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Ameliorative potential of gallic acid on the activation of ROS and down-regulation of antioxidant enzymes in cardiac tissue of rats infused with advanced glycation end products

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ABSTRACT

Advanced glycation end-products (AGEs) are implicated in the pathogenesis of diabetic complications including cardiac dysfunction and heart failure. We aimed to investigate the effects of circulatory AGEs on the oxidative stress parameters in the rat hearts, and also to examine the protective role of gallic acid (GA) in attenuating the AGEs-induced oxidative stress. The experimental Wistar rats were infused with *in vitro* synthesized rat serum albumin-derived AGEs intravenously at a dose of 50mg/kg/day, for 30 days with or without GA. The control animals received equal amount of unmodified rat serum albumin (RSA) intravenously for 30 days. The rats treated with AGEs alone exhibited increased plasma levels of creatine kinase (CK) ($p < 0.01$), and lactate dehydrogenase (LDH) ($p < 0.001$), when compared with controls. In parallel, a significant increase in the levels of the oxidative stress makers lipid peroxides (LPO) ($p < 0.001$), and protein carbonyls (PCO) ($p < 0.01$) were found in AGEs-RSA infused rat hearts compared with control rat hearts. The antioxidant enzymes analysis revealed a significant decrease ($p < 0.01$, respectively) in the activities of superoxide dismutase (SOD) and catalase (CAT) in AGEs-RSA infused rat hearts compared with control rat hearts. Gallic acid co-treatments (administered oral gavage daily at a dose of 25 mg/kg) normalized the decreased levels of antioxidant enzymes in the cardiac tissue on AGEs infusion. The results of the present study provide *in vivo* evidence that the circulating AGEs induce oxidative stress in the heart, and GA attenuates AGEs-mediated cardiotoxicity.

Key words: Advanced glycation end products, oxidative stress, antioxidant enzymes, gallic acid.

INTRODUCTION

Cardiovascular disease accounts for 80% of the mortality associated with diabetes mellitus (DM). Recent studies suggested that hyperglycemia is the major factor behind the cardiovascular complications in DM (Battiprolu et al., 2010). Along with the osmotic stress via the increased glucose flux through the polyol-sorbitol pathway; hyperglycemia also increases the formation of advanced glycation end-products (AGEs). Advanced glycation end-products commonly arise from the reaction of reducing sugars, such as glucose and short chain aldehyde, with amino groups. Once formed, these AGEs are very stable and often accumulate in the body especially in the long lived tissues such as collagen and other matrix proteins (Hartog et al., 2007). In addition, the AGEs are believed to mediate their pathogenic effects by interacting and up-regulating their receptors, such as receptor for AGE (RAGE). Through interacting with its membrane receptor, AGEs induces diverse signaling cascade, including induction of reactive

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oxygen species (ROS) and inflammatory cytokines, which consequently lead to apoptosis and compromised cardiovascular function (Ahmed, 2005; Coughlan et al., 2009; Méndez et al., 2010). Diabetic mice carrying targeted disruption of RAGE showed significantly decreased levels of mitochondrial and cytosolic ROS formation compared with normal diabetic mice (Tan, 2010). Treatment with AGE-Albumin significantly enhanced the DNA-binding activity of nuclear factor kappa-B (NF- κ B) in retinal endothelial cells (RMECs) and also increased the adhesion of leukocytes to RMEC monolayers (Moore et al., 2003). In addition, it has been shown that AGE-RAGE-induced cytosolic ROS facilitates mitochondrial superoxide production in cultured kidney cells (Coughlan et al., 2009). AGEs had also been shown to participate in the endothelial dysfunction via neutralizing nitric oxide production (Soro-Paavonen et al., 2010). The above described studies provide evidence for the role of advanced glycation pathway in the development and progression of diabetic complications, and suggest that AGE/RAGE signaling pathway induces oxidative stress.

