronary artery bypass surgery (control group), total 56 subjects were included in the study. Long saphenous vein biopsies were taken from distal part of the veins (below knee). All biopsies were taken from stages III and IV patients according to Hach classification [19]. The distribution of patients was performed by randomization. Patients were divided into four groups. Of 18 patients in group 1 (45.2 ± 15.7), 9 were female (50%) and other 9 were male (50%), who have not used any medication within the last six weeks. Operational indications of the patients were composed of pain, edema, venous stasis or cosmetic problems. The complaints of all patients were continuing for at least a year.

Of 14 patients in group 2 (44.0 ± 12.9), 6 were female (43%) and other 8 were male (57%), who have used diosmin-hesperidin 1000 mg/day (2 × 500 mg) for at least six weeks prior to the operation.

Of 14 patients in group 3 (46.9 ± 11.6), 6 were female (43%) and other 8 were male (57%), who have used calcium dobesilate for at least six weeks prior to the operation. The usual dose was as 2 × 500 mg. The distribution of patients in the first three groups according to CEAP classification is presented in Table I. There was no statistical difference between the groups in terms of CEAP classification.

Of 10 patients in group 4 (59.9 ± 10.9), 4 were female (40%) and other 6 were male (60%). All patients with coronary artery disease included in all of which preoperative Doppler USG examination revealed a normal anatomy and functioning superficial and deep venous systems. Doppler USG examinations of the veins were conducted in transverse and longitudinal axes. Presence of backflows was validated by breathing

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Table I: Distribution of patients according to CEAP classification

	CEAP Class 2	CEAP Class 3	CEAP Class 4
Group 1	10/18 (55.5%)	5/18 (27.7%)	3/18 (16.8%)
Group 2	8/14 (57.2%)	4/14 (28.5%)	2/14 (14.3%)
Group 3	8/14 (57.2%)	3/14 (21.4%)	3/14 (21.4%)

and augmentation maneuvers. Presence of intraluminal thrombus was also evaluated. Deep venous insufficiency was detected in 8 (17%) of 46 patients with varicose veins. The percentage of patients in each group who had deep venous insufficiency; three patients (37.5%) in group 1, two patients (25%) in group 2, and three patients (37.5%) in group 3. Venous thrombosis was not detected in any of our patients. Demographic and clinical features of the patients are presented in Table II.

Histopathologic examination

Tissue specimens obtained during the procedure were fixed in 10% formalin after gross examination of the vein one random sample was taken from both control group and varicose vein and embedded in paraffin cut into 5 µm sections and stained with Hematoxylin and Eosin (H-E). All H-E slides were reviewed by the same pathologist. 1 slide was selected from each case and same block used for immunohistochemistry and TUNEL method. Additionally, Massons's trichrome and elastic stains were done in both control group and varicose vein and then we compared staining pattern. Control group assumed to be containing normal amount of connective tissue.

Immunohistochemical study

Formalin fixed paraffin embedded tissues were cut into 4 µm sections deparafinized in xylene and dehydrated. The immunoperoxidase procedure was performed using the Streptoavidin-biotin-peroxidase method. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minute. Antigen retrieval was performed using 0.01 M sodium citrate buffer through microwave processing. Primary antibodies (antibcl-2 clone:124- Diagnostic Biosystem (DBS), anti-bax clone: 2D2- DBS, anti-p53, clone: D07-DBS) were applied for 2 hours at room temperature. The antibody reactions were visualized using 3 amino-9-ethylcarbazole (AEC). Sections were weakly counter stained with Mayer's hematoxylin. Tonsil for bcl-2, Hodgkin lymphoma for bax and colonic carcinomas for p53 were used as a positive control. Sections were assessed for each biopsy in contigous high-power fields (400 × magnification) and the proportion of positively staining cells were recorded by examining 1000 cells.

