Comparative Research on Anti-Inflammatory Effects of Different Dietary Antioxidants on Alcohol-Induced Damage in Gastric Cells

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ABSTRACT
Objective: Antioxidants prevent ill-effects of free radicals through regulation of different pro-and anti-inflammatory enzymes of the cell which have association with the pathophysiology of alcohol-induced gastropathy. This study was aimed to explore the mechanism in gastric adenocarcinoma (AGS) cell line and protection by dietary antioxidants e.g. catechin, resveratrol, quercetin, curcumin and 6-gingerol. Methods: AGS cells were exposed to 2.5% ethanol for varied time span in presence and absence of antioxidants. Cytotoxicity was assessed by MTT assay and annexin V-propidium iodide staining. The damages in cellular morphology were observed by DAPI and EtBr-acridine orange staining. Changes in ROS generation were examined through confocal microscopy. Western blots were performed using cell extracts to investigate the changes in expression level of COX-1, -2, catalase, superoxide dismutase (SOD), iNOS, MMP-9 and TIMP-1. Immunofluorescence study was done to cross check the expression of COX-2. Results: A concentration-dependent cytotoxicity was observed in the cell viability on ethanol exposure which was reduced by antioxidant treatment. Antioxidants reduced the inflammation by downregulating the expression of COX-2, iNOS and MMP-9 and by upregulating the expressions of catalase, SOD and TIMP-1 significantly. Catechin and quercetin demonstrated most prominent cytoprotection amongst the five, followed by resveratrol. These results were corroborated with MTT assay, DAPI and EtBr-acridine orange staining. Curcumin and 6-gingerol did not show any significant effect. Conclusion: Dietary antioxidants protect AGS cells from oxidative stress by maintaining the homeostasis between oxidant-antioxidant and protease-antiprotease ratio.

Key words: Dietary antioxidants, Gastric inflammation, Ethanol, ROS, AGS.

INTRODUCTION
The imbalance between aggressive and cytoprotective factors in stomach result in acid-pepsin secretion, excessive formation of Reactive Oxygen Species (ROS), mucous secretion, cellular regeneration and microvascular dysfunction paving the path for gastric ulceration. Ethanol-induced gastric inflammation involves the interplay of multiple molecular mechanisms products of arachidonate metabolism (e.g. leukotrienes) and mast cell secretory products. Prostaglandins and sulphydryl compounds have been demonstrated to shield from stomach injury by ethanol, therefore, play a role in maintaining the gastric mucosal integrity.

Alcohol abuse is a pan-societal problem worldwide and its ill effects on health are well known. Chronic alcoholism brings about inflammatory cell infiltration, cellular exfoliation, mucosal oedema, gastrointestinal hemorrhage, cirrhosis, recurrent infections and even malignancy. Ethanol triggers apoptosis in macrophages, neutrophils and thymocytes, thus causing widespread cellular damage and an immunocompromised state. Alcohol ingestion hampers absorption of micronutrients and impairs the efficiency of digestive enzymes. Ethanol-induced apoptosis has been reported both in vivo and in vitro and an effect of oxidative stress. Gastric epithelial exposure to ethanol lead to protein and macromolecular destruction, causing mucosal hyperemia, necrosis, oedema and mucosal or submucosal hemorrhage in gastric tissues. ROS attack vital cellular components like lipid, proteins, carbohydrates and nucleic acids, initiating aberrant downstream signaling or stimulation of apoptosis. Biological events such as inflammation, carcinogenesis, ageing, stroke appear to involve ROS. It also acts as an intracellular second messenger. When sublethal stress occurs, the epithelial cells alter their metabolic functions and regulate production of cytokines, infective agents and other adverse responses which may lead to cell necrosis or apoptosis. Depending on the cellular milieu, cell death accompanies activation of metabolic pathways as caspase, ceramide, altered gene expression and mitochondrial dysfunction. Many of these pathways
accompany secondary production of oxidants which appear to contribute to the magnitude of cell death. For example, overexpression of antioxidants like intracellular superoxide dismutase (SOD) reduces cell death.\textsuperscript{12-13} Alternatively, the depletion of antioxidant reserves exacerbates cell death by a variety of stimuli.\textsuperscript{14} Altered intracellular redox balance with antioxidant reserve depletion is associated with activation of transcription factors like NF-κB, TNFα, IL-1β, which are associated with inflammation, proliferation and apoptosis.\textsuperscript{25}

