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Author: Mohammed Jainuddin Yousuf Elangovan Vellaichamy

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PROTECTIVE ACTIVITY OF GALLIC ACID AGAINST GLYOXAL -INDUCED RENAL FIBROSIS IN EXPERIMENTAL RATS

Mohammed Jainuddin Yousuf and Elangovan Vellaichamy*

Department of Biochemistry, University of Madras, Guindy Campus, Chennai- 600 025, Tamil Nadu, India.

***Corresponding Author:**

Dr. Elangovan Vellaichamy

Department of Biochemistry

University of Madras

Guindy Campus, Chennai, India- 600 025

Tel: +91-44-22202734

Email: vellaie@gmail.com, vellaie@unom.ac.in

First Author:

Mr. Mohammed Jainuddin Yousuf

Department of Biochemistry

University of Madras

Guindy Campus, Chennai, India - 600 025

Email: jainuddin.y@gmail.com

Abstract

This study was designed to evaluate the protective activity of gallic acid (GA) against glyoxal (GO) an advanced glycation intermediate-induced renal fibrosis in experimental rats. Glyoxal (i.p) at a dose of 15 mg/Kg body weight/day for 4 weeks induces renal fibrosis. GA was administered orally (100 mg/Kg body weight/day) along with GO for 4 weeks. The anti-fibrotic activity of GA was analyzed by measuring the collagen synthesis and deposition in renal tissues using mRNA expression analysis and Masson trichrome staining (MTS), respectively. The nephroprotective potential of GA was assessed by quantifying the markers of kidney damage such as serum blood-urea-nitrogen (BUN), creatinine (CR) and alkaline phosphatase (AP). Moreover, basement membrane damage in renal tissues was analysed by periodic acid Schiff's (PAS) staining. GA co-treatment markedly suppressed the GO-induced elevation in mRNA expression of *collagen I* and *III*, *MMP-2*, *MMP-9* and *NOX* ($p<0.05$, respectively) genes as compared with GO alone infused rats. In addition, GA co-treatment significantly attenuated the GO -induced elevation in serum markers such as BUN, CR and AP levels ($p<0.05$, respectively). Furthermore, GA co-treatment restored back the decreased renal super oxide dismutase (SOD) activity ($p<0.05$) thereby assuage the reactive oxygen species (ROS) generation, and maintained the normal architecture of glomerulus. The present study clearly indicates that GO -induces renal fibrosis by enhancing GO/receptor of advanced glycation end product (RAGE) induced ROS generation and GA effectively counteracted GO-induced renal fibrosis by its ROS quenching and anti-glycation activity.

Keywords

Glycation, Glyoxal (GO), AGE intermediate, Gallic acid (GA), renal hypertrophy, Fibrosis.

1. Introduction

Glyoxal (GO) is a well known physiological α-oxoaldehyde, and is involved in the formation of advanced glycation end products (AGE) (Lange *et al.,* 2012). α-oxoaldehydes are implicated as an important causative factor of renal dysfunction (Subramaniam *et al.,* 2012; Matsumura *et al.,* 2013). Studies have shown that the α-oxoaldehydes are capable of interacting with membrane proteins, nucleotides, and phospholipids (Thornalley, 2008). Besides being a natural metabolite of glucose, GO can be formed by lipid peroxidation, nucleic acid oxidation and degradation of glycated proteins (Chetyrkin *et al.,* 2011; Turk, 2010). GO is also present in foods products, beverages and widely used in industrial chemicals (Arribas-Lorenzo and Morales, 2010). The reactive carbonyl group of GO can react with amino group of proteins to form covalent adducts known as AGEs (Singh *et al.,* 2014). It has been reported that the circulatory levels of α-oxoaldehydes are increased in hyperglycemic patients due to an imbalance in the intracellular glucose metabolism (Ogawa *et al.,* 2010).

Irreversible forms of AGEs are capable of altering the structure and functions of membrane proteins as well as contractile proteins (Snow *et al.,* 2007). An increased circulatory and tissues levels of AGEs have been attributed to the secondary complication of diabetes (Peppa *et al.,* 2003). We have previously demonstrated that infusion of GO induces renal damage and modulates the redox potential in experimental rats, most likely via receptor for AGEs (RAGE) mediated pathway (Subramaniam *et al.,* 2012). *In vitro* studies have shown that, AGE-RAGE-ROS signaling contribute to the release of proinflammatory cytokines and growth factors that are implicated in the pathogenesis of diabetic complications (Liu *et al.,* 2009).

