Cumulative antioxidant defense against oxidative challenge in galactose-induced cataractogenesis in Wistar rats

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Keywords: Aldose reductase, Antioxidant enzymes, Cataract, Curcumin, Galactose, Oxidative damage, Vitamin-E

Natural dietary ingredients are known for their antioxidant activity. Of such, curcumin, the active principle of turmeric, at 0.01% in the diet proved as pro-oxidative in galactose-induced cataract in vivo. The purpose of this study was to investigate the effect of vitamin E (VE), a well-known antioxidant, in combination with curcumin on the onset and maturation of galactose induced cataract. Periodic slit-lamp microscope examination indicated that in combination with vitamin-E, 0.01% curcumin (G-IV) delayed the onset and maturation of galactose-induced cataract. Biochemical analyses revealed that combined treatment of 0.01% curcumin and vitamin-E diet exhibited an efficient antioxidant effect, as it inhibited lipid peroxidation and contributed to a distinct rise in reduced glutathione content. The results indicate that natural dietary ingredients are effective in combination rather than the individual administration as they are complementing each other in reducing the risk of galactose induced cataract.

Keywords: Aldose reductase, Antioxidant enzymes, Cataract, Curcumin, Galactose, Oxidative damage, Vitamin-E

Cataract, opacity of the eye lens, is the leading cause of blindness worldwide. Various factors such as nutritional deficiencies or inadequacies, diabetes, sunlight, environmental factors, smoking and lack of consumption of antioxidants are known to increase the risk of cataract. Oxidative damage plays an important role in the opacification of the lens. The oxygen free radicals disturb cellular homeostasis through protein modification, and lipid peroxidation. Like other organs, the lens has a well-designed system of defense against oxidation. It uses primary defenses of non-enzymatic (i.e. glutathione, vitamin-E, vitamin-C and carotenoids) and enzymatic (i.e. superoxide dismutase, glutathione peroxidase, catalase) to neutralize free radicals and repair, recover or degrade the molecules that are damaged. Daily intake of foods containing micronutrients that quench/scavenge oxidants may act as cataractostatic. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) also known as diferuloylmethane is a major component of food flavoring agent, turmeric (Curcuma longa), and has been reported to be anticarcinogenic and anti-inflammatory. Curcumin has significant antioxidant activity both, in vitro and in vivo. Protective influence of curcumin against selenium induced cataract in rats has been reported. In addition to these properties, it has hypolipidemic, antidiabetic/hypoglycemic activities as well. Suryanarayana et al. have reported that dietary curcumin at very low levels (0.002% in the diet) delays galactose-induced cataract in rats; however, a higher level (>0.01%) proved to be pro-oxidative and thereby enhancing galactose induced cataract model in rats. Vitamin E (VE) is a lipid soluble membrane bound molecule and it scavenges/quenches free radicals and reduces oxidative damage to the lens tissues. Many case-control studies have revealed that low levels of plasma VE are associated with ocular damage (i.e. early cortical lens opacity, macular degeneration) and unhealthy aging. There are studies where the liposome delivered VE prevented the progression of cataractogenesis in the rats that received 25% galactose. More importantly it prevents the DNA damage caused by curcumin through oxyradicals.

Therefore, it is hypothesized that VE in combination with curcumin may prevent/delay cataract more effectively than either of the treatments alone. Therefore, in the present study the combined...
action of curcumin and vitamin-E has been studied in delaying of cataract in Wistar rats.

**Materials and Methods**

*Materials*—Curcumin, vitamin-E (vit-E), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), cumene hydroperoxide, DL-glyceraldehyde, O-phthalaldehyde (OPT), glutathione, N-ethylmaleimide (NEM), 2,4-dinitrophenylhydrazine, guanidine hydrochloride and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Galactose was from Merck (Germany). All other chemicals and solutions were of analytical grade and purchased locally.

*Animals/experimental design*—Male Wistar-NIN rats (21-24 days) having an average bodyweight of 35-40g, were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad and randomly assigned into 10 groups of 8-10 animals each.

Diet composition of each group is given in Table 1. Parallel controls were maintained for curcumin (both levels) and vitamin-E with normal stock AIN-93 diet. The Departmental Animal Ethics Committee approved animal care. All the animals were caged separately with similar ambient environment and the diurnal rhythm was maintained throughout the course of the study. The daily food intake and body weights (weekly) were monitored. The experiment was carried out for 26 days.

