Effect of curcumin on galactose-induced cataractogenesis in rats

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Purpose: Curcumin, the active principle of turmeric, has been shown to have both antioxidant and hypoglycemic activity in vitro and in vivo. The purpose of this study was to investigate the effect of curcumin on the onset and maturation of galactose induced cataract.

Methods: Sprague-Dawley rats (21 days old) were divided into 5 groups. The control group (A) received an AIN-93 diet, the galactose group (B) received 30% galactose in the diet, the test groups (C and D) received the B group diet plus 0.002% and 0.01% curcumin respectively, and group (E) received the control diet plus 0.01% curcumin, all for a period of 4 weeks. Cataract progression due to galactose feeding was monitored by slit lamp microscope and classified into 4 stages. At the end of the experiment biochemical parameters such as lipid peroxidation, aldose reductase (AR), sorbitol dehydrogenase (SDH), reduced glutathione, protein content, and protein carbonyls were measured in the lens. Advanced glycated end products (AGE) and protein oxidation were measured by AGE and tryptophan fluorescence respectively. Crystallin profile was analyzed by size exclusion chromatography (HPLC).

Results: Slit lamp microscope observations indicated that curcumin at 0.002% (group C) delayed the onset and maturation of cataract. In contrast even though there was a slight delay in the onset of cataract at the 0.01% level (group D), maturation of cataract was faster when compared to group B. Biochemical analysis showed that curcumin at the 0.002% level appeared to exert antioxidant and antiglycating effects, as it inhibited lipid peroxidation, AGE-fluorescence, and protein aggregation. Though the reasons for faster onset and maturation of cataract in group D rats was not clear, the data suggested that under hyperglycemic conditions higher levels of curcumin (0.01%) in the diet may increase oxidative stress, AGE formation, and protein aggregation. However, feeding of curcumin to normal rats up to a 0.01% level did not result in any changes in lens morphology or biochemical parameters.

Conclusions: These results suggest that curcumin is effective against galactose-induced cataract only at very low amounts (0.002%) in the diet. On the other hand at and above a 0.01% level curcumin seems to not be beneficial under hyperglycemic conditions, at least with the model of galactose-cataract.
METHODS

Materials: Curcumin, NADPH, NADH, 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), DL-glyceraldehyde, lithium sulphate, β-mercaptoethanol, glutathione, BSA, 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, butylated hydroxy toluene (BHT), O-pthalaldehyde (OPT), and EDTA, were obtained from Sigma Chemical Company (St. Louis, MO). Galactose was from Sisco Research Laboratory Pvt. (SRL) Chemicals (India). All other chemicals and solvents are of analytical grade.

Experimental design and dietary regimen: Male Sprague-Dawley rats (21 day old) having an average body weight of 39±3.72 g (obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad) were randomized in five groups, eight to twelve animals in each group. Each group was fed a different diet as follows; Group A: Animals in this group were fed a normal stock diet based on the AIN-93 formula [19] (n=8); Group B: Animals were fed 30% galactose in the above diet (n=12); Group C: Animals in this group received the B-group diet containing 0.002% curcumin (n=12); Group D: Animals in this group received the B-group diet containing 0.01% curcumin (n=12); Group E: Animals were kept on the AIN-93 stock diet containing 0.01% curcumin (n=6).

Animal care: Animal care and protocols were in accordance with and approved by the Institutional Animal Ethics Committee. Animals were housed in individual cages in a temperature and humidity controlled room having a 12 h light/dark cycle. All of the animals had free access to water. Food intake (daily) and body weights (weekly) were monitored. Animals were fed for 24 days. By that time all the animals in group D had developed mature cataract.

Slit lamp examination and cataract classification: Eyes were examined every other day using a slit lamp biomicroscope (Kowa Portable, Japan). Initiation and progression of lenticular opacity was graded in four stages as documented...
previously [20]. These stages were: Clear or stage 0; no vacuoles present or clear lens, stage I; vacuoles of less than one-third of the lens radius, stage II; 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 III; vacuoles extending up to two-thirds of the radius from the periphery (nuclear opacity may be seen), stage IV; 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 collection and processing:** At the end of the experiment animals were sacrificed by CO$_2$ asphyxiation and lenses were dissected by the posterior approach and stored at -70°C until further analysis.

A 10% homogenate was prepared from 2-3 pooled lenses (depending on the assay) in aqueous buffers. All the biochemical parameters were analyzed in the soluble fraction of the lens homogenate (15,000 x g at 4°C) except for lens MDA which was estimated in the total homogenate.

