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Preventive and Curative Effects of *Zingiber officinale* Extract against Histopathological and Ki-67 Immunohistochemical Changes of Glycerol-Induced Acute Renal Failure in Rat

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The purpose of this study was to investigate the protective effects of ginger extract on the glycerol-induced acute kidney failure. Forty eight male Sprague-Dawley rats which divided randomly into four equal groups of 12 animals each; Group I: Normal; Group II: Received ginger extract at a dose of 400 mg kg⁻¹ b.wt. per day by gavage; Group III: Received hypertonic glycerol (50%, v/v in sterile saline) was injected i.m. at a dose of 10 mL kg⁻¹ and Group IV: Ginger extract, given orally at a dose of 400 mg kg⁻¹ b.wt. per day for 30 days and injected i.m. with 10 mL kg⁻¹ of hypertonic glycerol. The results showed that serum creatinine, Na⁺ and blood urea nitrogen were markedly increased in glycerol-treated rats and creatinine clearance was decreased. It is proved that ginger extract reduced their levels significantly. This study confirmed the glycerol-induced acute renal failure in terms of inflammatory infiltrate with clusters of neutrophil cells, sever hemorrhage, necrosis of some renal tubule cells together with karyolytic and pyknotic nuclei. Also, degenerated glomeruli and widening of the urinary space were noticed, deposition of intraluminal PAS positive hyaline casts and loss of the apical brush borders and decreased Ki-67 proliferation marker. The labeling index of Ki-67 in this study showed that in control group (47.3+4.6), in ginger extract group (68.7+5.16), in ARF group (35.4+4.3) and ARF treated with ginger extract group (68.9+6.2) were positively stained. However, all these adverse effects were reversed by ginger supplementation. Thus, this study suggests that ginger can be used as a nephro-protective nutrient and protect the kidney from glycerol-induced damage.

Key words: Acute renal failure, ginger, Ki-67, immunohistochemistry

INTRODUCTION

The kidney is one of the major organs involved in whole-body homeostasis, with its major functions being the excretion of waste of metabolites, blood pressure regulation and metabolism of lipid, secretion and degradation of hormones and the production and utilization of systemic glucose (Gai *et al.*, 2014). It is well known that chronic renal impairment is further complicated by high blood pressure (Boudville *et al.*, 2006), deranged carbohydrate metabolism (Mak and De Fronzo, 1992) and dyslipidemia (Cheung *et al.*, 1993). Moreover, Chronic Renal Failure (CRF) has been reported to be associated with increased serum bile acid levels and alterations in the bile acid balance (Jimenez *et al.*, 2002).

The incidence and prevalence of the common dysfunction Chronic Renal Failure (CRF), is on the increase, both in developing and developed countries, compelling a very expensive and rising demand on health-care systems already burdened by scarcity of resources (James *et al.*, 2010; Nugent *et al.*, 2010). The disease is progressive in nature, requires involved and frequently expensive management, causes serious complications such as diabetes, stroke, cardiovascular diseases and other diseases and has no satisfactory treatment (Smart and Titus, 2011).

In the recent time, many natural products are being used to protect the tissues from various drugs or chemicals-induced toxicities. Among the natural products, *Zingiber officinale*, ginger commonly known (Ahmed *et al.*, 2008). Ginger is an underground rhizome of plant belonging to the family of Zingiberaceae and it is considered a usual ingredient of diet worldwide (Sertie *et al.*, 2005). Moreover, ginger is well known all over the world especially for its use in disorders of the gastrointestinal tract such as constipation, dyspepsia, nausea and vomiting (Foster, 2012). It was reported that *Zingiber officinale* has medicinal properties against digestive disorders, rheumatism and diabetes (Afzal *et al.*, 2001). Ginger extract possesses anti-oxidative characteristic, since it can scavenge hydroxyl radicals and superoxide anion (Krishnakantha and Lokesh, 1993). Akhani *et al.* (2004) reported that *Zingiber officinale* pretreatment inhibited the induced hyperglycemia and hypoinsulinaemia. Other investigators have showed that the hypolipidemic effect of ginger (Joshi and Jain, 2014). Furthermore, Ajith *et al.* (2008) studied the protective effect of ginger extract against the induced nephrotoxicity and renal failure. Ginger is also recommended by the traditional herbalist in South Asia for using in cardiopathy, high blood pressure (Duke, 2010). Its use was recorded in early

Chinese text and documented in ancient Greek, Roman and Arabic medical literature (Bhandari *et al.*, 2001).