Recently studies have been initiated to identify molecules that are capable of suppressing AGEs formation and its secondary complication in diabetic patients (Thornalley, 2003). Aminoguanidine (AG) is a well-known synthetic inhibitor of AGEs formation, and it was reported to confer protection against AGE-mediated diabetic nephropathy (Ravelojaona *et al.*, 2007). However, AG exhibits deleterious side-effects such as gastrointestinal disturbances and abnormalities in liver function besides its beneficial counteractions against AGEs (Thornalley, 2003). At this juncture, natural products have generally been proven to be relatively safe for human consumption, as compared to synthetic compounds. There, has been an increasing interest in the use of natural plant compounds, as anti-glycating agents (Elosta *et al.*, 2012).

Experimental studies on natural polyphenols such as, naringenin and its glycosides, quercetin and epicatechin exhibit a significant anti-glycation property (Zhang *et al.*, 2014). Gallic acid (GA) (3,4,5-trihydroxybenzoic acid), a natural antioxidant, has been reported to confers free radical scavenging activity (Stanley *et al.*, 2009), anti- inflammatory (Kroes et al., 1992) anti-hyperglycemic properties (Bak *et al.*, 2013). Gallic acid was reported to contain three hydroxyl groups bonded to the aromatic ring in an ortho position exhibits the strongest free radical, scavenging activity (Sroka and Cisowski, 2003). The ortho substitution of hydroxyl groups to the aromatic ring is good for anti-oxidant and free radical scavenging activity of phenolic acids (Borde *et al.*, 2011). We have previously reported that GA effectively counteracted AGE-induced cell proliferation and oxidative stress in H9C2 (2-1) rat cardiomyocyte cells, (Subramaniam *et al.*, 2012). Hence, in the present study, we made an attempt to study the beneficial counter actions of GA against GO, an intermediate metabolite of AGEs induced renal fibrosis in experimental rats.

2. Materials and Methods

2.1. Animals

Experimental protocol pertaining the use of adult male Wistar rats weighing about 120 - 150 g used in this study and has been approved by the Institutional Animal Ethics Committee. Animals were housed in plastic cages and kept at 25°C with 12:12 hour light: dark schedule. All animals were fed standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum*.

2.2. Drugs and Chemicals

Glyoxal, Bradford's reagent and GA was purchased from Sigma Aldrich (MO, USA). All the other chemicals used were of analytical grade.

2.3. Experimental Design

Animals are divided into four groups of 6 animals each. Group 1: Control group animals received a daily injection of saline during experimental period. Group 2: Animals received intraperitoneal injections of glyoxal (15 mg/kg BW/day) for 4 weeks (GO). Group 3: Animals receive an intraperitoneal injection of GO (15 mg/kg BW/day) along with oral administration of Gallic acid (100 mg/Kg BW/day) (GO+GA). Group 4: Animals received GA alone orally (100 mg/Kg BW/day) during the experimental period.

2.4. Biochemical Analysis

After the experimental period the blood sample was collected and centrifuged at 1,500g for 10 min at 4°C. The clear supernatant was used for further serum biochemical investigations, Blood urea nitrogen (BUN) (Rahmatullah and Boyde, 1980), serum creatinine (CR) (Tietz, 1987) and serum alkaline phosphatase (AP) (Watanabe *et al.*, 1967). Kidney tissues were homogenized in Tris-HCl buffer (100 mM, pH 7.4) using Teflon homogenizer and centrifuged at 12,000g for 30 min at 4°C. The supernatant obtained was used for further studies. Protein content of renal tissue extract was assessed by standard Bradford's method (Bradford, 1976). The total collagen

content was quantified by modified protocols of Bergman and Loxley (Bergman and Loxley, 1970). Thiobarbituric acid reactive substance, an index of lipid peroxidation (LPO) was performed using protocols of Ohkawa *et al.,* (Ohkawa *et al.*, 1979). Superoxide dismutase (SOD) activity was determined using the methods of Kakkar *et al.,* (Kakkar *et al.,* 1984).

