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Review

Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract

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Abstract

Free radicals and oxidative stress play a crucial role in the pathophysiology of a broad spectrum of cardiovascular diseases including congestive heart failure, valvular heart disease, cardiomyopathy, hypertrophy, atherosclerosis and ischemic heart disease. We have demonstrated that IH636 grape seed proanthocyanidin extract (GSPE) provides superior antioxidant efficacy as compared to Vitamins C, E and β-carotene. A series of studies were conducted using GSPE to demonstrate its cardioprotective ability in animals and humans. GSPE supplementation improved cardiac functional assessment including post-ischemic left ventricular function, reduced myocardial infarct size, reduced ventricular fibrillation (VF) and tachycardia, decreased the amount of reactive oxygen species (ROS) as detected by ESR spectroscopy and reduced malondialdehyde (MDA) formation in the heart perfusate. Cardiomyocyte apoptosis detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining. In concert, the proapoptotic signals mediated by JNK-l and c-fos proteins were also reduced suggesting that the novel cardioprotective properties of GSPE may be at least partially attributed to its ability to block anti-death signaling mediated through the proapoptotic transcription factors and genes such as *JNK-1* and c-*JUN*. In a separate study, GSPE pretreatment significantly inhibited doxorubicin-induced cardiotoxicity as demonstrated by reduced serum creatine kinase (CK) activity, DNA damage and histopathological changes in the cardiac tissue of mice. Concentration-dependent efficacy of GSPE was also assessed in a hamster atherosclerosis model. Approximately 49 and 63% reduction in foam

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cells, a biomarker of early stage atherosclerosis, were observed following supplementation of 50 and 100 mg GSPE/kg body weight, respectively. A human clinical trial was conducted on hypercholesterolemic subjects. GSPE supplementation significantly reduced oxidized LDL, a biomarker of cardiovascular diseases. Finally, a cDNA microarray study demonstrated significant inhibition of inducible endothelial *CD36* expression, a novel cardioregulatory gene, by GSPE. These results demonstrate that GSPE may serve as a potential therapeutic tool in promoting cardiovascular health via a number of novel mechanisms.

Keywords: Proanthocyanidins; In vitro; In vivo; Ischemia–reperfusion injury; TUNEL; c-JUN; JNK-1; Histopathology; Oligonucleotide microarray; CD36

1. Cardiovascular disease, free radicals, French Paradox and grape seed proanthocyanidin extract

1.1. Cardiovascular disease and free radicals

The cardiovascular disease is considered as the number one cause of death in the world. It comprises its own set of pathologies, chief among which are atherosclerosis, arteriosclerosis, hypertension, congestive heart failure, cardiomyopathy, coronary heart disease, hypertrophy, arrhythmias, ventricular fibrillation (VF), ventricular tachycardia (VT), myocardial infarction, and stroke [1–6]. Cardiovascular disease is also the chief cause of mortality among menopausal women [2]. It is worthwhile to mention that ischemic heart disease continues to be the major cause of cardiac death, and ventricular fibrillation is responsible for the development of sudden cardiac death [4]. A number of factors keep contributing to the epidemic scope of cardiovascular disease, which involves physical, emotional, environmental, and chemical stresses of modern life [5]. In addition, inactivity and obesity, sedentary life styles, bad food habits, tobacco smoking, and excessive alcohol consumption may contribute significantly to cardiovascular dysfunction [5].

Oxygen free radicals are implicated in the pathophysiology of diverse cardiovascular disease [1–3,6]. It has been well-documented that increased post-ischemic susceptibility to free radical damage results from the build-up of a strongly reducing environment during ischemia along with a decreased antioxidant capacity [3,6]. Free radical and oxidative stress also appear to be a common mediator of apoptosis, directly or via the formation of lipid peroxidation and lipid hydroperoxides [3,6].

1.2. French Paradox and cardioprotection

Epidemiological studies demonstrate that the consumption of red wine is beneficial in the prevention of cardiovascular diseases, which ultimately conceptualizes the term “French Paradox” [7–9]. French people consume high fat diets, exercise less, and smoke more, however, their death from cardiovascular disease is much less than in the US and other westernized societies [7,8]. Polyphenolic constituents including oligomeric proanthocyanidins and resveratrol, in red wine have been demonstrated to be the root cause of cardioprotective effects of “French Paradox” [8,9].