**Biochemical estimations:** Lens MDA was estimated as TBA reacting substances (TBARS) as described by Buyan, et al. [21]. Aldose reductase (AR) and sorbitol dehydrogenase (SDH) activities were estimated according to the methods described by Hayman, et al. [22] and Gerlach, et al. [23] respectively. GSH content was estimated by the spectrofluorometric method using OPT to yield a fluorescent complex as described by Hissin, et al. [24]. Protein carbonyl content of soluble protein was measured using the 2,4-DNPH method and was expressed as the ratio of moles of DNPH reduced (average E$_{365}$ nm = 21.0 mM$^2$/cm$^2$/mg of protein according to Uchida, et al. [25].

**Crystallin distribution profiles by HPLC:** Crystallin distribution in the soluble protein fraction was performed by gel filtration chromatography on a 600x7.5 mm TSK-G2000 SW column (TOSOH Co., Japan) using a Shimadzu HPLC system. The column was equilibrated with 0.1 M sodium phosphate buffer pH 6.7 containing 0.1 M sodium chloride at a flow rate of 1 ml/min. Soluble protein samples (20 µl of 1 mg/ml solution) were loaded onto the column and protein peaks were detected at 280 nm.

**Advanced glycation related fluorescence:** Advanced glycation end product (AGE) fluorescence was measured in soluble protein (0.3 mg/2 ml in 0.05 M sodium phosphate buffer, pH 7.4). Fluorescence spectra were obtained from 400-500 nm with excitation at 370 nm in a Perkin-Elmer spectrophotometer [26].

**Tryptophan fluorescence:** Tryptophan fluorescence was measured in the soluble protein fraction (0.15 mg/ml in 0.05 M sodium phosphate buffer, pH 7.4) as an indicator of protein oxidation and conformational changes. The fluorescence spectra were obtained at excitation 295 nm and emission between 310-400 nm.

**Protein estimation:** The protein content of lens tissue (total and soluble) was estimated by the method of Lowry using BSA as the standard.

**Statistical analysis:** ANOVA was used, with a p<0.05 considered significant.

### RESULTS

**Food intake and body weights:** There was no significant effect of feeding of either galactose or galactose plus curcumin (0.002% and 0.01%) on food intake and body weight of the

### Table 1. Effect of curcumin on lens protein content in galactose induced cataract

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (mg/lens)</td>
<td>14.21±0.82</td>
<td>9.08±2.48$^*$</td>
<td>9.21±2.24$^*$</td>
<td>6.09±1.59$^*$</td>
</tr>
<tr>
<td>Soluble protein (mg/lens)</td>
<td>10.11±0.35</td>
<td>5.48±1.03$^*$</td>
<td>6.09±2.16$^*$</td>
<td>2.35±1.58$^*$#</td>
</tr>
<tr>
<td>%Soluble Protein</td>
<td>71</td>
<td>60</td>
<td>66</td>
<td>38</td>
</tr>
</tbody>
</table>

The data presented below are the means±SD (n=5). The asterisk denotes that the data are significantly different from Group A and the sharp mark (“#”) denotes that the data are significantly different from Group B.

### Table 2. Effect of curcumin on lipid peroxidation, glutathione and activities of polyol pathway enzymes in galactose-induced cataract in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS n mol/g lens</td>
<td>10.98±1.43</td>
<td>17.28±1.00$^*$</td>
<td>14.94±1.42$^*$</td>
<td>17.02±1.57$^*$</td>
</tr>
<tr>
<td>GSH µmol/lens</td>
<td>11.45±0.29</td>
<td>5.53±0.22$^*$</td>
<td>7.30±0.16$^*$</td>
<td>4.43±0.16$^*$</td>
</tr>
<tr>
<td>AR</td>
<td>33.57±2.53</td>
<td>42.60±1.54$^*$</td>
<td>37.98±4.89</td>
<td>39.96±5.98$^*$</td>
</tr>
<tr>
<td>SDH2</td>
<td>4.86±0.47</td>
<td>5.41±1.86</td>
<td>5.32±1.05</td>
<td>5.59±1.03</td>
</tr>
</tbody>
</table>

The data presented below are the means±SD (n=5). The asterisk denotes that the data are significantly different from Group A. One µmoles NADPH oxidized/hr/100 mg protein and two µmoles NADH oxidized/hr/100 mg protein.