Green tea is the most popular beverage worldwide consumed by 80% of population, and its beneficial effects are enormous in several ways. It reduces blood glucose level; improves sensitivity to insulin and it also enhances antioxidant defenses (Serisier et al., 2008). Furthermore, green tea inhibits the formation of AGEs in an *in vitro* bovine serum albumin (BSA)/glucose system, and development of fibrosis in diabetic hearts (Babu et al., 2007). Gallic acid (3, 5, 4- trihydroxy benzoic acid), a food component that is especially abundant in green tea, is a polyphenol which possess strong antioxidant, anti-inflammatory, antimutagenic and anticancer activities (Priscilla et al., 2009). Studies have suggested that gallates have membrane binding property thereby prevents the tissue damage (Shahrazad et al., 2001).

Though several reports describing the effects of glycated molecules on redox status and cell homeostasis, there are no investigations carried out on the direct effects of circulating AGEs on the heart. Hence, we aimed to investigate the chronic effects of circulating AGEs on oxidative stress parameters in the heart tissue of Wistar rats. We also studied the protective role of gallic acid (GA) in restoring the changes mediated by AGEs.

MATERIALS AND METHODS

Chemicals

Rat serum albumin (RSA), Gallic acid and D-Glucose were purchased from Sigma Chemical Co., (St. Louis, MO, USA). All other reagents used in the study are analytical grade.

Animals

Male Wistar rats (6–8 weeks old) were used throughout the study. The animals were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India. All animals were fed standard pellet diet (Gold Mohr rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum*. They were maintained in a controlled

environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. The protocol of the experiment was approved by our institutional animal ethical Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (IAEC No.01/070/09).

Preparation of rat serum albumin derived-AGEs

AGEs-RSA was prepared as previously described with slight modifications (Andreea et al., 2008). Rat serum albumin (100 mg/ml) and d-glucose (1.6 M) have been co-incubated in sterile PBS; pH 7.4 was filtered through 0.2 μ m filter and kept in dark for 6-8 weeks at 37°C. RSA alone incubated at the same condition without glucose served as control. The density of brown color formed which is the typical physical appearance of AGEs. After incubation, the free sugar was removed by extensive dialysis against PBS, pH 7.4 for 48 h. The final protein concentration was determined using Bradford reagent. The formation of AGEs was confirmed by specific fluorescence at 355/460 and 320/380 nm, which reflects the formation of pentosidine and carboxy methyl lysine (CML) (Rasheed et al., 2009). The prepared AGEs-RSA were lyophilized and kept at -20°C until further use.

Experimental groups

The experimental rats were divided to into five groups, each group comprising of 6 rats.

- Group I served as normal control received vehicle alone;
- Group II animals were administered with RSA (50 mg/kg body weight/per day i.v) for the period of 30 days;
- Group III animals were administered with RSA-AGEs (50 mg/kg body weight/per day i.v) for the period of 30 days (Thomas et al, 2005);
- Group IV animals were co-treated with gallic acid by oral gavage daily at a dose of 25 mg/kg body weight, subsequent to infusion of RSA-AGEs (50 mg/kg body weight/per day i.v) ; and
- Group V animals were treated with gallic acid alone by oral gavage daily at a dose of 25 mg/kg body weight for 30 days.

The experiment was terminated at the end of 30 days and the animals were anesthetized with ketamine and xylazine and killed by cervical dislocation, blood sample was collected and the plasma was separated for the biochemical analysis.

Preparation of heart tissue homogenate

At the end of experimental period, heart from control and experimental groups of rats were excised and washed with ice-cold saline to remove blood. For the biochemical estimations, the left ventricular heart tissues were homogenized in ice-cold Tris-HCl buffer (100 mM, pH 7.4) using Teflon homogenizer, and centrifuged at 12,000g for 30 min at 4°C. The supernatants were separated and used for the biochemical estimations. The protein content in the tissue homogenate was estimated using a Bradford reagent kit.

