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International Journal of Current Research in Life Sciences
Vol. 10, No. 01, pp.3403-3408, January, 2021

ISSN: 2319-9490

RESEARCH ARTICLE

EFFECT OF TETRAHYDROCURCUMIN ANALYSIS OF FLUORESCENCE OF COLLAGEN IN EXPERIMENTAL DIABETES

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Received 17th November, 2020; Accepted 10th December, 2020; Published 30th January, 2021

ABSTRACT

Collagen is a protein containing several dibasic amino acids and has a slow turnover rate and is a strong candidate for extensive modification by glycation. Crosslinking is important in stabilizing the collagen fibrils. Cross linking contributes to the tensile strength of tissues such as tendon by decreasing the permeability and elasticity of extracellular matrix. Extensive cross linking of collagen however causes changes in its structure and the mechanical properties can be pathological. Such changes have been reported in aging and in various pathological conditions including diabetes.*Curcuma longa* is commonly used in the treatment of diabetes by ayurvedic physicians. Curcumin is a biologically active component isolated from the rhizome of *Curcuma longa* that possess antihyperglycemic activity, hypolipidemic action and anti - renal lesion effect. The use of curcumin is recommended for prevention of advanced glycatedendproducts (AGEs) accumulation and the associated complications of diabetes. Tetrahydrocurcumin (THC) is one of the major colorless metabolite of curcumin. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin.In diabetic rats, hydroxyproline and collagen content as well as its degree of cross-linking were increased, as shown by increased extent of glycation, collagen-linked fluorescence, neutral salt collagen, decreased acid and pepsin solubility.Administration of THC for 45 days to diabetic rats significantly reduced the accumulation and cross-linking of collagen.

Key words: Tetrahydrocurcumin, Curcumin, *Curcuma Longa*, Diabetes, Collagen.

Citation: Pidaran Murugan. 2021. "Effect of tetrahydrocurcumin analysis of fluorescence of collagenin experimental diabetes" *International Journal of Current Research in Life Sciences*, 10, (01), 3403-3408

INTRODUCTION

Collagen is present in all types of multicellular animals and is probably the most abundant animal protein in nature. It is estimated that collagen accounts for about 30% of the total human body protein. Collagen is located in the extracellular matrix of connective tissues (Royce and Steinmann, 1993). Collagen interacts with cells through the integrin cell receptors and mediates cellular adhesion and migration. Important roles for collagen have been identified in development, wound healing, platelet aggregation and aging. Its commercial importance in leather and the production of gelatin and glue have long been recognized. More recently, it is being used as a basis for biomaterials. Examples of its biomedical applications include injectable collagen to lessen facial wrinkles and defects; surgical collagen sponges to increase blood clotting and artificial skin for the treatment of burns.

Structure and properties: The classification of an extracellular matrix protein as a collagen is based on the presence of a domain with the distinctive triple-helical conformation.

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The collagen triple helix consists of three polypeptide chains, each of which adopts an extended polyproline II-like helix. The three chains are supercoiled about a common axis and linked by hydrogen bonds (Nimni, 1988). Recently, the detailed features of this conformation determined by X-ray crystallography, confirming the general structure and showing an extensive network of ordered water hydrogen bonded to the triple helix (Prockop and Kivirikko, 1995). The triple-helical conformation requires unique amino acid sequence features. Glycine is the smallest amino acid, must be present as every third residue. A high content of the sterically restricted imino acids, proline and hydroxyproline, is also necessary to stabilize the extended helix. Hydroxyproline provides additional stability through water-mediated hydrogen bonds. Collagen is the only animal protein other than elastin, with significant amounts of hydroxyproline and hydroxylysine residues. It is easy to identify a collagen triple helix from its amino acid sequence pattern of repeating glycine-X-Y sequences and glycine-proline-hydroxyproline is the most frequent tripeptide sequence (Brodsky and Ramshaw, 1997).