In addition, phytochemical reports have shown that the main constituents of ginger are Zingerone, Paradol, Gingerol and Shogaols. It was reported that 6-gingerol and 6-shogaol are the major Gingerol and Shogaol present in the rhizome (Saeid *et al.*, 2010).

Hence, in the current study an attempt was made to examine the nephro-protective effect of ginger extract against glycerol-induced renal failure and tissue injuries.

MATERIALS AND METHODS

Forty eight male Sprague-Dawley rats, weighing 180-210 g were used in the present study. They were divided randomly into four equal groups of 12 animals each, having free access to water and standard chow diet:

- **Group I:** Normal controls, received orally sterile saline for 30 days and injected with 10 mL kg⁻¹ of normal saline (0.9% NaCl) i.m.
- **Group II:** Received ginger extract at a dose of 400 mg kg⁻¹ b.wt. per day by gavage, for a period of four weeks
- **Group III:** Hypertonic glycerol (50%, v/v in sterile saline) was injected i.m. at a dose of 10 mL kg⁻¹. Injection volumes were divided equally between two hind limbs
- **Group IV:** Ginger extract, given orally at a dose of 400 mg kg⁻¹ b.wt. per day and injected with 10 mL kg⁻¹ of hypertonic glycerol i.m. for 30 days (Guidet and Shah, 1989)

Blood samples for urea nitrogen, creatinine, the creatinine clearance and Na level determination were taken from the heart and subsequently kidney was removed. All biochemical investigations were done by Auto Analyzer Apparatus (Beckman Coulter, U.S.A).

For histopathological studies, the right kidney was isolated immediately after sacrificing the animal and washed with saline. The renal specimens were fixed in neutral buffered formalin solution (10%), dehydrated in ethanol, cleared in xylene and embedded in paraffin wax and sections of 5 µm in thickness were obtained, deparaffinized, hydrated and stained with haematoxylin and eosin. Sections of the kidney were examined in blind fashion for hemorrhagic and hyaline casts and necrosis in all treatments.

Renal sections were estimated for tissue damage using the Periodic Acid-Schiff (PAS) reaction using criterion procedures. The cross sections of kidney containing the cortex and medulla were measured objectively by a pathologist for the severity of cellular damage. The PAS stained sections of glycerol injected

and treated with ginger extract animals were compared to control animals. Evaluation was conducted based on the following criteria: Tubular dilation, cast in lumen, inflammatory infiltration cells and cell swelling/enlargement.

For immunohistochemistry, paraffin sections of 5 µm thickness were mounted on siliconized slides (S3003, Dakoc cytometry, Carpinteria, CA, USA), dried overnight at 58°C in an oven, deparaffinized in xylene and rehydrated through a series of ethanol and washed with water. Then sections were immersed in citrate buffer (Retrieval solution; pH 6) and heated in a pressure cooker for 3 min. After that, sections were washed out in distilled water and then, treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase. Sections were incubated with primary nonspecific polyclonal antibody anti-Ki-67 antigen (Thermo scientific, U.S.A) with dilution at 1:50 for 60 min. The amplification system used was Envisions System (Dako cytometry, Carpinteria, CA, USA). Signal amplification was achieved with dextran-polymer coated with peroxide and a color was developed by incubation with diaminobenzidine tetrachloride (DAB) and hydrogen peroxide. Counter staining was done with Harris' hematoxylin. Slides were cover-slipped with resin. A slide from a well-known Ki-67 staining was run in parallel as an additional control. On considering 1000 cells in five histological field with manual counting, the Ki-67 Labeling Index has been counted as the percentage of positive cells (brown precipitate restricted to the nuclei).

