The Efficiency of Mitochondria-Targeted Curcumin in Reducing Thyroid Mitochondrial Oxidative Damage

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Abstract: *Mitochondria are the crucial site for the cellular energy production. The antioxidant has less therapeutic success to overcome mitochondrial toxicity produced by oxidative stress due to mitochondrial inability in accepting antioxidant. In the present investigation, we synthesized selective mitochondrial targeted curcumin for easy transport to mitochondria of the thyroid gland. Our observation shows the strong antioxidant effect of mitochondrial targeted curcumin against lipid peroxidation and mitochondrial transition induced by Escherichia coli. Mitochondria-targeted curcumin significantly improved endogenous glutathione level in the mitochondria, its protect the motochondrial defense system against oxidative damage. Thus it is concluded that mitochondria-targeted curcumin protects mitochondria against E.coli toxicity of thyroid gland, By lowering the oxidative damage, increasing the level of reduced glutathione and preserving the mitochondrial integrity.*

Keywords: Mitochondria, mitochondria-targeted curcumin, reactive oxygen species, oxidative stress, membrane permeability transition

1. Introduction

The thyroid gland and its associated hormones T3, T4 play an essential role in controlling the body's metabolic activity, development and antioxidant defenses (1-3). Alterations in the normal levels of T3, T4 cause clinical abnormalities and biochemical dysfunctions are to affect mitochondrial respiration producing free radicals (4-6), specially ˙OH (7,8). Thyroid activity was depressed when bacterial exotoxins injected in mice, rats, and rabbit (9).

Mitochondria plays a vital role in a number of important metabolic activities like amino acid biosynthesis, fatty acid oxidation, lipid metabolism, homeostasis of steroid hormones, urea cycle, apoptosis and intermediate metabolic pathways (10). The mitochondrial dysfunction leads to an alteration in energy metabolism and more generation of reactive oxygen species (ROS) (11). Abnormally high level of ROS may increase lipid peroxidation (LPO), damage to nucleic acid and oxidized necessary proteins, thus leading to damage cellular organelles. One of the most efficient ways to reduce the oxidative damage is to use compounds, which has antioxidant property. Although several conventional non-enzymatic antioxidants such as vitamin-E and vitamin-C have little or less protective value (12,13). One of the potential explanations for this response may be antioxidant therapies has non-specific nature (14,15). Consequently, antioxidants show limited potential and they may not accumulate in sufficient amount inside the mitochondria to reduce oxidative damage. Thus, efficient mitochondrial targeted antioxidants need to develop which have higher permeability towards mitochondrial membrane (16-19).

However, some of the studies have been demonstrating that bacterial cell and their metabolic product present in the extraneous environment of the host might influence thyroid function (20). *Escherichia coli* produced antithyroid compounds in cell-free and broth cultures (21-23), Paracolon *Bacillus* has to produce an enzyme that converts progoitrin to goitrin (24). *E.coli* and Cholera produced an enterotoxin that stimulates cyclic AMP in the thyroid gland and produced antithyroid compound (25).

The naturally occurring *Curcuma longa* (turmeric) have major phenolic compound curcumin which is a powerful immunomodulatory agent which has anti-inflammatory, antioxidant, anti-cancerous, anti-amyloid and food preservative properties (26-28). The curcumin 1 to 5 gm/kg of body weight do not cause any adverse effect (29) and long-term administration of curcumin to rodents leads to hyperproliferation of thyroid epithelial cells (30). The present study is to evaluate the level of oxidative damage and the level of the entire key antioxidant enzyme in mice thyroid gland induced by *E.coli*. We also report the protective effects of curcumin on the thyroid gland of mice.

2. Materials and Methods

Chemicals and reagents
All the following chemicals were of analytical grade supplied by Merck, Hi-Media, and Sigma chemical Co. USA. Triphenylphosphonium oxide (TPP), HCl, 48 % Hydrobromic acid (HBr), ethyl acetate, ethanol, dimethosulpho-oxide (DMSO), GSH (Glutathione reduced), riboflavin, Phenazine methosulphate (PMS), lithium lactate, H_2O_2 (30%), NADH, NADPH, thiobarburetic acid (TBA), trichloroacetic acid (TCA), nitroblue tetrazolium (NBT).

Animal protocol and design
Female adult Swiss albino mice weighing 28-32 gm were obtained from the college of veterinary sciences and animal husbandry Mhow (22.55º N, 75.75º E, M.P), India. The Ethical approval was taken from ADINA Institute of Pharmaceutical Sciences (Registration No. 1546/PO/E/S/11/CPCSEA), Sagar (23.88º N, 78.73º E) with international guidelines for care and use of laboratory animals. All animals (n=30) were housed at 25±2º C with 12h light and dark cycle. The whole animals were divided into three groups and each group consists of ten mice. *E.coli*

culture was obtained from Institute of Microbial Culture Chandigarh India (MTCC- 68).

