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Effect of Curcumin on the Advanced Glycation and Cross-linking of Collagen in Diabetic Rats

*G. B. Sajithlal, Pandarinathan Chithra and Gowri Chandrakasan**

Department of Biochemistry, Central Leather Research Institute, Adyar, Chennai–600 020, India

ABSTRACT. A close association between increased oxidative stress and hyperglycemia has been postulated to contribute significantly to the accelerated accumulation of advanced glycation end products (AGEs) and the cross-linking of collagen in diabetes mellitus. In the present work, we report the influence of curcumin, an efficient antioxidant, on the level of AGEs and the cross-linking of collagen in diabetic rats. Diabetic rats were given curcumin (200 mg/kg body wt) orally for a duration of 8 weeks. The antioxidant status in serum and the level of AGEs, cross-linking and browning of collagen in tail tendons and skin were investigated. The oxidative stress observed in diabetic rats was reduced significantly by curcumin administration. Nonenzymic antioxidants such as vitamin C, vitamin E, and glutathione were maintained at near normal values in curcumin-treated diabetic animals. Similarly, the accumulation of lipid peroxidation products in diabetic serum was reduced significantly by curcumin. Accelerated accumulation of AGE-collagen in diabetic animals, as detected by ELISA, was prevented by curcumin. Extensive cross-linking of collagen in the tail tendon and skin of diabetic animals was also prevented to a greater extent by curcumin treatment. A correlation between the level of AGEs and collagen cross-linking was noted, suggesting the involvement of advanced glycation in cross-linking. It was also noted that the preventive effect of curcumin on the advanced glycation and cross-linking of collagen was more pronounced than its therapeutic effect. However, the Maillard reaction fluorescence in both tail and skin collagen remained unaltered by curcumin. This study confirms the significance of free radicals in the accumulation of AGEs and cross-linking of collagen in diabetes. It supports curcumin administration for the prevention of AGE-induced complications of diabetes mellitus. BIOCHEM PHARMACOL **56**;12:1607–1614, 1998.

KEY WORDS. glycation; oxidation; collagen cross-linking; diabetes; curcumin; Maillard reaction

AGEs† have been implicated in the pathogenesis of various complications of diabetes mellitus [1, 2]. Collagen, a protein with a long half-life, is highly susceptible to advanced glycation *in vivo*. Since collagen is an important constituent of most of the tissues that are affected during diabetes, modifications of this protein may play a critical role in the complications of diabetes. Evidence for an important role for nonenzymatic glycation in the cross-linking of collagen in diabetes has accumulated [3–5]. *In vitro* incubation of collagen with glucose at concentrations that reflect those observed in hyperglycemia results in cross-linking and browning. However, the covalent binding of glucose to the amino group of a protein alone is not sufficient to account for structural changes observed in diabetic collagen [6, 7]. Oxygen radicals formed during glucose oxidation, and glycated protein oxidation may be involved directly in the formation of AGEs and collagen cross-linking. *In vitro* studies demonstrate that the presence of oxygen is indispensable for the advanced glycation and cross-linking of collagen [8, 9]. Antioxidative conditions and free radical scavengers have been proven to inhibit the formation of AGEs and the cross-linking of collagen.

Curcumin, an active principle isolated from turmeric (*Curcuma longa*), has been reported as an efficient antioxidant [10]. It was shown to be an anti-inflammatory agent and possesses membrane-stabilizing properties [11]. *In vitro* experiments have shown that curcumin effectively inhibits the accumulation of reactive oxygen species [12]. Curcumin was also shown to inhibit the formation of proinflammatory compounds such as prostaglandins and leukotrienes [13].

Since reports from many laboratories have suggested the significance of oxidation reactions and free radicals in the formation of AGEs, in the present work, we have studied the effect of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats. Experiments were designed to study both the preventive and therapeutic effects of curcumin.

MATERIALS AND METHODS

Materials

Curcumin (>98% pure), streptozotocin, hydroxyproline, thiobarbituric acid, collagenase (type VII), BSA, RNase,

* Corresponding author: Tel. 91-44-4911386; FAX 91-44-4911589.

