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# Sulodexide counteracts endothelial dysfunction induced by metabolic or non-metabolic stresses through activation of the autophagic program

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**Abstract. – OBJECTIVE: Endothelial dysfunction (ED) predisposes to venous thrombosis (VT) and post-thrombotic syndrome (PTS), a long-term VT-related complication. Sulodexide (SDX) is a highly purified glycosaminoglycan with antithrombotic, pro-fibrinolytic and anti-inflammatory activity used in the treatment of chronic venous disease (CVD), including patients with PTS. SDX has recently obtained clinical evidence in the "extension therapy" after initial-standard anticoagulant treatment for the secondary prevention of recurrent deep vein thrombosis (DVT). Herein, we investigated how SDX counteracts ED.**

**MATERIALS AND METHODS: Human umbilical vein endothelial cells (HUVEC) were used. Metabolic and non metabolic-induced ED was induced by treating with methylglyoxal (MGO) or irradiation (IR), respectively. Bafilomycin A1 was used to inhibit autophagy. The production of reactive oxygen species (ROS), tetrazolium bromide (MTT) assay for cell viability, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay for cell apoptosis, Real-time PCR and Western blot analysis for gene and protein expression were used.**

**RESULTS: SDX protected HUVEC from MGO- or IR-induced apoptosis by counteracting the activation of the intrinsic and extrinsic caspase cascades. The cytoprotective effects of SDX resulted from a reduction in a) ROS production, b) neo-synthesis and release of pro-inflammatory cytokines (TNFα, IL1, IL6, IL8), c) DNA damage induced by MGO or IR. These effects were reduced when autophagy was inhibited.**

**CONCLUSIONS: Data herein collected indicate the ability of SDX to counteract ED induced by metabolic or non-metabolic stresses by involving the intracellular autophagy pathway. Our experience significantly increases the knowledge of the mechanisms of action of SDX against ED and supports the use of SDX in the treatment of CVD, PTS and in the secondary prevention of recurrent DVT.**

*Key Words:*
Sulodexide, Endothelial dysfunction, Venous thrombosis, Autophagy, Diabetes, Irradiation, Reactive oxygen species, Inflammatory cytokines.

## Introduction

Venous thrombosis (VT) is the most frequent, significantly mortal and morbid vascular disease[1] that contributes to a substantial economic

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burden[2]. Endothelial dysfunction (ED) has been shown to be the common denominator for the onset and recurrence of VT, and persistent ED has been shown to promote the post-thrombotic syndrome (PTS), a long-term disabling condition that occurs as a result of DVT[3]. Endothelial cells (EC), forming more than just a wallpaper, contribute to maintain normal vascular tone and blood fluidity by regulating: (1) systemic blood flow, (2) tissue perfusion, (3) recruitment and extravasation of pro-inflammatory leukocytes through the expression of cell adhesion molecules and the production of cytokines, (4) activation of platelets and the coagulation cascade and (5) the recanalization of obstructive fibrin clots that permits reconstitution of blood flow and prevents recurrent VT[4]. Their alteration triggers a chronic inflammation associated with an increase in vasoconstrictor and pro-thrombotic products leading to ED and then inducing VT[5,6]. In particular, the aberrant generation of reactive oxygen species (ROS) has been recently shown to reduce the vascular tone, increasing total peripheral resistance and promoting hypertension, and to induce the endothelial activation that finally triggers a local inflammatory and immune response responsible for ED[6-8]. In this context, ROS have been shown to damage many components of the vascular wall such as the endothelial glycocalyx, whose destruction has been shown to trigger ED[9-11]. The endothelial glycocalyx is a network of membrane-bound proteoglycans, glycosaminoglycan and glycoproteins synthetized by EC that, by covering the endothelium luminally, regulates nitric oxide-dependent vasorelaxation, controls vascular permeability, attenuates leukocyte-vessel wall interactions, modulates the inflammatory and thrombotic state of the vascular wall, fulfilling a vasculoprotective role[3,7,8,11,12]. ROS, by acting as degrading factors, modify the glycocalyx proprieties and induce ED, which in turn perturbs the ability of EC to produce and maintain a functional glycocalyx. Thus, counteracting ROS production, pro-inflammatory cytokine synthesis and maintaining glycocalyx functions could represent strategic therapeutic opportunities to treat ED-related diseases such as VT, and to prevent the related recurrences. Sulodexide (SDX) is a natural highly purified mixture of natural glycosaminoglycans, composed by 80% fast-moving heparin (6-8000 Dalton) and 20% dermatan sulfate, which exhibits antithrombotic and pro-fibrinolytic activities and affects normal hemostasis to a lesser extent than heparin with a very low risk of bleeding[13,14]. SDX exerts its actions by reconstructing the glycocalyx[15-17], modulating the coagulation cascade[13,14,18] and preventing the release of pro-inflammatory cytokines and metalloprotease from white blood cells[13,19]. Because of these properties, SDX is largely used in chronic venous disease (CVD)[13,14,17] including patients with post-thrombotic syndrome (PTS)[17,20], and it has recently obtained important clinical evidence in the "extension therapy" after initial-standard anticoagulant treatment for the secondary prevention of recurrent DVT[21-24]. In this paper for the first time we investigated the ability of SDX to prevent ED induced by methylglyoxal (MGO), a diabetes-related metabolite, or by ionizing radiation (IR), both shown to promote ED[25,26] and predispose to VT[27,28]. In these "*in vitro*" models we showed for the first time that SDX counteracted the accumulation of ROS, the production of pro-inflammatory cytokines such as tumor necrosis factor-alfa (TNF-α), interleukins 1 (IL-1), 6 (IL-6) and 8 (IL-8), thereby protecting EC from stress-induced DNA damage and apoptosis-mediated death.