The pathogenesis of gastric inflammation is still not clear, although imbalance between oxidants and antioxidants play a crucial role. Literature suggest that cells modulate protein synthesis at both transcriptional and translational levels and thereby alter metabolism and function.\textsuperscript{16-18} Several enzymes exist inside the cell to neutralize free radicals. SOD catalyzes the transformation of superoxide radicals. Catalase and glutathione peroxidase neutralizes H₂O₂ and fatty acid radicals. Disruption of the activity of these enzymes results in free radical accumulation, which in turn, causes lipid peroxidation and tissue damage. These evidences prompted us to hypothesize that the critical balance between pro-inflammatory and anti-inflammatory proteins and cytokines play an essential role in gastric cytoprotection.

Matrix Metalloproteinases (MMPs) are zinc and calcium dependent endopeptidases released by inflammatory cells.\textsuperscript{19} Apart from extracellular matrix remodeling, MMP-9 regulates the activity of numerous cytokines, chemokines, receptors, growth factors and cell adhesion molecules essential to inflammation.\textsuperscript{20} The activity of MMP-9 is closely regulated on multiple levels including the endogenous inhibitor tissue inhibitors of metalloproteinase (TIMP)-1. This protease and anti-angiogenesis are produced in the gastrointestinal tract by several structural cells and their balance is critical for many physiological processes in the gut.\textsuperscript{21}

Epidemiological studies indicate a role of fruits and vegetables in guarding against diseases, particularly cardiovascular and inflammatory. Nutrients, providing such protection, include antioxidants and dietary fibers. There is a strong body of opinion that diseases may be caused by, or their development accelerated by, an imbalance between the overproduction of free radicals and antioxidant activity, which is also known as oxidative stress. A number of low molecular mass compounds with a range of solubility properties can act as antioxidants. Dietary polyphenols are considered beneficial because of their protective role in the pathogenesis of multiple systemic diseases associated to oxidative stress.\textsuperscript{22} This may help to prevent free radical mediated cytotoxicity and apoptosis.\textsuperscript{23} This possibility was addressed in this study by examining the role of five important dietary antioxidants, catechin, resveratrol, quercetin, curcumin and 6-gingerol, commonly used in Indian subcontinent and south-east Asia, in inhibiting ethanol-induced gastric damage by limiting epithelial inflammation in response to oxidative stresses and its association with different pro and anti-inflammatory enzymes in vitro.

**MATERIALS AND METHODS**

**Cell culture**

The human gastric adenocarcinoma cell line AGS was procured from American Type Culture Collection Centre (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS) and 20μg/mL penicillin and 20μg/mL streptomycin. These cells were grown in a humidified incubator containing 5% CO₂ and 95% air at 37°C. For experiments, cells were seeded (2×10⁴ cells/well) into 6 well plates and cultured for 24 hrs. Serum-free conditions were used to avoid potential flavonoid-protein interaction. 2.5% ethanol (~25 mM) was added to cells for 6-24 hrs as damaging agent. Protection studies were done with antioxidants prior addition of ethanol.

**Evaluation of cell viability: MTT assay**

Cytotoxicity of 2.5% ethanol and antioxidants was determined via colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.\textsuperscript{26,27} AGS (1 × 10⁴ cells/well) were cultured in a 96 well plate at 37°C. On confluence, a set of wells were treated with 2.5% alcohol media only and in other sets, five different antioxidants were added 12 hr prior addition of alcohol media. After the treatment, 100 µL of 0.5% (w/v) of MTT dissolved in 1X PBS was added to each well and the cells were incubated. The media were aspirated out and 150 ml of DMSO was added to each well to dissolve the formazan crystals. Absorbance of each sample was analyzed at 490 nm on a microplate reader (Robonik, India). Cell viability (%) was calculated by dividing the absorbance of samples obtained from cells incubated with ethanol and different antioxidants by the absorbance of samples obtained from cells incubated with culture medium only (Control) and multiplying this ratio by 100.\textsuperscript{28} Data were presented as the mean of three experiments run in duplicate.