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method was used to demonstrate the presence of apoptotic cells. Apoptag (Chemicon) was used as a primary antibody. Following deparaffinization and inactivation of endogenous peroxidase with 3% hydrogenperoksidase, the slides were incubated with 20 µg/ml proteinase K (applipere; Oncor, Gaithersburg, Md) for 15 minutes. The following steps were performed according to the manufacturer's instructions of TUNEL assay using Apoptag plus peroxidase insitu apoptosis detection. Finally, the slides

Table II: Demographic and clinical features of patients

	Age (Years)	Gender (M)	Gender (F)	Smoking (+)	Disease duration (Years)
Group 1	45.2 ± 15.7	9/18 (50%)	9/18 (50%)	9/18 (50%)	14.2 ± 2.1
Group 2	44.0 ± 12.9	6/14 (42.9%)	8/14 (57.1%)	5/14 (35.7%)	15.1 ± 1.9
Group 3	46.9 ± 11.6	6/14 (42.9%)	8/14 (57.1%)	7/14 (50%)	14.8 ± 2.0
Group 4	59.9 ± 10.9	6/10 (60%)	4/10 (40%)	5/10 (50%)	None

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were incubated with diaminobenzidine. Counterstaining of the specimens was done with 1% methyl green for 10 minutes. Macrophages within the germinal center of tonsil used as positive control. Each specimen were evaluated in light microscopy at high magnification (× 400). TUNEL positive cells were counted at 10 different fields of each specimen by the same observer and the apoptotic index was calculated by examining 1000 cells.

Statistical analyses

Data were entered using SPSS 10.0 package program. In statistical analyses, Chi-Square test was used for qualitative variables; Kruskal-Wallis test and Mann Whitney-U test were used for quantitative variables. In statistical interpretations $p < 0.05$ was considered as significant.

Results

There was no significant difference among the groups in respect to the demographic features such as gender, smoking and accompanying diseases. In the evaluation of groups in regard to age status with the exception of control group, group 4, there was no difference among groups 1, 2 and 3. There was also no statistically significant difference among groups 1, 2 and 3 with respect to the presence of familial history of varicose veins which suggest a genetic predisposition (Table II). According to the age of disease there was no statistically difference among the study groups (groups 1, 2 and 3). Presence of coronary artery disease in group 4 normally produces a statistical difference with regard to the presence of accompanying diseases in other groups.

Histopathologic examination

None of the HE-stained sections displayed necrosis in any group. Augmentation of intimal connective tissue and intimal thickening were observed in 14 patients in group 1, 8 patients in group 2, 10 patients in group 3 and 2 patients in group 4. Additionally, complete disappearance of internal elastic lamina was seen in 12 patients in group 1, in 13 patients in group 2, in 11 patients in group 3 and in 2 patients in group 4 whereas degeneration characterized by fragmentation was observed in 6 patients in group 1, in 1 patient in group 2, in 3 patients in group 3 and in 2 patients in group 4. Statistical evaluation of intimal connective tissue augmentation and intimal thickening on venous wall revealed significant difference between groups 1 and 4 ($p = 0.005$), and between groups 3 and 4 ($p = 0.03$). There exist statistically significant differences between groups 1 and 4 ($p = 0.048$), groups 2 and 4 ($p = 0.001$) and groups 3 and 4 ($p = 0.01$) with respect to the degeneration of internal elastic lamina. Results indicate a better condition for the patients in group 4 than in other three groups which also suggest an important role for the disappearance or degeneration of elastic lamina in varicose veins.

Molecular mediators of apoptosis

Apoptag

Apoptag staining was found to be positive in total 7 cells of 3 patients in group 4 and in 1 cell of 1 patient in group 3, signifying the presence of apoptosis (Fig. 1). Apoptag staining of all groups were seen in adventitial layers. The other groups did not exhibit Apoptag staining. The number of cells committing apoptosis was found to be statistically different in group 4 compared to the other groups ($p = 0.026$). The percentages of Apoptag positiveness was calculated as 30% in group 4, 7.1% in group 3 and 0% in other groups (Table III). This suggests that normal venous wall differs in respect to the presence of Apoptag staining compared to varicose veins.

