Effect of lycopene against gastroesophageal reflux disease in experimental animals

Arvind Kumar Giri, Jitendra Kumar Rawat, Manjari Singh, Swetlana Gautam and Gaurav Kaithwas*

Abstract

Background: Lycopene is a robust antioxidant with significant antiulcer activity. Henceforth, the present study was ventured to elucidate the effect of lycopene on experimental esophagitis.

Methods: Groups of rats were subjected to forestomach and pylorus ligation with subsequent treatment with lycopene (50 and 100 mg/kg, po) and pantoprazole (30 mg/kg, po).

Results: Treatment with lycopene evidenced sententious physiological protection when scrutinized for pH, acidity (total and free), volume of gastric juices and esophagitis index. Lycopene further embarked diminishing effect on oxidative stress through synchronizing lipid and protein peroxidation along with regulating the enzymatic activity of SOD and catalase. Lycopene also modified the levels of immunoregulatory cytokines (IL-1β and IL-6) favourably.

The dose dependent efficacy of lycopene in the current experimental condition was also attested when exemplified morphologically through scanning electron microscopy.

Conclusion: From the current line of evidences, it was concluded that lycopene can impart momentous protection against experimental esophagitis by wrapping up the reactive oxygen species and through dual inhibition of the arachidonic acid pathway.

Keywords: Anti-inflammatory, Dual inhibitors, Esophagitis, GERD, Interleukin-6, Oxidative stress, Pantoprazole

Background

Gastroesophageal reflux disease (GERD) is a complex anarchy with the potential for developing esophagitis, esophageal strictures and barretts esophagitis [1]. The situation commences due to reflux of gastric content into esophagus implying to mucosal devastation. Inductive agents in the refluxate are mainly responsible for mucosal damage in GERD. The agents provoke a negotiator release from mucosal and submucosal cells evoking inflammatory reactions resulting in visceral hypersensitivity along with other associated symptoms [2,3]. Abnormal antirefluxate barrier and luminal clearance mechanism results in prolonged contact of refluxate to esophageal mucosa and appears to be responsible for the morphological changes in the esophageal membrane of GERD patients [4,5]. Proton pump inhibitors (PPI) and histaminergic - 2 (H-2) blockers are the first line therapies in clinical management of GERD. Due to relapsing nature of disease, clinical management of GERD is difficult and require prolonged therapy [6]. Moreover, weak inhibitory activity in early phase and less effectiveness of therapy within initial hours of dosing are additional causes for therapeutic relapse in GERD [7].

Some classical inflammatory products like prostanoids and reactive oxygen species (ROS) are considered being critical negotiators in the pathogenesis of clinical GERD [8]. Participation of ROS through free radicals and inflammatory mediators including various cytokines is a well studied phenomenon in case of experimental esophagitis as well [9]. Lycopene is one of the most robust antioxidant with singlet oxygen quenching ability. Lycopene can upregulate the antioxidant electrophile/antioxidant response elements and is reported to have significant antiinflammatory potential as well [10]. In addition to above stated, lycopene is reported to have significant inhibitory effect on the gastric acid secretion followed by efficacy against H. pylori infection. Recently, lycopene was also evaluated for its efficacy in oral submucosal fibrosis [11-13]. In view of above and in quest of our continuous search for therapeutic alternatives for GERD, we hypothesize that lycopene by
virtue of its antioxidant and antiinflammatory property can
demonstrate a significant protection in management of
GERD, and same was explored through present preclinical
investigation.

Methods

Drugs and chemicals
Lycopene was received as a gift sample from Herbo
Nutra Pharmaceuticals Limited, New Delhi, India and
Pantoprazole (Palozac, Ajanta Pharma Private Limited,
Mumbai, India) was procured from the local market. All
other chemicals used were of analytical grade and pro-
cured from Himedia Laboratories, Mumbai, India.

Animals
Albino wistar rats (100–140 gm) were obtained from the
central animal house and kept in polypropylene cages
under standard condition of temperature (22 ± 5°C) with
12 h light/dark cycle and commercial pellet diet with
water ad libitum. The experimental protocol was ap-
proved by Institutional Animal Ethics Committee (IAEC)
(United Institute of Pharmacy, Naini, Allahabad, U.P,
India) (Approval No.UIP/IAEC/2014/FEB/01).

