Effect of Extract of Pomegranate (*Punica granatum* L.) on Glycated Protein-iron Chelate-induced Toxicity: An *In Vitro* Study on Human Umbilical-vein Endothelial Cells

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The binding of advanced glycation endproducts (AGEs) to a cell-surface receptor for AGEs (RAGE) induces the formation of reactive oxygen species (ROS), which have been causally implicated in the pathogenesis of diabetic vascular complications. Pomegranate fruit extract (PFE) contains, a naturally occurring polyphenolic compound reported to possess potent radical-scavenging and antioxidant properties and to display significant cardiovascular protective action. In this study, we investigated whether PFE could inhibit glycated protein-iron chelate-induced toxicity by interfering with ROS generation in human umbilical-vein endothelial cells (HUVEC). Glycated fetal bovine serum (GFBS) was prepared by incubating fetal bovine serum (FBS) with high-concentration glucose. Stimulation of cultured HUVECs with 50 mM 1 ml of GFBS significantly enhanced lipid peroxidation and decreased antioxidant enzyme activities and levels of phase II enzymes. However, preincubation of the cultures with PFE resulted in a marked decrease in the level of lipid peroxide (LPO) and increase in the levels of antioxidant enzymes in a PFE concentration-dependent manner. These results demonstrate that PFE could inhibit LPO and enhance the antioxidant enzyme status in GFBS-iron chelate exposed endothelial cells by suppressing ROS generation, thereby limiting the effects of the AGE–RAGE interaction. Hence, PFE may have therapeutic potential in the prevention and treatment of vascular complications in diabetic patients.

Key words —— glycated protein, iron, human umbilical-vein endothelial cell, pomegranate fruit extract, antioxidant enzymes

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia and hyperlipidaemia, both of which predispose affected individuals to long-term micro- and macrovascular complications of which cardiovascular disease is the most serious consequence. During hyperglycaemia, body proteins undergo increased glycation where glucose reacts non-enzymatically with protein amino groups to form a labile Schiff base that rearranges to a stable Amadori product. This Amadori product undergoes further reactions involving dicarbonyl intermediates such as 3-deoxyglucosone and ethylglyoxal to form complex, heterogeneous, fluorescent and crosslinked structures called advanced glycation endproducts (AGEs). The accumulation of crosslinked AGEs in body tissues are believed to be responsible for the long term complications of diabetes and ageing.[1] However, the major AGE *in vivo* is carboxymethyllysine (CML), which is not a crosslink but is formed by oxidative breakdown of Amadori products;[2] and its level increases two-fold in the skin of diabetic patients.[3] In the presence of oxygen and transition metals, glucose can undergo autoxidation (autoxidative glycation) as can Amadori products (glycoxidation) to produce free radicals capable of damaging proteins, lipids and nucleic acids.[4] Indeed, diabetes mellitus and ageing are associated with a build up of tissue AGE, increased oxidative stress, and a decline in antioxidant status.[5] Furthermore, circulating serum AGE can interact with cellular receptors (termed RAGE) to

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activate nuclear factor-kappa B (NF-kB), which in turn generates proinflammatory molecules and oxidative stress,[6] The involvement of advanced glycation endproducts in diabetic complications and ageing has prompted a search for compounds capable of inhibiting their formation.[7] Recent studies suggest that compounds with combined anti-glycation and antioxidant properties offer maximum protection against glucose-induced cellular damage.[8]

