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MODULATORY EFFECTS OF CURCUMIN ON ANTIOXIDATIVE ENZYMES IN SUBMANDIBULAR GLAND OF MALE MICE DURING AGING.

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ABSTRACT

Antioxidative effect of curcumin was examined to determine its therapeutic role in mice experimentally exposed to D-galactose induced oxidative stress. Curcumin treatment (30mg/kg body weight daily for 30 days, orally) along with and also after treatment of 5% D-galactose [0.5ml (sc)] was found to have a protective effect on D-galactose induced oxidative stress parameters namely Superoxide dismutase(SOD), Catalase (CAT), Glutathione peroxidase (GPx) activities in the submandibular salivary glands. Post treatment with curcumin in curative group I increased the level of all the three antioxidative enzymes in submandibular glands (SMG), while in curative (45 Days) group II the levels declined. Study indicates that curcumin treatment has a protective effect therefore can be used as therapeutic agent. Our study indicates that curcumin besides being an effective antioxidant may exert its protective effect through its routine dietary intake against oxidative stress. However, prolonged administration may cause damage due to its accumulation in cells.

KEYWORDS: **Aging, Curcumin, Submandibular gland, Antioxidative enzymes.**

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INTRODUCTION

Aging is the time dependent deterioration of the physiological functions; leading to cell's inability to withstand external and internal stress. The causative factors for the time dependent deleterious process of aging are yet not defined and no single adequate molecular explanation for aging is currently available[1]. In recent years, many theories have been proposed to account for this process, out of which the free radical theory of aging by Harman *et al.,* [2] seems to be promising. Herman proposed that highly reactive derivatives of oxygen, the free radical are produced during normal metabolism. Despite the fact that there is continuous production of free radicals, which increases with aging, our body possesses several defence systems that are constituted by enzymes and free radical scavengers[4]. The antioxidants superoxide dismutase [5,] tocopherols [6], catalase and glutathione peroxidase [7] limits the production of free radical damage to tolerable level. This theory predicts that the rate of aging is dependent on the level of oxidative stress i.e. the balance between pro-oxidant and antioxidants and the consecutive oxidative damage. According to the free radical theory of aging one might expect the activity of antioxidant enzymes to be altered with increasing age. There are a variety of SOD (EC 1.15.1.1) isoenzymes in mammalian cells. Manganese- containing SOD (Mn-SOD) exists in mitochondria of various cells, while copper and zinc containing SOD (Cu/Zn SOD) exists in cytosol [8]. Catalase (CAT) (EC1.11.1.16) mainly exists in the peroxisome and reacts not only with hydrogen peroxide by activating its decomposition into water and oxygen but also with hydrogen doners[9]. Glutathione peroxidase (GPx) (EC 1.11.1.9) catalyzes a reduction in various hydroperoxidases, including hydrogen peroxide through glutathione, thereby protecting mammalian cells against oxidative damage of the cytosol and mitochondria [10]. Curcumin, a yellow pigment from *C. longa* is a major component of turmeric commonly used as spice, and food coloring material exhibits anti-inflammatory, antitumor and antioxidant properities. So in the present study we evaluated the effect of dietary supplementation of curcumin on the antioxidant defense enzymes i.e. SOD, CAT and GPx. Also, the study examines the change in antioxidative enzymes of SMG during induced stress and recovery by curcumin supplementation.

MATERIAL AND METHODS

Swiss albino male mice (*Mus musculus*) of age six months, weighing about 50-55gm. were used for present investigation for treatment groups. Animals were maintained in the plastic cages in AC animal house (CPCSEA/233) under 12:12 hr. L: D cycle. The animals were provided with pellet food from 'Pranav Amrut food (Sangli, Maharashtra, India) and water *ad libitum*. Experiments were conducted in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and experimental protocols were approved by the institutional animal ethics committee (CPCSEA/233). Thirty mice were divided into following five groups of six animals each:

Group I*: *Control group: Animals were injected subcutaneously with 0.5 ml sterile water/day /animal for 30 days.

Group II: D-galactose induced group: The mice were injected subcutaneously with 5% D-galactose 0.5 ml /day/animal for 30 days to induce aging.

Group III: Protective group: The mice were injected subcutaneously with 0.5 ml of 5%D-galactose /day/animal along with; curcumin dissolved in honey was fed orally for 30 days at the dose of 30mg /kg body wt /day[11]. The co-treatment of curcumin was carried out to study its effect on induced aging and oxidative stress.

***Group IV*: *Curative group I*:** The mice were injected with 0.5ml of 5% D-galactose (sc) for 30 days, and then for next 30 days were fed orally curcumin dissolved in honey 30 mg/kg body wt /day, to study the recovery by curcumin.