## Materials and Methods

### Cell Culture and Treatments

Human umbilical vein endothelial cells (HUVEC) (Clonetics™, San Diego, CA, USA) were cultured as already described[26]. SDX (0.1 to 5 mg/L) was provided by Alfasigma S.p.A. (Bologna, Italy), MGO (400 mM)[29] and BafA1 (50 nM)[30] by Sigma-Aldrich (Milan, Italy). Radiation was delivered as previously described[26]. The dose rate was approximately 1.3 Gy/min and the applied dose was 4 Gy. The absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany). Briefly, for the experiments HUVECs were seeded in a 96-well microplate at an appropriate density of cells/well and then either treated with SDX at the concentrations indicated in the figures or pretreated with SDX and then treated with MGO or IR. Subsequently, the plates were incubated at 37°C for the times indicated in the figures or figure legends.

### Measurement of Cell Viability, Apoptosis, Caspase Activity, Superoxide Anion Production and Cytokine Secretion

MTT (ab211091) and TUNEL (ab66108) assays from Abcam (Cambridge, MA, USA), were used to measure cell viability and apoptosis, respec-

tively. ROS production was assessed by using the Chemiluminescence Superoxide Anion Assay Kit (CS1000) from Sigma-Aldrich (Milan, Italy). Caspase-Glo®-3/7 (G8090), -8 (G8200) and -9 (G8211) assay from Promega Corporation (Madison, WI, USA), were used to measure Caspase 3, 8 and 9, respectively. The levels of TNF-a, IL-1, IL-6 and IL-8 were measured by using Quantikine ELISA Kit from R&D Systems Inc. (McKinley Place NE, MP, USA). At the end of the experiments, the absorbed dose was measured using a Duplex dosimeter (PTW, Freiburg, Germany). The optical density value was reported as the percentage of variable measured in relation to the control group.

### Gene Expression Analysis

Real-time reverse transcription polymerase chain (RT-PCR) was performed as already described[31]. Total ribonucleic acid (RNA) was prepared using the RNeasy kit (Qiagen, Valencia, CA, USA) and reverse transcribed into cDNA (complementary deoxyribonucleic acid) by means of the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative Real-time PCR was performed on an ABI 7900HT system using SYBR- Green Mastermix (SuperArray, Frederick, MD, USA). PCR products were verified by melting curves and were run on a 2% agarose gel to confirm the appropriate size. The threshold cycle (CT) values for each gene were normalized to expression levels of β-actin, as already described[31]. The following primers were used: β-actin: FW-5'-AGAAAATCTGGCACCACACC-3', RW-5'-AGAGGCGTACAGGGATAGCA-3; IL-1: FW-5'-CAGGATGAGGACATGAGCAC-3', RW-5'-CTCTGCAGACTCAAACTCCA-3'; IL-6: FW-5'-TTCGGTACATCCTCGACGGC-3', RW-5'-ACCAGAAGAAGGAATGCCCAT-3'; IL-8: FW-5'-TCCTGATTTCTGCAGCTCTGTG-3', RW-5'-GTCCAGCAGAGCTCTCTTCCAT-3'; TNFa: FW-5'-TTGACCTCAGCGCTGAGTTG-3', RW-5'-CCTGTAGCCCACGTCGTAGC-3'; beclin-1: FW-5'-ACCGTGTCACCATCCAGGAA-3', RW-5-GAAGCTGTTGGCACTTTCTGT-3'.

### Protein Expression Analysis

Immunoblotting was conducted as previously described[32-35] with the following antibodies: anti-H2A histone family member X (H2AX, clone M-20), anti-phospho-H2AX (g-H2AX, clone 3C10), anti-sequestosome 1 protein (SQSTM1/p62, clone D3), beclin 1 (BECN1, clone H300) and a-tubulin (clone B7) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (Bethyl Laboratories Inc., Montgomery, TX, USA) were used for enhanced chemiluminescence (GE Health Life Sciences, Piscataway Township, NJ, USA) detection. Signals from protein bands were digitally acquired and quantified using the Chemidoc XRS system (Bio-Rad, Hercules, CA, USA).

### Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (SPSS software version 12.0.1; SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± standard deviation (SD) of triplicate determinations, with $p < 0.05$ considered statistically significant.