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and in beverage forms as juice and wines.[9] Commercial pomegranate juice shows potent antioxidant and anti-atherosclerotic properties attributed to its high content of polyphenols including ellagic acid (EA) in its free and bound forms [EA glycosides (EAGs)], gallotannins and anthocyanins (cyanidin, delphinidin, and pelargonidin glycosides), and other flavonoids (quercetin, kaempferol, and luteolin glycosides).[9–14] The most abundant of these polyphenols is punicalagin an implicated as being the bioactive constituent responsible for N50% of the juice's potent antioxidant activity.[9] Punicalagin is abundant in the fruit husk and during processing is extracted into pomegranate fruit extract (PFE) in significant quantities reaching levels of N2 g/l juice.[9,13–15] We are interested in the potential health benefits of phytochemicals and in evaluating the multifactorial effects and chemical synergy of multiple compounds, as found naturally in their unique compositions in foods compared to single purified active compounds.[16] Because pomegranates are widely consumed and shown to have potential benefits for human health[10,17] in this present study we investigated the antioxidant activities (inhibition of lipid peroxidation) of its polyphenols against glycated protein-iron chelate-induced toxicity in human umbilical-vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Cell lines —— HUVECs were obtained from Kurabo, Osaka, Japan. Fetal bovine serum (FBS) was purchased from Sera-Tech Zellbiologische Produkte GmbH, Salvator, Germany. The content of glucose in FBS was 0.63 mg/ml. The following enzyme assay kits were used: for GPx and GR, from Trevigen, Gaithersburg, MD, U.S.A.; for SOD, from Dojindo Molecular Tecnologies, Inc., in Kumamoto, Japan; and for catalase, from Cayman Chemical Company, Ann arbor, MI, U.S.A. Reduced glutathione and α-tocopherol were obtained from Nacalai Tesque, Kyoto, Japan.

Glycation of FBS —— For the glycation of FBS, 100 ml of FBS was incubated with 50 mM glucose at 37°C for 50 days under sterile conditions. After the incubation, the mixture was dialyzed against 67 mM phosphate buffer (pH 7.4) at 4°C. The dialyzate was used as glycated FBS (GFBS) after having been concentrated by use of Aquacide II (Calbiochem-Novabiochem Corp., La Jolla, CA, U.S.A.). The extent of glycation of GFBS was assayed with a commercial kit (Nippon Roche, Tokyo, Japan) and expressed as Fructosamine value (FV). The FV of GFBS prepared was 12 mM, and that of original FBS was 0.16 mM.

Preparations of PFE —— PFE was prepared as follows: the whole fruit was cut in half and squeezed in an electric lemon-squeezer, and the resulting juice was then centrifuged at 2500 rpm for 10 min. The supernatants from the centrifugation step of the PFE were recovered, microfiltered, aliquoted and immediately stored at −20°C.

Cultivation of HUVEC —— HUVEC were purchased from Kurabo. HUVEC from a vial in which the cells arrived were cultured in 5 ml of endothelial medium-2 (EG-2) supplemented with 10% (v/v) fetal bovine serum, gentamicin sulphate (50 mg/ml)/amphotericine-B (50 μg/ml) in addition to human recombinant fibroblast growth factor B (hFGF-B; 5 μg/ml), human recombinant epidermal growth factor (hEGF; 10 μg/ml), hydrocortisone (1 mg/ml), and heparin (10 mg/ml) in 25-cm^2 culture flasks. The cells cultures were kept at 37°C in 5% CO_2 and 95% air, humidified incubator. Once the cultures had reached 80% confluence, the cells were viewed under a microscope to ascertain the condition of the cultures (*i.e.*, confluence, mitotic activity). For subsequent subculturing, cells were seeded at 2.5×10^3 cells/cm^2. Cultured cells at the 3rd to 8th passage were used for the experiment.

The experimental design was as follows: the cells were divided into 10 groups with 5 flasks in each group. Group I was treated with 15% FBS; and Group II, with GFBS (70 μM). Group III and IV were treated with FBS+$FeCl_3$ (20 μM) and GFBS+$FeCl_3$ (20 μM) respectively; and Groups V, VI, and VII, with GFBS (70 μM) +PFE (250 μg/ml); FBS+$FeCl_3$+PFE (250 μg/ml) and GFBS+ $FeCl_3$ (20 μM)+PFE (250 μg/ml), FBS+ $FeCl_3$+PFE (250 μg/ml) respectively. Groups VIII, IX and X received GFBS (70 μM), +PFE

(500 μg/ml), FBS+ $FeCl_3$+PFE (500 μg/ml), GFBS +$FeCl_3$ (20 μM) +PFE (500 μg/ml) respectively. After 48 hr incubation, the samples were collected from all the experimental groups by detaching the cells from the flask by using a rubber policeman with phosphate buffer and then centrifuged at $250 \times g$ for 10 min. The cell pellets were dissolved in the same buffer and homogenized by using a sonicator, and again centrifuged at $10000 \times g$ for 15 min at 4°C. The supernatant was used for subsequent assays. Another portion of the supernatant was subjected to centrifugation at $10000 \times g$ for 60 min at 4°C to obtain microsomes for use in assays for cytochrome c reductase and glutathione S-transferase.

