Introduction

Diabetes has been considered an independent serious risk factor for development of myocardial dysfunction *(Fein et al., ١٩٨٠ and Shiomi et al., ٢٠٠٣)*. Diabetic cardiovascular problems represent the leading cause of death in diabetic patients *(Aronson et al., ١٩٩٧ and Borges et al., ٢٠٠٦)*.

Diabetic metabolic dysregulation is characterized by reduction of glucose utilization and reliance most exclusively on fatty acids to generate energy, disturbed oxygen metabolism with increased production of oxygen radicals such as reactive oxygen species (ROS) *(Halliwell, ١٩٩١ and Bocci, ٢٠٠٥)*, lipid oxidation products (LOP) *(Leinonen et al., ١٩٩٧ and Schleicher et al., ١٩٩٧)* and insufficient antioxidants *(Szaleczky et al., ١٩٩٩ and Bocci, ٢٠٠٥)* altering oxidant/antioxidant balance and producing oxidative stress disorder *(Ye et al., ٢٠٠٣)*.

Diabetic chronic oxidative stress could possibly be among other factors the linking element between many diabetic complications *(Du et al., ٢٠٠٠ and Nishikawa et al., ٢٠٠٠)* mediates structural and functional cardiovascular derangement, impairment of

various intracellular signaling pathways *(Feuvray, ٢٠٠٤)* and dysfunction in cardiac myocytes *(Suematsu et al., ٢٠٠٢)* as well as neutrophils adhesion to endothelial cells *(Kokura et al., ١٩٩٩)*.

Antioxidants might represent a new addition to the measures used in diabetic patients for preventing oxidative stress complications *(Haidara et al., ٢٠٠٦)*. However, use of conventional exogenous antioxidants cannot rehabilitate cells to actively increase intra-cellular antioxidant capacity to achieve a therapeutic result in contrast to medical ozone therapy *(Salvemini et al., ١٩٩٩ and Bocci, ٢٠٠٥)*.

In the light of rising of numbers of cardiac interventions invoking transient ischemia then reperfusion, the incidence of reperfusion injury *(Zahler et al., ٢٠٠٠)* described at least in part as a misguided inflammatory response resulting from transient oxidative stress production of excess oxygen by-products or ROS *(Belke et al., ٢٠٠٠)* is gaining ever-increasing relevance for development of protective strategies.

However, beneficial effects of reperfusion therapies have been limited by ischemic damage that

occurs before reperfusion and during reperfusion injury *(Bucciarelli et al., ٢٠٠٦)*.

A powerful tool to render an organ resistant to subsequent severe ischemia and reperfusion is confronting it to brief episodes of ischemia or oxidative stress ascribed as "ischemic preconditioning" *(Ma et al., ١٩٩١; Schreck et al., ١٩٩١ and Zahler et al., ٢٠٠٠)*. Brief levels of oxidative stress produces transient levels of ROS which can reduce subsequent injury to reperfused vessels *(Akimitsu et al., ١٩٩٦)* and cardiomyocytes *(Zhou et al., ١٩٩٦)* after submitting them to ischemia and can reduce leukocytic adhesion to vascular wall *(Kubes et al., ١٩٩٨)*.

Also, transient levels of ROS that can confer protection or adaptation to oxidative stress and control diabetic complications *(Al Dalain et al., ٢٠٠١)* can be adopted by intermittent controlled ozone therapy ascribed as "oxidative preconditioning" *(Leon et al., ١٩٩٨ and Candelario et al., ٢٠٠١)* analogous to ischemic preconditioning *(Murry et al., ١٩٩٠)* which act by inducing a transient calculated oxidative stress by ROS and LOP that activate many cellular responses *(Viebahn, ١٩٩٩ and Bocci, ٢٠٠٥)*.

Aim of the work

The present study is designed to assess the possible cardiac protective effect of oxidative conditioning by medical ozone and ischemic preconditioning by femoral artery ligation, in streptozotocin-diabetes. Also, the consequence of ischemic insult is evaluated after short- and long-term preconditioning mechanisms.

Cardiovascular Disease In Diabetes

Diabetes is considered as a risk factor for cardiovascular disease *(Laakso, ١٩٩٩)* as well as for myocardial dysfunction *(Fein et al., ١٩٨٠)*. Diabetic cardiovascular problems represent the leading cause of death in diabetic patients *(Aronson et al., ١٩٩٧ and Borges et al., ٢٠٠٦)*.

Diabetes is characterized by increased production of oxygen radicals such as reactive oxygen species (ROS) *(Halliwell, ١٩٩١ and Bocci, ٢٠٠٥)* and lipid oxidation products (LOP) *(Leinonen et al., ١٩٩٧ and Schleicher et al., ١٩٩٧)*.