Statistical analysis: Values are expressed as Mean±Standard Deviation (SD). Differences between groups were evaluated by analysis of variance (ANOVA) with appropriate correction for multiple comparisons (SPSS 21 for windows®).

RESULTS

According to Table 1, serum creatinine, BUN and Na levels were significantly elevated ($p<0.01$) in Glycerol-treated group than the control group. On the other hand, Ginger+Glycerol group showed significant decrease ($p<0.05$) in serum creatinine, BUN and Na levels compared with the Glycerol-treated group. In case

creatinine clearance analysis, the significantly decreased ($p<0.01$) in Glycerol-treated group when compared with control. The improvement was shown in glycerol injected animals and treated with ginger extract.

Hematoxylin and eosin histopathological investigations: The kidney sections of both control and ginger-treated group exhibited normal renal architecture (Fig. 1a-b). On the other hand, the kidneys of glycerol-treated group displayed acute renal failure lesions including inflammatory infiltrate with clusters of neutrophil cells, sever hemorrhage, necrosis of some renal tubule cells together with karyolytic and pyknotic nuclei. Also, degenerated glomeruli and widening of the urinary space were noticed (Fig. 1c-e). The renal sections in ARF treated with ginger extract displayed an improvement in the renal alterations (Fig. 1f) and a marked decrement in the lesions of glycerol-induced renal failure.

Periodic Acid-Schiff (PAS) staining was performed to examine histopathological changes in kidneys of the experimental animal groups. Interestingly within the renal cortex, the glycerol injected animals group exhibited dilated distal tubules. Tubular hyaline casts within the lumen compared exhibited an increase in the kidney control rat. In addition, prominent epithelial cell swelling in the dilated distal tubules was associated with loss of protein. Also, glycerol injection causes tubular epithelial injury in the form of marked dilated tubular lumen, deposition of intraluminal PAS positive hyaline casts and loss of the apical brush borders, cellular apoptosis and inflammatory infiltrate was shown clusters of neutrophil cells where these effects were almost reversed by the administration of ginger (Fig. 2a-f).

Ki-67 antibody was murine monoclonal antibody that reacts with the proliferation-associated Ki-67 antigen and has been shown to correlate with DNA synthesis in renal tissues. Positive expression of Ki67 by immunohistochemistry was detected as a brownish precipitate in the nucleus. The labeling index of Ki-67 in this study showed that in control group (47.3±4.6), in ginger extract group (68.7±5.16), in ARF group (35.4±4.3) and ARF treated with ginger extract group (58.9±6.2) were positively stained (Fig. 3a-f). The significant correlation between ARF group when compared with control group ($p<0.05$).

Table 1: Statistical analysis of renal biochemical concentrations in different experimental animal groups

Groups	Creatinine	Creatinine clearance	BUN	Na level
GI	0.86±0.04	0.58±0.03	8.5±0.55	1.83±0.12
GII	0.87±0.80	0.59±0.03	8.1±0.66	1.75±0.09
GIII	2.86±0.28**	0.07±0.02**	15.0±0.68**	4.30±0.56**
GIV	1.19±0.15*	0.32±0.05*	11.0±0.74	2.25±0.29

Data was expressed as Mean±SD. Significant differences in control group vs. treated groups are * $p<0.05$, ** $p<0.01$, BUN: Blood urea nitrogen, GI: Control, GII: Ginger, GIII: Glycerol and GIV: Ginger+Glycerol