2.5. Histological Examinations

Kidney tissue was fixed in 4% paraformaldehyde solution and embedded in paraffin wax. The sections obtained from kidney tissues were stained using hematoxylin and eosin (H&E) staining (Luna, 1968), Periodic acid Schiffs (PAS) staining (Thompson 1966) and Masson's trichrome staining (Luna, 1968) to observe the histological changes.

2.6. PCR analysis

Total cellular RNA was isolated by Trizol method based upon the method developed by Chomczynski and Sacchi (2006). To remove genomic DNA contamination, RNA samples were treated with RNase-free DNase I at 37°C for 30 minutes. The integrity of RNA was visualized by distinct 28 S and 18 S bands in 1.5% agarose gel electrophoresis. PCR was performed using GeNei M-MuLV RT-PCR kit along with the primers listed in Table 1.

2.7. Gel activity assays

The activity of MMPs was detected using gelatin zymography (Neely *et al.,* 1997). MMPs activity was visualized as clear distinct bands against blue background and was quantified by scanning densitometry. The antioxidant gel activity of SOD was determined by recommended protocols. Tissue extracts were separated in 10% native PAGE and the gel was stained in Riboflavin-NBT solution gives clear SOD bands against the blue-purple background of the gel (Beauchamp and Fridovich, 1971).

2.8. Immunostaining

The tissue sections were dewaxed; rehydrated using series of alcohol and were incubated for antigen retrieval in citrate buffer (pH 6.0). Following antigen retrieval process, the sections were incubated with Goat polyclonal IgG RAGE, Rabbit polyclonal IgG MMP-2, MMP-9, and Mn-SOD (1:100 each) primary antibodies diluted in 1% BSA in PBS for 2 h at room temperature. The sections were washed in PBS and incubated with its specific secondary HRP conjugated antibody at a dilution 1:250 for 1 h at room temperature. The peroxidase activity was visualized by treating the slides with DAB and was counterstained using Meyer's hematoxylin.

2.9. Immunoblot

Proteins were separated using SDS-PAGE and transferred to PVDF membrane, the membrane was processed with Goat polyclonal IgG RAGE (1:1000), Rabbit polyclonal IgG MMP-2 (1:500), MMP-9 (1:500) primary antibodies followed by HRP-conjugated antibody (1:2500) and developed using DAB solution.

2.10. Reactive Oxygen Species

Kidney tissue samples were prepared for dichlorodihydrofluorescein (DCF) fluorescence according to previously described method (Montoliu *et al.*, 1994). Formation of dichlorodihydrofluorescein (DCF) was measured using a fluorometer with excitation and emission wavelengths of 488 nm and 525 nm respectively.

2.11. Statistical Analysis

Statistical analysis was performed using GraphPad Software (San Diego, CA). The results are presented as means ± SEM. Statistical differences between groups were determined by one-way analysis of variance (ANOVA) with Students t test.

3. Results

3.1. Physiological parameters

Table.2. depicts the KW/BW ratio, which is an indicative of hypertrophic growth was increased (36%) significantly in GO infused rats as compared with control. Whereas, animals co-treated with gallic acid exhibits protection against GO- induced hypertrophy.

3.2. Biochemical Parameters

Table.3. shows the levels of Blood Urea Nitrogen, serum creatinine and serum alkaline phosphatase in control and experimental group of animals. Animals co-treated with gallic acid showed a significant decline ($p<0.05$) in the levels of serum biochemical markers as compared with GO alone treated rats. Animals received GA alone did not show any significant changes in the serum biochemical parameters as compared with that of the control animals.

3.3. Morphology

Figure.1. depicts the renal sections from control and experimental group of animals. Degenerating glomerulus and tubular epithelial cells were seen in GO treated animals indicated by arrows (panel b) However, animals co-treated with GA (panel c) showed a protection against glomerular damage as compared with the animals treated with GO alone. No significant change was observed in the gallic acid alone treated animals (plate d).