*Lenticular examination and cataract classification*—To evaluate the role of antioxidants, individually and in combination, in galactose-induced cataract, lenses were examined on alternate days and opacities observed were graded into four stages. Clear or stage 0; no vacuoles present or clear lens, stage 1; vacuoles of less than one third of the lens radius, stage 2; vacuoles located at the periphery of the lens occupying an area of between one-third and two-thirds of the radius from the periphery, stage 3; vacuoles extending up to two-thirds of the radius from the periphery (nuclear opacity may be seen), stage 4; vacuoles cover the entire lens, which appears white to the naked eye. The incidence of cataract appearance was expressed as the percentage of total lenses in each group.

*Lens extraction and processing*—At the end of the experiment, animals were sacrificed by CO2 asphyxiation and lenses were extracted and stored at -70°C till further analysis. A 10% homogenate was prepared and malondialdehyde (MDA) levels were estimated in the total homogenate. The rest of the biochemical parameters were carried out with 25,000 g supernatant.

*Biochemical estimations of oxidative stress markers and antioxidant enzymes*—MDA was estimated as TBA reacting substances (TBARS) as described by Buyan et al. Reduced and oxidized glutathione were estimated by the spectrofluorometric method using O-phthalaldehyde (OPT) to yield a fluorescent complex as described by Hissin and Hilf. Aldose reductase activity was estimated according to Hyman et al. Superoxide dismutase (SOD; E.C.1.15.1.1) activity was assayed by monitoring the rate of inhibition of pyrogallol reduction according to Marklund and Marklund. The activity of glutathione peroxidase was assayed spectrophotometrically using cumene hydroperoxide and GSH as substrate according to Martinez et al. The activity of glutathione reductase was assayed spectrophotometrically using 2, 4-dinitrophenyl-hydrazine according to Uchida et al.

*Protein estimation*—The protein content of lens (total and soluble) was estimated as per Lowry et al. using BSA as the standard.

*Statistical analysis*—The differences between the control and treated groups were analyzed using one-way ANOVA, followed by Post Hoc test (multiple comparison tests).
The differences were considered significant if $P$ was at least $<0.05$.

**Results**

*Food intake and body weights*—During the entire course of study there was a negligible variation in food intake and body weight of animals in all groups, ($P>0.05$, data not shown).

*Lens morphology*—Results of slit lamp examination indicate that 84% of the lenses in G-II developed stage 2 cataract and rest of the lenses were in stage 3 cataract after 14 days (Fig.1). At the end of experiment (i.e. 26th day) 88% of the lenses developed mature cataract (stage-4) and 12% of the lenses were in stage 3 (Fig.2). Feeding of curcumin at the 0.005% level delayed the onset of galactose induced cataract marginally as there were 78% of the lenses in this group (G-V) demonstrated stage 2 cataract while 14% were at stage 1 cataract and 8% of the lenses were at stage 3 cataract at the end of 14th day (Fig.1). Likewise, maturation of cataract was also not affected significantly by the 0.005% curcumin treatment (Fig.2). Feeding of curcumin at the higher dose (0.01%) slightly delayed the onset of cataract (7% were at stage 1 and 82% were at stage 2 and 11% at stage 3, Fig.1) and maturation of the cataract was faster than with galactose feeding alone (91% were at stage 4 and 9% at stage 3, Fig. 2). While VE treatment alone was more effective than either of the two curcumin doses in terms of delaying both onset and maturation of galactose-induced cataract (Figs 1, 2).
The combination of vitamin E with curcumin was very effective in delaying the onset and maturation of cataract; interestingly the effect was greater in combination with the higher level of curcumin (Figs 1, 2). Lenses from G-I and G-VIII-X animals were clear during the entire experimental period (data not shown).

**Biochemical analysis of oxidative markers and antioxidant enzymes**—There was a significant drop in protein content (total and soluble) in G-II when compared to G-I (Table 2). The antioxidant treatment individually did not result in much change in the protein content between the different groups (Table 2). However, the percentage of soluble protein increased significantly with combined treatment of curcumin and VE (G-IV and G-VI) (Table 2).