1.3. Antioxidants and grape seed proanthocyanidin extract

Novel antioxidants may offer an effective and safe means of counteracting some of the problems and bolstering the body’s defenses against free radicals and cardiovascular disease [10]. In particular, novel antioxidants can neutralize harmful free radicals and their noxious tissue- and organ-damaging effects as well as enhance the body’s antioxidant status [10,11]. Proanthocyanidins are naturally occurring polyphenolic compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark. Grape seed proanthocyanidins, a combination of biologically active polyphenolic flavonoids including oligomeric proanthocyanidins, have been demonstrated to exert a novel spectrum of biological, pharmacological, therapeutic, and chemoprotective properties against oxygen free radicals and oxidative stress [12,13]. We

have evaluated the cardioprotective ability of a novel IH636 grape seed proanthocyanidin extract (GSPE), which was obtained from InterHealth Nutraceuticals (Benicia, CA). GSPE was a natural, standardized, water–ethanol extract from California red grape seeds. High-pressure liquid chromatography (HPLC) analyses in conjunction with gas chromatography–mass spectrometry (GC–MS) demonstrated that GSPE contains approximately 75–80% oligomeric proanthocyanidins and 3–5% monomeric proanthocyanidins.

1.4. GSPE and free radical scavenging properties

In our laboratory, a significant amount of research has been conducted on GSPE. In the in vitro model, GSPE exhibited concentration-dependent dramatic scavenging ability towards biochemically generated superoxide anion, hydroxyl and peroxyl radicals, and provided significantly better scavenging as compared to Vitamins C and E [14,15]. GSPE also exhibited excellent dose-dependent protective ability against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced hepatic and brain lipid peroxidation, and DNA fragmentation, and peritoneal macrophage activation in mice, and provided significantly better protection than Vitamins C, E and β-carotene [16]. GSPE also demonstrated significant protection against smokeless tobacco-induced oxidative stress, DNA damage and apoptotic cell death in a primary culture of human oral keratinocytes, and provided better protection as compared to Vitamins C and E, singly and in combination [17]. GSPE exerted significant cytotoxicity towards human breast, lung and gastric adenocarcinoma cells, while enhancing the growth and viability of normal cells [18]. GSPE also exhibited significant protection against chemotherapeutic drugs-induced cytotoxicity towards normal human liver cells by modulating apoptotic regulatory genes *bcl-2*, *c-myc* and *p53* [19], and provided excellent in vivo protection against structurally diverse drug- and chemical-induced multiorgan toxicity [20]. GSPE protected against acetaminophen-induced hepatotoxicity by dramatically enhancing the expression of *bcl-*X_L gene in the liver tissue [21]. These studies demonstrate that GSPE is a potent scavenger of free radicals, bioavailable and provide significant protection towards multiple target organs against structurally diverse drug- and chemical-induced toxic manifestation [20–22].

2. Protection against myocardial ischemia–reperfusion injury, ventricular fibrillation (VF), ventricular tachycardia (VT), and cardiomyocyte apoptosis by GSPE

2.1. GSPE-induced protection against myocardial ischemia–reperfusion injury, alleviation of cardiovascular function, and protection against VF and VT in rats

The protective ability of GSPE was assessed against myocardial ischemia–reperfusion injury. Male Sprague–Dawley rats (275–300 g) were provided with food and water ad libitum until the start of the experimental procedure. Rats were randomly assigned to one of the two groups, control and GSPE. Half of the rats were fed GSPE (100 mg/kg per day for 3 weeks) orally in water while the other half were only given water. A group of rats were also given a lower dose of GSPE (50 mg/kg per day). At the end of the treatment period, rats were anesthetized, hearts were excised, aortic canulation was performed and hearts were perfused in the retrograde Langendorff mode as described by us earlier [15]. Hearts were then subjected to 30 min of global ischemia followed by 2 h reperfusion. The first 10 min of reperfusion was in the retrograde mode to allow for post-ischemic stabilization and thereafter in the antigrade working mode to allow for assessment of functional parameters, which were recorded at 30, 60 and 120 min of reperfusion.

Cardiac functional assessment and direct measurements of heart rate, developed pressure (defined as the aortic systolic minus end diastolic pressure), and the first derivative of the aortic pressure (dp/dt_{max}) were made at each time point. The incidence of reperfusion-induced ventricular fibrillation and ventricular tachycardia were determined as described by us earlier [23]. Infarct size estimation was performed using 1% triphenyl tetrazolium staining (TTC), while myocardial oxidative stress was determined by estimating malondialdehyde (MDA) using HPLC [15].