Diabetes is characterized by insufficient antioxidants *(Szaleczky et al., ١٩٩٩ and Bocci, ٢٠٠٥)* that alters oxidant/antioxidant balance *(Ye et al., ٢٠٠٣)* which could link between many diabetic complications *(Du et al., ٢٠٠٠ and Nishikawa et al., ٢٠٠٠)*.

Vascular tissue damage in diabetes is due to glucose-mediated four mechanisms: increased advanced glycated end products (AGE) formation, polyol

pathway, activation of protein kinase and hexosamine pathway flux *(Brownlee, ٢٠٠١)* and due to increase free fatty acids-mediated mechanisms *(Brownlee, ٢٠٠٤)*.

Cardiac endothelium:

Endocardial endothelium and pulmonary endothelium constitute central vascular endothelium *(Rosenberg and Aird, ١٩٩٩)* which contains the largest source of endothelial enzymes and endothelial mediators acting through specific receptor signaling pathways *(Brutsaert, ٢٠٠٣)*.

Vascular endothelial cells produce nitric oxide (NO) which regulates vasodilatation, anticoagulation, leukocyte adhesion, smooth muscle proliferation and antioxidative capacity of endothelial cells *(Rakhit and Marber, ٢٠٠١)*.

Moreover, vascular endothelium influences greatly synthetic and motor functions of the underlying smooth muscle and similar regulatory mechanisms and vessel wall cross-talk exist between pericytes and microvascular endothelium *(Chakra-varthy and Gardiner, ١٩٩٩)*.

Endothelial activation and dysfunction:

Cardiac endocardial endothelium (EE) and myocardial capillary endothelium (MyoCapE) are involved in modulating myocardial growth, contractile performance and rhythmicity *(Brutsaert, ١٩٩٠; Brutsaert and Andries, ١٩٩٢ and Andries et al., ١٩٩٦)*.

Evidences suggest that EE syncytium exhibits strong electrochemical coupling between EE cells *(Beny, ١٩٩٩)* that function as a sensor that amplify release of endothelium-derived paracrine substances which likely can cause modulation of interstitial ionic homeostasis and disturbances in excitability and conduction of subendocardial myocardial layers including terminal purkinje fibers and dense subendocardial plexus exhibiting a positive chronotropic effect and or arrhythmia *(Hassanabad et al., ١٩٩٨)*.

Moreover, EE functions as an autocrine or paracrine organ-oriented modulator of cardiac contractile performance, rhythmicity and growth, mostly right ventricular EE *(Arai et al., ١٩٩٩)*.

Right ventricular EE cell to cardiomyocyte number ratio is high, so right ventricular pump performance will be adjusted to incoming blood-born

signals of mixed venous blood *(Brutsaert, ٢٠٠٣)* unlike left ventricular EE which optimizes cardiac pumping performance through coronary circulation and MyoCapE *(Brutsaert, ٢٠٠٣)*.

Vascular endothelial cells contain nitric oxide synthase (NOS) which synthesizes NO *(Moncada et al., ١٩٨٩)*.

Three NOS isoforms are characterized: type I neural constitutive (ncNOS), type II inducible (iNOS) and type III endothelial constitutive (ecNOS). All three NOS isoforms are present in human myocardium *(Kelly et al., ١٩٩٦)*.

Low level of endogenous basal non-stressed physiological conditions produces NO almost exclusively by MyoCapE, EE and coronary vascular endothelium *(Schulz et al., ١٩٩١ and Andries et al., ١٩٩٨)* which causes +ve inotropic action *(Mohan et al., ١٩٩٦)*.

Nitric oxide is known to be a modulator of biological phenomena from cell signal to effector and from physiology to pathology. NO is an important physiological regulator in maintenance of cell physiology from immunomodulation to calcium signaling. Low NO concentrations are associated with

cytostasis and high concentrations are associated with cytotoxicity. Balance between cytostatic and cytotoxic effects of NO may lie in tissue concentration in which NO produced, particular NO synthase isoform is activated and complex interaction with other ROS, is particularly pertinent in context of ischemia-reperfusion (I/R) *(Rakhit and Marber, ٢٠٠١)*. A cardioprotective role was addressed to NO mainly by myocardial preconditioning and modulating mitochondrial function *(Cassina and Radi, ١٩٩٦)* and antioxidant effects *(Siow et al., ١٩٩٩)*.

Endogenous nitric oxide (NO) synthesis during ischemia reperfusion was found to protect isolated guinea pig hearts versus apoptotic cell death *(Czarnowaska et al., ٢٠٠١)*.