Galactose fed rats (G-II) showed a significant elevation in MDA levels (measured as TBARS) and a decrease in glutathione peroxidase (GPx) and glutathione reductase (GR) activities respectively when compared to controls (G-I) (Table 2), indicating an increase in lipid peroxidation. In addition, we have also observed a remarkable decrease (47%) in reduced glutathione (GSH) level and a rise in oxidized glutathione (GSSG) level in galactosemic animals compared to controls (Table 2). Curcumin administration at lower dosage (0.005%) to galactose fed rats prevented the changes in GSH, GSSG, MDA, GPx and GR levels (Table 2); and in contrast, at higher dosage (0.01%), there was an increased elevation in the oxidative insult similar to that was found in the G-II rats (Table 2). However, G-VI (0.005% curcumin+15mg VE/100gm diet) showed inhibition of carbonyl formation (Table 2).

The specific activity of aldose reductase, a key enzyme in polyol pathway, was significantly higher in G-II than G-I (Table 2). Curcumin administration at lower dosage (0.005%) to galactose fed rats prevented the changes in GSH, GSSG, MDA, GPx and GR levels respectively and decreased MDA and GSSG levels compared to G-VII (15mg VE), G-V and G-II respectively (Table 2), indicating a defensive action against oxidative insult. Interestingly, the higher dosage of curcumin in combination with vit-E was more effective in decreasing the fluctuations of GSH, GSSG, MDA, GPx and GR in galactose treated rats, as compared to all other groups (Table 2).

The AR activity was marginally decreased in the rats that received vitamin-E (Table 2).

**Protein carbonyls**—Protein carbonyl content was significantly higher in G-II compared to G-I (Table 2). Combined treatment of 0.01% curcumin + 15mg VE/100gm diet showed inhibition of carbonyl formation (Table 2).

In the groups VIII, IX and X there were no noticeable changes in lens morphology and antioxidant enzymes in relation to the control group.

**Discussion**

Cataract is a multifactorial disorder, and many causative agents are involved which generate active oxygen species intraocularly. The highly reactive species derived from the univalent reduction of oxygen appear to be mainly responsible for the oxidative damage. These effects can be decreased by nutritional supplements. Hence, attempts were made to study the prophylactic nature of anti-cataract agents in the management of cataract. The observations of the present study support the hypothesis that by using right kind of antioxidants from nutritional sources a definite delay in the progression of cataractogenesis can be realized.