No differences were observed in baseline function between control and GSPE-fed groups. GSPE-fed rat (100 mg/kg body weight, p.o.) hearts displayed significant recovery of post-ischemic myocardial function, which was clearly demonstrated by significant differences in the developed pressure readings throughout the reperfusion period [15]. At 120 min of

reperfusion, differences between developed pressure readings were remarkable (48.5 ± 7.7 mmHg versus 73.0 ± 6.1 mmHg). The maximum first derivative of the developed pressure showed a similar trend. GSPE-fed animals (100 mg/kg body weight, p.o.) showed markedly higher aortic flow. At 120 min of reperfusion, aortic flow data were very impressive (17.6 ± 4.2 ml/min versus 30.8 ± 2.7 ml/min). Infarct mass as well as infarct size (percentage infarct of total volume at risk) were remarkably reduced in the GSPE-fed group (100 mg/kg body weight, p.o.) as compared to the control group (0.209 ± 0.017 g versus 0.163 ± 0.012 g and 24.6 ± 0.8% versus 18.7 ± 1.0%). MDA formation was performed as a biomarker of myocardial oxidative stress, which was also significantly reduced in the GSPE-fed group. GSPE pretreatment significantly decreased the amount of reactive oxygen species (ROS) in the heart as demonstrated by ESR spectroscopy [23].

GSPE also caused a striking reduction in the incidence of reperfusion-induced VF and VT. Following treatment of the rats with 50 and 100 mg/kg of GSPE, the incidence of reperfusion-induced VF was reduced from its control value of 92 to 42% ($P < 0.05$) and 25% ($P < 0.05$), respectively. No significant reduction in VT was observed in rats treated with 50 mg/kg of GSPE. However, at 100 mg/kg of GSPE, VT was reduced by 68% ($P < 0.05$) [23].

Thus, GSPE-fed rat myocardium was more resistant to ischemia–reperfusion injury as compared to the control animals. The GSPE-fed group showed better post-ischemic ventricular recovery and a reduced incidence of reperfusion-induced VF and VT as compared to the corresponding control animals. Development of reperfusion-induced arrhythmias and cardiac injury could contribute to a massive production of hydroxyl radicals in the reperfused isolated myocardium. Thus, GSPE strongly interfered with the hydroxyl radical formation and oxidative stress. This was further evident in studies in which the presence of hydroxyl radicals were assessed [15,23]. The formation of hydroxyl radicals in the heart perfusate in GSPE-treated animals was significantly lower as compared to the control animals, which was demonstrated by both ESR and HPLC techniques [15,23]. Furthermore, we assessed oxidative stress by monitoring MDA. A dramatic increase in the MDA concentration was significantly higher in the control group, which was significantly reduced in GSPE-treated hearts. The antioxidants are known to interfere with the free radical formation and antioxidant reserve and antioxidant enzyme capacity are significantly reduced following ischemia and reperfusion. The loss of key antioxidant enzymes and antioxidant status downregulates the overall antioxidant reserve of the myocardium, and makes the heart susceptible to ischemia–reperfusion-induced injury. The reduced antioxidant defense cannot provide protection against increased activities of ROS and oxidative stress.

2.2. Inhibition of cardiomyocyte apoptosis, and downregulation of proapoptotic genes c-JUN and JNK-1 by GSPE in rats

To examine apoptosis, cardiomyocytes were obtained by a well-established technique [24] and immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method in conjunction with an antibody against myosin heavy chain for specifically identifying apoptotic cardiomyocytes [24]. To quantity the abundance of JNK-1 and c-JUN proteins, Western blot analysis was performed using respective antibodies followed by densitometric scanning [24].

GSPE significantly reduced the incidence of cardiomyocyte apoptosis as demonstrated by TUNEL assay. The number of apoptotic cells was significantly higher (23%) in the ischemic–reperfused myocardium compared to the control hearts. GSPE treatment significantly reduced the number of apoptotic cells by approximately 69% compared to the ischemic–reperfused hearts.

Thirty minutes of ischemia followed by 2 h of reperfusion significantly enhanced the amount of the protein levels of JNK-1 and c-JUN, which were dramatically reduced by GSPE pretreatment. The JNK-1 level was reduced by approximately 57%, while the c-JUN level was reduced by approximately 54%, respectively [24].