†*Abbreviations:* AGEs, advanced glycation end products; PMSF, phenymethylsulfonyl fluoride; and RNase, ribonuclease.

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Freund's complete and incomplete adjuvants, peroxidase-conjugated anti-rabbit IgG, *o*-phenylenediamine, PMSF, iodoacetamide, benzamidine hydrochloride, and pepsin were obtained from the Sigma Chemical Co. HEPES and EDTA were purchased from S.D. Fine Chemicals. PBS was obtained from Hi Media. All other reagents and solvents used in this study were of the highest analytical grade, unless otherwise indicated.

Animals and Experimental Design

Male Wistar rats weighing about 150–200 g were made diabetic by a single intraperitoneal injection of streptozotocin (60 mg/kg body wt) dissolved in 0.1 M citrate buffer. After 3 days, rats with blood glucose levels above 18.0 mM were divided randomly into two groups. One group received curcumin (suspended in saline) at a dose of 200 mg/kg body weight orally, once a day, for a period of 8 weeks. The other group received saline for the same period. Similarly age-matched male control rats were also divided into two groups. One group received curcumin, and the other group remained without curcumin. To study the preventive effect, curcumin administration was started from day 3 of the induction of diabetes. To investigate the therapeutic effect, curcumin treatment was begun 4 weeks after the onset of diabetes. The total experimental period was 8 weeks in the preventive study group and 12 weeks in the therapeutic group. The curcumin treatment period was maintained constant in both groups (8 weeks). All rats were allowed free access to water and rat chow. Body weight and blood glucose were monitored regularly once every 3 weeks. Blood glucose was measured by the glucose oxidase method. Glycohemoglobin was measured by boronate affinity chromatography [14]. At the end of the experimental period, the animals were killed under ether anesthesia following a 12-hr fast. The tails and skin of the experimental animals were removed and stored frozen at −70° until used. Blood collected was immediately processed and analyzed.

Analysis of Antioxidant Status

Lipid peroxidation was assessed in the serum using thiobarbituric acid reagent as described by Yagi [15]. Nonenzymic antioxidants were estimated in serum. Reduced glutathione was measured by the method described by Ellman [16]. Vitamin C was estimated by the method of Omaye *et al.* [17]. Vitamin E was analyzed by the method of Emmerie and Engel [18].

Antigen Preparation and Immunization

AGE-RNase prepared by incubating RNase (20 mg/mL) with 0.5 M glucose in 0.2 M phosphate buffer (pH 7.4) for 3 months was used as the antigen for immunization. Two female white rabbits were immunized by subcutaneous administration of 100 μg AGE-RNase in Freund's complete adjuvant. This was followed by four booster doses in incomplete adjuvant. Antibody response was checked by Ouchterlony double diffusion and non-competitive ELISA.

Competitive ELISA for AGEs

Test samples for ELISA were prepared as follows. Tail tendons and skin samples were washed thoroughly to remove all the unbound materials. Then the samples were digested by collagenase (1:50) for 48 hr at 37°, and centrifuged; the supernatant was used for the assay. Hydroxyproline estimation of the supernatant was carried out as described by Woessner [19].

Measurements of AGEs were performed by competitive ELISA as described by Makita *et al.* [20] with a few modifications. AGE-BSA prepared by incubating BSA (50 mg/mL) with 0.5 M glucose in 0.2 M phosphate buffer, pH 7.4, for 6 months was used as the standard.

Microtiter plates (96 wells) were coated with AGE-BSA by adding a 0.1-mL solution of AGE-BSA (10 μg/mL) to each well and incubated overnight at 4°. Wells were washed three times with PBS containing 0.1% Tween 20 (PBS-Tween). Then the wells were blocked by incubation for 1 hr with 0.1 mL of 0.5% gelatin. After washing three times with PBS-Tween, 100 μL of antisera (1:2000), preincubated overnight at 4° with either the standard or the test antigen, was added and incubated for 2 hr at 37°. The wells were then washed three times with PBS-Tween and developed with peroxidase-conjugated anti-rabbit IgG, utilizing *o*-phenylenediamine as the substrate. Absorbance was measured at 492 nm in a computerized ELISA reader (Bio-Rad). The results were expressed as AGE units. The absorbance corresponding to 1 μg of AGE-BSA standard was arbitrarily fixed as one AGE unit.