## Results

### Sulodexide Protects HUVEC from Methylglyoxal- or Irradiation-Induced Apoptotic Cell Death by Increasing Autophagy

Increasing doses of SDX (in a range between 0.1 to 5 mg/L) did not affect HUVEC viability (Figure 1A) or induce cell death (Figure 1B). The maximum concentration of SDX found in the plasma of treated patients in a steady state condition (1.5 mg/L)[13] was used for the following experiments. SDX significantly counteracted the reduction in cell viability (Figure 2A) and increase in apoptosis (Figure 2B) induced by MGO or IR. These cytoprotective effects started 48 hours after SDX pre-treatment and reached maximum efficiency in 72-hour pretreated cells (Figure 2A and B, SDX+MGO *vs*. MGO and SDX+IR *vs*. IR). In 72-hour pretreated cells, SDX increased cell viability by 41% ± 6 and 53% ± 8 (Figure 2A) and reduced apoptosis by 49% ± 7 and 57% ± 9 (Figure 2B) in MGO and IR treated cells, respectively. No statistically significant differences were described in 12- or 24-hour SDX pretreated cells (Figure 2A and 2B). 72 hours of SDX pretreatment was used for the following experiments. SDX restrained MGO-induced activation of caspase 8 and caspase 3 by 44.3% ± 8.2 and 43% ± 6, respectively, and IR-induced activation of caspase 8, caspase 9 and caspase 3 by 32% ± 8, 52% ± 9 and 39% ± 9, respectively (Figure 2C). SDX upregulated Beclin 1 and downregulated p62 basal

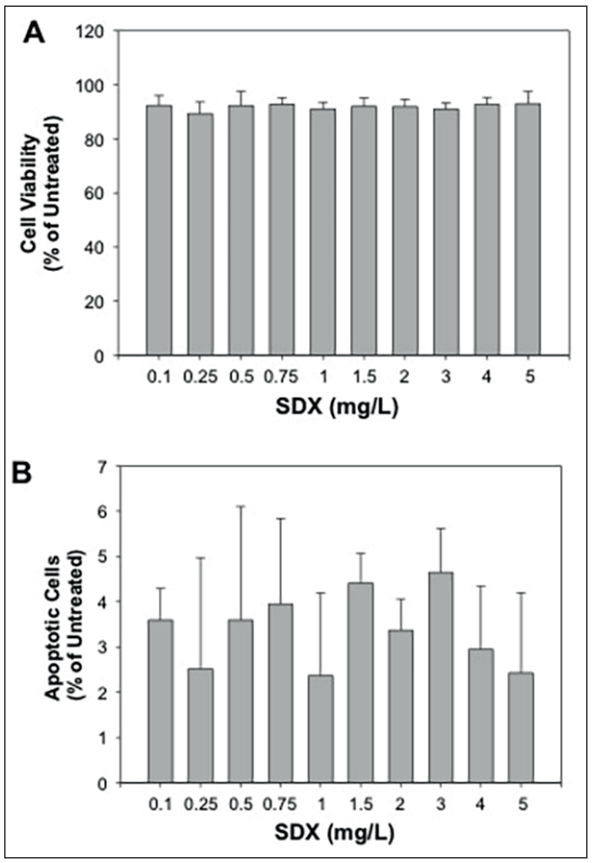


**Figure 1.** Effects of increasing doses of SDX on HUVEC viability and apoptosis-mediated cell death. Dose-dependent effect of SDX on viability ***(A)*** and apoptosis ***(B)*** of HUVEC after 72 hours of treatment. Cell viability was measured by MTT and apoptosis by TUNEL assay. Results are representative of three independent experiments ±SD.

protein expression (Figure 3A, SDX). MGO and IR significantly downregulated Beclin 1 protein expression levels while MGO, but not IR, significantly increased p62 expression (Figure 3A, MGO or IR). MGO and IR treatment counteracted the upregulation of Beclin 1 induced by SDX (Figure 3A, SDX+MGO or SDX+IR). Treating cells with BafA1 counteracted the cytoprotective effects of SDX (Figure 3B and 3C). The presence of BafA1 reduced the ability of SDX to counteract the MGO- or IR-induced reduction in cell viability by 42.8% ± 6 (Figure 3B, BafA1+SDX+MGO *vs*. SDX+MGO) and 55.2% ± 7 (Figure 3B, BafA1+SDX+IR *vs*. SDX+IR) and in apoptosis by 71.5% ± 8 (Figure 3B, BafA1+SDX+MGO *vs*. SDX+MGO) and 74.6% ± 11 (Figure 3B, BafA1+SDX+IR *vs*. SDX+IR). BafA1 itself significantly reduced HUVEC viability by 28.3% ± 7 (Figure 3B, BafA1 *vs*. Untreated) and increased the percentage of apoptotic cells by 18.5% ± 7 (Figure 3A, BafA1 *vs*. Untreated).