Group V Curative group II: Mice of in this group received 0.5 ml 5% D-galactose (sc) per day for 30 days and then curcumin dissolved in honey was fed orally to these D-galactose induced aged male mice at a dose of 30mg/kg body weight daily for next 45 days. After completion of the dose, the animals were sacrificed by cervical dislocation; SMG were dissected out, blotted and weighed and homogenized in respective homogenization medium and used for following estimation. Measurement of total SOD activity was performed according to Beauchamp and Fridovich [12] by calculating percentage of formazan dye formation. The catalase mediated decomposition of H_2O_2 was estimated directly at 240 nm with a modified method of Luck [13.] Glutathione peroxidase activity was assayed spectrophotometrically by using Beers and Sizer method [14,] glutathione oxidation was recorded at 240 nm in presence of sodium azide. Protein concentration in samples was determined by the method of Lowry'[15] using bovine serum albumin as the standard. Gel electrophoresis- 10% - Native polyacrylamide slab gel electrophoresis was performed as described by Laemmli [16] method. Samples were solubilized in sample buffer. Samples (30 µl/slot), were subjected to electrophoresis at 150 V. Gel was stained according to Beauchamp and Fridovich [12] method.

RESULTS

The mean ± SD values of SOD, CAT and GPx activities in submandibular glands of male mice in relation to the oxidative stress and changes in the enzyme activities due to dietary supplementation of curcumin is summarized in Table.

Table

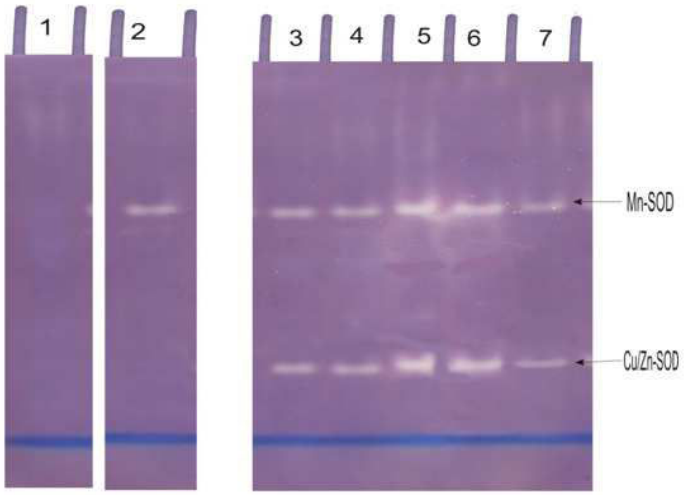
The effect of extract of Curcuma longa on SOD, CAT and GPx activities in submandibular glands of D galactose –induced aged male mice. (Values are mean ± S.D.) (Enzyme activity expressed in unit/mg protein)

Sr.No	Treatment	Age of animal (in Weeks)	SOD Activity	Statistical Significance	CAT Activity	Statistical Significance	GPx Activity	Statistical Significance
1	Control (5)	24	1.4974±0.1396	-------	1.4812±0.0179	-------	1.1267±0.0176	--------
2	D-galactose induced (5)	24	0.9606±0.03477	1:2 P<0.001	0.9732±0.0180	1:2 P<0.0001	0.1412±0.0127	1:2 P<0.0001
3	Protective (5)	24	1.2942±0.0043	2:3 P<0.0001	1.2914±0.0145	2:3 P<0.0001	1.2914±0.025	2:3 P<0.0001
4	Curative I(5)	28	1.3058±0.0124	2:4 P<0.0001	1.2956±0.0027	2:4 P<0.0001	0.5640±0.0161	2: 4 P<0.0001
5	Curative II(5)	30	0.9586±0.01383	2:5 P>0.05 (NS)	0.9704±0.0179	2:5 P=0.8126 (NS)	0.265±0.0178	2:5 P<0.0001

P< 0.05-Significant, P< 0.0001- Highly Significant, p>0.5- Non Significant (NS)
(Numbers in parenthesis denotes number of animals)

The highest activity of all the three antioxidative enzymes was observed in control group mice. A statistically significant decrease in all the three enzyme (SOD, CAT, GPx) activities was found in the submandibular gland of D -galactose induced mice (p<0.001). The activities of SOD, CAT, GPx were elevated in SMG, only for groups which were fed with curcumin daily for 30 days along with D-galactose and also after the D galactose treatment was over, when compared with the D-galactose induced group. This increase was observed in the protective and curative group I studied whereas, in SMG, the decrease was observed for group-curative group II. The electrophoretic separation of isozyme of SOD in control group showed presence of two clearly colourless separate bands of Mn-SOD and Cu/Zn-SOD. In D-galactose treated group mice SMG, intensity of both isozymes were reduced as compared to control group. Oral administration of curcumin in protective and curative group I considerably increased the intensity of both bands. (Figure no. 1).

Figure No. 1



Legends to figure
Lane 1: Sample of SMG control group mice. Sample treated with H_2O_2.
Lane 2: Sample of SMG control group mice. Sample treated with DDC.
Lane 3: Sample of SMG control group mice.
Lane 4: Sample of SMG from D-galactose treated group.
Lane 5: Sample of SMG from protective group.
Lane 6: Sample of SMG from curative I group.
Lane7: Sample of SMG from curative II group.