Diabetic endothelial dysfunction:

Endothelial damage is a major cause of associated vascular complications *(Inguchi et al., ٢٠٠٠)* in diabetes and ischemia reperfusion *(Okada et al., ١٩٩٨ and Qi et al., ١٩٩٨)*. NO reacts with superoxide in vascular endothelial cells to form peroxynitrite ($ONOO^-$) which causes tissue injury *(Beckman et al., ١٩٩٠; Ishida et al., ١٩٩٦ and Wang and Zweier, ١٩٩٦)*, NO can inhibit oxygen radical

pathways e.g., xanthine oxidase and leukocyte NADPH oxidase *(Lee et al., ٢٠٠٠)*.

Tumor necrosis factor-alpha (TNF-α) levels can induce iNOS *(Habib et al., ١٩٩٦; Torre et al., ١٩٩٦; Kasai et al., ١٩٩٧; Satoh et al., ١٩٩٧ and Sasayama et al., ١٩٩٩)* which provokes further TNF-α expression *(Kalra et al., ٢٠٠٠)* in cardiomyocytes and cardiac endothelium *(Sigusch et al., ٢٠٠٠)* causing cardiac depression *(Oral et al., ١٩٩٧; Kubota et al., ١٩٩٧ and Bozkurt et al., ١٩٩٨)*.

TNF- α is a key mediator of reperfusion injury released early after I/R *(Squadrito et al., ١٩٩٣ and Kupatt et al., ١٩٩٩)* which increases IL٦ and IL٨ secretion that chemotactically activate leukocytes *(Tritto et al., ١٩٩٧)*.

Hemodynamic outcome in streptozotocin (STZ)-induced diabetic rats is implicated to be resulted from reduced subendocardial coronary perfusion which impaired subendocardial coronary reserve *(Borges et al., ٢٠٠٦)* and sympathetic innervation of heart and vessels *(Fazan et al., ١٩٩٧; Fazan et al., ١٩٩٩; Schann et al., ١٩٩٧ and Akiyama et al., ١٩٨٩)*, as well as decreased cardiac output *(Ren and Bode, ٢٠٠٠)*.

Postperfusion increased expression of angiotensin II receptor type I (ATI-R). Angiotensin II and catecholamines *(Imai et al., ١٩٩٢ and Ito et al., ١٩٩٣)* could have enhanced ET-١ release which induce superoxide radicals mediating post ischemic endothelial dysfunction, P-selectin expression and neutrophil adhesion *(Ergul, ٢٠٠٢ and Duda et al., ٢٠٠٦)*.

Cardiac endothelial cells ATI-R is activated in diabetic rats by oxidative stress which could contribute to superoxide-mediated NO activation *(Mehra et al., ٢٠٠٥)* resulting in peroxynitrite formation which is toxic to cardiac myocytes *(Ishida et al., ١٩٩٦)*. This represents a preferential site for neutrophil activation, increased adhesion molecules expression *(Sohn et al., ٢٠٠٣)* and inflammatory cytokines as TNF-α and IL١ which themselves potentiate neutrophil activation *(Takahashi et al., ٢٠٠١)*.

Diabetic cardiomyopathy:

Trost et al., (٢٠٠٢) proved that STZ model is a valuable tool to study diabetic cardiomyopathy using mice as an animal model in which extensive focal endomyocardial necrosis is observed in ١٢ weeks

diabetic rat *(Akula et al., ٢٠٠٣)* while diabetic hearts in acute phase are more resistant to irreversible cell damage *(Ravingerova et al., ٢٠٠٣)*.

There is a body of evidence showing a predominant decrease in number of β١-adrenergic receptor in chronic diabetes *(Maeda et al., ١٩٩٥; Savarese and Berkowitz, ١٩٩٧ and Dincer et al., ٢٠٠١)* while during development of diabetes *(Maeda et al., ١٩٩٥ and Fazan et al., ١٩٩٧)* an increase in number of β١-adrenergic receptors of short duration was demonstrated *(Austin and Chess-Williams, ١٩٩٢ and Uekita et al., ١٩٩٧)*.

Extracellular signal regulated kinase V٢ (ERKV٢) which protects rat myocardium against heart injury was reduced by approximately ١/٣ in long-term diabetes unlike short-term diabetes *(Xu et al., ٢٠٠٤)*.