Induction of reflux esophagitis
Groups of male rats (n = 6), fasted for 36 h received normal
saline (3 ml/kg, p.o.) (Sham control), pantoprazole (30 mg/
kg, p.o.), lycopene (50 mg/kg and 100 mg/kg, p.o) [14].
Esophagitis was induced (except in sham control) by ligat-
ing the fore stomach and pylorus with 2–0 silk suture. After
12 h, the animals were sacrificed by cervical dislocation.
The chest was opened with a midline incision, esophageal
and stomach tissue was removed. Stomach was opened
along the greater curvature and esophagus was dissected
out by extending the dissection line along the major axis.
The tissue was washed with normal saline and examined
for lesion. Severity of erosions was scored using Table 1
and esophagitis index was calculated by dividing the total
score by ten. Volume of gastric juices, total acidity, free
acidity and pH was measured as described previously [15].
Schematic representation of the procedure to be followed
for pylorus and forestomach ligation has been already pub-
lished by our laboratory [16].

Estimation of oxidative stress paradigms
Tissues were homogenized in ice cold 0.01 M Tris- HCl
buffer (pH 7.4) and subjected to the estimations of tissue
glutathione (GSH), superoxide dismutase (SOD), catalase,
thiobarbituric acid reactive substances (TBARs) and protein
carbonyl using the methods previously established at our
laboratory [17-20]. Tissue supernatant was further sub-
jected for estimation of interleukin-1ß (IL-1ß) (K0331212P)
and interleukin-6 (IL-6) (K0331229P) using radioimmuno-
assay kits (Koma Biotech inc, Seoul, Korea).

Morphological evaluation
Esophageal tissues were evaluated morphologically through
scanning electron microscopy using the method established
at our laboratory. Briefly, samples were fixed in glutaralde-
hyde (2.5%) followed by washing in phosphate buffer
(0.1 M) at 4°C. Post washing, the tissues were further fixed
in osmium tetroxide (1%) with subsequent washing with
phosphate buffer (0.1 M). Subsequently, samples were
dehydrated using increasing concentration of acetone (30%,
50%, 70%, 90%, 95%, and 100%). Samples were air dried
and mounted to aluminium stub with adhesive tape and
observed under scanning electron microscope (JEOL-
JSM-6490LV) [21].

In-vitro COX and LOX inhibition assay
Lycopene and pantoprazole were assayed for COX-1,
COX-2 and 15-LOX inhibitory activity using a COX-
inhibitor screening kit (Catalog No.760111) and LOX-
inhibitor screening kit (Catalog No. 760700); Cayman
Chemical Company, USA, following manufacturer's proto-
col. Stock solution of lycopene (10% CMC) and pantopra-
zele were prepared in water for injection and further
dilutions were made up to a concentration of 1 µg/ml. Per-
centage inhibition was calculated by comparing the absorb-
ance intensities, measured spectrophotometrically with a
ELISA plate reader (ALERE Microplate Reader, AM-2100)
at 590 and 490 nm for COX and LOX respectively. Test
was performed in triplicate.

Statistical analysis
All data are presented as mean ± SD and analyzed by
one way ANOVA followed by Bonferroni test for possible
significance identification between the various groups.
P < 0.05, P < 0.01, P < 0.001 were considered statistically sig-
ificant. Statistical analysis was carried out using Graph
pad instat software (3.2), San Diego, California.

Results
The present inquisition was ventured to arbitrate the
effect of lycopene on pylorus and forestomach ligation
induced reflux esophagitis in rats. Esophagitis control group
was accorded with esophageal inflammation, edema, le-
sions and oral administration of lycopene incomparably
ruled out the reflux esophagitis.