Bcl-2 expression:

Positive stating was observed for Bcl-2 expression in 75, 17 and 4 cells in groups 4, 3 and 2, respectively (Fig. 2). We did not observe positive staining for bcl-2 in group 1. Immunohistochemically staining of this protein bcl-2 expression in group 4 produces significant statistical difference compare to groups 1, 2 and 3 ($p = 0.0002$). Bcl-2 expression in group 3 was found to be significantly different compared to group 1 ($p = 0.023$). The percentage of Bcl-2 expression in groups was calculated as 70%, 35.7%, 7.1% and 0% in groups 4, 3, 2 and 1, respectively (Table IV). The reason of higher percentages in group 4 suggests Bcl-2 expression is higher in

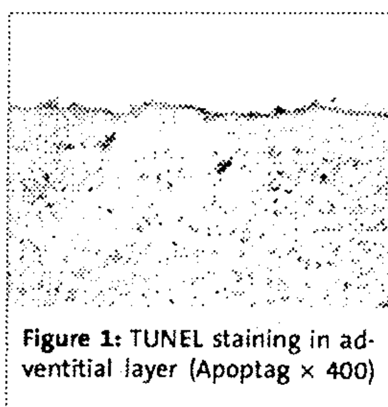

Figure 1: TUNEL staining in adventitial layer (Apoptag × 400)

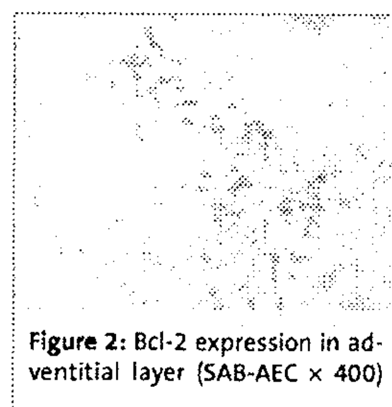

Figure 2: Bcl-2 expression in adventitial layer (SAB-AEC × 400)

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Table III: Distribution of Apoptag staining of patients according to the groups

	Number of patients	Number of cells	%
Group 1	0	0	0
Group 2	0	0	0
Group 3	1	1	7.1
Group 4	3	7	30

Table IV: Distribution of patients in terms of Bcl-2 expression according to the groups

	Number of patients	Number of cells	%
Group 1	0/18	0	0
Group 2	1/14	4	7.1
Group 3	5/14	17	35.7
Group 4	7/10	75	70

normal venous wall. The difference between group 3 and 1 could be explained by regulatory effect of calcium dobesilate on apoptosis by inducing diminished or absent Bcl-2 expression.

Expression of bax and p53

None of the samples displayed staining with proapoptotic protein, bax. Samples also did not display nuclear staining for p53.

Discussion

Apoptosis is a programmed cell death which takes place in cellular development and homeostasis. Dysregulated apoptosis is implicated in the development and pathogenesis of numerous diseases such as atherosclerosis, cardiovascular diseases, neurodegenerative disorders and cancer [20, 27, 28].

In recent years some studies focused on dysregulated or impaired apoptosis in patients with varicose veins in which the etiopathogenesis is still not clear [3, 4, 13, 38]. The common conclusion of these studies is that apoptosis could take part in the development of varicose veins. In our study, in addition to investigating the role of apoptosis in the development of varicose veins, we investigated the effects of calcium dobesilate and diosmin-hesperidin, the drugs used in varicose veins, on apoptosis.

Apoptosis is a dynamic process in which proapoptotic and antiapoptotic proteins are involved [1, 27]. Bax, bak, bid and bad are proapoptotic proteins, whereas Bcl-2, Bcl-xl and Bcl-w are antiapoptotic proteins [1, 27]. The expression and activities of these proteins are effected by cellular injury, growth factors and survival factor from other cells [1]. If there is no cellular injury and in the presence of normal survival factor, antiapoptotic members of Bcl-2 protein family suppresses apoptosis [1]. On the contrary, if there is cellular injury or lack of survival factors, apoptosis, the programmed cell death is triggered [1].