Analysis of Tendon Breaking Time

The thermal stability of collagen was assessed according to the method of Yue *et al.* [21]. Collagen fibers were cut in 5-cm lengths weighing 2 to 2.5 mg. Each fiber was suspended by a clip with a weight of 5 g attached, and was immersed in a glass tube containing 7.0 M urea kept in a waterbath maintained at 40°. The mean breaking time was taken as tendon breaking time.

Analysis of Tendon Collagen Solubility in Acid

For measuring the amount of acid-insoluble collagen, the tail tendons were removed, dissected free of adhering tissues, and washed thoroughly in PBS containing protease inhibitors: 1.0 mM each of EDTA, PMSF, iodoacetamde, and benzamidine hydrochloride. All operations were carried out at 4°. The washed tendons at a concentration of 2 mg wet weight/mL of 0.05 M acetic acid were stirred at 4° for 24 hr. The mixture was homogenized in a polytron homogenizer and stirred for an additional 24 hr. The suspension was then centrifuged at 9000 *g* for 60 min at 4° (Hitachi refrigerated centrifuge). The collagen in the clear

TABLE 1. Body weight and glycemic status of experimental animals

	Body wt (g)	Glycohemoglobin (%)	Blood glucose (mM/L)
Preventive study group			
Control	238 ± 24	4.1 ± 0.36	8.1 ± 0.79
Control curcumin treated	230 ± 22	4.2 ± 0.38	8.3 ± 0.8
Diabetic	114 ± 12*	12.7 ± 1.3*	24.3 ± 2.1*
Diabetic curcumin treated	124 ± 11.5	12.1 ± 1.23	25.2 ± 2.4
Therapeutic study group			
Control	242 ± 26.1	4.2 ± 0.34	7.8 ± 0.7
Control curcumin treated	238 ± 24.6	4.1 ± 0.37	8.0 ± 0.7
Diabetic	108 ± 11.3*	13.1 ± 1.28*	26.1 ± 2.4*
Diabetic curcumin treated	112 ± 12	13.0 ± 1.3	24.8 ± 2.6

Values are the means ± SD of 12 experimental animals.
*$P < 0.01$ vs control group animals.

supernatant was defined as acid-soluble collagen. It was precipitated by bringing the supernatant to 1.0 M sodium chloride and recovered by centrifugation, redissolved in 0.05 M acetic acid, lyophilized, and weighed.

Analysis of Collagen Cross-linking in the Skin

Analysis of cross-linking of collagen in the skin was carried out by the fractionation of collagen as described by Miller and Rhodes [22]. After dehairing and washing the skin samples thoroughly in saline, lipids were removed using chloroform and methanol (2:1). Sequential extractions with individual solvents were carried out with 0.05 M Tris · HCl (pH 7.5) containing 1.0 M sodium chloride for the extraction of monomeric collagen. Protease inhibitors such as PMSF (1.0 mM), ethylmaleimide (1.0 mM), and EDTA (2.0 mM) were included. Acid-soluble collagen was extracted in 0.5 M acetic acid (pH 3.0) under similar conditions. The residue left after acid extraction was then digested using acetic acid containing pepsin (1:10, w/w). Collagen present in the remaining residual tissue was taken as insoluble collagen. All operations were carried out at 4°. Collagen content in each fraction was estimated by measuring the amount of hydroxyproline [19].

Analysis of Fluorescence

Fluorescence was measured by the method described by Monnier *et al.* [23]. Approximately 3.0 mg tissue was finely minced in PBS and centrifuged at 1500 *g* for 10 min. The pellet was washed with distilled water, and the lipids were extracted with 5.0 mL of chloroform methanol (2:1, v/v) overnight. The samples were rehydrated by the addition of 2.0 mL of methanol and 0.5 mL of distilled water, and then centrifuged; the pellet was washed twice with methanol, three times with distilled water, twice with 0.02 M HEPES (pH 7.5) containing 0.1 M $CaCl_2$ (buffer H), and stored overnight at 4° in buffer H. Then the buffer was removed, and the pellet was resuspended in 3.5 mL of buffer H containing 1200 units of collagenase. Four drops of toluene was included to prevent bacterial growth. The digestion was carried out for 48 hr at 37°. A blank containing collagenase in buffer H was included. The digest was centrifuged at 1500 *g* for 30 min, and the clear supernatant containing digested collagen was used for assays of fluorescence and hydroxyproline content. Fluorescence was measured against distilled water at 440 nm upon excitation at 370 nm and was corrected for collagenase blank.