Lycopene (50 and 100 mg/kg) momentarily repressed
esophagitis in experimental animals as illustrated through
conspicuous reduction in the free acidity, total acidity,

Table 1 Scoring of erosion and severity

| Erosion (mm) | 1 or less | 1-2 | 2-3 | >3 |
|-------------|----------|----|----|----|
| Score       | 1        | 2  | 3  | 4  |
Table 2 Effect of pantoprazole and lycopene on pH, volume of gastric juices total acidity, free acidity and esophagitis index in experimental animals

| Group   | Treatment                               | Intestinal pH | Volume of gastric juices (ml/100 g) | Total acidity (mEq/l) | Free acidity (mEq/l) | Esophagitis index |
|---------|-----------------------------------------|---------------|------------------------------------|-----------------------|----------------------|------------------|
| Group-I | Sham control (Normal saline, 3 ml/kg,p.o) | 3.55 ± 0.31***| 2.58 ± 0.38 (14.00)              | 29.39 ± 0.97***       | 20.99 ± 1.31***     | 0.38 ± 0.35***   |
| Group-II| Esophagitis control (Normal saline, 3 ml/kg,p.o) | 2.73 ± 0.28   | 3.00 ± 0.45                      | 36.26 ± 0.65          | 33.02 ± 0.98        | 3.03 ± 0.34      |
| Group-III| Lycopene (50 mg/kg,p.o)                  | 3.65 ± 0.23   | 1.57 ± 0.12 (47.66)              | 30.05 ± 0.93***       | 26.32 ± 1.14***     | 1.33 ± 0.12***   |
| Group-IV| Lycopene (100 mg/kg,p.o)                 | 4.00 ± 0.34***| 1.87 ± 0.12 (37.66)              | 28.26 ± 0.79***       | 25.79 ± 1.09***     | 1.17 ± 0.16***   |
| Group-V | Pantoprazole (30 mg/kg,p.o)              | 3.92 ± 0.30***| 1.35 ± 3.40 (55.00)              | 26.99 ± 1.36***       | 22.32 ± 1.14***     | 0.77 ± 0.16***   |

Each group contains six animals. Values are represented as mean ± SD. Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test (**P < 0.01, ***P < 0.001). Values in parenthesis represent percentage inhibition.

Table 3 Effect of pantoprazole and lycopene on biochemical markers of oxidative stress in experimental animals

| Group   | Treatment     | Glutathione (mg %) | Superoxide dismutase (unit of SOD/ mg of protein) | Catalase (nmol H$_2$O$_2$/min /mg of protein) | Thiobarbituric acid reactive substances (nmol of MDA/mg of protein) | Protein carbonyl (nmol/ml) |
|---------|---------------|--------------------|---------------------------------------------------|---------------------------------------------|-----------------------------------------------------------------|---------------------------|
| Group-I | Sham control  | 4.71 ± 0.42***     | 110.39 ± 66.13                                   | 4.45 ± 0.40***                              | 1.20 ± 0.06***                                                  | 48.18 ± 1.82***          |
| Group-II| Esophagitis    | 3.69 ± 0.10        | 64.21 ± 31.76                                    | 2.58 ± 0.71                                 | 5.17 ± 0.01                                                    | 162.12 ± 1.74            |
| Group-III| Lycopene      | 4.78 ± 0.10***     | 71.70 ± 17.90                                    | 4.05 ± 0.78***                              | 3.28 ± 0.07***                                                  | 129.39 ± 5.04***         |
| Group-IV| Lycopene      | 4.02 ± 0.20        | 64.68 ± 15.09                                    | 4.10 ± 0.37***                              | 3.03 ± 0.03***                                                 | 112.50 ± 3.00***         |
| Group-V | Pantoprazole  | 4.26 ± 0.05***     | 67.69 ± 27.06                                    | 3.44 ± 0.38                                 | 2.84 ± 0.04***                                                 | 98.26 ± 3.07***          |

Each group contains six animals. Values are represented as mean ± SD. Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test (**P < 0.01, ***P < 0.001). Statistical significance compared between lycopene (50 mg/kg) and lycopene (100 mg/kg) using one-way ANOVA followed by Bonferroni test (P < 0.001).
pathogenesis of GERD and lycopene was observed to have marked effect on that through decrease in acidity (total and free), gastric volume and thereby subsequent increase in pH. After lycopene treatment modulation in pH and acidity of gastric content was also reflected through decrease in esophagitis index. Physiological changes as observed in the present study, suggest, positive modulation in GERD by lycopene. Precedent studies have unfolded the role of free radicals in pathogenesis of GERD in experimental animals with

