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Generation of plasmatic oxidative damages in patients with chronic venous insufficiency

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Chronic venous insufficiency (CVI) is a chronic human disease, represents an important social and health problem. The aim of the present study is to investigate and compare: 1) the plasmatic levels of endogenous antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx_1)); 2) lipid peroxidation (malondialdehyde (MDA)) levels; and 3) ongoing oxidative stress damages in patients at different stages of Chronic Venous Insufficiency (CVI) (clinical classification as CEAP, C2- C4 stage) and in CEAP-C2 patients, complicated by Type 2 diabetes mellitus (Type-2 DM), compared to healthy volunteers.

The investigation was conducted in Medical Faculty in collaboration with University Hospital of Stara Zagora, Bulgaria. In the current study were included 25 healthy volunteers (as control group, **CG**) and 52 diagnosed CVI patients, divided into two groups: 1) **CVI group**- n=32 (clinical classification as CEAP, C2- C4 stage); 2) **CVI+ Type-2 DM group** – n= 5 with Type 2 diabetes mellitus. Written informed consent was obtained from all the subjects. All patients were investigated for plasmatic GSH and GPx_1 levels, SOD, CAT and MDA profile.

Plazmatic GSH and GPx_1 activity was no statistically significantly decreased in CVI patients and statistically significantly decreased in CVI+Type 2 DM compared to CG (p<0.05), indicating induced OS. Lipid peroxidation levels were statistically significantly increased in CVI+Type 2 DM patients (p<0.05). SOD and CAT activities were statistically significantly decreased in CVI (p<0.05) and CVI+Type 2 DM (p<0.05), compared to the non-diabetic CG.

Increased MDA levels and decreased GSH, Gpx_1, SOD and CAT activities observed in CVI and CVI+ Type-2 DM patients suggest that OS plays an important role in the pathogenesis of varicose vein damages.

Key words: CVI, CVI+Type-2-DM, pathogenesis, OS.

INTRODUCTION

Chronic venous insufficiency (CVI) is a chronic human disease known since antiquity. This human disease represents an important social and health problem in industrialized countries and affects the quality of life [1-4]. CVI clinically manifests with various stages of varicose vein, associated with specific pathogenetic changes such as: 1) venous dilation et elongation; 2) deformity; 3) valvular venous incompetence; 4) blood stasis; 5) venous hypertension and hypoxia [1, 5]. Martinez-Zapata *et al.* [6] noticed that in patients with CVI, veins were unable to blood transport directly to the heart. Also, it was registered that patients with CVI problem were impossible to regulate the temperature and hemodynamic reserve. The different stages of CVI are determined by specific clinical manifestations: pain, paraesthesia, spasms, numbness, edema, varicose veins, skin pigmentation, varicose wounds and skin atrophy signs [6]. The CVI usually treatment depends on the progression of the disease and includes non-invasive and invasive [6, 7] methods.

Recent years experimental and clinical data have shown that highly toxic reactive oxygen species (ROS) is the one of the primary factors stimulating vascular disorders and changes in vascular endothelial cells, particularly in hypertension, diabetes, and several cardio-vascular disease [8, 9]. Increased ROS production and redox-signaling acted as pathophysiological role in endothelial damages or inflammation, and consequently on endothelial cell migration and angiogenesis [10]. Smith et al. in their group of patients discovered that the ROS generated metabolites cause neutrophils and monocytes activation in the vein wall and leads to endothelial damage [11]. In addition, increased oxidative stress and destructive ROS oxidations contribute to the chronic pathological damages in veins: progression of valvular incompetence and altered plasmatic stress levels [4, 12-13]. Plasma and blood cells interacting with all organs and tissues in the body reflect the exact oxidative status of the patient. Plasma samples contains sources of reactive metabolites produced by damaged venous tissue and activated inflammato ry cells [4, 14].