Thus, GSPE reduced cardiomyocytes apoptosis in the ischemic–reperfused heart. Previous studies have demonstrated that JNKs are activated during the reperfusion of ischemic myocardium [25]. At least two members of the JNK family, JNK-1 (46 kDa) and JNK-2 (55 kDa), can phosphorylate c-JUN on specific N-terminal serine residues [26]. Previous observations are supported by the results in the present

study by documenting that JNK-1 is activated by ischemia–reperfusion. Induction of the expression of c-JUN in the ischemic–reperfused and preconditioned myocardium in our study is also consistent with previous findings [25]. Ionizing radiation and most DNA-damaging agents that activate JNK can also generate free radicals. The only kinases that phosphorylate the serine 63 and 73 of c-JUN in vivo are JNKs, which can phosphorylate a variety of cytoplasmic and nuclear proteins [27]. Additionally, JNK activates the tumor suppressor *p53* gene [28], a proapoptotic transcription factor that suppresses the anti-death gene *bcl-2* and enhances *bax* induction [29]. JNK can also antagonize the function of the anti-apoptotic protection of *bcl-2* through phosphorylation [30]. Thus, this is consistent with earlier reports [15,24], and further suggests that GSPE can reduce cardiomyocyte apoptosis through the inhibition of ischemia–reperfusion-induced activation of proapoptotic genes *JNK-1* and *c-JUN*.

3. Protection against doxorubicin-induced cardiotoxicity

3.1. Doxorubicin and GSPE treatment in mice

Adult male ICR (CD-1) mice weighing 30–40 g (3 months old) were obtained from Harlan–Sprague–Dawley Inc. (Indianapolis, IN, USA). There were four experimental groups as described earlier [22]: a control group (receiving saline only), a GSPE alone group (100 mg/kg per day, p.o. for 9 days), a doxorubicin-treated group (20 mg/kg, i.p. for 48 h) and a GSPE plus doxorubicin-treated group (100 mg GSPE/kg per day, p.o. for 9 days and a 20 mg/kg, i.p. dose of doxorubicin was administered on day 7, 1 h after regular GSPE exposure). All animals were sacrificed 48 h after doxorubicin exposure [22].

3.2. Protective ability of GSPE against doxorubicin-induced alterations in serum chemistry (CK, ALT and BUN activities), DNA fragmentation and morphological changes

Control and GSPE treatment alone did not induce any increase in creatine kinase (CK), ALT and BUN activities or morphological changes [22]. Doxorubicin treatment alone induced a significant increase in serum creatine kinase activities (a highly reliable biomarker of cardiac injury) that exceeded control (1009±24 U/l) by approximately six-fold (5949 ± 612 U/l) [22]. It is very important to emphasize that a six-fold increase in serum CK activity is considered toxicologically significant and may threaten severe injury including mortality. GSPE pretreatment followed by doxorubicin treatment resulted in dramatic cardioprotection (1437 ± 193 U/l). Doxorubicin induced a six-fold increase in serum ALT activity (a biomarker of hepatotoxicity) in the absence of any increase in BUN level [22]. A six-fold increase in serum ALT is toxicologically insignificant and may not cause any danger to animal health and survival. GSPE pre-exposure totally abolished doxorubicin-induced increases in CK and ALT activities close to control animals.

Doxorubicin alone also caused significant damage to the cardiocellular chromatin architecture leading to DNA fragmentation. Doxorubicin induced a 1.8-fold higher cardiac DNA damage compared to control, while GSPE pretreatment completely blocked doxorubicin-induced DNA fragmentation [22]. In DNA agarose gel electrophoresis, doxorubicin-induced a ladder-like pattern of endonucleolytic DNA degradation, which was also completely abolished following GSPE pretreatment (figure not shown) [22].

GSPE treatment did not cause morphological change in the architecture of the cardiac cells. In histopathological assessment, GSPE pretreatment provided significant protection against doxorubicin-induced cardiotoxicity (Fig. 1). Morphologically, doxorubicin alone induced limited myocytolysis, condensed nuclei, condensed and fragmented apoptotic nuclei, deformed myocytes, owl's eye-shaped configuration, overall depletion of glycogen, and numerous intercellular spaces (Figs. 1 and 2). Tissue sections of GSPE+doxorubicin closely resembled either control or GSPE alone treated tissues. Residual necrosis was minimal (Fig. 1). Thus, GSPE provided excellent protection against doxorubicin-induced cardiotoxicity as demonstrated both by biochemically and histopathologically [22].