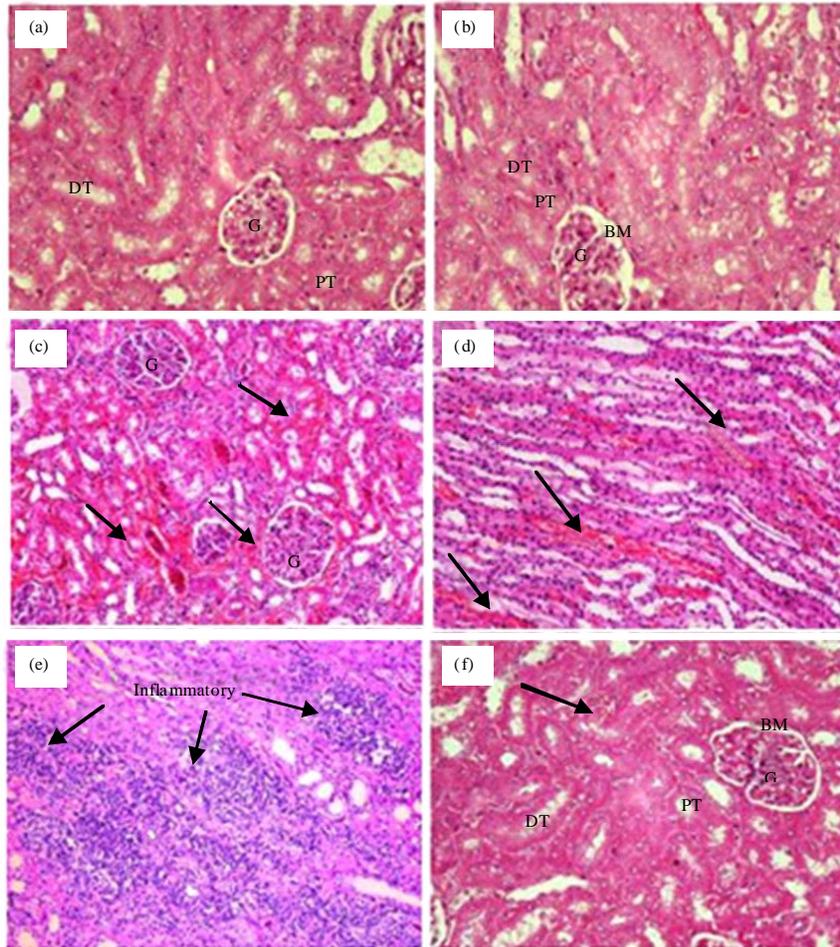


Fig. 1(a-f): Micrograph of transverse section of kidney (a and b) Control and ginger extract treated rats showed normal glomeruli (G), basement membrane (BM), proximal (PT) and distal tubules (DT), (c-e) Renal sections of ARF rats, showed destructured tubules associated with degenerated glomeruli, hemorrhage (arrows), congested blood vessel and inflammatory cell infiltration and (f) Renal sections of ARF rats treated ginger extract for 8 weeks showed the normal arrangement of tubules, displayed an improvement in the renal alterations and a marked decrement in the lesions of glycerol-induced renal failure (H and E×250)

DISCUSSION

The general model for studying ARF is obtained that the rat was at single intramuscular injected glycerol stage. The disease that follows intramuscular injection of glycerol in the rat is similar to the ARF seen in crush injury and the renal histological consequences such as tubule injury are similar to those observed clinically (Xing *et al.*, 2014). Therefore, glycerol injection model was employed in the present study to investigate the effect of ginger extract against ARF. In the rat model of glycerol-induced ARF, it is generally considered that the degree of renal failure is correlated with the duration of dehydration. In general, the syndrome of ARF was characterized by a rapid reduction in the ability of the

kidney to eliminate waste products. A functional definition of impairment includes: (1) An accumulation in the normal end-products of nitrogen metabolism either from the diet or normal tissue catabolism, reacted in a rising creatinine and BUN; (2) Inadequate tubular resorption of sodium, bicarbonate loss and excretion of water, resulting in edema (Rao *et al.*, 2015).

Based on the biochemical and preliminary analyses in plasma (Table 1), it was shown that renal function in ginger extract treated rats was comparable to that in control and ARF+ginger extract treated rats, indicating that the ginger extract reduced ARF lesions. Earlier studies based that ginger components can improve the anti-oxidant capacity under various drug-induced oxidative stress conditions in kidney