The enzymatic and non-enzymatic antioxidant systems were used in the body to neutralize the toxic damages caused by increased free radicals

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production. To enzymatic systems were referred glutathione (GSH), glutathione peroxidase (GP_X), superoxide dismutase (SOD), catalase (CAT), and to non-enzymatic: water and fat-soluble vitamins [15]. Moreover, in chronic inflammatory processes, the antioxidant and pro-oxidant systems balance is impaired, and this may lead to an increase plasma lipid peroxidation and reduces antioxidant activity [15]. Several contemporary studies were focused mainly on antioxidant enzyme systems and direct determination of malondialdehyde (MDA) and uric acid (UA) concentration levels. These studies confirm that elevated ROS increase oxidative stress parameters in plasma and specific varicose vein segments [4, 12, 16-17].

The aim of the present study is to investigate and compare the ongoing oxidative stress damages in patients at different stages of Chronic Venous Insufficiency (CVI) (clinical classification as CEAP, C2- C4 stage) and in CEAP-C2 patients, complicated by Type 2 diabetes mellitus (Type-2 DM), compared to healthy volunteers. For this purpose, we studied (1) the plasmatic activity of endogenous antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx1)); and 2) the end products levels of lipid peroxidation, measured by malondialdehyde (MDA), as an oxidative stress biomarkers.

EXPERIMENTAL MATERIALS AND METHODS

Patient and study design

In this study were included 37 patients (32 women and 5 men) with CVI, aged range between 23–60 years. All patients were CVI diagnosed and considered for varicose vein operation after a thorough physical examination and Doppler ultrasound (ESAOTE, LCD- 0051, 50-60 Hz EIZO NANAO Corp. 2011, Italy) of the venous system of the lower extremity. The CVI patients demonstrated venous reflux in the greater saphenous vein: 60% with C4 stage and 40% with C2 stage. The operations were performed at the Clinic of Vascular surgery, University hospital of Stara Zagora, Bulgaria in the period February 2017 to June 2018. Patients with other disease were excluded of the experiment. Patients were divided into 3 different groups according to the clinical severity of the CVI disease and the complications caused by type 2 diabetes: **1) Group 1** (**CVI**) consisted of n=32 patients with CVI clinical symptoms, each of them fell into C2 and C4 clinical stages, with varicose veins without registered concomitant diseases; 2) **Group 2** (**CVI + DM2**) consisted of n=5 patients with clinical symptoms of CVI, each of whom fell into, C2 and C4 clinical stages, with changes in the skin characteristics: pigmentation, lipodermatosclerosis, and complicated by type 2 diabetes mellitus (*disease duration up to 10 years*); 3) **Group 3** (control group, **CG**) consisted of n=25 healthy individuals (7 men and 18 women), aged 25–62, without any CVI signs and other concomitant diseases. All patients were with normal diastolic and systolic blood pressure (mmHg), nonsmokers, with similar weights and similar social class. The study procedure was approved by the *Ethical Committee of the Medical Faculty of Stara Zagora (No4)*, and informed consent was obtained from all the participants.

Blood collection and plasma preparation

Venous blood (3.0 mL) was collected from CVI, CVI+DM2 and healthy donors in vacutainer tubes (ACT-2, Germany) containing K_2EDTA (1.5 mg/ 1ml) and after 25 minutes, the tubes were centrifuged at 4000 rpm for 10 min at 4°C. 12 hrs fasting samples were collected during the operation procedure. 1 ml of plasma samples was separated and stored at –20°C until further assay was done.

Reagents and apparatus

All the reagents used in this study (thiobarbituric acid (TBA, 85%); ELISA commercial kits (Sigma Aldrich Company, Catalog No. CS0260, 2–8°C; and CS0271, 2–10°C, respectively)) were of analytical grade and purchased from the Sigma Aldrich Company. The biochemical analyses were performed at UV–VIS spectrophotometer-400 (TERMO Sci., RS232C, Stratagene, USA) and ELISA spectrophotometer (Uриd-660 A, Germany).