These studies in conjunction with our previous studies [20–22] demonstrate the protective ability of GSPE with modulation of anti-apoptotic gene *bcl-X_L* and modification of several other molecular targets such as DNA damage/repair, lipid peroxidation, and

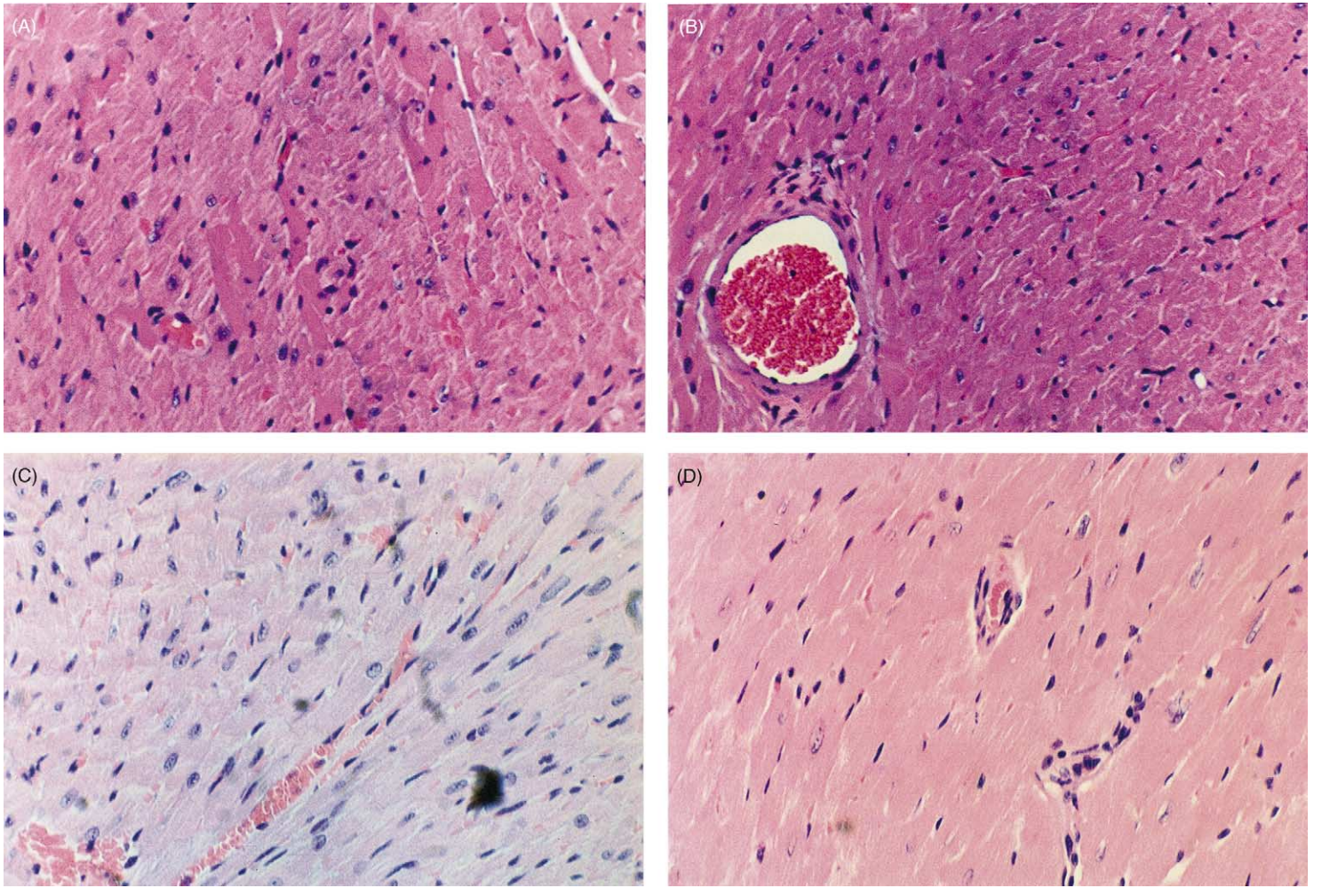

Fig. 1. Light photomicrographs of paraffin-embedded H&E-stained mouse heart sections. (A) Representative mouse heart section (100×) from control animals, no pathologic changes present. (B) Representative heart section (100×) from GSPE-treated animals (100 mg/kg, p.o. for 9 days), no pathologic features present. (C) Representative heart section (100×) from animals treated with doxorubicin (20 mg/kg, i.p. for 48 h). (D) Representative heart section (100×) from mouse treated with GSPE (100 mg/kg, p.o. for 9 days) and doxorubicin (20 mg/kg, i.p. for 48 h). Near complete protection was observed by GSPE on doxorubicin-induced changes.