Assay of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) —— The viability of HUVECs were assed by MTT assay.

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed using 20% sodium dodecyl sulfate (SDS) solution, which releases the formazan crystals. These crystals are solubilized by *N*,*N*-dimethyl formamide (DMF) present in the solubilizer. The colour developed is then determined in an ELISA reader at 620 nm.

HUVECs cells were plated separately in 96 well plates at a concentration of 1×10^4 cells/well. After 48 hr, cells were washed twice with 100 μl of serum-free medium and starved for 1 hr at 37°C. After starvation, cells were treated with different concentrations of PFE (50–300 μg) for 48 hr. At the end 44 hr of the treatment, cells were incubated with 100 μl RPMI-1640 medium containing MTT (0.5 mg/ml), for 4 hr at 37°C in a CO_2 incubator. The 50% inhibitory concentration value (IC50) of PFE was identified in HUVE cells.

Measurement of Protein —— Protein was measured by using a DC Protein Assay Kit from Bio-Rad, Ann arbor, U.S.A. The method is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay.[18)]

Measurement of Lipid Peroxidation —— Lipid peroxide (LPO) level were measured with a Determiner LPO (Kyowa Medics Co., Ltd., Tokyo, Japan), based on the basic principle well documented by Yagi.[19)]

Determination of Activities of Antioxidant Enzymes —— Activity of GPx was measured by using a Bioxytech GPx-340 assay kit from OxisResearch, Ann arbor, U.S.A. according to the instructions provided with the kit. The GPx-340 assay is an indirect measure of the activity of c-GPx. Oxidized glutathione, produced upon reduction of organic peroxide by c-GPx, is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm providing a spectrophotometric means for monitoring GPx enzyme activity. Activity of glutathione reductase was measured by using a Glutathione Reductase assay kit from Trevigen. The assay was performed per the instruction manual. The oxidation of NADPH to $NADP^+$ is monitored as a decrease in absorbance at 340 nm. This rate of decrease is directly proportional to the glutathione reductase activity. The level of SOD activity was measured with a SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.) according to the protocol given by the supplier. Catalase activity was measured by using a Cayman chemical assay kit, and the assay was performed as per the instruction manual. Enzyme activity was then standardized to mg protein.

Cytochrome c (Cyt c) Reductase Assay —— This colorimetric assay was conducted with a kit from Sigma (St Louis, MO, U.S.A.), and all reagents required for performing the assay were included in the assay kit. The assay was performing according to the manufacturer's instructions. In brief, cell lysates were treated with Cyt c in presence of NADPH. The reduction of Cyt c was measured at 550 nm.

Glutathione S-transferase (GST) Assay —— Glutathione S-transferase assay was done with a Cayman Chemical Company kit. This assay is based on measuring the conjugation of 1-choloro-2,4-dinitrobenze (CDNB) with reduced glutathione in the microsomal fraction. The conjugation is accompanied by an increased absorbance measured at 340 nm. The GST activity was expressed as $nmol^{-1}ml^{-1}$.

Statistical Analysis —— The results were expressed as the mean ± S.D., and the statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (GraphPad software, La Jolla, CA. Instat, U.S.A.). $p < 0.001$, $p < 0.01$ and $p < 0.05$ levels were considered significant.

RESULTS

As shown in Fig. 1, PFE treatment of HUVECs resulted in 90, 82, 74, 66, 53 and 46% decrease in cell viability at the doses of 50–300 μg/ml of PFE, respectively. Figure 2 (A–J) shows the phase-contrast micrographs of HUVECs treated with various combinations of additives. In Fig. 2A, the normal architecture of the HUVECs incubated with FBS is seen. In comparisons, treatment with GFBS resulted in injury of cells as evidenced by degeneration cell shrinkage and individual cell necrosis (Fig. 2B). The addition of $FeCl_3$ to FBS cultures (Fig. 2C) led to some cellular degeneration; whereas with GFBS cultures massive necrosis and expanded intracellular space and shrinkage of cells (Fig. 2D) occurred. When GFBS cultures were treated with 250 μg/ml PFE (Fig. 2E) the cells showed less injury than without it, indicating some protection by the extract; and when PFE 500 μg/ml was used, the injury was even less (Fig. 2H) from iron toxicity. Figure 2F shows that the extract at 250 μg/ml protected against necrosis and shrinkage seen in the FBS-$FeCl_3$ cultures (Fig. 2C) and was even more protective at 500 μg/ml (Fig. 2I). At 250 μg/ml (Fig. 2G), PFE did not reduce much the toxic damage due to GFBS plus iron; whereas at 500 μg/ml (Fig. 2J) it was much more effective.