**DAPI staining**

Cells were grown on coverslips to ~50% confluence. After treatment with 2.5% ethanol for 12 hrs, or with different antioxidants, cells were stained with DAPI (4,6-diamidino-2-phenylindole); washed and observed under confocal microscope at 60X magnification, for nuclear fragmentation, shrinkage or condensation. The cellular damage (or protection) was reflected by nuclear state.

**EtBr-Acridine Orange (AO) staining**

EtBr-AO staining combines the differential uptake of fluorescence DNA binding dyes AO and EtBr and the morphologic aspect of chromatin condensation in the stained nucleus, which allows to distinguish between viable, apoptotic and necrotic cells. Viable cells possess uniform bright green nuclei. Early apoptotic cells show bright green areas of condensed or fragmented chromatin in the nucleus and necrotic cells show uniform bright orange nuclei. After the exposure time, 100µg/mL of EtBr-AO was added to the cell monolayer in each separate well and the plates were incubated. The stained cells were then observed under an Olympus fluorescence microscope at 20X magnification.

**Immunofluorescence staining**

AGS cells were cultured on glass coverslips separately. Cell monolayers were fixed in 2% paraformaldehyde in PBS. The fixative was removed and remaining reactive sites were blocked with 0.1M glycine/PBS. The cells were then treated with 0.2% (v/v) Triton X-100/PBS containing 0.1% (w/v) BSA. Cells were then incubated with respective primary antibody (1:200 dilution) followed by washing and incubation with rabbit anti-goat Texas Red conjugated secondary antibody. Cover slips were mounted with anti-fading agent and observed under confocal microscope at 60X magnification.

**Mitochondrial ROS detection**

Intracellular ROS level in treated AGS cells were measured using a kit, obtained from Life Technologies and ROS micrographs were taken by Andor confocal microscope. The assay was based on 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H₂DCFDA), a fluorogenic marker for ROS in live cells.

**Apoptosis study**

To confirm the viability and apoptosis, AGS cells were analyzed by flow cytometry, using Fluorescein Isothiocyanate (FITC)-conjugated annexin
V using a kit from Biologend. 10' cells/plate were seeded and cell apoptosis were induced by alcohol treatment for 12 hrs. Both detached and attached cells were collected for FITC-annexin V and PI staining. The cell suspension was transferred to a 5mL culture tube, FITC (5 µL) was added into each tube and incubated. Then 5ul of PI was added and again incubated. Early and late apoptotic cell populations were visualized by constructing a dot plot with the aid of Fluorescence Activated Cell Sorter (FACS).

Western blotting
Cells were lysed using RIPA buffer and the protein concentration of the lysate was measured using Lowry method. Proteins from the media or lysed cell extract were run (200µg) in 8% nonreducing SDS-PAGE and transferred to nitrocellulose membrane. Then the membranes were incubated with different antibodies at 4°C overnight followed by chemiluminiscent secondary antibodies at room temperature. The immune complexes were detected using enhanced chemiluminiscence reagent and developed using BioRad ChemiDoc. The intensity of each bands corresponds to the level of expression of the different proteins.

Statistical analysis
All data were obtained in duplicate and triplicate to calculate mean and standard deviation. Significant differences between means were evaluated by an analysis of variances. MTT assay data were fitted using Sigma plot. Statistical analysis was performed using Student-Newman-Keuls test (ANOVA) and Student’s t test as noted in the text. P value <0.05 was considered statistically significant.