Statistical Analysis

Data are presented as the means ± SD of 12 experimental animals in each group. Statistical analysis was done using Student's *t*-test, with a *P* value of less than 0.01 considered significant. Correlation between the level of AGEs and collagen cross-linking was analyzed by calculating the correlation coefficient.

RESULTS

The body weight, blood glucose, and glycohemoglobin levels of experimental animals are shown in Table 1. The glycemic status and body weight of the diabetic animals were unaltered by curcumin treatment. Table 2 depicts the antioxidant status of the experimental animals. Diabetic animals showed increased lipid peroxidation and reduced nonenzymic antioxidants such as vitamin C and glutathione. The lipid-soluble antioxidant vitamin E was increased in the diabetic serum. The administration of curcumin reduced the increased oxidant stress observed in diabetic animals. Curcumin also showed both preventive and therapeutic effects on diabetes-induced increased lipid peroxidation. The levels of nonenzymic antioxidants such as vitamin C, glutathione, and vitamin E were maintained at near normal values in curcumin-treated diabetic animals.

AGEs were identified and estimated by competitive ELISA using polyclonal antibodies raised against AGE-RNase. To minimize background readings and to ensure maximum cross-reactivity between AGE-antibody and antigen, in this study we utilized AGEs from three different proteins. Antibodies raised against AGE-RNase were used to detect AGE-collagen, using AGE-BSA as the standard. This procedure ensures that the absorbance obtained is due almost completely to AGE-antibody antigen reaction, al-

TABLE 2. Lipid peroxidation and antioxidant levels in the serum of experimental animals

	Lipid peroxidation (μmol/L)	Vitamin E (μg/mL)	Vitamin C (μg/mL)	Glutathione (μg/mL)
Preventive study group				
Control	6.8 ± 0.7	1.6 ± 0.1	18.0 ± 1.6	274 ± 27
Control curcumin treated	6.1 ± 0.5	1.7 ± 0.1	18.3 ± 1.8	268 ± 28
Diabetic	16.3 ± 1.8*	2.8 ± 0.3*	11.3 ± 1.2*	163 ± 15.9*
Diabetic curcumin treated	8.2 ± 0.8†	1.8 ± 0.1†	16.3 ± 1.5†	253 ± 26†
Therapeutic study group				
Control	7.1 ± 0.8	1.7 ± 0.1	19.6 ± 1.9	271 ± 26.5
Control curcumin treated	7.3 ± 0.7	1.6 ± 0.1	19.6 ± 1.8	262 ± 26.5
Diabetic	18.3 ± 1.9*	3.0 ± 0.28*	9.2 ± 0.9*	132 ± 14*
Diabetic curcumin treated	8.6 ± 0.8†	1.9 ± 0.1†	17.3 ± 1.8†	246 ± 25.2†

Values are the means ± SD of 12 experimental animals.
*$P < 0.01$ vs control group animals.
†$P < 0.01$ vs diabetic group animals.

though it is widely accepted that the AGE-antibody does not cross-react with the native protein. The levels of AGE-collagen measured in the tail tendon and skin of experimental animals are given in Table 3. It may be noted that curcumin prevented diabetes-induced accumulation of AGEs in tail tendons and skin collagen samples. However, the therapeutic effect of curcumin on the levels of AGEs was not as effective as observed in the preventive study group. In the latter group, curcumin showed more than 40% inhibition, whereas in the therapeutic group only 15% inhibition was noted.