Collagen types: At least 19 distinct molecules have been classified as collagens and specific types are associated with particular tissues. These collagens form the characteristic fibrils seen by electron microscopy with a 67-nm repeating

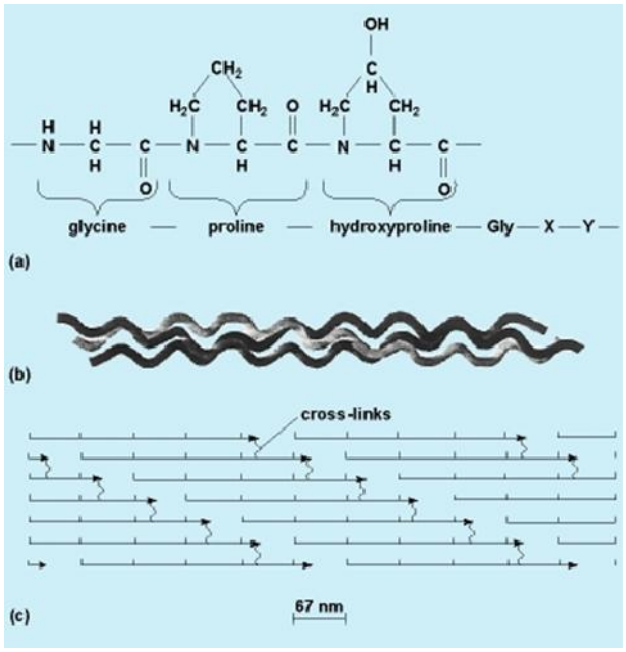

Fig. 1. Levels of collagen structure (*a*) Typical amino acid repeating sequence. (*b*) Triple-helical molecular structure. (*c*) Staggered arrangement of molecules which generates the 67-nm periodic fibrils

banding pattern. Type I is the most common fibril-forming collagen. Its fibrils make up the mineralized matrix in bone, the strong parallel bundles of fibers in tendon and the plywood like alternating layers in the transparent cornea. Type II is the major fibril-forming collagen in cartilage, while type III is found in blood vessels and skin, together with type I. The family known as fibril-associated collagens with interrupted triple helices or FACIT collagens, decorates the outside of the 67-nm periodic fibrils. The FACIT collagens appear to link fibrils and bind to other matrix components. Basement membranes serve to separate cell layers and act as filtration barriers, contain a distinctive group of collagens, denoted as type IV collagens. Type IV collagens have many breaks in the repeating glycine-X-Y pattern of their triple helix. These collagens are organized into a network or meshlike sheet structure. In the kidney glomerulus, the network based on type IV collagen acts as a filter to determine, which molecules will pass from the blood into the urine.

Glycation and cross linking of collagen: Glucose and other sugars also act as cross-linking agents of the extracellular matrix (ECM). Collagen has a long biological half-life and the level of non enzymic glycosylation or glycation, increases gradually with aging (Bailey *et al*., 1995) or in hyperglycemic conditions such as diabetes (Monnier *et al*., 1986). Reducing sugars (glucose, fructose, etc.) bound to free protein amino groups and go through a series of reactions to form a class of heterogeneous, non enzymic sugar-amino adducts that are called AGEs (Bailey *et al*., 1995). The diabetes associated changes in collagen function in the basement membranes are documented to be the biochemical link between persistent hyperglycemia and diabetic microvascular disease. These modifications are associated with decreased solubility, increases in fluorescence, thermal stability and mechanical strength. An increase in AGE-modified collagen has been detected in diabetic and ageing rats (Tomasek *et al*., 1994).

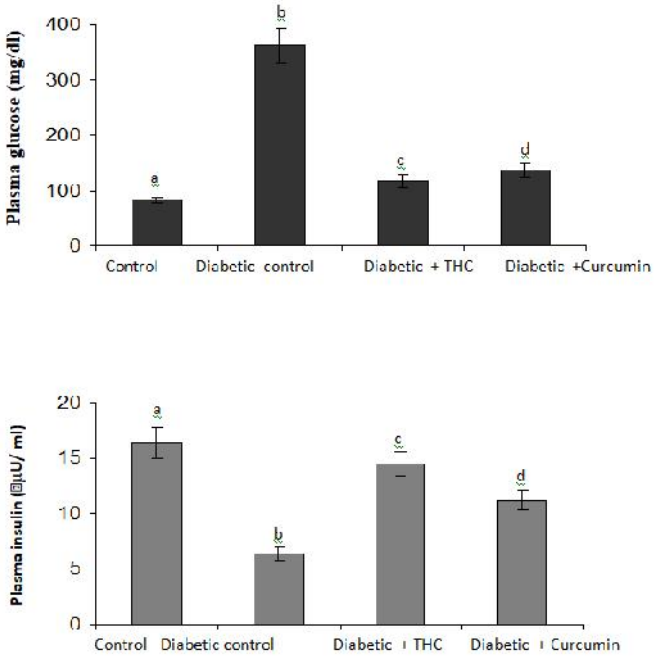

Figure 2 and 3. Changes in the levels of glucose and insulin in normal and experimental rats