### *Sulodexide Counteracts Reactive Oxygen Species, Pro-Inflammatory Cytokine Release and DNA Damage Induced by MGO or IR by Involving the Autophagy Mechanism*

SDX counteracted MGO-induced ROS production by 33% ± 6 (Figure 4A, SDX+MGO *vs*. MGO) and IR-induced ROS production by 58% ± 9 (Figure 4A, SDX+IR *vs*. IR). These effects were significantly nullified by pretreating cells with BafA1 (Figure 4A, BafA1+SDX+MGO *vs*. SDX+MGO and BafA1+SDX+IR *vs*. SDX+IR). SDX reduced the neo-synthesis (Figure 4B) and release (Figure 4C) of pro-inflammatory cytokines induced by MGO (TNF-α 81% ± 13, IL-1 68% ± 11, IL-8 43% ± 12, Figure 4B, SDX+MGO *vs*. MGO) or IR (TNF-α 52% ± 4, IL-1 37% ± 8, IL-8 52% ± 9, Figure 4B, SDX+IR *vs*. IR). SDX reduced the MGO- or IR-induced release of TNF-α by 67% ± 12 and 22% ± 4, IL-1 by 38% ± 9 and 49% ± 3, and IL-8 by 34% ± 5 and 41% ± 6 (Figure 4B, SDX+MGO *vs*. MGO and SDX+IR *vs*. IR). Although SDX reduced IL-6 gene expression and release induced by IR by 38% ± 7 and 59% ± 7, no significant effects were described in MGO-treated cells (Figure 4B and 4C, SDX+MGO *vs*. MGO and SDX+IR *vs*. IR). BafA1 restrained the effects of SDX, restoring the gene expression and release of TNF-α, IL-1, IL-6 and IL-8 induced by MGO (Figure 4C, BafA1+SDX+MGO *vs*. SDX+MGO) or IR (Figure 4B, BafA1+SDX+IR *vs*. SDX+IR). DNA damage, investigated by assessing the phosphorylation of H2AX at Ser 139 (γ-H2AX), showed that SDX prevented the accumulation of γ-H2AX induced by MGO or IR, effects nullified by BafA1 treatment (Figure 5).

## Discussion

VT is increasingly recognized as an important cause of morbidity and mortality that drastically affects quality of life and productivity, causing a dramatic increase in healthcare costs[1,2]. Several pathophysiological risk factors have been related to VT and among them, ED is certainly one of the most important. Endothelium is a complex tissue and each component plays a key role in regulating vascular homeostasis[36] by controlling systemic blood