Collagen content was assessed by Masson's trichrome staining was shown in Figure.1 (f) depicts the collagen deposition in the kidney tissues of GO received animals. However, animals co-treated with GA (panel g) showed decrease in the collagen content in the renal tissue as compared with GO treated animals.

Periodic Acid Schiff's staining of the kidney tissue shows the thickening of the basement membrane was evidenced by an increase in the PAS stain in the tubular basement membrane, and in the glomerulus in GO group animals (panel j). However, a decrease in the PAS stain was observed in the GO+GA group of animal (panel k) in the degenerating tubules. No significant

changes in the glomerulus and tubules were observed in control and GA control group animals panel (i) and (l).

3.4. Collagen content

Figure.2 (A) depicts total collagen deposition in the kidney tissues of control and experimental group of animals. An increase in the collagen accumulation in the kidney tissues of GO group animals ($p<0.01$) was observed as compared with control group animals. On the other hand, a significant decrease in the total collagen content was evidenced in the kidney tissues of GO+GA group animals ($p<0.05$) as compared with GO treated animals. No significant change was observed in the animals treated with GA alone.

Figure.2 (B) explains the mRNA expression profile of collagen I and collagen III gene in the kidney tissues of control and experimental group of animals. It was evidenced that an increase in the expression of collagen I ($p<0.01$) and collagen III ($p<0.05$) gene in the GO group animals was observed as compared with control group animals. Animals co-treated with GA showed a marked reduction in the expression profile of collagen I and III genes. No significant change was observed in GA alone treated animals.

3.5. Metalloproteinase accumulation

Figure.3 (A) exhibits the gelatinase activity in the kidney tissues of control and experimental group animals. MMP-2 and -9 activity was observed to be increased in the GO treated animals ($p<0.001$ and $p<0.01$). However, animals co-treated with GA showed a significant reduction in the gelatinase activity in the renal tissue as compared with GO treated animals. No significant change was observed in the animals treated with GA alone.

Figure.3 (B) depicts the mRNA expression profile of MMP-2 and -9 genes in the kidney tissue of control and experimental group animals. GO treated animals showed increased mRNA

expression of MMP-2 and -9 ($p<0.01$, respectively) as compared with control animals. Conversely, animals co-treated with GA showed a significant decrease in the expression levels of the MMP-2 and -9 ($p<0.05$, respectively) in the kidney tissue as compared with GO treated animals.

3.6. NADPH oxidase and ROS levels

Figure.3 (C) depicts the mRNA expression levels of NOX in the kidney tissues of the experimental group of animals. GO group animals showed an increase in the expression levels of NOX ($p<0.01$) as compared with control animals. Animals co-treated with GA exhibited a significant reversion in the levels of NOX as compared with GO treated animals.

Figure. 3 (D) depicts the ROS generation in control and experimental group animals. GO treated animals showed an increase in the ROS generation in the renal tissue extract ($p<0.001$) as compared with control group animals. However, GA co-treated rats showed a significant reduction in the ROS generation as compared with GO alone treated rats.

3.7. Immunostaining

Figure.4 shows MMP-2 and MMP-9, RAGE and Mn SOD protein expression in the renal tissue sections of control and experimental group of rats. An increased expression of MMP-2 and MMP-9 in the glolmerulus (panel b and f) was observed in the animals treated with GO alone. However, there was comparatively decreased in the levels of MMP-2 and MMP-9 in the glomerulus (panel c and g) of the GO+GA group animals. The photograph of glomerulus from the control and GA control group animals showed no significant changes. RAGE serves as a gateway for AGEs to induce cellular damage (Wada and Yagihashi, 2005) was found to be increased in the GO administered group of animals Figure.4 (Panel j). The expression of RAGE was reduced in the GA co-treatment group of animals (Panel k) which shows that GA may

possess anti-AGEs property in renal injury. No change in the glomerulus was observed in the control and GA group animals. The immune expression of antioxidant enzymes superoxide dismutase was measured using immunostaining Figure.4 shows the detection of Mn-SOD activity in experimental group animals and it was observed that there was a decrease in the expression of MnSOD levels in GO alone administered group (Panel n) as compared with control group animals (Panel m) and animals receives GO along with GA reveals decreased levels of MnSOD in the kidney tissue (Panel o) which explains that natural bioflavanoid GA might be an alternate source in delaying renal injury caused by food derived glycotoxins.