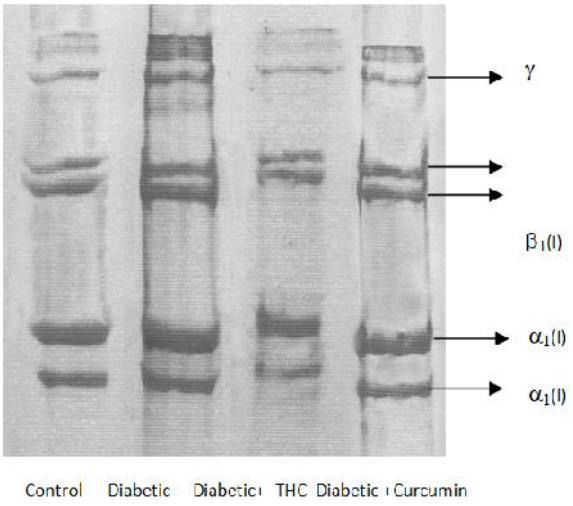

Figure 4. SDS gel pattern of acid soluble collagen from tail tendon in control and experimental rats

The alterations caused by AGE- modifications of collagen may play a role in the pathogenesis of various complications in poorly controlled diabetic patients (Katayama *et al*., 1996).

Diabetic complications due to collagen modifications: During diabetes, collagen undergoes an extensive modification and finally leads to the development of diabetic complications. Two main factors play an important role in the post translational modifications of collagen. Firstly, the accumulation of browning product or AGE's by the long half-life period or slow turnover of collagen, rendering it highly susceptible to advanced glycation. Secondly, the structural and functional alterations of collagen that occur in tissues like joints, arteries, retina and renal glomerular system that are severely affected during diabetes. Atherosclerosis, thickening of basement membranes, increased arterial wall stiffness, decreased lung elasticity, sclerosis of renal glomeruli, stiffening of heart, periarticular rigidity and osteoarthritis (Kilo *et al*., 1972; Minaker, 1987) are the pathological changes which involve collagen in diabetes.

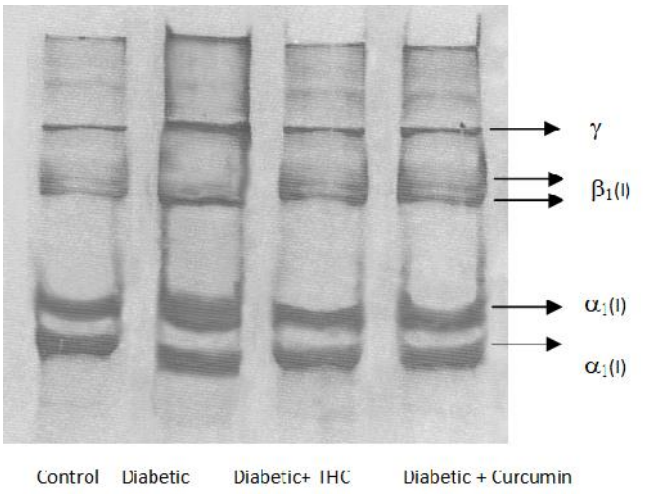

Figure 5. SDS gel pattern of pepsin soluble collagen from tail tendon in control and experimental rats

Accelerated aging occurs in diabetes when collagen becomes increasingly cross-linked, less soluble, thermally stable and resistant to enzyme digestion (Hamlin and Kohn, 1971) with advancing age. It has been reported that collagen in various tissues from diabetic individuals is less soluble in acid and pepsin (Schnider and Kohn, 1982) and less digested by collagenase and CNBr (Hamlin *et al*., 1975; Kohn, 1983).