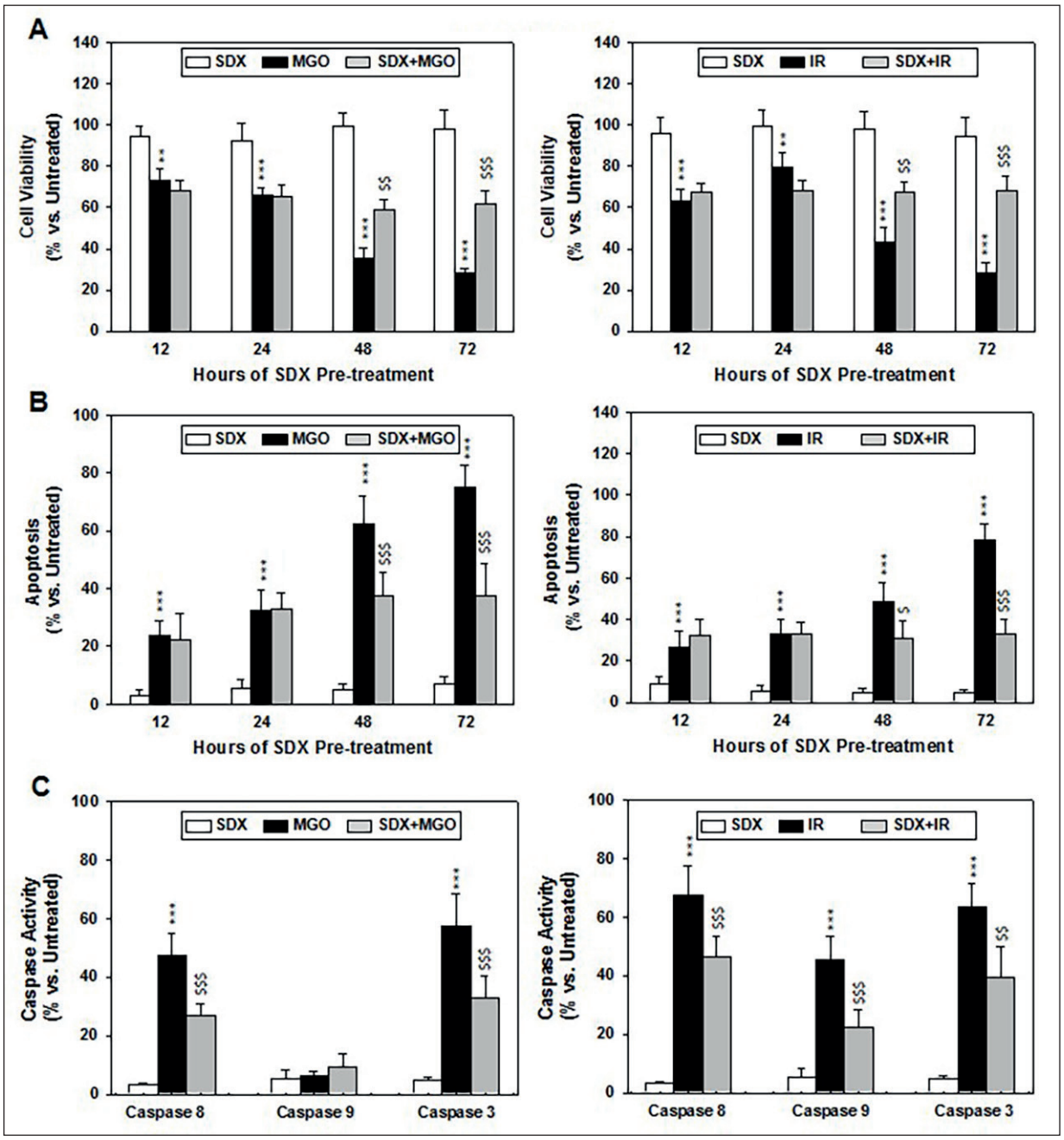


**Figure 2.** Effects of SDX in preventing MGO or IR reduction in cell viability and increase in apoptosis. HUVEC were pretreated with SDX for 12, 24, 48 or 72 hours and then treated with MGO or IR; cell viability and apoptosis were measured 72 hours later by MTT or TUNEL assay, respectively. ***(C)*** HUVEC treated with SDX for 72 hours and then treated with MGO or IR; Caspase 8, 9 and 3 activity were measured 24 hours later. Results are representative of three independent experiments ± SD. (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs.* Untreated, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ or $^{\$\$\$}p < 0.001$ *vs.* MGO or IR).

flow and tissue perfusion, the movement of fluid, ions and other macromolecules, controlling the recruitment and extravasation of pro-inflammatory leukocytes in response to tissue damage and participating in the blood coagulation system[4,36]. In this context, the glycocalyx, a membrane-bound mixture of proteoglycans, glycosaminoglycan and glycoproteins that luminally covers endothelium, has been shown to actively participate and play a key role in EC functions[9,10]. Thus, loss of the glycocalyx has been shown to promote ED, which in turn,