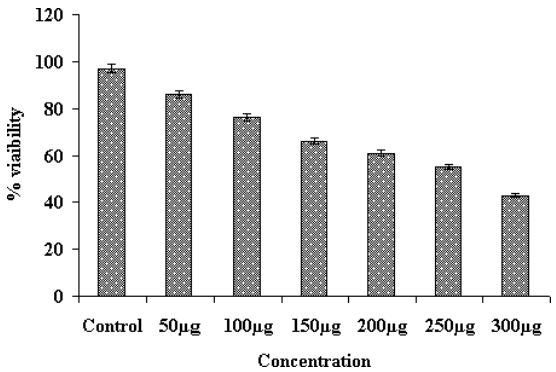

Fig. 1. Effect of PFE on Viability of HUVECs by MTT
Cell survival was determined after 48 hr of incubation. Results represent the mean of 5 independent experiments.

The lipid peroxide levels were measured as the Malon dialdehyde (MDA) concentration and expressed as nmol/ml mg^{-1} protein (Fig. 3). In the

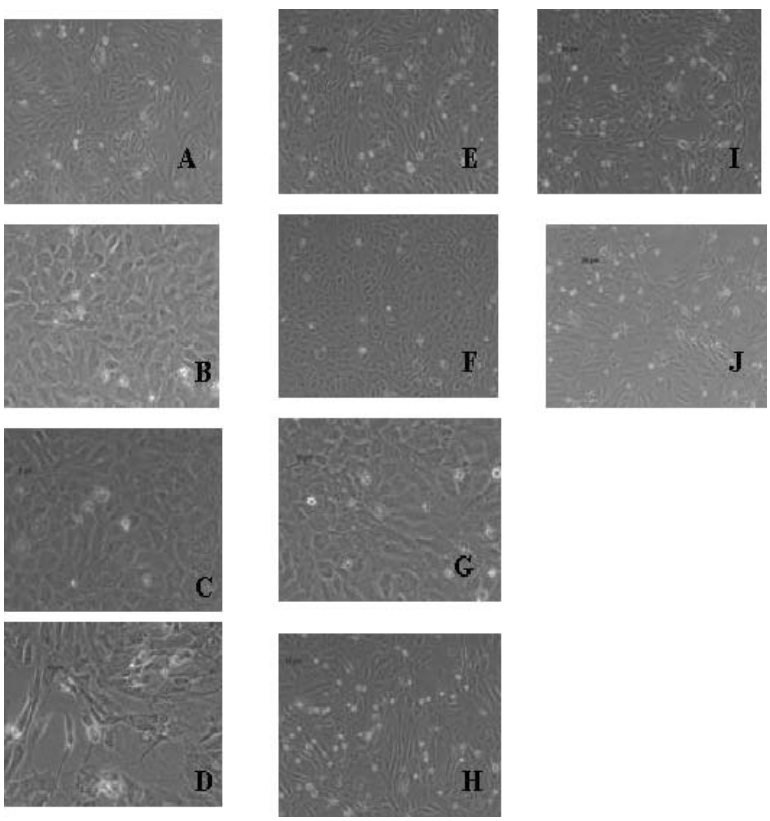

Fig. 2. Phase-contrast Micrographs of HUVECs Treated as Indicated in the Legend
A: FBS, B: GFBS, C: FBS+$FeCl_3$, D: GFBS+$FeCl_3$, E: GFBS+PFE (250 μg/ml), F: FBS+$FeCl_3$+PFE (250 μg/ml), G: GFBS+$FeCl_3$+PFE (250 μg/ml), H: GFBS+PFE (500 μg/ml), I: FBS+$FeCl_3$+PFE (500 μg/ml), J: GFBS+$FeCl_3$+PFE (500 μg/ml).