Despite the limited current knowledge of cell turn-over on the vessel wall, recent studies implicate dysregulated apoptosis in the development of restenosis secondary to intimal hyperplasia following angioplasty and venous grafting [14, 30]. The association of dysregulated apoptosis and varicose veins was first suggested in a study by Ascher et al. [3] in which they proposed a relation between varicose veins and abnormal changes in the cell cycle of vessel wall and disturbances in DNA repair mechanisms [21]. Subsequent studies supported the assumption of apoptosis suppression in vessel wall of varicose veins [4, 13, 38]. Inhibition of apoptosis in vessel wall could translate into a longer survival and dysfunction of cells. Dysfunctional cells with longer survival might cause weakness and dilatation in vessel wall.

In the context of the knowledge given above, one or more factors involved in apoptotic process of varicose disease may facilitate the progression of the disease. In the literature, Bcl-2 expression is reported to be a protective factor against vascular apoptosis [6, 34]. Bcl-2 protein is thought to support cellular viability by regulating lipid peroxidation, cellular redox state and intracellular calcium balance [10, 22, 27]. Also in our study positive Bcl-2 expression was found to be higher in the control group. Lack of staining in group 1 suggests missing bcl-2 protein expression may have a role in development of varicose veins. Consistent with the literature, despite monitoring Apoptag positivity in 3 patients in group 4, not observing it in any patient in group 1 is gave rise to thought that dysregulation of programmed cell death could play a role in the etiology of varicose veins.

We believe that imbalance of regulatory proteins in patients with varicose veins disrupts normal apoptosis. Damaged and dysfunctional cells, in the vessel wall do not commit apoptosis and hence could not be killed. Structural and functional deterioration of these cells may play a role in development of varicose veins.

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There exist two major theories about the occurrence mechanism of varicose veins [23, 25, 39] and one of which is the venous valve failure theory that explain vein dilation and the second is wall weakness theory that explains vessel wall enlargement. When considering the first theory, occurrence of varicose veins is contradictory to the theory despite the lack of insufficiency in sapheno-femoral junction of vena saphena magna (VSM) [23, 39]. On the other hand, lack of venous dilation in VSM used as in situ arterial graft after valve deformation despite exposure to arterial pressure attenuates the valve failure theory [39]. For this reason recent studies have conducted investigating the possible relation between vein dilation and disturbances in venous wall [4, 13, 24, 25, 38]. Common conclusions of these studies are impaired apoptosis, inhibition of cell cycle and loss of integrity between structural components of venous wall (smooth muscle, elastic fibers and collagen fibers) may deteriorate the structure of venous wall. All these pathologic events may occur in sapheno-femoral junction region. Wall weakness in this region may itself cause venous dilation and valve incompetence. In our study we found injury of elastic lamina and augmentation of intimal connective tissue in venous wall along the finding of suppressed apoptosis in varicose veins. Disturbances of venous metabolism causing extracellular matrix remodeling in venous wall are considered to be a first step mechanism of cellular injury in venous wall [17]. Injury to venous wall is mediated by free oxygen radicals produced by the result of deterioration of this metabolism. Free oxygen radicals may be a trigger factor for lipid peroxidation and cell damage in venous wall [17]. Consequently, degradation of glucose-aminoglycans and collagen increases [37]. In one study [16], it was demonstrated that alterations in venous connective tissue were important for the proteolytic activity of saphenous vein even in stages without valvular insufficiency. Consequently, levels of bcl-2, a regulatory protein for cellular lipid peroxidation, may be affected by lipid peroxidation in venous wall caused by free oxygen radicals. The imbalance between proapoptotic and antiapoptotic members of bcl-2 family might play an important role in the development of varicose veins. In our study we examined the effects of calcium dobesilate, and diosmin-hesperidin on apoptosis. Calcium dobesilate inhibits membrane lipid peroxidation in human erythrocytes and polymorphonuclear leukocytes in effect of free oxygen radicals and scavenges superoxide anions [18]. The drug is also used as an angioprotective agent like diabetic retinopathy and venous insufficiency for the treatment of vascular diseases [18]. It has been reported to decrease capillary permeability and fragility in diabetic retinopathy [18], decrease blood viscosity [18], inhibit platelet aggregation [18, 32] and decrease thrombus formation [31]. The effect of decreasing capillary permeability is thought to be related with its regulatory effect on collagen biosynthesis in basal membrane [40]. A clinical study on human peripheral mononuclear cells suggests that calcium dobesilate contributes to the regulation of apoptosis by lipid peroxidation [18]. The same study also suggests that calcium dobesilate, along human peripheral mononuclear blood cells, exerts antioxidant effects also in human polymorphonuclear cells, erythrocytes and bovine endothelial cells. This is accomplished by decreasing concentrations of superoxide anions and free oxygen radicals [18].