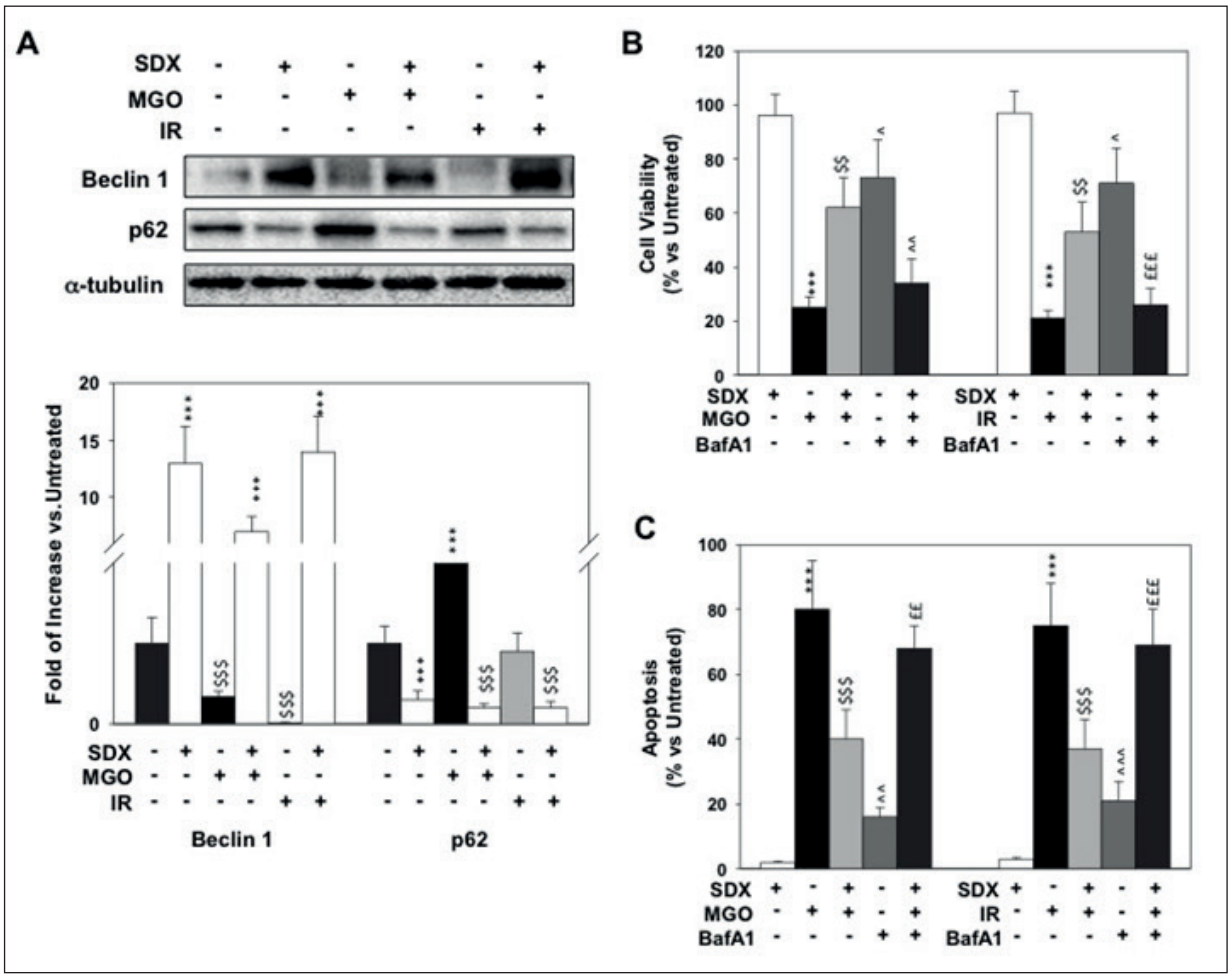


**Figure 3.** Effects of SDX in modulating the basal autophagic activity and its role in mediating SDX cytoprotective effects. ***(A)*** Cell lysates from HUVEC ± 72 hours of SDX ± MGO or IR were analyzed by immunoblotting with specific antibodies for indicated proteins; α-tubulin expression shows the loading of samples. Densitometric analysis of three independent experiments is reported below the blots (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs.* Untreated). ***(B*** and ***C)*** HUVEC were pretreated with SDX for 72 hours in the presence or absence of BafA1 and then treated with MGO or IR; cell viability and apoptosis were measured 72 hours later by MTT or TUNEL assay, respectively. Results are representative of three independent experiments ±SD. (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs.* Untreated, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ or $^{\$\$\$}p < 0.001$ *vs.* MGO or IR, $^{£}p < 0.05$, $^{££}p < 0.01$ or $^{£££}p < 0.001$ *vs.* SDX+MGO or SDX+IR).

by sustaining ROS production, establishes a self-sustaining vicious circle that supports and amplifies loss of the glycocalyx inducing blood cell recruitment and activation of an inflammatory status that finally promotes VT and related recurrences[3,5-8,37]. Thus, restoring the damaged glycocalyx represents a therapeutic strategy in counteracting VT. SDX is a highly purified glycosaminoglycan obtained from porcine digestive mucosa and it is composed of a mixture of 80% electrophoretically fast-moving heparin fraction with a molecular weight of about 7000 Da and affinity for antithrombin III, and 20% dermatan sulfate, with a molecular weight of 25,000 Da and affinity for the heparin II cofactor[13]. Due to its composition, SDX restores the vascular endothelial glycocalyx, shows an anti-thrombotic, profibrinolytic and anti-inflammatory action[3,13-19,38,39] and, for these reasons, it is used in the treatment of CVD[13,14,17], PTS[17,20] and in the secondary prevention of recurrent DVT[21-24]. Furthermore, other evidence suggests that SDX has an anti-oxidant action that counteracts ROS production[40,41] and a cell protection activity against cellular aging[42] and apoptosis[40,41]. However, although the anti-thrombotic,