3.8. Immunoblotting

Figure.5 depicts the protein expression of MMP-2, MMP-9 and RAGE inn control and experimental group animals. An increase in the levels of MMP-2 ($p<0.01$), MMP-9 ($p<0.05$) and RAGE ($p<0.01$) was observed in the renal tissues of GO treated animals, on the other hand animals receives GA along with GO showed a significant reversion of the MMP-2, MMP-9 and RAGE in the kidney tissues of experimental group animals. Animals receive GA alone showed no significant change as compared with the control group.

3.9. Antioxidant status

The results from the oxidative stress markers and the levels of antioxidant enzymes in the kidney tissue of experimental animals are represented in Figure.6. GO received animals induced an imbalance in the redox status, with increased levels of thiobarbituric acid reactive substances (TBARS) ($p<0.01$) and declining activities of SOD ($p<0.01$) was found as compared to the control animals; whereas, animals received GA express a reversion in the LPO and SOD levels as compared with the GO group. Figure.6 (C) shows the detection of Mn-SOD gel activity in renal tissue homogenate of the experimental animals. SOD activity was significantly decreased

in GO infused animal kidney tissue ($p<0.01$). However, animals receive GO along with GA has a remarkable increase in the levels of SOD as compared with that of the GO group ($p<0.05$).

4. Discussion

The results of the present study demonstrated that GO an intermediate metabolite of AGEs induces renal fibrosis and that the natural antioxidant GA effectively attenuated the GO-induced renal fibrosis probably by attenuating the GO-induced ROS generation in the kidney tissues of experimental rats.

The kidneys are the vital organs of circulatory system that are responsible for maintaining the electrolyte balance in the blood and removing the toxic materials from the blood. In the present study, glyoxal treated animals showed a loss of body weight (Table. 2) and renal hypertrophy (36%). AGEs and its intermediate glyoxal are considered as uremic toxins. These reactive molecules reported to accumulate in the tubular epithelial cells and mesangium of the kidneys, and induce glomerular damage (Sebekova *et al.*, 2001; Greven *et al.*, 2005; Nematbakhsh *et al.*, 2012). It has been shown previously that, morphological changes in the kidney may reflect the renal hypertrophy and chronic progressive nephropathy (Greaves, 2000). Hence, the observed loss of body weight and increase in the kidney weight/body weight ratio in the glyoxal treated rats was probably due to glyoxal-induced renal injury.

Serum creatinine, and to a lesser extent blood urea, detects renal dysfunction in many circumstances (Baek *et al.*, 1975). Creatinine is found to be a reliable marker in renal injury. The kidneys maintain the levels of creatinine by clearing it out from the body and elevation in the levels of serum creatinine shows malfunction of kidneys. Similarly urea is also a metabolic byproduct which can build up in the plasma during renal insult. The BUN-to-creatinine ratio generally provides more information about kidney function (Lip *et al.*,2013). In the present

study, GO-induced animals showed elevated levels of serum creatinine and BUN (Table. 2) ($p<0.05$). In contrast, animals received GA along with GO showed significantly decreased ($p<0.05$) levels of serum renal markers as compared to the GO treated animals. In addition, Alkaline phosphatase (ALP) was also elevated in the GO treated animals while GA treatment reverted back the elevated levels to near normal. ALP is a hydrolyze enzyme that dephosphorylates various molecules, most effectively operating in an alkaline environment. Pathologic conditions are most commonly associated with elevations in ALP (Eknoyan *et al.*, 2003). In the present study, the elevated levels of ALP might be due to the poor detoxification of glyoxal leads to liver discomfort and thereby results in the elevation in the ALP. Combined together, these results clearly indicated the nephroprotective role of GA against the GO induced changes in kidney.