Table 1. Effect of PFE on Antioxidant Enzyme Activities in HUVEC Treated with Glycated Protein-iron Chelate

Particulars	SOD (amount of enzyme that inhibits the antioxidants of pyrogallol by 50%)	CAT (μmol/min/mg protein)	GPx (μmoles of glutathione oxidized/min per each mg protein)	GR (nmoles of NADPH oxidized/min/mg protein)
FBS	98.65 ± 1.72	21.76 ± 1.42	0.67 ± 0.006	64.01 ± 3.64
GFBS	81.07 ± 3.93^{a*}	15.85 ± 2.34^{a*}	0.43 ± 0.004^{a*}	52.08 ± 2.09^{a*}
FBS+$FeCl_3$	85.34 ± 3.65^{a*}	17.09 ± 0.73^{a*}	0.49 ± 0.007^{a*}	54.31 ± 2.08^{a*}
GFBS+$FeCl_3$	71.32 ± 1.4^{b*}	10.18 ± 0.41^{b*}	0.37 ± 0.008^{b*}	47.69 ± 1.65^{b*}
GFBS+PFE (250 μg/ml)	89.1 ± 2.61$^{b\#}$	16.05 ± 0.85$^{b\#}$	0.52 ± 0.003$^{b\#}$	58.25 ± 2.51$^{b\#}$
FBS+$FeCl_3$+PFE (250 μg/ml)	92.68 ± 3.27$^{a@}$	20.3 ± 0.76$^{a\mathrm{NS}}$	0.58 ± 0.012$^{a\#}$	59.26 ± 1.29$^{a\#}$
GFBS+$FeCl_3$+PFE (250 μg/ml)	79.53 ± 1.92$^{a*b@}$	15.82 ± 1.12$^{a*b\mathrm{NS}}$	0.49 ± 0.007$^{a*b\mathrm{NS}}$	53.02 ± 1.38$^{a*b@}$
GFBS+PFE (500 μg/ml)	91.08 ± 2.88$^{b\#}$	17.33 ± 0.98$^{b@}$	0.55 ± 0.003^{b*}	59.07 ± 1.45^{b*}
FBS+$FeCl_3$+PFE (500 μg/ml)	93.15 ± 1.61$^{a\#}$	20.92 ± 1.19$^{a\mathrm{NS}}$	0.61 ± 0.072$^{a@}$	60.78 ± 2.14$^{a@}$
GFBS+$FeCl_3$+PFE (500 μg/ml)	86.37 ± 2.36^{a*b*}	19.18 ± 0.79$^{a\#b*}$	0.58 ± 0.045$^{a@b*}$	59.80 ± 1.65$^{a@b*}$

Values are expressed as mean ± SEM. of 5 determinations. $^{*}p < 0.001$, $^{\#}p < 0.01$, $^{@}p < 0.05$. NS = Not Significant. *a*: as compared with FBS, *b*: as compared with GFBS.

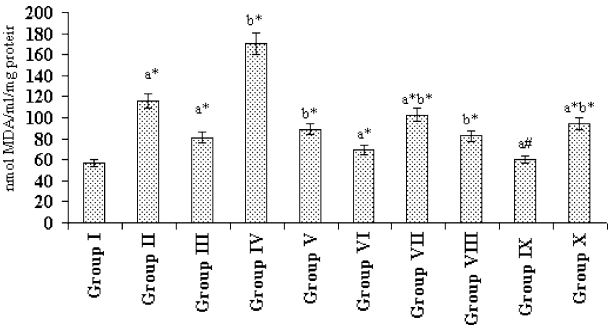

Fig. 3. Effect of PFE on LPO Levels in HUVEC Treated with Glycated Protein-iron Chelate

Group I: FBS, Group II: GFBS, Group III: FBS+$FeCl_3$, Group IV: GFBS+ $FeCl_3$, Group V: GFBS+PFE (250 μg/ml), Group VI: FBS+$FeCl_3$+PFE (250 μg/ml), Group VII: GFBS+$FeCl_3$+PFE (250 μg/ml), Group VIII: GFBS+PFE (500 μg/ml), Group XI: FBS+$FeCl_3$+PFE (500 μg/ml), Group X: GFBS+$FeCl_3$+PFE (500 μg/ml). Values are expressed as mean ± SEM. of 5 determinations. $^{*}p < 0.001$, $^{\#}p < 0.01$, $^{@}p < 0.05$. a: as compared with FBS, b: as compared with GFBS.