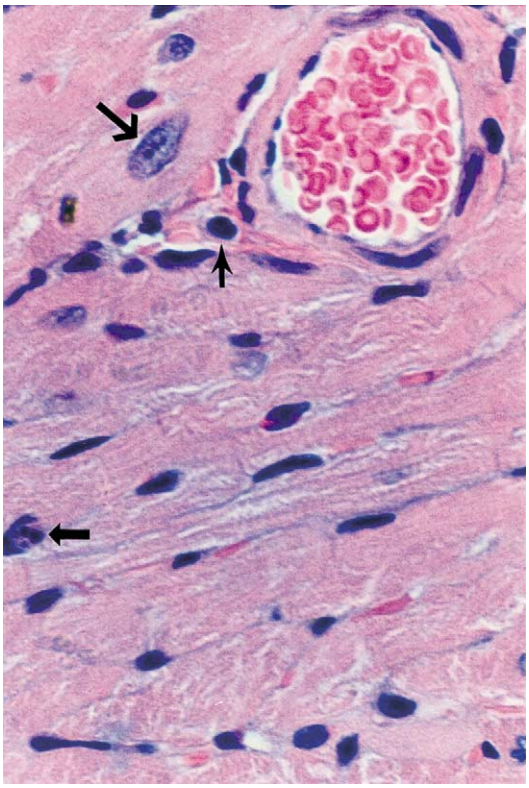

Fig. 2. Doxorubicin-induced apoptotic cell death in the cardiac tissue (1000×). (➞) Owl's eye-shaped configuration; (➔) condensed nuclei; (➡) condensed and fragmented apoptotic nuclei.

intracellular Ca^{2+} homeostasis. Especially, GSPE provide dramatic protection against acetaminophen-induced downregulation of *bcl-X_L* gene in the hepatic tissue and antagonized both apoptotic and necrotic cell deaths of liver cells. In a separate study, GSPE exhibited its' ability to inhibit cytochrome P450 2E1 in both in vitro and in vivo models [31], which is an additional cytoprotective attribute, in conjunction with the novel antioxidative, detoxifying, anti-apoptotic or anti-endonucleolytic potential of GSPE.

4. Beneficial role of GSPE in hamster atherosclerosis model

4.1. Hamster atherosclerosis model and treatment

The hamster model was selected because these animals have a similar lipid profile to hypercholesterolemic humans when fed a hypercholesterolemic diet of 0.2% cholesterol and 10% coconut oil for 10 weeks. After 10 weeks, these animals developed foam cells, which is a biomarker of the early stages of atherosclerosis [32].

Male, weanling, Syrian Golden hamsters were received from Charles River Breeding Laboratories (Wilmington, MA) and given commercial non-purified rodent chow (Ralston Purina, St. Louis, MO) for 4 weeks. They were then separated into groups, each with comparable average weights. The animals were housed in plastic cages, three or four animals per cage with a bedding of wood chips, in a temperature controlled room (20 °C) and a 12 h light/dark cycle. Experimental groups were given a high cholesterol diet containing 0.2% cholesterol and 10% coconut oil. The low GSPE food contained 0.88 g/kg of GSPE (equivalent to a dose of 50 mg GSPE/kg body weight) and the high GSPE food contained 1.76 mg/kg of GSPE (equivalent to a dose of 100 mg GSPE/kg body weight). After 10 weeks of feeding and following 12 h of food deprivation, the animals were anesthetized with pentobarbital and a cardiac puncture was performed. The blood was collected in an EDTA microcontainer and the plasma isolated and stored at −90 °C until assay was performed. The animals anesthetized were perfused with 10% formaldehyde in a phosphate buffered saline and the aorta was then isolated and prepared for histology as described [32]. The atherosclerotic index (percentage of aorta covered with foam cells), and triglyceride and cholesterol levels were determined as described earlier [32].

4.2. Amelioration of atherosclerotic index by GSPE in a hamster atherosclerosis model

The dose-dependent protective ability of GSPE was assessed. Atherosclerotic index (percentage of aorta covered with foam cells) was reduced by approximately 50 and 63% following supplementation of these animals with 50 and 100 mg/kg of GSPE, respectively, in conjunction with the hypercholesterolemic diet of 0.2% cholesterol and 10% coconut oil. Under these same conditions, total cholesterol was reduced by 25 and 23%, and triglyceride level was reduced by 10 and 34% following supplementation of 50 and 100 mg/kg GSPE, respectively (J.A. Vinson and D. Bagchi, unpublished results).

5. Effect of GSPE in a randomized, double-blind, placebo-controlled study in hypercholesterolemic human subjects

5.1. Human subjects, IRB approval, and treatment

Forty hypercholesterolemic human subjects (210–300 mg/dl) were recruited for a protocol approved by the Institutional Review Board (IRB) at Georgetown University Medical Center (Washington, DC, USA). The four groups of 10 patients each received either placebo bid, niacin-bound chromium (ChromeMate®) 200 μg elemental chromium bid, GSPE 100 mg bid, or a combination of niacin-bound chromium plus GSPE at the same dosage bid. Total cholesterol, LDL, HDL and triglycerides were measured as described earlier [33]. Autoantibodies to Ox-LDL, an indirect measure of oxidized LDL, were estimated using a non-isotopic ELISA technique [33].