Diabetic nephropathy (DN) is characterized by enlargement of the glomerulus and tubules (Thomas *et al.*, 2005). Previous studies have shown that, AGEs were found to be deposited in tubular epithelial cells and in the glomerular mesangium of kidneys (Vlassara *et al.*, 1994). It has been suggested that AGEs and its intermediates such as GO have the ability to cause cytotoxicity (Sliman *et al.*, 2010), altered cell morphology, (Thomas *et al.*, 2005, Shangari and O'Brien, 2004) and finally loss of cellular architecture in kidney tissues (Subramaniam *et al.*, 2012). The renal tissue sections from the glyoxal treated animals showed an increase in glomerular damage and basement membrane thickening. It explains the reason for the renal impairment in these rats. An increase in the accumulation of polysaccharides in the glomerulus which are indicated by arrows (Figure. 1) eventually affects the filtration capabilities of glomerulus as evidenced by an increased in PAS staining (Horobin and Kiernan, 2002). The gallic acid co-treated animals have shown a decreased PAS staining in the basement membrane,

and thereby minimize the severity of basement damage as compared with glyoxal received animals.

Matrix metalloproteinase (MMPs) are the family of endopeptidases involved in the collagen degradation. It has been shown that degraded products of MMPs are the catalyst for collagen synthesis (Parthasarathy *et al.,* 2013; Löffek *et al.,* 2011). Moreover, an increase in the expression of MMPs activity ultimately leads to fibrosis; whereas suppression of MMPs activity is eventually results in decreased accumulation of ECM proteins in tissues (Li *et al.,* 2000). Studies have shown that engagement of AGE-RAGE induces ROS generation, thereby accelerates matrix metalloproteinase activity in experimental animals (Cau *et al.,* 2011). In the present study, an increased MMP-2 and MMP-9 expression was observed in GO received animals suggested that GO-RAGE-mediated ROS generation might induce MMP-2 and -9 activation. Conversely, a significant decrease in the expression of MMP-2 and -9 ($p<0.05$) was observed in GO+GA treated group of animals. Thus, GA being a potential antioxidant might have scavenged the ROS thereby minimizing the production and accumulation of collagen in the GO treated renal tissues.

Receptor for advanced glycation end products (RAGE) serves as the surface receptor for the AGEs, and it is responsible for the downstream signaling of various pathological events. (D'Agati and Schmidt, 2010). It was reported that blockade or down-regulation of RAGE downstream signaling could be a therapeutic target for various diseases (Yamagishi and Takeuchi, 2004). Studies have showed that RAGE was minimally expressed during normal physiological conditions (Soro-Paavonen *et al.,* 2008). Studies have shown that interactions of AGE/RAGE stimulate ROS generation, which could promote RAGE expression, which warns to a vicious cycle between RAGE-downstream signaling pathways (Soro-Paavonen *et al.,* 2008).

Result of the present study has shown that GO treated animals exhibited an increased expression of RAGE. However, animals co-treated with GA showed decreased expression of RAGE, suggesting that GA counteracts GO, and thereby minimizing the GO-induced RAGE activation and further downstream signaling thus exhibited that it may act as a natural anti-glycation agent.

A subunit of NADPH oxidase (NOX) plays a key role in production of superoxide (O^{2-}) by transferring electrons across the membrane from NAD(P)H to molecular oxygen (Bedard and Krause, 2007). Activation of NOX results in mitochondrial dysfunction and impaired antioxidant status and ultimately leads to oxidative stress (Cai, 2006). Studies have suggested that the activation of RAGE induces NADPH oxidase and cellular ROS production in endothelial cells (Wautier *et al.,* 2001).Our studies support the hypothesis that AGE-RAGE interaction, play a key role in amplifying NOX and induces ROS generation in the renal tissue (Nishikawa *et al.,* 2000; Subramaniam *et al.,* 2013). Interactions of GO and RAGE stimulate the NADPH oxidase and cytosolic production of ROS within the renal tissues and this is inhibited by the natural anti-oxidant GA. Furthermore, our own recent study demonstrated the effectiveness of GA as an anti-glycating agent for AGE mediated macro vascular complications (Subramaniam *et al.,* 2012). In the present study, natural dietary flavonoid GA attenuates the expression of NOX in the kidney tissues by demonstrating its capability in GO/RAGE mediated NOX activation during micro vascular complications (Figure.3).