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animals during the entire course of the study (data not shown).

**Lens morphology:** Results of slit lamp examination indicate that all of the animals fed on the galactose diet developed stage II cataract after two weeks. (Figure 1). After four weeks 72% of the lenses developed mature cataract (stage IV) and 28% of the lenses were in stage III (Figure 2). Feeding of curcumin at the 0.002% level delayed the onset of galactose induced cataract since only 50% of the lenses in this group demonstrated stage II cataract while 33% were in stage I cataract and 17% of the lenses had not developed any lenticular opacity at the end of two weeks (Figure 1). Furthermore, maturation of cataract was also slowed by the 0.002% curcumin treatment, as only 58% of the lenses in this group demonstrated stage IV cataract and 42% were still in stage III after 24 days (Figure 2). Feeding of curcumin at the higher dose (0.01%) slightly delayed the onset of cataract (8% were in stage I and 92% were in stage II, Figure 1) and maturation of cataract was faster than with galactose feeding alone (100% were in stage IV, Figure 2). Lenses from group A and group E animals were clear during the entire experimental period (Figure 1 and Figure 2, data for group E animals not shown).

**Biochemical studies:** To investigate the possible mechanisms of differential effects of curcumin at different doses on galactose-induced cataract we investigated various biochemical parameters related to the oxidative stress/antioxidant system, the polyol pathway, protein oxidation and glycation, protein content, and crystallin distribution.

There was a significant decrease in both total and soluble protein in group B rats compared to animals in group A (Table 1). Feeding of curcumin at the 0.002% level did not improve the protein levels (both total and soluble), however there was a marginal improvement in the percentage of soluble protein compared to group B (Table 1). Feeding of curcumin at the 0.01% level, in contrast, led to a further decrease in total and soluble protein compared to group B animals (Table 1).

Lens MDA (measured as TBARS) levels were significantly higher in group B compared to group A, indicating an increased lipid peroxidation due to galactose induced cataract (Table 2). A remarkable decrease (52%) in reduced glutathione (GSH) levels in group B animals compared to group A (Table 2) further supports the oxidative damage observed in galactose cataract, which is in accordance with earlier studies. Curcumin at low levels prevented the changes in GSH and MDA levels due to galactose feeding, however at the 0.01% level curcumin had no influence on these parameters (Table 2).

The specific activity of AR, a key enzyme of the polyol pathway, was significantly higher in group B animals than group A (Table 2). Feeding curcumin at both levels (0.002% and 0.01%) resulted in a marginal decrease in AR activity compared to group B, but the decrease was not statistically significant (Table 2). The activity of sorbitol dehydrogenase (SDH), the second enzyme from the polyol pathway, was unaltered in all of the groups (Table 2).

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**Figure 3.** Tryptophan fluorescence of soluble protein in different groups. Protein (0.15 mg/ml) in 0.05 M sodium phosphate buffer pH 7.4 was excited at 295 nm and emission was monitored 300-400 nm. Data represent three observations.

**Figure 4.** Protein carbonyl content in soluble protein fraction of lens. Protein carbonyl groups present in 1 mg/ml of soluble protein fraction of different groups was assayed by reactivity to 2,4-DNPH as described in the Methods section. The asterisk indicates that the carbonyl content of group B is statistically significant from group A. Data are average of three values.
Tryptophan fluorescence: Tryptophan fluorescence spectra (Figure 3) showed a decrease in tryptophan fluorescence in group B compared to group A. Although curcumin at both levels (group C and D) significantly inhibited the decrease in tryptophan fluorescence when compared to group B, the inhibitory effect was pronounced only at the 0.002% levels (Figure 3).

Protein carbonyls: Protein carbonyl content was significantly higher in group B compared to group A (Figure 4). Feeding of curcumin at the 0.002% (group C) and 0.01% levels (group D) inhibited carbonyl formation (Figure 4).

Advanced glycation end product (AGE) fluorescence: AGE fluorescence (Figure 5) suggested that there was an increase in group B compared to Group A. Curcumin inhibited AGE fluorescence only at the 0.002% level (group C). In contrast AGE fluorescence was enhanced due to feeding of curcumin at the 0.01% level, along with galactose (Figure 5).