Biochemical plasma oxidative stress biomarkers

Reduced Glutathione (GSH) levels. Plasma GSH was assessed by ELISA commercial kit (Sigma Aldrich Company, Catalog No- CS0260, 2–8^0C), according to the method of Akerboom and Sies [18]. The GSH level of the plasma samples cause a continuous reduction of 5.5'-dithiobism (2-nitrobenzoic acid) (DTNB) was expressed as nanomoles of GSH per milliliter of protein.

***Glutathione peroxidase (GPx_1) levels*.** Plasma GPx_1 levels was assessed according ELISA commercial kit (Sigma Aldrich Company, EH0826, C6-323 Biolake, 1–4°C) [19]. The GPx_1 concentration expressed as ng glutathione peroxidase per milliliter of protein.

Catalase (CAT) activity. Catalase activity was assessed by using method described by Aebi H.,

1984 [20]. The activity was expressed as units per gram of hemoglobin.

***Superoxide dismutase (SOD) activity*.** Plasma SOD activity was assessed by using method described by Sun *et al.* [21]. The activity was expressed as units per gram of hemoglobin.

***Determination of malondialdehyde (MDA) concentration*.** Plasma MDA concentration, one of the final products of lipid peroxidation, was measured as a TBA acid (85%) assay by the method of Plaser et *al.* [22] which measure the MDA concentration in nmoles.

Statistical analysis

Statistical analysis was performed using Statistica 8.0, Stasoft, Inc., one-way ANOVA. The data were expressed as means ± standard error (SE). To compare the results and to determine statistically significant of control group with the results of CVI and CVI+DM2 patients have been used unpaired Student *t*- test. To define witch group are different from each other we have used LSD post hoc test. The value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

At this stage, different studies have shown that increased free radicals concentration and OS, promote damages to endothelial cell integrity, and leads to pathological changes in the varicose venous wall structure. In addition, the oxidation of proteins, lipids and DNA aided by toxic oxygen molecules, resulting in increased CVI oxidative stress levels [5]. Krzyściak and Kózka [16] accentuated that the CVI- oxidative damages degree correlates with clinical stage of vein wall disease. It could be supposed that oxidative disorders lead to changes in collagen content, fiber mass and weakness of the venous wall [23]. Fowkes *et al.* [24] drew attention to the fact that the number of women with CVI is higher than in males, and this ratio decreases as the age increases. In our study we demonstrated that CVI women (32 participants) were more amenable to oxidative stress toxic effects than CVI men (only 5 in the study). Besides oxidative changes, pregnancy, stale life or concerns about cosmetic defects, it may be the cause of the prevalence of females in the experiment [24].

During the chronic venous insufficiency cells generated high concentration of ROS including hydroxyl radicals (•OH), superoxide anions (O^{2}–), and hydrogen peroxide (H_2O_2) could influence the endogenous antioxidant system. Higher ROS levels lead to secondary risk factors such as diabetes, hypertension, etc. [4]. Reduced Glutathione and GSH-dependent enzymes were first endogenous protective antioxidants systems that activate several mechanisms counteract oxidative vascular damages [25]. Harlan *et al.* [26] commented the increased damage in cultured endothelial cells was caused by GSH depletion. Shimizu *et al.* [27] suggest that GSH-antioxidant protective effects were the result of lipid hydroperoxides detoxification and reduced free radical formations.

Total plasma GSH levels measured in CVI patient were no statistically significantly decreased compared to the CG ($p<0.03$, *t*-test). In patients with CVI+Type-2 DM the GSH levels were statistically significantly decreased, compared to the control ones ($p<0.000$, *t*-test), (**Fig 1**). In patients with CVI+Type-2 DM were statistically significantly decreased compared to the CVI patients and ($p<0.004$, *t*-test). There was statistically significantly difference in plasma GSH levels between two CVI groups ($p<0.05$, LSD).