Cardiac marker enzymes

The plasma levels of cardiac markers creatine kinase (CK) and lactate dehydrogenase (LDH) were estimated by the methods of Okinaka et al., (1961), and King et al., (1965), respectively. The CK enzyme activity is expressed as μmol of phosphorus liberated/mg of protein/h. The LDH activity is expressed as μmol of pyruvate liberated/mg of protein/h.

Oxidative markers and Antioxidant enzymes

The levels of lipid peroxide (Niehaus et al., 1968) and protein carbonyls (Levine et al., 1990) and the activities of enzymatic antioxidants superoxide dismutase (Kakkar et al., 1978), catalase (Sinha, 1972), and non-enzymatic antioxidant glutathione (Ellman, 1959) were determined in the left ventricular heart tissue homogenate of control and experimental groups of rats.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0; GraphPad Software, San Diego, USA). The results are presented as mean \pm SEM. The statistical differences between the groups were determined by Student's *t* test. The probability value of $p < 0.05$ was considered significant.

RESULTS

The levels of cardiac markers creatine kinase (CK) and lactate dehydrogenase (LDH) in the plasma of control and experimental group of animals are represented in Table 1.

Table 1: Effect of AGEs and gallic acid on the levels of creatine kinase (CK) and lactate dehydrogenase (LDH) in the plasma of control and experimental group of rats.

Groups	LDH	CK
Group I (Control)		87 \pm 5
	916 \pm 15	
Group II (RSA-Infused)	927 \pm 18 ^{NS}	93 \pm 6 ^{NS}
Group III (AGEs-Infused)	1332 \pm 37 ^{***}	170 \pm 9 ^{**}
Group IV (AGE + GA co-treatment)	934 \pm 20	98 \pm 8
Group V (GA)		92 \pm 3 ^{NS}
	925 \pm 11 ^{NS}	

Data are represented as Mean \pm SEM (n=6) per group. ** $P < 0.01$, *** $P < 0.001$, control vs AGE-infused. NS- non-significant, control vs RSA, AGE+ GA, and GA. CK activity is expressed as μmol of phosphorus liberated/mg of protein/h. LDH activity is expressed as μmol of pyruvate liberated/mg of protein/h.

Table 2: Effect of AGEs and gallic acid on the release of lipid peroxides (LPO) and protein carbonyls (PCO) in control and experimental group of rats.

Groups	LPO (nmoles MDA/mg protein)	PCO (nmoles/mg protein)
Group I (control)	4.2 \pm 1.6	2.1 \pm 0.6
Group II (RSA-Infused)	4.3 \pm 1.7 ^{NS}	2.4 \pm 0.5 ^{NS}
Group III (AGEs-Infused)	7.2 \pm 2.3 ^{***}	7.3 \pm 1.2 ^{**}
Group IV (AGE+GA co-treatment)	4.5 \pm 1.8 ^{NS}	2.6 \pm 0.8 ^{NS}
Group V (GA)	3.4 \pm 1.2 ^{NS}	2.2 \pm 0.6 ^{NS}

Data are represented as Mean \pm SEM (n=6) per group. ** $P < 0.01$, *** $P < 0.001$, control vs AGE-infused. NS- non-significant, control vs RSA, AGE+ GA, and GA. LPO is expressed as nmoles malondialdehyde (MDA) /mg protein and PCO is expressed as nmoles/mg protein.

Table 3: Effect of AGEs and gallic acid on the activity of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) in control and experimental group of rats.