GFBS-iron chelate-treated cells the lipid peroxidation level was significantly increased when compared with that for the cells incubated in FBS alone ($p < 0.001$). The treatment with PFE of GFBS-iron-chelate or FBS-iron chelate-treated cells resulted in a reduced LPO level. The treatment with the GFBS-iron chelate significantly reduced the SOD and catalase activities in HUVECs when compared with their levels in the control (GFBS) group; whereas the PFE protected against these reductions (Table 1).

The GFBS-iron treatment significantly reduced the GPx level in HUVEC when compared with the control group (GFBS) level ($p < 0.001$). Treatment of HUVECs with 250, or 500 μg/ml of PFE significantly altered the GPx levels to near normal when compared with the level for the GFBS-iron-treated group ($p < 0.001$). Treatment of HUVECs with GFBS-iron depleted the GR activity significantly ($p < 0.001$) when compared with the activity for the FBS treated group; and treatment with PFE at 250 or 500 μg/ml significantly protected against this depletion (Table 1).

Next, Cyt c reductase in the various cultures was evaluated. Cyt c reductase activity was greater in the GFBS-iron chelate treated HUVECs than in the GFBS-treated ones. This increase was significantly inhibited by PFE (Table 2). Treatment of HUVECs with the GFBS-iron chelate also significantly ($p < 0.001$) reduced the GST acitivity when compared with the activithy for the FBS treatment group. Again PFE prevented this reduction and actually, marginally increased it (Table 2).

DISCUSSION

Glucose and Amadori products can undergo autoxidation in the presence of transition metals to generate free radicals. Similarly, Amadori products undergo metal-catalyzed oxidation (glycoxidation), generating reactive dicarbonyl compounds capable of forming AGEs and free radicals.[4] Free radicals can damage proteins, lipids, and nucleic acids and might contribute toward tissue damage in di-

Table 2. Effect of PFE on Cyt c Reductase and GST Activities in HUVEC Treated with Glycated Protein-iron Chelate

Particulars	Cyt c reductase (nmol of Cyt reduced min/mg protein)	GST (nmol/min/mg protein)
FBS	12.78 ± 0.93	9.72 ± 0.63
GFBS	$34.02 \pm 1.15^{a*}$	$4.72 \pm 0.28^{a*}$
FBS+$FeCl_3$	$21.45 \pm 1.08^{a*}$	$5.48 \pm 0.21^{a*}$
GFBS+$FeCl_3$	$42.67 \pm 1.24^{b*}$	$3.91 \pm 0.14^{b*}$
GFBS+PFE (250 μg/ml)	$28.87 \pm 0.92^{b*}$	$6.48 \pm 0.61^{b*}$
FBS+$FeCl_3$+PFE (250 μg/ml)	$16.2 \pm 0.76^{a*}$	$8.29 \pm 0.32^{a*}$
GFBS+$FeCl_3$+PFE (250 μg/ml)	$29.94 \pm 1.12^{a*b@}$	$6.97 \pm 0.61^{a*b@}$
GFBS+PFE (500 μg/ml)	$19.82 \pm 0.98^{b*}$	$7.38 \pm 0.52^{b*}$
FBS+$FeCl_3$+PFE (500 μg/ml)	$14.62 \pm 0.98^{a\#}$	9.03 ± 0.75^{aNS}
GFBS+$FeCl_3$+PFE (500 μg/ml)	$26.40 \pm 0.67^{a*b*}$	$7.42 \pm 0.56^{a*b*}$

Values are expressed as mean ± SEM. of 5 determinations. $^{*}p < 0.001$, $^{\#}p < 0.01$, $^{@}p < 0.05$. NS = Not Significant. *a*: as compared with FBS, *b*: as compared with GFBS.

abetes.[20] Increased glycation and accumulation of tissue AGEs can alter protein conformation and impair function by altering enzyme activity, modifying protein half-life, altering immunogenicity, and causing cross-linking of structural proteins.[1,21]

Recent studies have highlighted the benefits of using medicinal plants with combined antiglycation and antioxidant properties for the treatment of diabetic patients. Pomegranate juice is a popular drink worldwide. Commercial pomegranate juice (PFE) shows potent antioxidant and anti-atherosclerotic properties, which are attributed to its high content of polyphenols including EA in its free and bound forms (as ETs and EAGs), gallotannins and anthocyanins (cyanidin, delphinidin and pelargonidin glycosides), and other flavonoids (quercetin, kaempferol, and luteolin glycosides;[10,12] both of these properties are believed to reside in the EA component in PFE. In this study PFE inhibited AGE formation in a dose-dependent manner, as evaluated by its effects on LPO and antioxidant enzymes activities.