The extent of cross-linking of tail tendon collagen was assessed by tendon breaking time (Fig. 1) and acid insolubility measurements (Fig. 2). Corresponding to the decrease in the levels of AGEs, curcumin treatment produced a reduction in the cross-linking of collagen in diabetic animals. It was observed that 8 weeks of diabetes resulted in a significant increase in the tendon breaking time. Curcumin treatment significantly prevented this increase in tendon breaking time. Similarly, the increased accumulation of acid-insoluble collagen observed in tail tendon collagen was also prevented by curcumin treatment. As observed for AGE-collagen, the therapeutic effect of curcumin on collagen cross-linking was less pronounced compared with its preventive effect. In the preventive study, more than 50% inhibition of the increased accumulation of acid-insoluble collagen was observed. However, in the therapeutic study, only 20% inhibition was noted. A positive correlation ($r = 0.89$) between tendon breaking time and AGE-collagen in the tail tendons of curcumin-treated and untreated diabetic animals was noted. Similarly, the level of AGEs correlated well ($r = 0.96$) with the percentage of insoluble collagen in the skin of experimental animals.

Figures 3 and 4 depict the effect of curcumin on the cross-linking of skin collagen. The percentages of neutral salt-soluble and acid-soluble collagen were decreased, whereas pepsin-soluble collagen and insoluble collagen were increased in the skin of diabetic animals. The amount of monomeric collagen was reduced to 2% of the total collagen compared with 5% of that observed in normal animals. This may be due to increased cross-linking or decreased biosynthesis of collagen in diabetic skin or both. This shows that the overall cross-linking of collagen is

TABLE 3. Effect of curcumin on the increased accumulation of AGE-collagen in experimental animals

	AGEs (units/μg hydroxyproline)	
	Tail tendon collagen	Skin collagen
Preventive study group		
Control	3.2 ± 0.3	3.7 ± 0.3
Control curcumin treated	3.1 ± 0.28	3.3 ± 0.3
Diabetic	7.8 ± 0.8*	6.8 ± 0.7*
Diabetic curcumin treated	5.2 ± 0.5†	4.5 ± 0.4†
Therapeutic study group		
Control	3.4 ± 0.3	3.5 ± 0.4
Control curcumin treated	3.3 ± 0.33	3.4 ± 0.36
Diabetic	8.1 ± 0.8*	7.4 ± 0.67*
Diabetic curcumin treated	6.7 ± 0.6†	6.0 ± 0.5†

Values are the means ± SD of 12 experimental animals.
*$P < 0.01$ vs control group animals.
†$P < 0.01$ vs diabetic group animals.

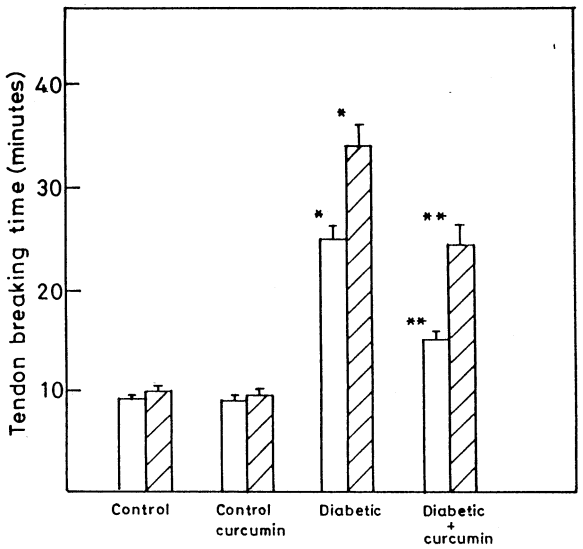

FIG. 1. Tendon breaking time measured in the tail tendons of experimental animals. Key: (□) curcumin treatment started from day 3 of diabetes induction, and (▨) curcumin treatment started after 4 weeks duration of diabetes. Values are the means ± SD of 12 experimental animals. Statistical significance: *P < 0.01 vs control group animals, and (**)P < 0.01 vs diabetic group animals.

increased in diabetic skin. Curcumin treatment prevented the increase in cross-linking of skin collagen in diabetic rats. As observed for tail tendon collagen, the effect of curcumin was more pronounced in the preventive study group than in the therapeutic study group.