Table 4 Effect of pantoprazole and lycopene on immunoregulatory cytokines in esophageal tissues of experimental animals

| S. No. | Treatment                          | IL-1β (pg/ml) | IL-6 (pg/ml) |
|--------|------------------------------------|---------------|--------------|
| Group-I| Sham control (Normal saline, 3 ml/kg, p.o.) | 758.98 ± 30.61*** | 17673.16 ± 196.4*** |
| Group-II| Esophagitis control (Normal saline, 3 ml/kg, p.o.) | 2595.9 ± 210.93 | 43429.33 ± 868.9 |
| Group-III| Lycopene (50 mg/kg, p.o.) | 755.68 ± 82.67*** | 25878.2 ± 405.1*** |
| Group-IV| Lycopene (100 mg/kg, p.o.) | 788.86 ± 38.19*** | 22055.31 ± 779.86*** |
| Group-V| Pantoprazole (30 mg/kg, p.o.) | 612.98 ± 44.47*** | 15611.14 ± 411.82*** |

Each group contains six animals. Values are represented as mean ± SD. Statistical significance compared to toxic control using one-way ANOVA followed by Bonferroni test (**P < 0.001). Statistical significance compared between lycopene (50 mg/kg) and lycopene (100 mg/kg) using one-way ANOVA followed by Bonferroni test (***P < 0.001).
Concomitant increase in levels of malondialdehyde (MDA) [22]. MDA is a stable product of lipid peroxidation and is a sensitive and reactive marker of membrane damage. MDA forms a colour complex with thiobarbituric acid (TBA) which can be scrutinized spectrophotometrically. In the present study, we observed momentous increase in MDA in esophagitis control, advocating active involvement of ROS after forestomach and pylorus ligation, which is in alignment to foregoing reports [21]. It would be apropos to mention that lycopene treatment momentously decreased lipid peroxidation as attested by decrease in MDA formation through TBA assay. GSH is the most profuse low molecular weight thiol and is an omnipresent tripeptide, involved in wide range of enzymatic reaction. The function of GSH in an oxidation reduction process is to act as reductant, resulting in formation of glutathione disulphide (GSSG). In first few hours of oxidative stress, free radicals damage leads to consumption of GSH, directing decreased GSH level and therefore it is considered a marker of short term oxidative stress [23]. Decreased levels of GSH represent its increased consumption by the cells as a consequence of ROS generation. Results from the present study depict that lycopene treatment significantly helped to restore the GSH in dose dependent manner, suggesting either restored biogenesis of GSH or decreased oxidative stress.

The SOD and catalase are antioxidant enzymes with induct radical scavenging activity. SOD interacts with superoxide radical to form H$_2$O$_2$, which is subsequently catabolised by catalase to molecular oxygen and water [24]. Catalase is a hemeprotein and protects the tissue from deleterious effects of highly reactive hydroxyl radicals [25,26]. In present study, a simultaneous decrease in SOD and catalase activity after ligation of pyloric end and forestomach was observed. This decrease in enzymatic activity of SOD and catalase could be attributed to increased utilization, in consequence to oxidative stress. Treatment with lycopene restored the diminished levels of catalase and SOD. The free radical attack can destroy all types of biological molecules including proteins, lipids and DNA. When proteins are oxidised either by α-amidation pathway or by oxidation of glutamide side chain, carbonyl group is produced on a protein side chain. Hence the occurrence of protein carbonyl content and amino acid residue is one of the most general indicators of protein oxidation. Therefore, protein carbonyl is universally used as marker of protein oxidation [27,28]. In the present study pylorus and forestomach ligation significantly increased protein carbonyl level, indicating protein oxidation. Subsequent treatment with lycopene was observed for decrease in protein carbonyl levels in esophageal tissues. From above line of evidences, one can derive that lycopene could be instrumental physiologically and biochemically in the management of esophageal reflux in experimental conditions.