In the literature, flavonoids (Diosmin-hesperidin) are known to protect tissue and cells efficiently against free oxygen radicals [8]. In one study the protective effect has been shown to be caused by clearance of tissues from free radicals and other oxidized products with the effect of this drug [12]. This drug is also protective against neutrophil-mediated injury of venous wall [2]. It is also reported that this drug inhibits tumor growth and promotes apoptosis in patients with colon cancer [26]. Though flavonoids are known to protect tissue and cells against free oxygen radicals as calcium dobesilate, we have not shown regulation effect of flavonoids on apoptosis in our study. An experimental study [36] showed that; chronic elevation of venous pressure is associated with an inflammatory reaction in venous valves, a process that may lead to their dysfunction, reflux and upstream elevation of venous pressure. These effects are mitigated by the anti-inflammatory flavonoids (MPFF) in a dose dependent manner (50 mg/kg/day, and 100 mg/kg/day). According to this study, MPFF dose (1000 mg/day) may be insufficient for inhibition of lipid peroxidation and protection against occurrence of apoptosis-mediated varicose veins in our study.

Formation of lipid peroxidation, free oxygen radicals and superoxide anions has an important part in the initiation and progression of venous wall injury [17]. Free oxygen radicals by acting through myeloperoxidase and oxidases binding to cell membrane, activates neutrophils which in turn cause structural and functional injury of cell [9, 17, 35]. In our study prepared with the scope of instructions given in etiopathogenesis of varicose veins, demonstration of bcl-2 expression in group 3 patients using calcium-dobesilate for varicose veins compared to patients in group 1 is important. Finding this expression as statistically significant ($p = 0.023$) also might be related with protective role of calcium-dobesilate

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against cellular injury. Decrease in cellular injury and dysregulation of apoptosis may result in sustained cellular viability as a consequence of bcl-2 expression. This effect of calcium dobesilate may be caused by decrease in lipid peroxidation and clearance of free oxygen radicals and superoxide anions in tissue. Therefore we think that regulation of regulatory proteins in apoptosis might in turn maintain normal apoptotic process. We consider that regulated apoptosis has a protective effect against development and progression of primary varicose veins.

In conclusion, we consider dysregulated apoptosis may be play a role an important factor in the etiopathogenesis of varicose veins. While considering mitochondrial apoptotic pathway (intrinsic) as a primary step for dysregulated apoptosis, we believe that a dysregulation in any step of apoptosis might trigger development of varicose veins. The results of our study suggest that use of calcium dobesilate in patients with varicose veins and with the risk of developing varicose veins might have the protective potential against occurrence of apoptosis-mediated varicose veins.

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Correspondence address

Erkan Iriz, MD
Cardiovascular Surgery
Gazi University Medical School
Senlik Mah. Sedefciler Sok. No. 46/10
TR-06500 Ankara, Turkey
E-mail: erkaniriz@gmail.com

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