Crystallin distribution: To investigate possible alterations in crystallin profiles due to galactose-induced cataract and the influence of curcumin addition, soluble proteins were analyzed by gel filtration. The distribution profile by HPLC showing a decrease in β- and γ-crystallin and the appearance of a low molecular weight peak in group B compared to group A, an indication of protein modification/degradation in galactose cataract (Figure 6). Supplementation of curcumin at the 0.002% level to galactose fed rats normalized the alterations in crystallins (Figure 6). However, feeding of curcumin at the 0.01% level to diabetic rats resulted in some alterations which were very distinct from group B. A further decrease in γ-crystallin and an increase in the high molecular weight peak (that includes α-crystallin) over the changes that were observed in group B was observed with treatment of the higher level of curcumin. All of the morphological and biochemical parameters in group E were similar to that of control animals (group A, data not shown).

Figure 5. AGE fluorescence of soluble protein in different groups. Protein (0.15 mg/ml) in 0.05 M sodium phosphate buffer pH 7.4 was excited at 370 nm and emission was monitored 400-500 nm. Data represent three such observations.

Figure 6. Distribution profile of crystallins. Distribution profile of crystallins in soluble fraction of group A (A), group B (B), group C (C) and group D (D) rats. Soluble protein (20 µl, 1 mg/ml in equilibration buffer) was loaded on TSK-G 2000 SW gel filtration columns and protein peaks were detected at 280 nm with a flow rate of 1 ml/min. Peaks representing α-, β-, and γ-crystallins, low molecular weight (LMW) and high molecular weight (HMW) fractions are indicated at their respective positions.
DISCUSSION

Curcumin, the yellow phenolic pigment present in turmeric, has been shown to have a wide range of biological activities in various systems both in vitro and in vivo, including antidiabetic/hypoglycemic [15,16] and antioxidant [9,10] properties. Oxidative stress has been considered to be a major factor in the development of different types of cataract including diabetic cataract [5-7,27]. Thus, the antioxidant and anti diabetic/hypoglycemic potential of curcumin merits investigation in the prevention or delay of cataract.

It has been shown previously that curcumin attenuates lens opacification induced by 4-HNE in organ culture [17]. Lenses collected from rats treated for two weeks with curcumin (75 mg/kg/day) were more resistant to 4-HNE induced opacification in organ culture compared to lenses from control animals. Though induction of one of the isozymes of GST (rGST8-8) was implicated in the above study, the actual biochemical mechanism(s) by which curcumin prevented cataractogenesis needs to be investigated. Later it was demonstrated that feeding curcumin in the diet also attenuated galactose-induced cataract in rats, as assessed by lens morphology (slit lamp microscopy) and lens light scattering (ALIT) [18]. Apoptosis was shown to be decreased by curcumin when compared to a galactose alone treatment group [18], although the biochemical mechanism(s) has not been elucidated. The dose dependency of curcumin effects were not addressed and only one dose (0.0025%) was studied, which corresponded to 1.4 mg/kg/day intake [18]. This study [18] was a follow-up of a previous study [17]. In the previous study animals were treated with 75 mg/kg/day curcumin, which was over 50 fold higher.

We have studied the effect of curcumin on galactose induced cataract in rats at two different doses (0.002% and 0.01%) and investigated the possible biochemical mechanism(s) related to diabetic cataract in order to better understand the anticataractogenic potential of curcumin. The reasons for selecting these two doses are based on an earlier study which indicated that a curcumin dose of 0.0025% provided only 30% protection based on light transmission data [18], hence a curcumin dose of less than 0.0025% may not be of much help in protecting lens damage. Also, in a pilot study we have used a higher dose (0.02%), which is 10-fold the dosage used in the earlier study and observed enhanced cataract progression compared to galactose-induced cataract in rats (unpublished results). Therefore, we have chosen the 0.002% and 0.01% levels for this study.

In the present investigation, galactose induced cataract in rats was used to study the biochemical basis for the protective effect of curcumin, as galactose cataract is an accepted model for diabetic cataract. Three possible mechanisms that may be involved in cataract formation as a result of hyperglycemia or hypergalactosemia are the polyol pathway, oxidation, and non-enzymatic glycation [28]. The relative contributions to galactosemic cataract by the polyol pathway resulting in osmotic balance [29] versus oxidative stress [30,31] remains under debate. With an increase in the severity of cataract, there will be a leakage of hydrolyzed crystallins from the lens into the aqueous humor. The total and soluble protein content in lenses in this study (Table 1) indicated that proteins are decreased in groups B, C, and D. when compared to group A. A similar trend was observed in the earlier reports with total protein content [27]. However decrease in protein was significantly prevented by a dosage of 0.002% curcumin compared to group B but not by a dosage of 0.01%.