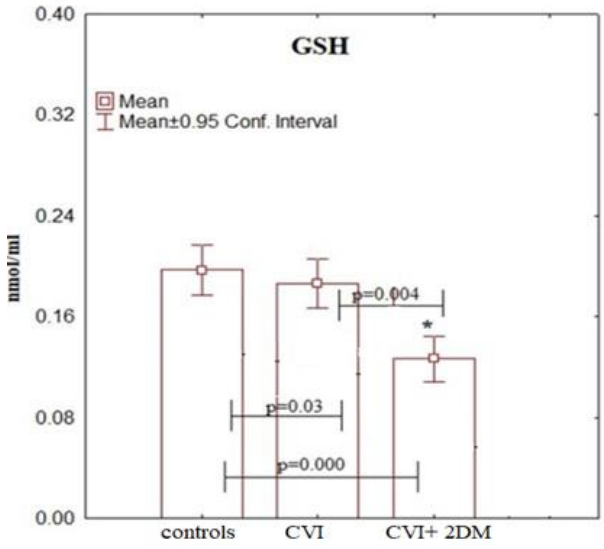

Figure 1. GSH activity in patients with CVI and CVI+Type-2 DM (n=37) (t-test; LSD post hoc); *considered statistically significant difference between control vs CVI groups, CVI+Type-2 DM ($p<0.05$); **considered statistically significant difference between CVI and CVI+Type-2 DM ($p<0.05$).

Franco *et al.* reported the lower levels of oxidized GSH are difficult to be measured in plasma [28]. In agreement with our results, Budzyn *et al.*, Horesca *et al.*, and Condezo- Hoyos *et al.* [4-5, 17] reported that uric acid and reduced GSH did not show differences between CVI patients and control group.

The superoxide radical ($\cdot O_2^-$) is easily converted into H_2O_2 and affects blood cells. Jonhson *et al.* [29] determine that three enzymes were involved in the blood cells oxidative protection: glutathione peroxidase-1 (Gpx_1), peroxyderoxin-2 ($Prdx_2$) and

catalase. Gpx_1 enzyme except that eliminated endogenous H_2O_2, also protect blood cells against organic peroxides [29, 30].

Glutathione peroxidase-1 content measured in plasma was statistically significantly decreased in both CVI (p=0.03, t-test) and CVI+ Type-2 DM (p=0.000, t-test), compared to the controls (**Fig 2**). In patients with CVI+ Type-2 DM significantly lower levels of Gpx_1 compared to CVI in plasma were also found (p=0.000, t-test). There was statistically significantly difference in plasma Gpx_1 levels between two CVI groups (p<0.05).

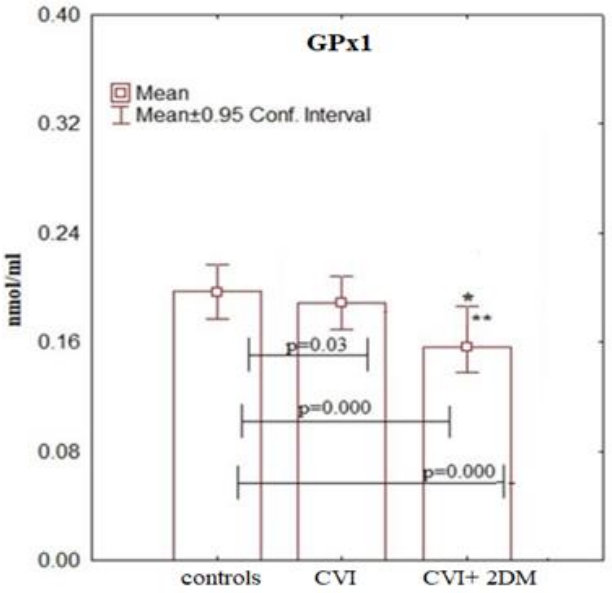

Figure 2. GPx_1 activity in patients with CVI and CVI+Type-2 DM (n=37); (t-test; LSD post hoc); *considered statistically significant difference between control vs CVI groups, CVI+Type-2 DM (p<0.05); **considered statistically significant difference between CVI and CVI+Type-2 DM (p<0.05).