Interaction of AGE/RAGE mediated oxidative stress alters the antioxidant status of the renal system in diabetic nephropathy (Rosca *et al.,* 2005), and this would alter the activity of enzymatic defense mechanisms, ultimately contributing to excess cellular ROS accumulation (Kalim *et al.,* 2010). Studies have shown that decrease in the activity of super oxide dismutase (SOD) leads to the accumulation of ROS generation and thereby leads to the amplification of

lipid peroxidation (LPO) (Kakkar *et al.*, 1995). Previous studies by Huang *et al.*, showed that flavanoids has the ability to suppress AGEs mediated oxidative stress via RAGE (Huang *et al.*, 2006). Recent studies showed that increased collagen synthesis and MMPs activity are related to SOD status and ROS formation (Giftson *et al.*, 2010). In the present study we observed that more toxic role of GO in the induction of oxidative species with depletion of antioxidant enzyme (Figure. 6) and increase in the ROS levels (Figure 3). Animals exhibited a significant ($p<0.01$) increase in the level of lipid peroxides (LPO) with declining activities ($p<0.01$) of antioxidant enzymes such as superoxide dismutase (Mn-SOD). On the other hand, animals co-treated with GA reduces the LPO activity probably due to its metal chelating properties (Mirjana Andjelkovic *et al.*, 2006) and thereby restoring the levels of SOD to near normal levels might be due to the action of GA on superoxide, hydroxyl and alkoxyl radical coupled to attenuate the oxidative stress which eventually reduces cellular damage (Mitic *et al.*, 2010). The experimental evidence suggests that AGEs formed from GO and methylglyoxal contribute to collagen cross linking. In addition, AGE/RAGE downstream signaling stimulates ROS and negatively regulates the antioxidant status, which collectively induced the collagen synthesis and MMP mediated ECM rearrangement and subsequently fibrosis. However, GA co-treatment along with GO inhibited these changes and improved the GO-induced fibrotic complications in experimental Wistar rats.

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Figure Legends

Figure 1

Kidney sections from the control and experimental group animals were stained with H & E; Masson trichrome and Periodic Schiff's stain (n=6/group). H&E stain (a-d). Panel a: normal control; Panel b: GO infused rats; Panel c: GO+GA treated rats; Panel d: GA control animals. Masson's trichrome to visualize collagen deposition (e-h). Panel e: normal control; Panel f: GO infused rats shows increase blue staining reflects interstitial fibrosis, panel g: gallic acid treated renal sections GO+GA shows mild increase in the blue stain in the tubules. Panel h: GA control rats. PAS staining (i-l) visualize basement membrane damage. Panel i: normal control; Panel j: GO alone infused rats; Panel k: GO+GA group animals; Panel l: GA control animals, renal sections were shown at 40X magnification. Scale bar- 50µm.

Figure 2

(A) Shows the total collagen content in the kidney tissue of experimental animals. Increased in the collagen content in GO group animals compared with the control group and there was a significant decrease in the collagen levels of GO+GA group animals as compared with the GO group. No significant change was observed in the GA group compared with the control group animals. (B and C) represents an increased mRNA expression of Collagen I and Collagen III in GO group animals and there was a significant decrease in the mRNA expression levels of GO+GA group animals. GAPDH was used as internal control. Results were expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; **P<0.01 and NS- Non significant.

Figure.3

Zymogram analysis of MMP-2 and -9 activity showing activity of MMP-2 and MMP-9 in the tissue extracts. Gels were stained with 0.25 % Coommassie brilliant blue and destained until the clear lytic bands were visible. Results were expressed as Mean±SEM (n=6/group). Significance

is indicated as *P<0.05; **P<0.01; ***P<0.01 and NS- Non significant. (B) mRNA expression of MMP-2 and -9 in control and experimental group animals. GAPDH was used as internal control. Results were expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; **P<0.01 and NS- Non significant. (C) mRNA expression levels of NOX-4 in control and experimental group animals. GAPDH was used as internal control. Results were expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; **P<0.01 and NS- Non significant. (D) The figure shows the spectroflourimetric analysis of ROS generation in control, GO, GO+GA and GA treated tissue extract. Results were expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; ***P<0.001 and NS- Non significant.