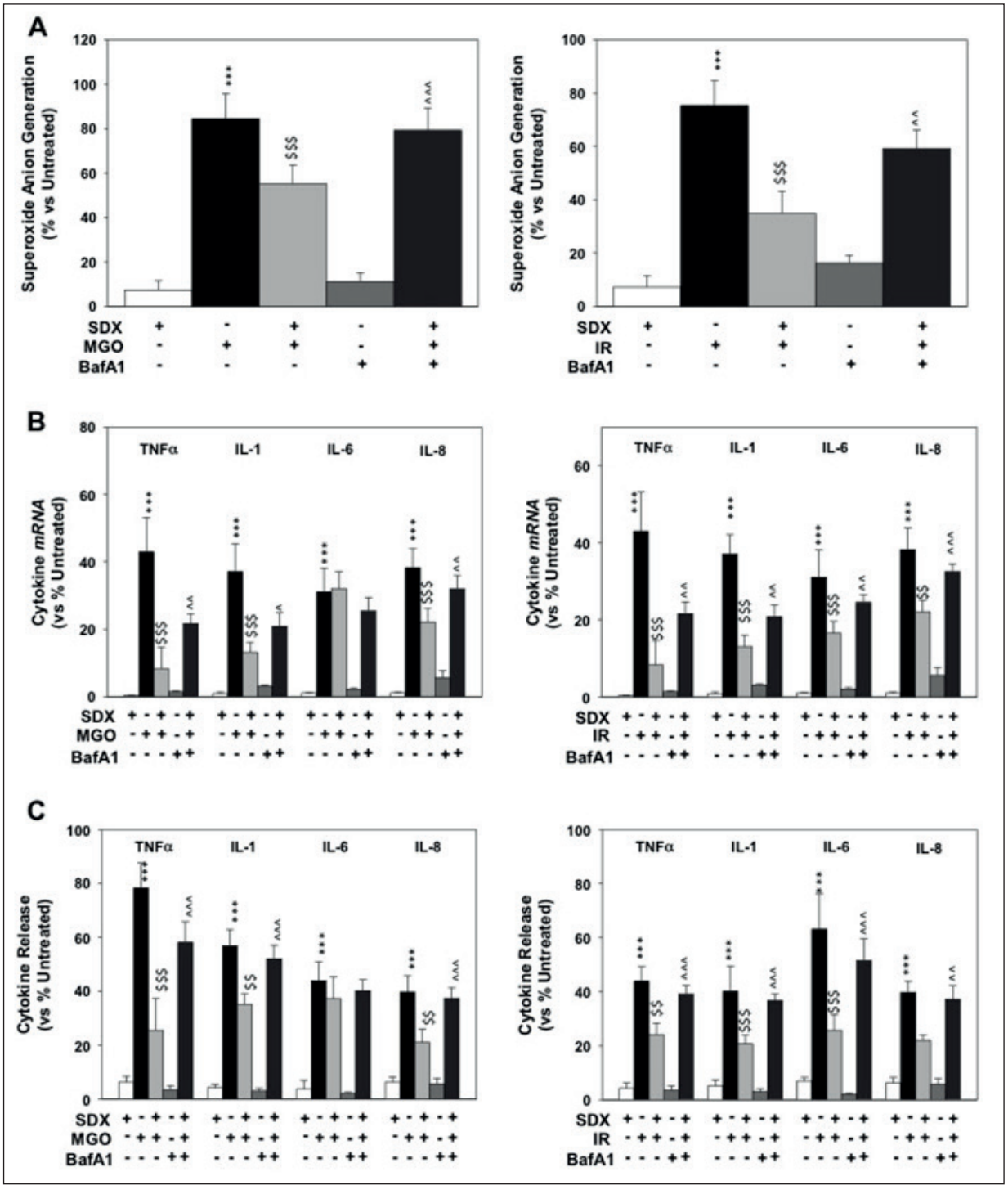


**Figure 4.** Effects of SDX and related induced autophagy in modulating MGO- and IR-mediated production of ROS, TNF-α, IL-1, IL-6 and IL-8. HUVEC were pretreated with SDX 72 hours in the presence or absence of BafA1 and then treated with MGO or IR; ***(A)*** ROS production, (***B)*** TNF-α, IL-1, IL-6 and IL-8 gene expression and ***(C)*** release were measured 24 hours later. Results are representative of three independent experiments ±SD. (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs.* Untreated, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ or $^{\$\$\$}p < 0.001$ *vs.* MGO or IR, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ or $^{\wedge\wedge\wedge}p < 0.001$ *vs.* SDX+MGO or SDX+IR, $^{£}p < 0.05$, $^{££}p < 0.01$ or $^{£££}p < 0.001$ *vs.* SDX+MGO or SDX+IR).

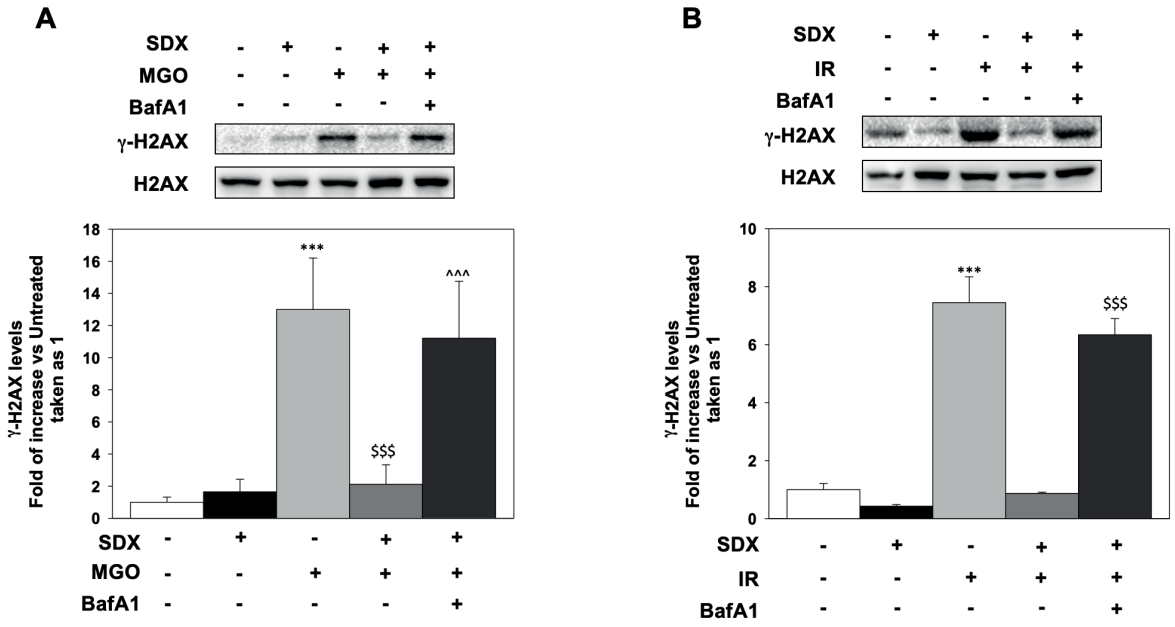


**Figure 5.** Effects of SDX and related induced-autophagy in preventing the MGO- or IR-induced DNA damage. Cell lysates from HUVEC ± 72 hours of SDX ± BafA1 ± MGO ***(A)*** or IR ***(B)*** were analyzed by immunoblotting with specific antibodies for indicated proteins; α-tubulin expression shows the loading of samples. Densitometric analysis of three independent experiments is reported below the blots (*$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ *vs.* Untreated, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ or $^{\$\$\$}p < 0.001$ *vs.* MGO or IR, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ or $^{\wedge\wedge\wedge}p < 0.001$ *vs.* SDX+MGO or SDX+IR).