Several experimental diabetic studies revealed the abnormalities in mechanical properties of tail tendons such as tensile behaviour, stress-strain behaviour, crimp length and tendency towards a larger fibril diameter (Galeski *et al*., 1977). The extensive crosslinking of collagen play a main role in functional and structural alterations of renal, vascular and cardiac tissue (Paul and Bailey, 1996). *Curcuma longa* is commonly used in the treatment of diabetes by ayurvedic physicians. Curcumin is a biologically active component isolated from the rhizome of *Curcuma longa* that possess antihyperglycemic activity (Arun and Nalini, 2002), hypolipidemic action (Suresh Babu and Srinivasan, 1997) and anti - renal lesion effect (Suresh Babu and Srinivasan, 1998). The use of curcumin is recommended for prevention of advanced glycatedendproducts (AGE) accumulation and the associated complications of diabetes (Sajithlal *et al*., 1998).

Tetrahydrocurcumin (THC) is one of the major colourless metabolite of curcumin. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin (Majeed *et al*., 1995 and Sugiyama *et al*., 1996). Curcumin is rapidly metabolized during absorption from the intestine, yielding THC (Ravindranath and Chandrasekara, 1980), which has shown the strongest antioxidant activity among all curcuminoids (Osawa *et al*., 1995). Several studies in experimental animals indicated that THC also prevent(s) cancer (Lin and Lin-Shiau, 2001), protect(s) against inflammation (Nakamura, 1998 and Hong *et al*., 2004), atherosclerotic lesions (Naito *et al*., 2002) and hepatotoxicity (Pari and Murugan, 2004). In our previous study, we have demonstrated the antidiabetic effect of THC in streptozotocin (STZ) induced diabetic rats (Pari and Murugan, 2005). To our knowledge, so far no other biochemical investigations has been carried out on the effect of THC in analysis of fluorescence of collagen in experimental diabetic rats. The present investigation was carried out to study the effect of THC on analysis of fluorescence of collagenin rats with STZ and nicotinamide induced diabetes.

MATERIALS AND METHODS

Animals: Adult male albino Wistar rats (8 weeks), weighing 180 to 200 g bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, were used. All animal experiments were approved by the ethical committee (Vide. No: 284, 2005), Annamalai University and were in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 24 ± 2^{o} C, humidity of 45 to 64%. During the whole experimental period, animals were fed with a balanced commercial diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*.

Drugs and chemicals: THC was a gift provided by Sabinsa Corporation, USA. Curcumin was purchased from Sigma chemicals company, St Louis, USA. All other chemicals and biochemicals were of analytical grade.

Induction of diabetes: Non-Insulin dependent diabetes mellitus was induced (Masiello *et al*., 1998) in overnight fasted rats by a single intraperitonial injection (i.p) of 65 mg/kg body weight STZ, 15 min after the i.p administration of 110 mg/kg body weight of nicotinamide. STZ was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal saline. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h and then on day 7 after injection. The animals with blood glucose concentration more than 200 mg/dl will be used for the study.

Experimental design: In the experiment, a total of 24 rats (18 diabetic surviving rats, 6 normal rats) were used. The rats were divided into four groups of six each, after the induction of STZ diabetes. The experimental period was 45 days. Group I: Normal rats. Group II: Diabetic control rats. Group III: Diabetic rats given THC (80 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days. Group IV: Diabetic rats given curcumin (80 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days (Arun and Nalini 2002). At the end of 45 days, the animals were deprived of food overnight and sacrificed by decapitation. Blood was collected in tubes containing potassium oxalate and sodium fluoride mixture for the estimation of blood glucose. Plasma was separated for the estimation of insulin.The tail of normal and experimental rats were removed and stored frozen at - 80^{0}C until used.

Statistical analysis: The data for various biochemical parameters were analyzed using analysis of variance, and the group means were compared by Duncan's multiple range test. Values were considered statistically significant if $p < 0.05$.

Analysis of fluorescence of collagen: Fluorescence of collagen in the tail tendon was determined by the method described by Monnier *et al*. (1986). AGEs exhibit a yellow-brown pigmentation and a characteristic fluorescence pattern, with excitation in the range 350-390 and fluorescence emission at 440-470.