Groups	SOD (Units/mg protein)	CAT (Units/mg protein)	GSH ($\mu\text{g}/\text{mg}$ protein)
Group I (Control)	5.5 \pm 0.9	53 \pm 11	14 \pm 3
Group II (RSA-Infused)	5.2 \pm 0.8 ^{NS}	52 \pm 13 ^{NS}	13 \pm 5 ^{NS}
Group III (AGE-Infused)	3.2 \pm 0.4 ^{**}	36 \pm 9 ^{**}	6 \pm 2 ^{**}
Group IV (AGE+GA co-treatment)	4.8 \pm 0.8 ^{NS}	48 \pm 8.2 ^{NS}	14 \pm 4 ^{NS}
Group V (GA)	5.1 \pm 0.7 ^{NS}	55 \pm 5.9 ^{NS}	13 \pm 5 ^{NS}

Data are represented as Mean \pm SEM (n=6) per group. *** $P < 0.001$, control vs AGE-infused. NS- non-significant, control vs RSA, AGE+ GA, and GA. SOD: Activity is expressed as units in enzyme concentration required for 50% inhibition of NBT reduction/minute. CAT: Activity is expressed as units in μmol of hydrogen peroxide consumed/minute.

The rats infused with AGEs alone (Group III) exhibited a significant increase in the levels of CK ($p < 0.01$) and LDH ($p < 0.001$) as compared with Group I and Group II rats. Whereas, animals co-treated (Group IV) with GA exhibited a significant decrease in the plasma levels of CK and LDH as compared with Group III rats, which is probably due to the protective nature of gallic acid. No significant changes in the levels of CK and LDH were observed in the rats treated with RSA (Group II) and GA (Group V) alone as compared with control rats (Group I).

The results presented in Table 2 shows the levels of lipid peroxide (LPO) and protein carbonyls (PCO) in the left ventricular heart tissue extract of control and experimental group of rats. The rats infused with AGEs alone (Group III) exhibited a significant increase in the left ventricular tissue levels of LPO ($p < 0.001$) and PCO ($p < 0.01$) as compared with Group I and Group II rats. Whereas, animals co-treated (Group IV) with GA exhibited a significant decrease in the left ventricular tissue levels of LPO and PCO as compared with Group III rats, No significant changes in the levels of LPO and PCO were observed in rats treated with RSA (Group II) and GA (Group V) alone as compared with control (Group I) rats.

Table 3 depicts the activities of enzymatic antioxidants (SOD and CAT), and the levels of non-enzymic antioxidant (GSH) in the left ventricular heart tissue of control and experimental group of rats. The rat hearts infused with AGEs alone (Group III) showed a significant decrease in the activities of SOD ($p < 0.01$) and CAT ($p < 0.01$) as compared with Group I and Group II rat hearts. Whereas, animals co-treated (Group IV) with GA exhibited a significant increase in the left ventricular tissue levels of SOD and CAT as compared with Group III rats, No significant changes in the activities of SOD and CAT were observed in rats treated with RSA (Group II) and GA (Group V) alone. Similar trend was observed in the reduced glutathione levels. The rats infused with AGEs alone (Group III) exhibited a significant decrease in the left ventricular tissue levels of GSH ($p < 0.01$) as compared with Group I and Group II rats. Whereas, animals co-treated (Group IV) with GA exhibited a significant increase in the left ventricular tissue levels of GSH as compared with Group III

rats, No significant changes in the levels of GSH was observed in rats treated with RSA (Group II) and GA (Group V) alone as compared with control (Group I) rats.

DISCUSSION

The results of the present study demonstrate that oxidative markers levels are increased in the AGEs infused rat hearts but are decreased in gallic acid (co-treated) treated rat hearts. AGEs are a heterogeneous group of compounds formed from the non-enzymatic glycosylation reaction between reducing sugars and amino group of proteins. Elevated circulating and myocardial levels of AGEs have been reported in diabetic patients (Iacobellis et al., 2004), and suggested to contribute to the development and progression of heart failure (Ahmed, 2005).