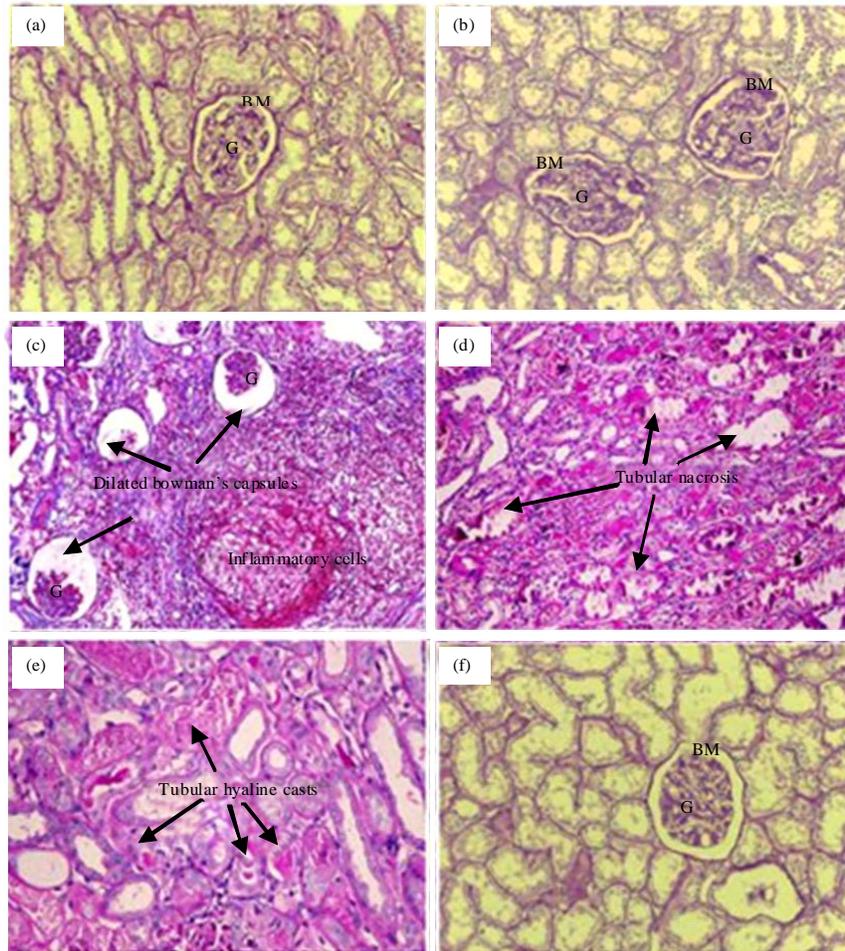


Fig. 2(a-f): Micrograph of renal section (a and b) Control and ginger extract treated rats showed normal glomeruli (G), basement membrane (BM), (c-e) Renal sections of ARF rats, showed dilated Bowman's capsule space, tubular necrosis, inflammatory cells infiltration and tubular hyaline casts and (f) Renal sections of ARF rats treated ginger extract showed the normal glomeruli and basement membrane (PAS×250)

(Ramudu *et al.*, 2011). As a major finding of the present study, we demonstrated that glycerol-induced detrimental effects in kidney cells were recovered with ginger extract treatment. In addition to these findings, glycerol-induced increase in renal functions (Creatinine, BUN, Na^+ level) and decrease in creatinine clearance were significantly attenuated by ginger supplementation in the kidney of rats. The results of this study indicate that extract of ginger alone rendered significant protection against glycerol-induced nephrotoxicity which was evident from the lowered serum urea and creatinine levels. These findings are consistent with previous studies that suggested that the chemopreventive properties of ginger may lie in its ability to regulate viability and cell function (Pan *et al.*, 2008).

Further evidence for glycerol-induced acute renal failure was clearly observed in our histopathological

studies. As shown in the photomicrograph (Fig. 1) severe degenerative changes in tubules, diffused cellular infiltration all over the parenchyma and severe congestion of blood vessel, renal cortex and medulla cortical interstitial hemorrhage were observed in glycerol treated group. Photomicrograph with combination treatment glycerol and ginger extract illustrates that damaged tubules and glomeruli appeared to be restored and only mild degeneration of tubular epithelium was noticed. From these histopathological observations it is clear that ginger is beneficial in restoring the glycerol-induced tissue damages in rats' kidney. Also, the clusters of inflammatory cells were shown in glycerol injected group in hematoxylin and PAS stains. On the other hand, the ginger extract inhibits activation of neutrophil cells by inhibiting the pro-inflammatory chemokine and cytokine release. The present results support the previous studies