Collagen-linked fluorescence levels measured in the tail tendons and skin of experimental animals are given in

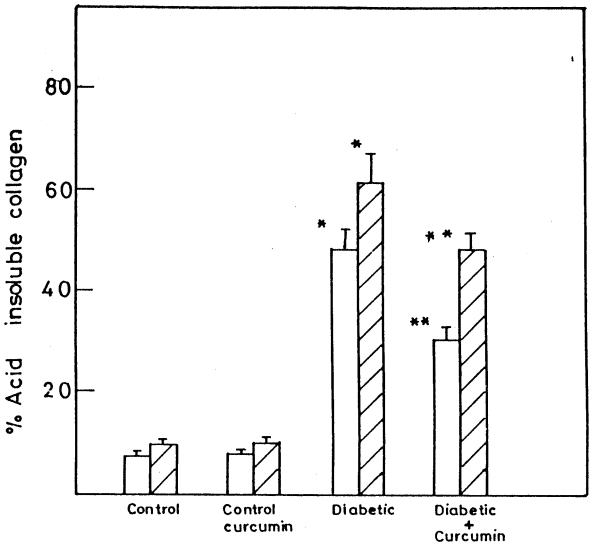

FIG. 2. Percentage of acid-insoluble collagen in the tail tendons of experimental animals. Key: (□) curcumin treatment started from day 3 of diabetes induction, and (▨) curcumin treatment started after 4 weeks duration of diabetes. Values are the means ± SD of 12 experimental animals. Statistical significance: *P < 0.01 vs control group animals, and (**)P < 0.01 vs diabetic group animals.

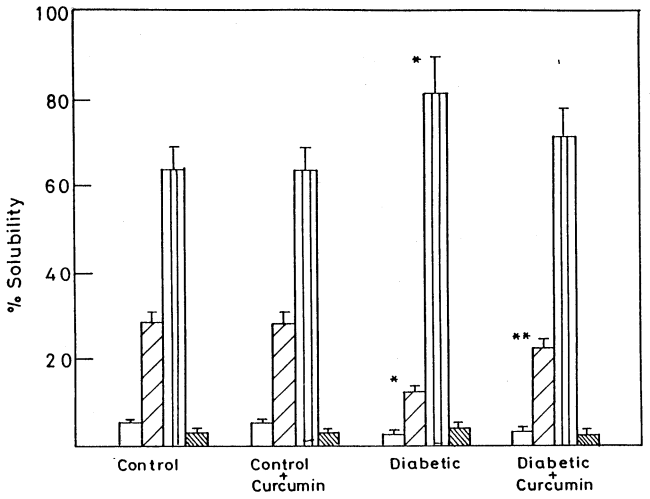

FIG. 3. Percentage of soluble collagen observed in the skin of experimental animals (curcumin treatment started from day 3 of diabetes induction). Key: (□) neutral salt soluble, (▨) acid soluble, (▥) pepsin soluble, and (▧) insoluble collagen. Values are the means ± SD of 12 experimental animals. Statistical significance: *P < 0.01 vs control group animals, and (**)P < 0.01 vs diabetic group animals.

Table 4. Collagen obtained from diabetic animals showed increased fluorescence. Curcumin treatment did not show any effect on the increased fluorescence of diabetic collagen.

DISCUSSION

The purpose of the present investigation was to study the effect of the antioxidant curcumin on the increased AGEs and accelerated cross-linking of collagen in diabetes. Since