The curcumin attenuates lens opacification by 4-HNE in organ culture. The dose dependent nature as well as biochemical mechanism of curcumin against galactose and streptozotocin-induced cataractogenesis was also reported. However, in galactose model higher dose was not protective rather deleterious as it enhanced cataractogenesis. This may perhaps be due to the prooxidative nature of curcumin at higher dose under galactosemic but in hyperglycemic conditions. Therefore, a combination of curcumin and VE was attempted to assess their efficacy in the oxidative stress in treating the galactose-fed rats. Based on the hypothesis the effect of curcumin in combination with VE was assessed in cataractogenesis. The galactose-fed rats showed higher oxidative insult mediated through excessive accumulation of reducing sugars, which contribute to the hastening up of cataractogenesis. As a
Table 2—Effect of curcumin and vitamin-E on protein content, lipid peroxidation, levels of glutathione, antioxidant/detoxifying enzymes, aldo reductase and protein carbonyls in galactose-induced cataract in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total protein (mg/lens)</th>
<th>Soluble protein (mg/lens)</th>
<th>TBARS (nmol/g lens)</th>
<th>Reduced GSH (μmol/lens)</th>
<th>Oxidized GSH (GSSG) (μmol/mg protein)</th>
<th>GR (GSH-R) (Units/mg protein)</th>
<th>GPx (Units/mg protein)</th>
<th>SOD (Units/mg protein)</th>
<th>Alt (Units/mg protein)</th>
<th>Protein carbonyls (nmols/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Cont.)</td>
<td>15.46±0.94</td>
<td>11.30±1.74</td>
<td>10.80±0.09</td>
<td>12.18±0.96</td>
<td>0.161±0.028</td>
<td>0.268±0.013</td>
<td>0.58±0.062</td>
<td>4.74±0.25</td>
<td>22.49±0.526</td>
<td>2.55±0.23</td>
</tr>
<tr>
<td>II (30% Gal.)</td>
<td>8.65±1.32*</td>
<td>4.96±0.99*</td>
<td>17.28±1.71*</td>
<td>5.84±0.92*</td>
<td>0.414±0.014*</td>
<td>0.102±0.007*</td>
<td>0.17±0.027*</td>
<td>3.04±0.78*</td>
<td>37.54±0.443*</td>
<td>8.22±0.494*</td>
</tr>
<tr>
<td>III (30% gal. + 0.01% cur.)</td>
<td>7.66±1.04*</td>
<td>4.03±0.6*</td>
<td>16.85±0.67*</td>
<td>5.15±0.14*</td>
<td>0.403±0.023*</td>
<td>0.140±0.012*</td>
<td>0.20±0.207*</td>
<td>2.80±0.20*</td>
<td>36.23±0.506*</td>
<td>6.84±0.17*</td>
</tr>
<tr>
<td>IV (30% gal. + 0.01% cur.+ 15 mg VE/100g diet)</td>
<td>14.41±0.84</td>
<td>10.55±0.59</td>
<td>11.58±0.97*</td>
<td>11.16±0.58*</td>
<td>0.165±0.021*</td>
<td>0.243±0.013*</td>
<td>0.55±0.082</td>
<td>3.38±0.11*</td>
<td>26.31±0.782</td>
<td>2.65±0.183</td>
</tr>
<tr>
<td>V (30% gal. + 0.005% cur.)</td>
<td>10.39±1.30*</td>
<td>6.75±0.43*</td>
<td>14.33±0.35*</td>
<td>8.65±0.31*</td>
<td>0.265±0.017*</td>
<td>0.193±0.014*</td>
<td>0.31±0.061*</td>
<td>3.97±0.10*</td>
<td>31.06±0.187</td>
<td>4.85±0.227*</td>
</tr>
<tr>
<td>VI (30% gal. + 0.005% cur.+ 15mg VE/100g diet)</td>
<td>13.49±1.81* #</td>
<td>9.47±0.31*</td>
<td>12.66±0.49*</td>
<td>10.47±0.63*</td>
<td>0.202±0.021*</td>
<td>0.232±0.008*</td>
<td>0.48±0.643*</td>
<td>4.27±0.09*</td>
<td>28.51±0.585*</td>
<td>3.28±0.226*</td>
</tr>
<tr>
<td>VII (30% gal+15mg VE)</td>
<td>12.75±0.89*</td>
<td>8.55±0.40*</td>
<td>13.40±0.52*</td>
<td>9.46±0.31*</td>
<td>0.233±0.018*</td>
<td>0.201±0.008*</td>
<td>0.40±0.640*</td>
<td>4.69±0.10</td>
<td>31.64±0.437*</td>
<td>4.02±0.115*</td>
</tr>
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The asterisk (*) denotes that the data are significantly different from Group-I. This (#) indicates that the data is not significantly different from group-IV at >0.05. a One unit of GR defined as μmol NADPH oxidized/min. b One unit of GPx defined as μmol GSSG formed/min. c One unit of SOD represents the amount of enzyme required for 50% inhibition of pyrogallol reduction/min. d Data are expressed as μmol NADPH oxidized/hr/100mg protein.
result, the protein content (total and soluble) was decreased in the test group in relation to control.

The main culprit in galactose-induced cataract is accumulation of sugar alcohols, which results in osmotic disequilibria followed by altered levels of oxidant scavengers. In the present study, aldose reductase (AR) activity in G-II was significantly increased, which is indicative of oxidative stress. In the antioxidant treated groups, AR activity was inhibited moderately and this in agreement of the observation of Kinoshiita\textsuperscript{32}.

An imbalance in redox state of the lens glutathione system in galactose group, was observed which is indicative of oxidative insult caused by galactose feeding. These findings are suggestive of a clear and pronounced oxidative damage that has set in group-II induced by galactose. However, in the test groups that received the antioxidants, the redox system was protected except in group-III; the effect was more pronounced in 0.01% curcumin+VE received group.

Superoxide dismutase decreased in the galactose group is indicative of oxidant activity\textsuperscript{33}. Unlike other parameters, SOD activity was not improved with combined treatment rather, vit-E alone was much effective. At this stage, the mechanism(s) of Vitamin E in quenching the pro-oxidative nature of higher dose of curcumin, thus making it effective in delaying the cataract, is not known. This needs further investigation. Nonetheless, biochemical analyses suggest that curcumin (0.01%) in combination with vitamin-E appears to decrease oxidative stress under galactosemic conditions. Therefore, an antioxidant therapy, i.e. daily intake of combination supplements that can delay/prevent cataractogenesis is suggested.

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