Reagents: Phosphate buffered saline, N-2-hydroxyethylpiperazine N-2 ethane sulphonic acid: 0.02 M, pH 7.5, Calcium chloride: 0.12 M, Type VII collagenase.

Procedure: Approximately 3 mg tissue was finely minced in phosphate buffered saline and centrifuged at 2,000 rpm for 10 min. The pellet was washed with distilled water and the lipids were extracted with 5 ml of chloroform: methanol (2:1 v/v) for an over night. The samples were rehydrated by adding 2 ml of methanol and 0.5 ml of distilled water, then centrifuged and the pellet was washed twice with methanol, three times with distilled water, twice with 0.02 M N-2-hydroxy-ethylpiperazine N-2 ethane sulphonic acid containing 0.12 M calcium chloride (buffer H) and stored over night at 4°C in buffer H. The buffer H was then removed and the pellet was resuspended in 3.5 ml of buffer H containing type VII collagenase. Four drops of toluene was added to prevent bacterial growth. The digestion was carried out for 48 h at 37°C. A blank containing collagenase in buffer was included. The digest was centrifuged at 2000 rpm for 30 min. The clean supernatant containing digested collagen was used for the assay of fluorescence and hydroxyproline content. Fluorescence (Hitachi spectrofluorimeter) was measured against distilled water at 440 nm upon exitation at 375 nm and corrected for collagenase blank. The level of fluorescence in the tail tendon was expressed as arbitary units/µmol of hydroxyproline.

RESULTS

Figure 2 and 3 shows the level of plasma glucose and plasma insulin in normal and experimental groups. The level of plasma glucose was significantly increased whereas the level of plasma insulin was significantly decreased in diabetic rats. Oral administration of THC and curcumin to diabetic animals significantly reversed all these changes significantly. The effect of THC was more potent than curcumin. Figures 4 and 5 illustrate the SDS-gel pattern of acid soluble and pepsin-soluble collagen in tail tendon of control and experimental rats. The increased bandwidth of β components in diabetic collagen, while THC and curcumin supplemented diabetic group showed lesser bandwidth.

DISCUSSION

Non enzymicglycation and subsequent AGE formation inevitably occur during chronic diabetes and have been suggested as a primary cause of diabetic late complications (Brownlee and Lilly, 1993). In the present investigation, treatment with THC showed significant antihyperglycaemic activity. The antihyperglycaemic activity of THC is due to release of insulin from the existing β-cells of pancreas. In our study, the levels of hydroxylproline and total collagen are elevated in the tail tendons of diabetic rats, which could be due to increased glucose and non enzymicglycation. In addition, prolyl hydroxylase, an ascorbic acid dependent enzyme, is required to maintain the normal properties of collagen. The activity ofprolyl hydroxylase has been reported to alter in diabetic rats. This alteration is mainly due to the reduction in the concentration of plasma and tissue ascorbic acid in diabetes. In our study, we have also observed a significant reduction in the concentration of ascorbic acid in plasma and tissue of diabetic rats. The decrease in ascorbic acid concentration and thereby altered prolyl hydroxylase could be responsible for the alterations of collagen observed in diabetic rats. Significant increase in the concentration of ascorbic acid in THCand curcumintreated diabetic rats may be responsible for the activation of prolyl hydroxylase, which inturn maintain the collagen content. Experimental evidence indicates that collagen in diabetes undergoes extensive chemical modifications that results in decreased solubility, decreased susceptibility to enzymes, increased stability and accelerated cross-linking (Reiser, 1998). These modifications of collagen have received considerable attention, since collagen is an important constituent of most of the tissues that are damaged in diabetes. Collagens are especially exposed to glycation because they contain several lysine, hydroxylysine and arginine residues with free amino groups. Further they have a slow turnover rate and are exposed to ambient level of glucose (Reiser, 1998; Brownlee *et al*., 1998). AGE crosslinking causes proteins that are normally flexible to become rigid. The cells, tissues and blood vessels become stiff and increasingly dysfunctional (Vasan *et al*., 2003). In healthy individuals, this process occurs slowly as the body ages. In diabetic patients, the rate of AGE accumulation and the extent of protein cross-linking are accelerated due to exposure to highly elevated concentrations of glucose (Reiser, 1998). ROS acts as a fixative that couples both glycation and cross-linking of collagen (Vasan *et al*., 2003). Oxygen radicals play an important role in the formation and accumulation of AGE (Sajithlal *et al*., 1998). Thus it is well documented that free radical scavengers and metal chelators can inhibit the formation of AGE and cross-linking of proteins both *in vitro* and *in vivo* (Vasan *et al*., 2003).