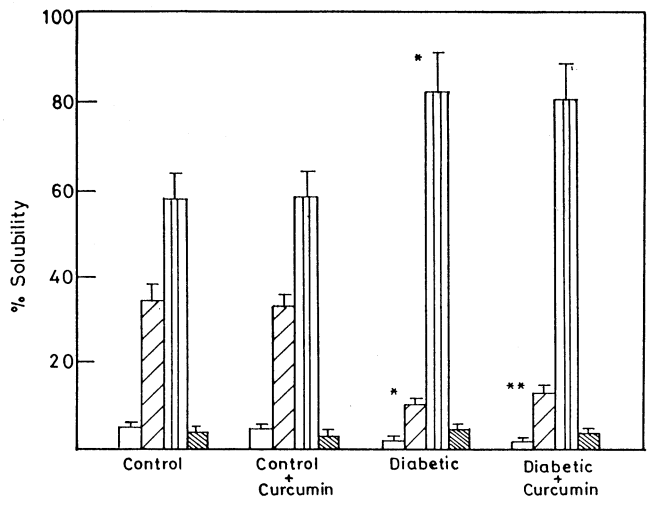

FIG. 4. Percentage of soluble collagen observed in the skin of experimental animals (curcumin treatment started after 4 weeks duration of diabetes). Key: (□) neutral salt soluble, (▨) acid soluble, (▥) pepsin soluble, and (▧) insoluble collagen. Values are the means ± SD of 12 experimental animals. Statistical significance: *P < 0.01 vs control group animals, and (**)P < 0.01 vs diabetic group animals.

TABLE 4. Level of fluorescence measured in the tail tendon and skin collagen of experimental animals

	Fluorescence (AU/μmol hydroxyproline)	
	Tail tendon collagen	**Skin collagen**
Preventive study group		
Control	28.4 ± 3.0	31.0 ± 4.1
Control curcumin treated	26.0 ± 2.0	34.6 ± 3.8
Diabetic	58.6 ± 6.1*	88.0 ± 9.1*
Diabetic curcumin treated	57.0 ± 5.2	91.0 ± 9.8
Therapeutic study group		
Control	26.3 ± 2.8	36.0 ± 4.1
Control curcumin treated	27.0 ± 3.8	33.2 ± 4.3
Diabetic	61.0 ± 6.8*	94.0 ± 10.3*
Diabetic curcumin treated	63.0 ± 7.1	91.0 ± 9.8

Values are the means ± SD of 12 experimental animals. AU = arbitrary units.
*$P < 0.01$ vs control group animals.

a clear idea of the entire constituents of AGEs is still in its infancy and the pathogenic role of the structurally characterized AGEs is not well established, polyclonal anti-AGE antibodies were used to check the presence of AGEs instead of any other monoclonal antibody. Moreover, experimental evidence indicates that the competitive ELISA gives good results [24].

Although the level of nonenzymic antioxidants, such as vitamin C and glutathione, was decreased in diabetic animals, the level of vitamin E, a lipid-soluble antioxidant, was increased. Studies from other laboratories also have reported elevated levels of vitamin E in diabetes [25–27]. As suggested by Aoki *et al.* [27], this may be due to the increased intake of vitamin E per unit weight or the increase in serum lipid levels in diabetic rats or both. The increase may also be due to an alteration in metabolism or the storage of vitamin E by diabetic rats compared with the controls [28].

The insignificant effect of curcumin on hyperglycemia and body weight of diabetic rats observed in this study agrees well with a recent report on the effect of curcumin on the progression of experimentally induced diabetes in rats [29]. As curcumin treatment reduced the increased oxidant stress in diabetic animals, collagen fibrils in these animals were exposed to the same amount of hyperglycemia and for the same duration as in untreated control diabetic animals, but to a reduced oxygen radical environment. This underscores the significance of oxygen radicals in the advanced glycation and cross-linking of collagen *in vivo*.

The covalent reaction between glucose and collagen may not be the only factor involved in the formation of AGEs and cross-linking observed in diabetes. It has been proposed that both free glucose [30] and protein-glucose adduct [31] undergo oxidation in the presence of trace amounts of metal ions, generating free radicals and reactive carbonyls. These reactive oxygen species and carbonyls may contribute significantly to the increased cross-linking of collagen. Elgawish *et al.* [32] demonstrated that hydrogen peroxide is directly involved in glucose-induced cross-linking of collagen. Metal oxidation of protein–glucose adduct also results in the formation of fragmentation products like 3-deoxyglucosone, which further increase the cross-linking process [33]. In agreement with earlier *in vitro* studies [8, 9], we observed that the administration of an efficient antioxidant, curcumin, resulted in the prevention of increased accumulation of AGEs and cross-linking of collagen in diabetic rats. Fu *et al.* [8, 9] have shown that the incubation of collagen with glucose under a reduced supply of oxygen or under antioxidative conditions results in the inhibition of carboxymethyllysine and pentosidine formation. Similarly, the increased cross-linking of collagen is also prevented under antioxidative conditions, suggesting the critical role of oxidation reactions. Similar results have been reported *in vivo* [27].