RESULTS

Ethanol-Induced cytotoxicity to cultured cells
The effect of increasing concentrations of ethanol (0.5–5%) at different time points on AGS cells were examined by MTT assay (Figure 1 A and B). Cytotoxicity was dependent on both the concentrations of ethanol applied and the duration of exposure. The earliest signs of injury were seen after 30 mins of exposure to 2.5% ethanol in the cultured cells where the percentage of cell survival was approximately 52%. MTT assay also revealed that beyond 30 min and 2.5% concentration of ethanol, the cellular damage was so intense (survivality below 40%) that it was difficult to recover. More than 60% cells died when 3% ethanol was applied for 30 min. In addition, the effect of five different antioxidants on varied concentrations was assessed for the longer span (up to 24 h). It was seen that all the antioxidants in 50 µM concentration for a maximum period of 24h did not reveal any major effect as represented in Figure 1D. The survival declined to 76% or lower when the compounds were applied in 100 µM as depicted in Figure 1C. So, 50 µM was selected as the working concentration of antioxidants for the entire study. In the following experiments to assess the role of antioxidants, 2.5% concentration of ethanol for 30 min was used as a cytotoxic agent. On exposure of 2.5% ethanol to AGS cells for 30 min, cell viability decreased by approximately 50%. When ethanol media was added to antioxidant pretreated cells, viability increased significantly. In presence of catechin, resveratrol and quercetin, percentage of viable cells were 82%, 78% and 77% respectively. Cumin and 6-gingerol did not provide significant protection in terms of cell survival against ethanol-induced damage.

Antioxidants maintained cellular morphology and nuclear integrity against ethanol-induced damage
AGS cells were exposed to 50 µM of antioxidants followed by 2.5% ethanol for 30 min. DAPI (Panel A) and EtBr-AO (Panel B) staining of those cells were depicted in Figure 2. The cells revealed a shrunken morphology along with chromatin aberration and fragmentation when treated with ethanol alone. Pretreatment of catechin, resveratrol and quercetin provided significant protection to the cells which were evident from DAPI staining. Catechin and resveratrol treated cells were quite comparable to the control ones. Panel B represented the various apoptotic states of cells stained with EtBr-AO cocktail. The treatment period was same with DAPI pictures. More than 70% cells became yellow and approximately 30% cells became orange on EtBr-AO staining which signified that the cells became pro-apoptotic to necrotic. Catechin and resveratrol protected the cells significantly by reducing the extent of apoptosis. In this view, it can be inferred that cumin and 6-gingerol did not provide protection to AGS cells against ethanol-induced cytotoxicity. Further experiments were done with all five antioxidants but the results were represented only for catechin, resveratrol and quercetin for brevity.

Antioxidants reduced ethanol-induced apoptosis
Reports suggest that ethanol intoxication is often associated with apoptosis. We present data corresponding to the effect of 2.5% ethanol in the degree of apoptosis and necrosis in AGS cells and the extent of protection provided by the antioxidants. Ethanol induced a significant increase in apoptosis 65% in comparison with the values obtained in control cells, which was 8% as revealed by the presence of an early marker of apoptosis annexin V. Interestingly, catechin, resveratrol and quercetin treatment significantly reduced apoptosis to 36%, 35% and 32% respectively as revealed by Figure 3. This observation suggests that pretreatment of dietary antioxidants could limit the gut pathology by limiting cell death mechanisms.

Effect of antioxidants on Inflammation-related proteins' expression during protection
To monitor the possible anti-inflammatory mechanism of different antioxidants, western blots were performed for some proteins COX-2, COX-1, iNOS, MMP-9, TIMP-1, SOD and catalase. Western blot of β-tubulin was conducted to confirm equal protein loading in all the blots. The increased inflammatory reaction in ethanol-treated cells suggested the involvement of 4.27 folds higher iNOS, 5 folds higher COX-2 along with approximately 2 folds higher MMP-9, both in pro and active forms. Figure 4A also illustrates that iNOS expression is reduced by cat-
Ethanol alters the mitochondrial membrane potential, thus overshoots the functioning of mitochondrial enzymes.