Several reports indicate the increase in collagen-linked fluorescence during exposure to high glucose levels *in vitro* and *in vivo* (Sajithlal *et al*., 1998). In the present study, the increase in fluorescence in collagen incubated with glucose is an indication of increased advanced glycation and it is also quantified as a measure of increased AGE (Stefek *et al*., 2000). AGE modifies and damage tissues in various ways in addition to forming cross-links. These modification and cross-linking actions of AGE, contribute to numerous complications associated with diabetes (Asif *et al*., 2000). ROS formed during glucose oxidation and glycated protein oxidation involved directly in the formation of AGE (Sajithlal *et al*., 1998). Many antioxidants have some AGE-inhibitory activity primarily by preventing the autoxidative pathways of AGE formation (Rahbar and Figarola, 2003). It is well documented that the chelating activity of AGE inhibitors and AGE breakers contribute to the inhibition of AGE formation and protection against diabetic complication (Price *et al*., 2001). THC reduced the collagen-linked fluorescence indicating its role in reducing AGE. THC was shown to antioxidant activity and provide protection from free radical damage (Murugan and Pari, 2006). In the present study, THC ameliorate the AGE linked fluorescence, which may be due to its antioxidant and free radical scavenging effect.

The collagen cross-linking in aging and hyperglycemia results in dysfunction of collagenous tissues those are responsible for the morbidity and mortality in age and diabetes, primarily renal, cardiovascular and retinal tissues (Paul and Bailey, 1996). Studies indicate that collagen cross-links significantly contribute to cardiovascular stiffening in human and experimental animals (Vasan *et al*., 2003). Free radicals generated by the oxidation of free glucose and protein–glucose adducts in the presence of trace amounts of metal ions may contribute significantly to increase in the cross linking of collagen (Ahmed *et al*., 1986; Wolff and Dean, 1987). H_2O_2 (Elgawish *et al*., 1996) and lipid peroxidation may also play an important role in the cross-linking of collagen.

THC and curcumin increase the solubility of tail tendon, which could be due to the decrease in the cross-linking of collagen. The cross linking of tail tendon collagen was assessed by the solubility of collagen. The percentage of neutral salt, acid and pepsin soluble collagen was decreased in the tail tendons of diabetic rats. As cross-linking proceeds, the solubility of collagen in neutral buffer and acid solution also changes. Highly cross-linked collagen becomes less soluble in the above solutions and can be released only by limited pepsin digestion (Wolff and Dean, 1987). Treatment with THC and curcumin had increased the solubility of collagen in neutral, acid and pepsin digestion, which could be associated with decreased cross-linking of collagen. This is evidenced by improved glycemic control and decreased extent of glycation.

The SDS-gel pattern of collagen confirms the structural alterations in collagen in diabetic rats. The band size of β-component of collagen in the diabetic rat was increased as compared to that of control rat. The relative abundance of high molecular weight collagen chain was demonstrated by the decreased ratio of α to β chains. β-chains are dimers in which the inter chain crosslinks are not disulfide bridges (Monnier *et al*., 1986). In the present study, a significant decrease in the ratio of αtoβ components of tail tendon collagen of diabetic rats was also observed. The increased intensity of β-component observed in diabetic rats in our study suggests that collagen chain are capable of enhanced intramolecular crosslinking since the β-component is a dimer of α-chains. In the present study, we investigated the effect of THC on glycation and cross-linking of tail tendon collagen and its effect was compared with curcumin. THC significantly reduced the glycation, AGE and cross-linking of tail tendon. The antihyperglycemic and antioxidant effects of THC may be ascribed for its beneficial activity and have therapeutic role in the prevention of glycation induced pathogenesis in diabetes mellitus and aging. The THC administration showed more effective than curcumin.

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