In addition to glucoxidation and glycoxidation, lipid peroxidation is also known to induce cross-linking of collagen. Malondialdehyde, an end product of lipid peroxidation, can react with the free amino groups of collagen and stimulate cross-linking. Fu *et al.* [34] observed that *in vitro* peroxidation of lipids in the presence of proteins results in the formation of the glycoxidation product carboxymethyllysine. The rate of lipid peroxidation is very high in diabetic animals and leads to the accumulation of peroxidation products. The observed decrease in the increased stability of collagen in diabetic rats by curcumin may be due to the inhibition of lipid peroxidation [35]. The thermal rupture time is much longer in the glycated tail tendon collagen treated with products of lipid peroxidation than in normal tail tendon collagen.

In addition to cross-linking, collagen-related fluorescence is altered by glycation. Along with increased cross-linking, diabetic collagen also exhibits increased Maillard reaction fluorescence. Although this is not understood, a correlation between the severity of diabetic complications and collagen-linked fluorescence has been reported [23, 36]. Similarly, pentosidine, a recently discovered AGE, also shows high correlation with the severity of complications in individuals with long-standing insulin-dependent diabetes [37]. However, the contribution of fluorescent products in the cross-linking of collagen is uncertain. Collagen-linked

fluorescence is not consistent with the extent of cross-linking [38, 39]. Aoki *et al.* [27] have shown that antioxidant vitamin E has no effect on the fluorescence of collagen in diabetes, although the cross-linking of collagen is partially prevented, as was observed in the present study. Although a correlation between collagen-linked fluorescence and cross-linking was not observed, a correlation between AGE level and cross-linking was noted, suggesting an interrelationship between AGE level and collagen cross-linking, rather than between fluorescence and cross-linking.

It was shown that, in addition to glucose, free radicals and lipid peroxides also play an important role in the development of collagen-linked fluorescence. Fujimori [40] has demonstrated the increased accumulation of fluorescent materials when collagen is incubated in the presence of chemically generated singlet oxygen. It appears that the reactive radicals formed during glycation and oxidation reactions can also have an influence on the development of fluorescence. Odetti *et al.* [41] have studied both glycation-induced fluorescence and malondialdehyde-induced fluorescence separately and find that malondialdehyde-induced fluorescence accumulates at a slower rate than glycation-induced fluorescence. Although most of these studies suggest a direct involvement of oxidation reactions in the development of collagen-linked fluorescence, the present study and an earlier report [27] clearly demonstrate the insignificant effect of antioxidants on the development of fluorescence during diabetes. However, the present study clearly indicates that oxidative reactions and lipid peroxidation play an important role in the formation of AGEs in diabetes. Future studies may reveal the significance of fluorescent products in diabetes mellitus. The inhibition of cross-linking of collagen by curcumin without a significant effect on fluorescence suggests the possibility that all the fluorescent products formed during diabetes may not be cross-linkers or that all the cross-linkers may not be fluorescent in nature.

The observation that the inhibitory effect of curcumin on the accumulation of AGEs was less pronounced in the therapeutic study group than in the preventive study group suggests that AGE-induced modifications of collagen are irreversible to a greater extent. It may be suggested that the modulation of oxidative stress in diabetic animals after prolonged hyperglycemia may not reverse tissue damage effectively.

In conclusion, we have studied the preventive and therapeutic effects of curcumin, an efficient antioxidant, on the advanced glycation and cross-linking of collagen in diabetic rats. Curcumin treatment significantly reduced the increased oxidative stress observed in diabetes. Increased accumulation of AGEs and cross-linking of collagen in tail tendon and skin of diabetic animals were prevented by curcumin, without any effect on Maillard reaction fluorescence. The use of curcumin is recommended for the prevention of AGE accumulation, and the associated complications of diabetes.

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