The major focus of this study was to examine the comparative preventive potential of some commonly used dietary antioxidants on ethanol-induced gastric inflammation in vitro. The primary mechanism underlying ethanol-induced gastric cell damage is the release of ROS, upregulation of some pro-inflammatory proteins and cytokines and eventually apoptosis. Antioxidants, therefore, reduces gastric inflammation by counteracting the effects of ROS production. The antioxidant properties of catechin, resveratrol and quercetin are well documented but the mechanism is not well understood in the context of restoring protease-antiprotease homeostasis as well as balancing the critical ratio of pro-inflammatory/anti-inflammatory balance.

The comparative evaluation of the anti-inflammatory role of some antioxidants on ethanol-induced gastric inflammation in vitro showed that catechin and quercetin were most effective in maintaining the intracellular ROS but not like catechin. Resveratrol and quercetin also reduced the intracellular ROS while catechin pretreatment restored the cellular morphology almost like control cells. Resveratrol and quercetin also reduced the intracellular ROS but not like catechin (Figure 6).

**DISCUSSION**

Ethanol treated cells showed higher production of ROS while catechin and resveratrol pretreatment significantly decreased ROS production in the cytoplasm of the cells, which was confirmed by counterstaining with nuclear stain Hoechst. Chromatin condensation, deformity in the nucleus, distortion in cellular shape and size were evident in ethanol treated cells whereas catechin pretreatment restored the cellular morphology almost like control cells. Resveratrol and quercetin also reduced the intracellular ROS but not like catechin.
dizes cellular organelles and macromolecules. The present study showed that catechin and resveratrol played a successful role in restoring the pool of endogenous antioxidants such as catalase and SOD.

The involvement of ROS in ethanol-associated apoptosis of gastric epithelial cells is emphasized by in-vitro studies which show that exogenous antioxidants exert both cytoprotective and anti-apoptotic effects against the damaging effects of ethanol.\(^2\) We monitored these effects by annexin V affinity and PI binding assay. The FACS data clearly demonstrated that early apoptotic cells are highly preponderant ~78% in the ethanol-treated group, while catechin and resveratrol treatment reduced it by 38% and 52% respectively.

Prevention of ethanol-induced cytotoxicity involves highly coordinated complex sequence of events and pathways encompassing scavenging of ROS, regeneration of cellular organelles with downregulation of oxidative stress. SOD and catalase act as endogenous cellular defense system to neutralize free radicals. SOD is also involved in the regulation of apoptosis in various immune cells. Our study showed that 2.5% ethanol treatment reduced the reserve of SOD as well as catalase by 2.1 and 6.2 folds whereas pretreatment of antioxidants restored the expression level very near to control. Catechin and resveratrol induces the expression of SOD more than the basal level in response to ROS which is very significant. It is noteworthy that quercetin did not influence the expression level of SOD or catalase in this context.

The current study demonstrated that the critical balance between pro-inflammatory and anti-inflammatory proteins play a critical role in maintaining the integrity of gastric epithelial cells against ethanol-induced damage, where catechin and resveratrol played a significant role in maintaining the homeostasis between pro- and anti-inflammatory proteins. MMP-9 and TIMP-1 are thought to be deeply involved in the pathogenesis of inflammatory diseases. Although the mechanism is still under investigation and not accurately deciphered, the higher ratio of MMP-9/TIMP-1 is considered a major theory to explain the onset of inflammation. Ethanol treated cells expressed increased level of MMP-9. Pretreatment of antioxidants reduced the ratio very near to control, thus reduced inflammatory response.

CONCLUSION

We investigated the gastroprotective effect of dietary antioxidants, especially catechin and resveratrol in ethanol-induced damage in AGS cells. The results revealed that pretreatment with appropriate concentrations of antioxidants (~50 µM) could significantly protect AGS cells which might be attributed to the capability of scavenging free radicals, inhibition of apoptosis, maintaining morphological integrity and restoring the pool of anti-inflammatory proteins and cytokines. This innovative approach is a sensitive way to evaluate the impact of dietary antioxidant on risk of gastric inflammation. Dietary modification of fruits, vegetables and non-
alcoholic beverages represents an effective, practical, low-cost means of preventing gastric ulcer and cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

COX: cyclooxygenase; ROS: Reactive oxygen species; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases; SOD: Superoxide Dismutase.

REFERENCES