The plasma levels of CK and LDH are the well known markers of cardiac damage. When myocardial cells are damaged or destroyed due to deficient oxygen supply or glucose or due to oxidative stress, the cardiac membrane becomes permeable or may rupture which results in leakage of enzymes (Shreesh et al., 2010). These enzymes enter into the blood stream thus increasing their concentration in the serum (Mathew et al., 1985). LDH is found to be released from the stunned or infarcted myocardial tissue (Robinson and Christenson, 1999). The release of the cardiac LDH into the blood circulation is used as an index of cardiac cell damage. LDH is the enzyme involved in the final step of anaerobic glycolysis. Increased activity of LDH in diabetes mellitus has been reported (Ajmani et al., 2011). The LDH system reflects the NAD⁺/NADH ratio, indicated by the lactate/pyruvate ratio in the cytosol. Normal LDH activity is indicative of improved channeling of (pyruvate) glucose by mitochondrial oxidation. In addition to LDH, plasma CK activity is a more sensitive indicator in early stage of myocardial ischemia, while peak rises in myocardial tissue (Shreesh et al., 2010). CK catalyzes the transfer of phosphate from creatine phosphate to adenosine diphosphate (ADP), producing ATP. Increased CK activity was observed in conditions like increased muscle activity, stress, or trauma (Robinson and Christenson, 1999). In the present study, the AGEs alone (Group III) infused animals exhibited higher plasma levels of these markers enzymes indicating that AGEs/RAGE signaling induces cardiac tissue damage. In line with the previous reports, animals treated with gallic acid showed a significant decrease in the level of these markers suggesting the cardio-protective role of gallic acid (Priscilla et al., 2009).

Anti-oxidants are substances that delay or inhibit oxidative damage to the target molecule. Cellular system is equipped with both enzymatic and non-enzymatic defenses to counteract the reactive oxygen species(ROS) (Kalim et al., 2010). SOD, CAT and GSH are the three primary antioxidant enzymes located in the heart, with different sites of action in cardiomyocytes. Superoxide dismutase can catalyze dismutation of O₂^{•-} into H₂O₂, which is then deactivated to H₂O by catalase. A decrease in the activity of these antioxidants can lead to an excess availability of superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂), which, in turn, generate hydroxyl radicals (•OH), resulting in initiation and

propagation of lipid peroxidation (Maritim et al., 2003). In addition, to these, glutathione is a major non-protein thiol in living organisms which involved in the maintenance of normal cell structure and function, probably through its redox and detoxification reaction. Reduced glutathione functions as free radical scavenger and in the repair of free radical caused biological damage (Valko et al., 2007).

In diabetes, free radicals are formed disproportionately during glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins (Khullar et al., 2010). This results in abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms (Lorenzi et al., 2010). In the present study, infusion of AGEs, decreased the levels of antioxidant enzymes in the cardiac tissues and these levels were found to be restored on GA treatment. Reduced activity of antioxidant enzymes, such as SOD, magnifies the generation of ROS leading to the damage of cellular organelles and enzymes and increased lipid peroxidation (Kakkar et al., 1995). Recent reports showed that non-enzymatic glycation of SOD leads to site-specific and random fragmentation of enzyme, accompanied by enzymatic inactivation (Jabeen et al., 2007). Depletion in the activities of antioxidant enzymes in the heart tissue of AGEs induced rats may be due to the increased utilization of these antioxidants for scavenging ROS. The activities of these enzymes were brought back to near normal values upon treatment with GA. This could be due to the direct action of GA on superoxide, hydroxyl and alkoxyl radical coupled with its ability to attenuate oxidative stress (Katiyar et al., 2001), which in turn reduces the cellular damage. Mitić et al., suggested that the ameliorative potential of GA could be due its powerful hydrogen-donating nature which attributed to the presence of the trihydroxyl group in its ring (Mitić et al., 2010). Taken together, the results of the present study provide *in vivo* evidence that the circulating AGEs induce oxidative stress in the heart, and GA attenuates AGEs-mediated cardiotoxicity, providing further evidence of a role for the advanced glycation pathway in the development and progression of diabetic cardiomyopathy.

Conflict of interest statement

None declared.

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