5.2. Human clinical study results in hypercholesterolemic subjects

Hypercholesterolemia, a significant cardiovascular risk factor, is a challenging problem to the health professionals. We evaluated the possible beneficial effects of GSPE in a randomized, double-blind, placebo-controlled study in 40 hypercholesterolemic patients. After 8 weeks of supplementation, the change in total cholesterol from baseline among groups was: placebo $-3.5 \pm 4\%$, niacin-bound chromium $-10 \pm 5\%$, GSPE $-2.5 \pm 2\%$ and combination of niacin-bound chromium plus GSPE $-16.5 \pm 3\%$ ($P < 0.01$ [33]). The major changes in LDL levels: placebo $-3.0 \pm 4\%$, niacin-bound chromium $-14 \pm 4\%$, GSPE $-1.0 \pm 2.0\%$, and combination of niacin-bound chromium plus GSPE $-20.0 \pm 6.0\%$ ($P < 0.01$) [33]. No significant changes were observed in the HDL or triglyceride levels in either of these groups. The GSPE supplemented subjects demonstrated greater decreases in the level of autoantibodies to oxidized LDL. Approximately -17.3 and 10.4% decreases in the level of autoantibodies to oxidized LDL were observed in the placebo and niacin-bound chromium group in comparison to -30.7 and -44.0% in the GSPE and a combination of niacin-bound chromium plus GSPE groups [33]. There was also a trend for those groups receiving GSPE to decrease autoantibodies to oxidized LDL greater than 50% over 8 weeks: placebo = 2/9, niacin-bound chromium = 1/10, GSPE = 6/10, and a combination of niacin-bound chromium + GSPE = 3/8 [33]. GSPE supplementation in conjunction with a novel niacin-bound chromium was demonstrated to favorably influence circulating cholesterol, LDL, and antibodies to oxidized LDL. Thus, GSPE may induce significant health benefits to hypercholesterolemic human subjects.

6. Effect of GSPE on cardioregulatory *CD36* gene: cDNA microarray analysis

6.1. Cardioregulatroy CD36 gene: an introduction

CD36 antibody was purchased from Immunotech (Westbrook, ME). CD36, also called glycoprotein IV, is an 88-kD transmembrane glycoprotein with a large extracellular domain. Originally found on platelets and monocytes, it is also expressed in microvascular endothelial cells and is responsible for neovascularization in vivo. CD36 is well known as an adhesion receptor for TSP-1 and *Plasmodium falciparum* parasitized erythrocytes, facilitates the binding of platelets to collagen, monocytes, and the subendothelium, and contributes to the activation of monocytes and platelets. As such, endothelial CD36 has pro-inflammatory functions [34–36]. cDNA microarray was performed using MICROMAX™ Human cDNA Microarray System I (NEN Life Science Products Inc., Boston, MA). A brief description of the process of differential gene expression analysis using cDNA Microarray System I have been provided below.

6.2. RNA extraction, cDNA labeling and hybridization

Primary human umbilical vein endothelial cells (HUVEC) were obtained from Cascade Biologics Inc. (Portland, OR). The cells were cultured in the medium (Medium 200 supplemented with low growth serum supplement, LSGS) supplied by Caascade Biologics as described earlier [37]. Total RNA was extracted from cells pretreated or not with GSPE (2 μg/ml, 24 h) and then activated with TNFα (20 ng/ml, 6 h) using TRIZOL (Gibco-BRL) [37]. The messenger RNA

(mRNA) in each pool, without further purification, was converted to Biotin and Dinitrophenyl (DNP) cDNA using reverse transcriptase and nucleotide analogs, for use as individually traceable gene targets in the assay [38]. The cDNA was mixed and simultaneously hybridized to the microarray in an overnight incubation. Biotin and DNP cDNAs were sequentially detected with a series of conjugate reporter molecules according to the tyramide signal amplification process. Ultimately, each of the two fluorescent labels (Cyanine 3 or 5) was associated with the hybridized genetic material from each of the two starting samples.