The crystallin profile of soluble protein indicates that there was a decrease in β- and γ-crystallin peaks and an increase in low molecular weight proteins in both the galactosemic (group B) as well as in the 0.01% curcumin (group D), indicating protein degradation/modification due to oxidative stress in these groups. Furthermore, a significant amount of higher molecular weight aggregated protein along with ε-crystallin was present in group D, suggesting that degradative proteins in turn may be involved in the formation of high molecular weight aggregates and this may be one of the reasons for faster maturation of cataract in group D. Such protein modifications were not observed in group C, indicating the protective effect of curcumin at lower levels.

Sugar cataract formation associated with diabetes and galactosemia has been linked to the aldose reductase (EC.1.1.1.21) catalysed production of sugar alcohols (polyols), sorbitol, and galactitol from glucose and galactose respectively [32]. SDH catalyzes the conversion of sorbitol to fructose in the presence of NADH [32]. Evidence has shown that there was a slight increase in SDH activity in galactosemic rats. In the present investigation AR activity was significantly increased in group B, however feeding of curcumin at both levels inhibited AR activity marginally (not significant). Also there was no influence of curcumin at both levels on SDH activity. In addition, the direct effect of curcumin on rat lens AR inhibition could be seen only at a very high concentration (IC₅₀ 150 μM), which was much higher than the amount used in this study (unpublished data). Therefore we did not measure polyol (galactitol) content in these animals. Nonetheless, our in vitro AR and SDH activity measurements with lens extracts may not completely rule out the possibility of curcumin influencing polyol levels.

There is substantial evidence that oxidative stress is at least partly responsible for galactosemic cataract, and that antioxidants can be effective inhibitors of cataractogenesis in this model [30,31]. In addition, during aging and progression of diabetes it has been observed that lens crystallins undergo extensive modifications such as oxidation and glycation [33,34]. Glycation may progress to browning, resulting in AGE formation which are highly fluorescent in the visible region [26,34,35]. A decrease in GSH along with increased MDA and protein carbonyls in the present study suggest an increased oxidative stress due to galactose feeding, as reported in the previous study [27]. These alterations are either normalized or minimized by 0.002% curcumin in the diet. In contrast, at 0.01% curcumin levels the oxidative related parameters are either unaltered or oxidative stress is increased (a significant decrease in GSH levels). Increase in AGE fluorescence in group B suggests that glycation may also play an important role in cataractogenesis by enhancing protein unfolding.
crosslinking, and finally aggregation. Decreased tryptophan fluorescence further supports the above possibility that there may be conformational changes. These changes are, however, prevented by 0.002% curcumin but not by 0.01% curcumin. In fact, AGE fluorescence was higher in group D compared to group B. Thus increased protein aggregation, as evidenced by the HPLC profile, may be the possible mechanism for faster maturation of cataract in group D.

Although the influence of curcumin on osmotic imbalance due to galactose feeding could not be ruled out, based on the data it appears that curcumin at a 0.002% level may prevent the oxidative stress and glycation related structural changes in galactose fed rats by exerting antioxidant and antiglycating effects, and thereby delay galactose-induced cataractogenesis. The studies of Hegde, et al., on a mouse model with low levels of AR and polyols showed the role of oxidative stress and glycation in diabetic cataract [36]. This data not only supports our findings but demonstrates the beneficial effects of curcumin. Nevertheless, it is not clear whether one of the actions (antioxidant properties) of curcumin is responsible for influencing other factors such as glycation and polyol levels or vice versa. The relative contribution of each of them in delaying cataractogenesis needs to be further dissected.

The reasons for faster onset and maturation of cataract in group D rats are not known at present. Nonetheless biochemical data suggest that higher levels (>0.01%) in the diet appears to increase oxidative stress, increased AGE formation, and protein aggregation under hyperglycemic conditions. However, feeding of curcumin to normal rats up to a 0.02% level has not resulted in any changes in lens morphology as well as biochemical parameters (unpublished data) and suggests that higher levels (0.01%) of curcumin may not be beneficial under diabetic conditions (at least galactosemia in the present study). Therefore, studies are underway to investigate the differential effects of curcumin in other animal models of diabetic cataract such as streptozotocin or alloxan induced diabetic cataract.

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REFERENCES