Decreased Gpx_1 levels probably could be explained by the low GSH content, in patients with CVI+Type-2 DM and the inactivation of antioxidant enzyme systems and severe oxidative stress changes occurring in the body. The GSH and Gpx_1 reduced activity found in our study could be considered as an ineffective adaptation of the endogenous antioxidants [31] to increased superoxide anion ($\cdot O_2^-$) concentration, produce by varicose veins [32] and type 2 diabetes. Similarly, decrease in intracellular GSH and Gpx_1 levels in patients with type 2 diabetes was reported previously by [31].

Regarding to antioxidant enzyme systems, SOD and CAT are basic enzymes responsible for hydrogen peroxide catabolism, key agent for ROS activation. The plasmatic CAT activity (**Fig. 3a**) was also statistically significantly decreased in the two CVI groups compared to the CG: for CVI vs CG $p \leq 0.000$, t-test; for CVI+ Type-2 DM vs CG (8723.77 ± 164.3 vs. 30027.81 ± 129.6, IU/gHb, $p \leq 0.000$, t-test). There was statistically significant difference between CVI and CVI+ Type-2 DM groups, p<0.05, LSD post hoc test.

The SOD activity (**Fig. 3b**) was statistically significantly decreased in the CVI group (CVI 750.42±41.86 vs. control: 1130±23.61, p=0.000, t-test) and in the CVI+Type-2 DM (CVI+ Type-2 DM 371.07±51.49 vs. control 1130±23.61, p<0.000, t-test), compared to CG. Moreover, a statistically significant two-fold decrease was observed in plasma SOD activity in CVI+ Type-2 DM group (CVI+ Type-2 DM 371.07±51.49 vs. control 1130±23.61, p<0.000, t-test) as compared to healthy subjects There was statistically significant difference between two CVI groups (p<0.05) and CVI groups and controls (p<0.05).

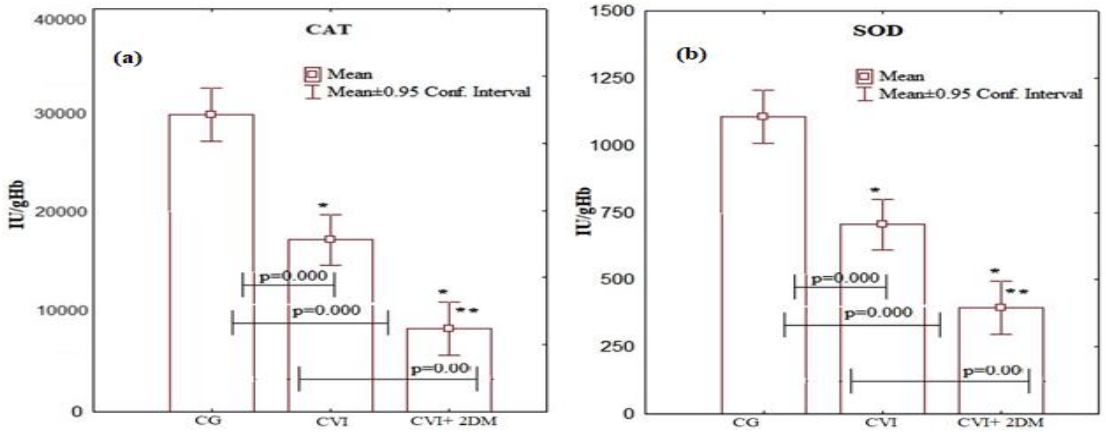

Figure 3. Plasma SOD and CAT activity in patients with CVI and CVI+Type-2 DM (n=37); (t-test, LSD); *considered statistically significant difference between control vs CVI groups (p<0.05); **considered statistically significant difference CVI and CVI+Type-2 DM (p<0.05).