Moreover, increased superoxide radicals in diabetic chronic oxidative stress could have induced focal mitochondrial DNA oxidative damage *(Rolo and Palmeira, ٢٠٠٦)* characterized by dissipation of membrane potential and calcium overload *(Rakhit and Marber, ٢٠٠١)* leading to calcium handling defect in cardiomyocytes *(Muscari et al., ٢٠٠٤)* together with cardiac sympathetic innervation impairment

frequently observed in diabetes *(Maeda et al., ١٩٩٥ and Fazan et al., ١٩٩٩)* and altered beta-adrenoceptor sensitivity *(Kaneko et al., ١٩٩١)* could have decreased cardiac contractility *(Saini et al., ٢٠٠٥)* and ejection fraction *(Joffe et al., ١٩٩٩)*.

Diabetic oxidative stress could have reduced activity of Ca/calmodulin-dependent sarcoplasmic reticulum Ca^{++} uptake, sarcoplasmic reticulum $Ca^{٢+}$/ calmodulin-dependent protein kinase II (Ca MkII) *(Osada et al., ٢٠٠٠)*, the expression of sarcoplasmic reticulum calcium-ATPase, Na^{+}/Ca^{++} exchanger *(Choi et al., ٢٠٠٢ and Brevig et al., ٢٠٠٦)* and responsiveness of contractile proteins to intracellular Ca^{++} concentration *(Wang and Morgan, ١٩٩٢ and De Keulenaer and Sys, ١٩٩٥)*.

Furthermore, low levels of cytosolic Ca^{++} can be attributed to reduce ryanodine sensitive Ca^{++} channel expression of cardiac myocytes in rats *(Choi et al., ٢٠٠٢ and Brevig et al., ٢٠٠٦)* and decreases inward Ca^{++} current which triggers ryanodine Ca^{++} channels to release Ca^{++} *(Choi et al., ٢٠٠٢ and Bers, ٢٠٠٢)* from sarcoplasmic reticulum.

ROS-induced decline in mitochondrial respiration could have increased mitochondria capacity to

produce ROS *(Petrosillo et al., ٢٠٠٣ and Saini et al., ٢٠٠٥)*, thus mitochondria can be both the first target and initiator of oxidative stress *(Christophe and Nicolas, ٢٠٠٦)*.

In addition, focal mitochondrial DNA oxidative damage-induced calcium defect in diabetes could have resulted from inhibition of eNOS which together with increased ET-١ *(Brunner et al., ١٩٩٧ and Hoshino et al., ٢٠٠٥)* could have interacted with NO and decreased NO availability.

Mitochondrial dysfunction also could result in persistent depression of energy production *(Du et al., ٢٠٠٠ and Nishikawa et al., ٢٠٠٠)* and cardiolipin damage mainly in mitochondrial complex I and III *(Paradies et al., ٢٠٠٤)*.

Oxidative stress:

Oxidative stress refers to circumstances in which ROS are excessively formed or when there are inadequate endogenous defences *(Hoeldtake et al., ٢٠٠٣)*. ROS include free radicals as superoxide ($^{*}O_{٢}$), hydroxyl ($^{*}OH$), peroxyl ($^{*}RO_{٢}$), hydroxyperoxyl ($^{*}HRO_{٢}$), hydrogen peroxide ($H_{٢}O_{٢}$) and hydrocholrous acid (HOCL) *(Turko et al., ٢٠٠١ and Evans et al., ٢٠٠٢)*.

West, (٢٠٠٠) proposed interaction between hyperglycemia and enhanced production of ROS. Enzymatic sources of diabetic oxidative stress include NOS, NADPH oxidase and xanthine oxidase *(Guzik et al., ٢٠٠٠; Guzik et al., ٢٠٠٢ and Aliciguzel et al., ٢٠٠٣)* while mitochondrial respiratory chain is non enzymatic source *(Green et al., ٢٠٠٤)*. ROS may injure cells by causing lipid peroxidation which triggers cell death *(Park and Lucchesi, ١٩٩٩)* that can be prevented when oxygen radicals are inactivated by specific scavengers *(Zweier and Villamena, ٢٠٠٣)*.

Antioxidants:

Antioxidant is any substance that when present at low concentrations prevents oxidation of oxidizable substrate *(Halliwell, ١٩٩١)*.

Examples of enzymatic antioxidants are: superoxide distmutase (SOD), catalase in lysosomes, glutathione peroxidase (GP) in mitochondria and xanthine oxidase enzyme. While non enzymatic antioxidants are vitamins A, C and E, glutathione, alpha lipoic acid, carotenoids, trace elements like copper, zinc, selenium, coenzyme Q١٠ (CoQ١٠) and cofactor like folic acid, albumin and vitamins B_1, B_2, B_6 and B_{12} *(Evans et al., ٢٠٠٣)*.