DISCUSSION

Evidences suggest that free radical generation is involved in physiological process of aging[17]. The free radical theory of aging suggests that oxygen free radicals interact with cellular macromolecules that result in cellular senescence and aging. D-galactose induced aging was used as an experimental model for studying aging and to design suitable strategies against aging. The exact cellular mechanism underlying D-galactose induced aging has not been well understood so far. Existing data indicate that the oxidative stress might be one of main possible reason. Oxidative stress co-exists with altered antioxidant systems both enzymatic and non-enzymatic. Hence we assessed the activities of most important anti-oxidant enzymes SOD, CAT and GPx in SMG of D-galactose induced aged male mice and also investigated the effect of curcumin on the same. Exposure of submandibular glands to D- galactose induced oxidative stress results in the decreased activities of SOD, CAT and GPx. The increase in SOD, CAT and GPx activities in SMG of protective and curative group I could be the protective response by submandibular cells to counteract the oxidative stress in the tissue. But decrease in antioxidative enzymes especially GPx in curative group II indicates damaging effect due to prolonged treatment of curcumin. Once superoxide radical is produced then hydrogen peroxide and hydroxyl radicals are continuously produced by a Haber-Weiss reaction and /or Fenton type reaction[18, 19]. SOD is a first line of defense against Cu-SOD, Zn-SOD, Mn-SOD [20] and extracellular SOD (EC-SOD) [21]. Mn-SOD is located in mitochondrial matrix [21] and Cu/Zn-SOD is found abundance in cytosol [22] both of which protect cells against oxidative injury. Effect of aging on the activities of SOD, CAT and GPx has been studied in a variety of organs and animals [23,24]. Previous studies on effect of age on antioxidative enzyme activity of brain have yielded confirming results and none of these studies have attempted to correct the potential for oxidative metabolism with enzyme activity [24, 25].
Studies of Vertenuchy *et al.,* [25] Carillo *et al.,* [26] Matsuo [27] implies that activities of catalase and SOD are relatively stable throughout life span. In contrast, studies of Del Maestro R *et*

al [24] indicated a marked and progressive decline in catalase activity in all of the brain regions studied. SOD has an antioxidant effect against the super oxide anion. SOD accelerates the dismutation of superoxide to H_2O_2 which in turn is removed by CAT [28]. Thus SOD can acts as a primary defense against superoxide anion and prevents further generation of free radicals [29]. The decreased SOD activity in SMG suggests that the accumulation of superoxide anion radical might be responsible for increased lipid peroxidation following D-galactose treatment which was evident in our previous study [30]. D-galactose acts directly or indirectly and alters oxidant status that makes submandibular gland acinar cells more susceptible to oxidative stress. Several studies have shown that D-galactose causes increased lipid peroxidation and decreased enzymatic activities in lysosomes, which is an agreement with to the data obtained in our study [31]. The activity of enzyme directly involved in oxidative stress scavenging namely SOD, was analyzed by native-PAGE in the same tissue used for above described spectrophotometric assay. The SOD electrogram indicated the presence of two bands in control SMG sample. The first band could be identified as Mn-SOD since it was not inhibited by DDC whereas the faster moving band was characterized as Cu/Zn-SOD since it was negatively affected by the inhibitor.

Curcumin has been reported to show antioxidative properties by one or more following interactions. Scavenging/neutralizing free radicals by oxygen quenching and making it less available for oxidative reaction and/or inhibition of oxidative cascade and preventing it's outcome and chelating and disarming oxidative prosperities of metal ion such as iron[32-34].Dietary curcumin is reported to inhibit superoxide anion generation and hydroxyl radical generation through preventing oxidation of Fe^{++} in Fenton's reaction, which generates .OH radicals [35]. Therefore the decrease in ROS threshold in curcumin treated animals. In present study, oral administration of curcumin along with D-galactose (protective group) and also after D-galactose treatment in curative group I considerably increased activities of SOD, CAT and GPx as compared to D-galactose treated group. Thus, though D-galactose reduced level of antioxidant enzymes, the treatment of curcumin again enhanced their activities. Therefore, in electrophoretic separation of SOD isozymes prominently colourless bands were visible after curcumin feeding indicating recovery of isozymes. These results supported our results of biochemical estimations. In the D-galactose treated group SMG sample, intensity of both SOD isozymes i.e. Mn-SOD and Cu-Zn-SOD was reduced as compared to control group. Both the bands appeared a little bluish, instead of clear colourless. This was because in absence if SOD, reduced riboflavin forms superoxide radical, which donates e- to NBT and blue formazon dye was formed. The unclear band in D-galactose treated group SMG sample indicates that there was decrease in both Mn-SOD and Cu-Zn-SOD in D-galactose treated group.

CONCLUSION

Administration of curcumin reversed the changes induced by D- galactose supporting the hypothesis that plant products are effective antioxidative agents. Supplementation of curcumin significantly enhances the antioxidative levels in submandibular glands. But its prolonged administration causes damaging effect which may be due to its accumulation in cells.

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