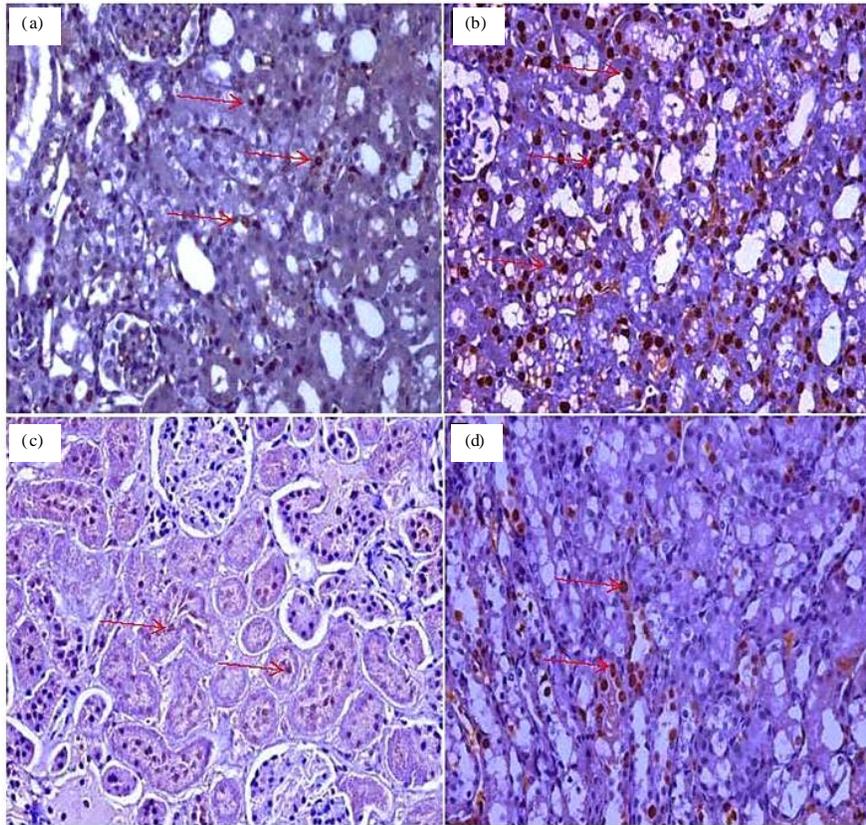


Fig. 3(a-d): Ki-67 immuno-micrograph of renal section (a and b) Control and ginger extract treated rats showed strong nuclear brown stain (arrows), (c) Renal sections of ARF rats, showed weak positive Ki-67 nuclear stain and (d) Renal sections of ARF rats treated ginger extract showed the moderate positive Ki-67 nuclear stain (Immunoperoxidase \times 250)

that reported the suppressive effect of ginger extract on expression of pro-inflammatory genes (Kim *et al.*, 2005).

When the kidney was damaged by glycerol injection, the tubular cells released in urine and no proliferation was done, but when the ARF animals treated with ginger extract, the kidney tissue recovers from acute damage, it depends on events sequence that include epithelial cell spreading and possibly migration to cover the exposed areas of the basement membrane, cell differentiation and proliferation to return cell number by differentiation, which results in restoration of the functional integrity of the nephron (Marenzi *et al.*, 2015). Under normal conditions, kidney proximal tubule cells divide at a low rate, as estimated by proliferative cell nuclear antigen Ki-67 immunoreactivity (Nadasdy *et al.*, 1994). This cell production balances the loss of tubular epithelial cells into the urine (Khejonnit *et al.*, 2015).

CONCLUSION

This study confirmed the glycerol-induced acute renal failure in terms of increased renal functions,

decreased creatinine clearance, damaged renal cells and decreased Ki-67 proliferation marker. However, all these adverse effects were reversed by ginger supplementation. Thus, this data suggests that ginger can be used as a nephro-protective nutrient and protect the kidney from glycerol-induced damage.

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