6.2.1. Detection and data processing

MICROMAX^{TM} Custom Scanning and Data Analysis Service was utilized. A laser detection system was used to scan and report the relative quantity of the two dyes at any given gene spot on the microarray. Finally, the differential scanning data were processed by computer to ascertain and profile the genes of interest as a result of the experiment. MICROMAX^{TM} Human cDNA Microarray System I array contains 2400 human genes. Differentially expressed genes were classified into Prosite categories. Prosite is a database of protein families and domains, which consists of biologically significant sites, patterns and profiles that help to reliably identify to which known protein family (if any) a new sequence belongs [39]. The cDNA Microarray System I is capable of detecting 1.2×10^5 molecules of Biotin or DNP cDNA. Taking into account theoretical values for amount of messenger RNA in a total RNA population, cDNA synthesis efficiency and overall detection efficiency of the system, it is possible to detect approximately 50% of the input messenger RNA with cDNA Microarray System I. Therefore, cDNA Microarray System I can theoretically detect as few as 1.2 copies per cell of a gene starting with RNA from 2.0×10^5 cells. Using the same experimental protocol, it has been demonstrated that signal saturation occurs at 3.0×10^7 molecules. Given the above factors, this value translates into 150 copies per cell of a gene from 2.0×10^5 cells.

6.3. Inhibitory effect of GSPE on cardioregulatory CD36 gene in cDNA microarray analysis

Potent anti-inflammatory properties of GSPE have been elucidated. Previous study in our laboratory have demonstrated the potent inhibitory effect of GSPE on TNFα-induced VCAM-1 expression in human umbilical vein derived endothelial cells (HUVEC) at low concentrations (1–5 μg/ml). Such effect was consistently observed in mRNA as well as protein analysis suggesting interception of TNFα-induced transcriptional control of adhesion molecule gene expression by GSPE [37]. In a model of endothelial cell leukocyte co-culture, the interaction between these two cell types was studied. GSPE treatment clearly downregulated TNFα-induced adherence of T lymphocytes to HUVEC [37]. To further explore the effects of GSPE on TNFα-induced gene expression profile in HUVEC, a transcriptome analysis has been performed. Two thousand and four hundred genes were screened using the MICROMAX^{TM} Human Full-Length cDNA Microarray (NEN Life Sciences, Boston, MA). Several anti-inflammatory and cardioprotective genes sensitive to TNFα have been identified to be inhibited in response to GSPE treatment (C.K. Sen, S. Roy and D. Bagchi, unpublished results). Of all the genes, *CD36* represents a major candidate. *CD36* has been identified as a potential Ox-LDL receptor and directly linked to foam cells and atherosclerosis.

Although not usually considered to be a signaling receptor, CD36 co-precipitates from platelets and endothelial cells with several src-related kinases. It has been demonstrated that CD36 is an essential mediator of the anti-angiogenic action of TSP-1 on endothelial cells in vitro. CD36 can produce a biological response in endothelial cells and the first identification of a receptor able to mediate the effects of a broad spectrum inhibitor of angiogenesis like thrombospondin-1. Taken together, the downregulatory effects of GSPE on endothelial cell CD36 expression may be viewed as an anti-inflammatory and pro-angiogenic effect [34,40,41]. This is inconsistent with our findings related to inducible adhesion molecule expression [37] and VEGF expression [42].

Furthermore, CD36 belongs to the class B scavenger receptor family, which includes the receptor for selective cholesteryl ester uptake, scavenger receptor class B type I, and lysosomal integral membrane protein II. An important pathologic function of scavenger receptors, related to macrophage foam cell formation and the pathogenesis of atherosclerosis, is recognition and internalization of oxidatively modified LDL. In 1993, Endemann et al. [43] first identified CD36

as a potential Ox-LDL receptor. It has been observed that breeding the CD36 deficiency onto a proatherogenic apoE-null background yields animals that are significantly protected from lesion development. Animals fed a western diet showed >70% reduction in aortic lesion size and distribution [44]. The result of inhibitory effects of CD36 correlates very well with the results of foam cell inhibition data in a hamster atherosclerosis model and a human clinical study conducted in hypercholesterolemic human subjects. Thus, our results showing the inhibition of inducible CD36 expression by GSPE warrant further investigation of the anti-atherogenic role of this natural product.

7. Conclusion

Taken together, these studies demonstrate that GSPE is bioavailable, a potent free radical scavenger, and exhibits novel cardioprotective properties. The novel mechanistic pathways of cardioprotection exerted by GSPE include its: (i) potent hydroxyl and other free radical scavenging abilities; (ii) anti-apoptotic, anti-necrotic, and anti-endonucleolytic potentials; (iii) modulatory effect on apoptotic regulatory *bcl-X_L*, *p53*, and c-*myc* genes; (iv) cytochrome P450 2E1 inhibitory activity; (v) inhibitory effects on adhesion molecules; (vi) modulatory effects on proapoptotic and cardioregulatory genes c-*JUN*, *JNK-1*, and *CD36*.

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