Figure.4

Immunostaining analysis of kidney sections from the control and experimental group animals (n=6/group). Panel (a-d) shows MMP-2, Panel (e-h) shows MMP-9, Panel (i-l) shows RAGE, Panel (m-p) shows SOD expressions in the kidney sections of control and experimental group of rats respectively. An increased MMP-2 and -9 expression is evidenced in (panel b and f, respectively) in GO treated rat renal sections. An increased RAGE and decreased SOD expression is evidenced in (panel j and n, respectively) in GO treated rat renal sections. GA treatment neutralizes GO induced MMPs (panel c and g, respectively) RAGE (panel k) and SOD (panel n) expressions in the renal tissue sections. Kidney tissue sections were observed at 40X Magnification.

Figure.5

Immunoblotting analysis of MMP-2, MMP-9 and RAGE. β-actin was used as internal control. Where, Lane C-control; Lane GO – Glyoxal (GO) alone infused rats; Lane GO+GA - Gallic acid (GA) along with GO group animals; Lane GA - GA control animals. Results were

expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; **P<0.01 and NS- Non significant.

Figure.6

(A) indicates the released Malondialdehyde-an indicator of LPO is expressed as nmoles/mg protein. (B) Activity is expressed as 50% inhibition of epinephrine auto-oxidation for SOD. (C) Native polyacrylamide gel activity assay for SOD activity in kidney tissue extract of experimental group animals. Where, Lane C-control; Lane GO – Glyoxal (GO) alone infused rats; Lane GO+GA - Gallic acid (GA) along with GO group animals; Lane GA - GA control animals. Results were expressed as Mean±SEM (n=6/group). Significance is indicated as *P<0.05; **P<0.01 and NS- Non significant.

Figure.7

Schematic outline of possible mechanism of glyoxal induced renal fibrosis and the protective mechanism of gallic acid.

Table.1: RT-PCR oligonucleotide gene-specific primers

Gene	**Primer sequences**	**Size (bp)**	**An. Tm (°C)**
Collagen-I	Sense 5'-TGCTGCTTGCAG TAACGTCG-3' Antisense 5'-TCAACACCATCTCTGCCTCG-3'	136	60.8
Collagen III	Sense 5'- AAAGGTGAAACTGGTGAACGTGGC-3'	578	61.3

	Antisense 5'- TCCATCTTGCAGCCTTGGTTAGGA- 3'		
MMP-2	Sense 5'- CTATTC TGTCAGCACTTTGG- 3' Antisense 5'-CAGACTTTGGTTCTCCAACTT-3'	309	53
MMP-9	Sense 5'- AGTTTGGTGTCGCGGAGCAC-3' Antisense 5'- TACATGAGCGCTTCCGGCAC-3'	754	57
NOX	Sense 5'- GGACCCCGATCCCAACTACGC-3' Antisense 5'- GCGCTTCCGAGAACGCTGGT-3'	298	59
GAPDH	Sense 5'-TCCACCACCCTGTTGCTGTAGC-3' Antisense 5'-TGGAAAGCTGTGGCGTGATG-3'	401	58

Table.2: Effects of glyoxal, an AGE intermediate on body weight, kidney weight and KW/BW ratio in control and experimental group of rats.

Parameters	Control	GO	GO+GA	
Body Weight (gm)	135±5.8	110±7.1^{a*}	125±5.31^{b*}	13
Kidney Weight (gm)	0.7±0.01	0.9±0.02^{a*}	0.79±0.03^{b*}	0.6
KW/BW ratio	0.52±0.02	0.81±0.02^{a*}	0.63±0.01^{b*}	0.4

Values in the table are expressed as mean±SEM, n=6; *p<0.05 a-GO Vs Control; b-GO+GA Vs GO; No significant NS-Control Vs GA

Table.3: Effects of glyoxal, an AGE intermediate on serum BUN, Creatinine, albumin and alkaline phosphatase in control and experimental group of rats.

Parameters	Control	GO	GO+GA	GA
BUN (mg/dL)	14±0.2	20±1.4^{a*}	16±0.8^{b*}	13±0.2^{NS}
Creatinine (mg/dL)	0.15±0.01	0.25±0.02^{a*}	0.2±0.02^{b*}	0.13±0.01^{NS}

Alkaline phosphatase (U/L)	187±9.8	239±21.1[a*]	197±11.9 [b*]	185±8.2 [NS]

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