profibrinolytic and anti-inflammatory abilities of SDX have been largely investigated, the molecular mechanisms involved are not completely understood nor is the ability of SDX to counteract both metabolic or non-metabolic stresses able to induce ED. Herein, we demonstrate for the first time that SDX prevented ED induced by metabolic and non-metabolic oxidative stresses such as MGO or IR, known to potentially promote VT[27,28], by restraining ROS production and pro-inflammatory cytokine release through activation of the autophagic program. Doses of SDX ten times higher than those physiologically found at the steady state condition in the blood of treated patients (1.5 mg/L)[13] did not induce any change in cell viability, confirming the extreme pharmacological safety of SDX. Starting with this data we have shown that after 2 days of daily treatment, SDX prevented stress-induced EC death, suggesting that the drug needed to promote significant changes accounting for its cytoprotective action. We found that the cytoprotection mediated by SDX depends on its ability to prevent stress-induced apoptosis-mediated cell death. Apoptosis occurs when cells are irreversibly damaged, through the activation of the extrinsic caspase 8/3- or intrinsic caspase 9/3-mediated cascades[43,44], known to be activated by MGO[45] and IR[26]. We found that SDX, already shown to prevent EC apoptosis induced by oxygen-glucose deprivation[41], also significantly counteracts the activation of the apoptotic program induced by MGO and IR. Thus, our data showed the cytoprotective effects of SDX indicating that this ability was independent from the metabolic or non-metabolic nature of the stress. Autophagy is a complex lysosomal catabolic process by which cells degrade or recycle their contents to maintain cellular homeostasis, adapt to stress, and respond to disease[46,47]. In particular, regarding EC, the literature indicates the cytoprotective effects of autophagy, the loss of which has been shown to be a central mechanism in inducing ED[47]. For example, in EC, shear stress-induced increases in NO production is markedly blunted in autophagy deficient cells and loss of autophagy promotes ROS-mediated ED and inflammatory cytokine production[46,47]. Our experiments revealed that SDX significantly upregulated the basal expression of Beclin-1, the main downstream effector of the autophagic pathway and

downregulated the expression of p62, the main inhibitor[48,49]. Thus, we supposed that the activation of the autophagic program by SDX has a cytoprotective function. Our hypothesis was demonstrated by using the autophagy inhibitor BafA1. BafA1 nullified the anti-oxidant and cytoprotective proprieties of SDX, confirming the hypothesis that increasing the intracellular endothelial autophagic flux could be one of the molecular mechanisms by which SDX protects EC and prevents ED. These data are consistent with recent studies that showed cardiovascular benefit from the upregulation of autophagy by some molecules[47,50,51]. When cells die they trigger an inflammatory response that participates in tissue repair but can also cause tissue damage; thus, inflammation contributes to the pathogenesis of a number of diseases[52]. Unlike healthy endothelium, ED promotes the release of significant amounts of cytokines that, by promoting and sustaining local chronic/systemic inflammation, finally increase the surface of ED and could predispose to VT[3,6]. SDX has been already shown to counteract inflammation in patients with chronic venous insufficiency by directly reducing the release of pro-inflammatory cytokines from EC[39,42]. However, our researches show for the first time in a ED cell model induced by metabolic and non-metabolic oxidative stresses, known to promote VT, that SDX: i) counteracted TNF-α, IL-1 and IL-8 neosynthesis and release induced by MGO or IR; ii) counteracted IL-6 neosynthesis and release induced by IR but not by MGO; iii) negatively modulated pro-inflammatory cytokine neosynthesis and release by activating the autophagic program. In particular, considering that TNF-α, IL-6 and IL-8 have been associated with an increased risk of DVT recurrence[53], our data support the possible role of SDX in the secondary prevention of recurrent DVT. Furthermore, the fact that SDX reduced the cytokine mRNA levels corroborate the previous hypothesis that this drug could induce significant intracellular changes, such as gene expression[54]. Moreover, the fact that in this "*in vitro*" model the anti-inflammatory action of SDX was found to be strictly correlated with authophagy confirms the key role of this program in mediating the cytoprotective effects of SDX. MGO and IR, directly or by inducing inflammation finally lead to cell death by causing DNA damage[55-57]. No data have been yet collected on the ability of SDX to protect the DNA from stress-induced damage and we found that SDX prevented phosphorylation of H2AX, known to be a specific marker for DNA damage[58].

## Conclusions

Although these data have been collected "*in vitro*", they clearly show the ability of SDX to protect EC from apoptosis and prevent ROS-mediated ED, counteracting both metabolic and non-metabolic toxic stresses potentially associated with an increased risk of VT. SDX seems to be able to block the vicious circle triggered by ROS and responsible for the chronic inflammation in venous disease. Our study increases the knowledge of the mechanisms of action of SDX against ED and supports the use of SDX in the treatment of CVD, PTS and in the secondary prevention of recurrent DVT.

### Conflict of Interest

Paolo Mattana is employed in Alfasigma. The other Authors declare that they have no conflict of interests.

### Acknowledgements

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### Ethics

This research did not include human and animal studies.

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