Significantly decrease on plasmatic SOD and CAT concentration in CVI patients and CVI+ Type-2 DM group are consistent with data have demonstrated by Condezo-Hoyos *et al.*, and Budzyń *et al.* [4-5]. In addition, our results indicates statistically significantly around two-fold decrease the levels of the endogenous antioxidant enzymes SOD, CAT, GSH, Gpx_1 in the CVI+ Type-2 DM group ($p<0.05$). These findings indicate that CVI+Type-2 DM abruptly exhaust the antioxidant enzyme system probably due to increased oxidative stress in B- cells. The elevated glycemic parameters in diabetic overweight CVI patients probably activated additional free radical formation and an increase in antioxidant/ pro-oxidant imbalance and oxidative disturbances. Several studies also reported decreased activity of the antioxidant enzymes in Type-2 DM [33, 34].

In contrast, lipid peroxidation levels (**Fig. 4)** were statistically significantly increased in the two CVI groups compared to the CG: for CVI vs CG (11.17±0.74 vs. 9.14±0.3 CG, IU/gHb, $p\leq0.003$, *t*-test), *t*-test; for CVI+Type-2 DM vs CG (13.517±0.94 vs. 9.14±0.3 CG, IU/gHb, $p\leq0.004$, *t*-test). There was statistically significant difference in lipid peroxidation between two CVI groups $p<0.03$, LSD post hoc test.

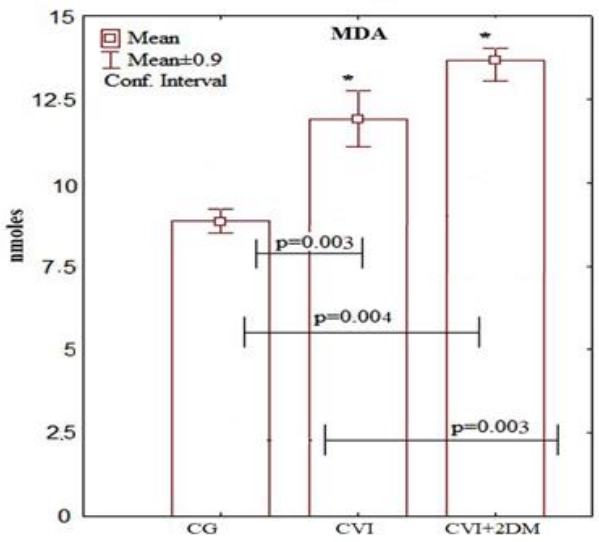

Figure 4. MDA levels in plasma in patients with CVI and CVI+Type-2 DM (n=37); (t-test, LSD post hoc); *considered statistically significant control vs CVI groups ($p<0.05$); **considered statistically significant CVI and CVI+Type-2 DM ($p<0.05$).

Statistically significantly lipid peroxidation increase has observed in both CVI groups in comparison to the healthy subjects. In parallel with this change, there was a decrease in the amounts of the endogenous antioxidants SOD, CAT, GSH, Gpx_1 during the same period. Moreover, the fact that CVI groups express statistically significantly MDA increase and decrease in endogenous enzymes as compared to control patients shows ongoing free radical oxidation and lipid peroxidation processes [35]. Similar results were observed by Condezo-Hoyos and colleagues [4], who detected an approximately double increase in MDA plasma levels in CVI (C2 stage) compared to healthy controls. Higher levels of lipid peroxidation was also reported in varicose veins homogenates and plasma by Krzyściak *et al.* [36], Budzyń *et al.*[5] and Shetty *et al.* [37], especially among women.

CONCLUSION

Oxidative stress processes were demonstrated in CVI and CVI+Type 2DM Bulgarian population using spectrophotometry and ELISA methods. It is important to note that increased lipid peroxidation and decreased endogenous GSH, Gpx_1, SOD and CAT activities observed in both CVI groups suggest that oxidative damages plays an important role in the pathogenesis of varicose vein damages. The antioxidant therapy application in the future, can lead to ROS and oxidative stress reduction in vain wall.

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***Ethical approval**: The study was approved by the Institutional Ethics Committee.*

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