Foregoing, preclinical and clinical studies on GERD have shown that the immune and inflammatory responses are characterized by specific cytokine and chemokines profiles (IL-1β, IL-2, IL-6 and many more). Moreover, reflux of acid into the esophagus, release immunoregulatory cytokines along with damage of mucosal lining; further strengthen the theory that GERD is an auto-immune disorder with

![Figure 2](image.png)

**Figure 2** The possible free radical scavenging mechanism for Lycopene. Lycopene scavenges free radical by three mechanisms: **a)** Adduct formation, **b)** Allylic H substraction, and **c)** Electron transfer system.
inflammatory participation [29-31]. Considering the physiological importance of immunoregulatory cytokines, we quantified IL-1β and IL-6 in the esophageal tissues. IL-1β is a member of IL-1 cytokine family and is mainly produced by the activated macrophages. IL-1β is involved in variety of activities including cell proliferation, differentiation and apoptosis. The IL-6 is secreted by various cells like T-cells, β-cells, microglia, fibroblast, endothelial cells, neurons and astrocytes. IL-6 is originally identified as β-cells differentiation factor and is synthesized in response to IL-1β. IL-6 has an important role in host reaction to inflammation which results in the synthesis of acute inflammatory proteins. IL-6 and IL-1β are proinflammatory cytokines involved in the acute phase of immune responses. Both IL-1β and IL-6 can stimulate the inflammatory and autoimmune response in many diseases like diabetes, cancer and esophagitis [32]. The current line of evidences suggest the over expression of immunoregulatory cytokines (IL -1β and IL-6) in response to forestomach and pylorus ligation (esophagitis control) which is in corrroboration to the precedent reports and also suggest utmost immunocompromised status [33]. The lycopene treatment restored the levels of IL-1β and IL-6 to a significant level further strengthening the hypothesis outlined in the current study.

The prolong contact of esophageal mucosal membrane with acid and pepsin due to the impaired antireflux barrier can lead to morphological changes in it and to investigate the same, esophageal tissues were investigated for morphological changes using SEM [4]. Significant ultrastructural changes, including dilation of intracellular spaces, extensive erosion of esophageal mucosa, detachment of epithelial layer and mucosal degeneration, as reputed by precedent studies, were also evident in the current experiment. Treatment with lycopene demonstrated significant protection through restoring the normal morphology of the esophageal tissues.

When contemplated for the in-vitro COX and LOX inhibition assay, lycopene evidenced a significant inhibition of COX-1 and COX-2 enzymes with considerable inhibition of 15-LOX, suggesting dual inhibition of the arachidonic acid pathway. It would be appropriate to mention that dual inhibitors of arachidonic acid metabolism are established to have excellent gastrointestinal safety profile and thereby lycopene could offer a fringe benefit in management of gastrointestinal disorders.

Lycopene is a non provitamin A carotenoid with polyisoprenoid structure [34,35]. It is a lipophilic compound with high number of conjugated diene and is one of the most robust antioxidant with a singlet oxygen quenching ability. Several lines of evidence suggest that lycopene can upregulate the antioxidant electrophile/antioxidant response element, which stimulates the production of phase II detoxifying antioxidant enzymes that protect the cells from ROS [10]. Lycopene can react with variety of radicals through adduct formation, electron transfer and allylic H substitution. The adduct genesis results in configuration of resonance-stabilised carbon centered peroxy radical, where a free radical can attach to the polyene chain (through highly conjugated double bonds of lycopene) to form a lycopene peroxyl (ROO-lycopene) radical adduct. ROO-lycopene may possibly react with O2 to form ROO-lycopene-OO' (Figure 2). Through electron transfer mechanism, lycopene can form radicals like cation radical, anion radical or alkyl radical depending upon the type of radical involved in the process. Another method through which lycopene can curb down the ROS is hydrogen abstraction (allylic H substitution) leading to formation of lycopene radical [36-38] (Figure 2).

As enumerated from the current experimental evidences, it became settled that lycopene can offer momentous protection against the experimental esophagitis, and the same could be attributed to its ROS scavenging along with antiinflammatory (dual inhibitory) potential. Lycopene is a well established antioxidant and is under clinical investigation for various ailments including prostate cancer. Authors would like to comment that the present preclinical finding further strengthen the candidature of lycopene against gastroesophageal cancer and other similar ailments.

Competing interests The authors declare that they have no competing interests.

Authors’ contributions AKG: Carried out the bench work. JKR and MS: Compiled the data, statistical analysis and organised the manuscript. SG: Contributed towards bench work and statistical analysis. GK: Designed the study and prepared the manuscript. All authors read and approved the final manuscript.

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