Lipid peroxidation has been used as an indirect measure of oxidative stress. The endproduct of stable aldehydes react with thiobarbituric acid (TBA) to form a thiobarbituric acid-malondialhyde adduct.[22] In previous studies, it was found that the LPO level was increased in HUVECs when the cells were incubated with glycated protein and iron.[23,24] In the present study treatment with PFE significantly ($p < 0.001$) reduced lipid peroxidation. The PFE extract protected against glycation-induced protein fragmentation probably because of its ability to chelate transition metals, thereby preventing autoxidative glycation and glycoxidation.

After PFE treatment, an increase in GR activity was observed. If there was a deterioration of the oxidative status and GR was inhibited, oxidized glutathione (GSSG) levels would most probably have been raised and not decreased as they were. It has also been described that certain polyphenols, namely, tannic acid and coumarins,[9,25] are able to increase GR activity;[26] and the presence of these or related polyphenols in PFE[27,28] may account for the observed increase in the GR activity. The same pattern of results was found for GPx, *i.e.*, increased activity after treatment with PFE. This enzyme catalyses the reduction of hydroperoxides at the expense of reduced glutathione (GSH). Catalase is also a peroxidase, and at the same time the most important enzyme involved in H_2O_2 degradation. Along with the increase in GPx activity there was also an increase in catalase activity in the PFE treated groups.

As they increase the same kind of substrates, GPx and catalase activities are often related.[29] This suggests that smaller amounts of hydroperoxides or H_2O_2 are being generated, which is also in agreement with the induction of SOD activity in PFE treated HUVECs. These enzyme catalyses the dismutation of O_2^-, producing H_2O_2, and the reduction on its activity may result from a decrease in superoxide production. It may seem reasonable to believe that endogenous antioxidant defences are lowered, as they are no longer required to act on an organism supplied with generous amounts of exogenous antioxidants. The decline in GR, GPx, SOD and catalase activities thus seems more compatible

with a general decrease in oxidative stress. Another glutathione-related enzyme is GST, a phase-II enzyme responsible for the detoxification of several substrates. This enzyme can also conjugate altered proteins and mediates protein repair mechanisms. Increased GST activities were observed in the cells treated with PFE. This increase could reflect the decrease in protein damage, which would, most likely, translate into less GST activity. Another explanation could be, as in the case of GR, that this enzyme is regulated in a competitive manner by polyphenols from pomegranate (*e.g.*, ellagic acid, tannic acid), as has been previously proposed.[30, 31] However, polyphenols are known to be able to modulate the endogenous antioxidant defence by interacting with the antioxidant response to oxidative injury management.[32, 33]

It is not clear, however, if the effects of PFE resulted from polyphenolic interference with enzymes, which could be consequence of a broader and more nonspecific action, connected to their antioxidant potential. Moreover, many factors need to be taken into account when examining the present results, as the degree of oxidative stress, the polyphenol class and concentration as well as the biological system studied may all introduce other elements of variability in the response to antioxidant ingestion.[34]

Thus, the overall condition of the HUVECs after 48 hr of PFE ingestion appears to be a state of reduced oxidative stress. This view is supported by the presumed decrease in protein and DNA damage, by the increase in activity of antioxidant enzymes (GPx, catalase, SOD and GST), most probably related to less production of oxygen reactive species. However, it should not be forgotten that the observed increased activity of antioxidant enzymes, along with higher levels of GSH, may still lead to vulnerability in the case of exposure to an insult or aggression, since other endogenous antioxidant defences are diminished in such situations. To clarify the pathophysiological meaning of these changes will require other approaches.

In conclusion, this study has indirectly shown that, *in vitro*, PFE can inhibit the nonenzymatic glycation of HUVECs. *In vivo* studies in normal animals and in animals with artificially induced diabetes are now required. This will help to define the physiological, pharmacological, chemical, and toxicity indices that might allow these antioxidant to be used safely and efficiently in humans in an attempt to minimize the nonenzymatic glycation of proteins, the hallmark of diabetic hyperglycemia and other chronic pathophysiological states.

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