3. Experimental Design

Synthesis of targeted antioxidant
Mitochondrial targeted curcumin synthesized by covalent linkage of curcumin with lipophilic cation. TPP (1.31 gm) reacted with HBr (350 ml) precursor to obtain lipophilicity (31). To synthesize the targeted derivative of curcumin, a solution of lipophilic cation refluxed with curcumin and evaporated to obtained mitochondrial targeted curcumin (mt-c) (32).

In vivo study
Reverse osmosis (RO) water 200 μl in (5×10^6) *E.coli*, and DMSO (2% v/v) (33) were injected in mice by intraperitoneal, after three days 100 μl mt-c (0.12 mg/100 μl), RO water and DMSO were induced in mice for seven days. Control group mice for each experimental setup were given simultaneously RO water and DMSO. The animals were euthanizing by decapitation for ten days from treatment. Thyroid gland with trachea was dissected out, washed in ice-cold saline (0.9% NaCl), and stored frozen at -80ºC for further studies.

Preparation of tissue extract
Thyroid gland extract was prepared in 0.02 M tris-Cl (pH 7.4) and homogenate (10% w/v). Homogenate was centrifuged at 1000 rpm for 10 min at 4º C. After the first centrifugation, the pellet was discarded and the supernatant fluid was recentrifuged at 12500 rpm for 20 min. So obtained was stored for the study of biochemical assay.

Biochemical estimation

Protein estimation
Protein content was measured by the method of (34).

Estimatimation of T3, T4, and TSH
Detection of T3, T4, and Thyroid stimulating hormone (TSH) measured using ELISA provided by The Calbiotech Inc. (California, USA) (35). All the assay were performed in triplicate.

Assay of lipid peroxidation
Lipid peroxidation was determined by the measuring of thiobarbituric acid reactive substance (TBARS) in terms of malonaldehyde (MDA) following as described the method (36) with some modification. Briefly, 1ml of the tris-maleate buffer of pH 5.9, and 10 μl of the tissue extract was incubated at 37º C for 30 minutes. After, 1.5 ml of TBA reagent was added and the mixture was incubated at boiling water (100-120º C) for 10 min. After cooling at room temperature pyridine: n butanol (3:1, v/v) mixture and 1N NaOH is added. The contents were thoroughly shaken and allowed to stand for 10 minutes. The photometric measurement was carried out at 548 nm and the level of lipid peroxidation was expressed as nmol MDA/g.

Assay of SOD and Catalase
The activity of superoxide dismutase (EC: 1.15.1.1) was determined method (37). The reaction mixture consisted of 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M), 300 μM NBT, 186 μM PMS, and 0.1 ml suitably diluted tissue extract. The reactions were started by addition of 780 μM NADH at 30° C and stopped after 90 s by the addition of 1 ml of TCA. The reaction mixture was stirred with 4 ml of n-butanol and allowed to stand for 10 min. The control set without tissue extract run simultaneously. The unit of the enzyme was defined as 50% inhibition of NBT/min and the activity was expressed as units/mg protein.

The Catalase activity (EC: 1.11.1.6) was measured as described (38), with some modification. Briefly, the reaction mixture 1 ml consisted of 0.067 M phosphate buffer (pH 7.0) and 0.003% H_2O_2. By the addition of diluted tissue extract, the reaction was started and a decrease in absorbance at 240 nm was recorded for 10 min. The activity of catalase was expressed as μmol of H_2O_2 consumed/min/mg protein.

Glutathione reductase assay
The activity of glutathione reductase (EC: 1.6.4.2) was measured following the method of (39). The reaction mixture (1 ml) containing 0.2 M potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 2 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was initiated by addition of 20 μl tissue extract and NADPH was recorded as a decrease in absorbance at 340 nm for 10 min. Nonspecific oxidation of NADPH was calibrated by the absorbance measured in the absence of GSSG. Unit of the enzyme was defined as μmol NADPH/min and the activity of the enzyme was expressed as units/mg protein.

Analysis of SOD and glutathione peroxidase by native PAGE
For SOD tissue extract 40 μg protein was loaded in each lane of 10 % native PAGE. After electrophoresis gels were in Coomassie blue staining solution for 30 minutes, after gels were deep in 2.5 mM NBT solution for 20 minutes and gels were deep in specific staining solution of SOD band as describe earlier (40). The staining mixture contains 28 μM riboflavin and 28 μM TEMED. After 20 minutes incubation in the dark, gels were exposed to fluorescent light to develop achromatic bands against dark blue background corresponding to SOD protein in the gel.

Glutathione peroxidase (EC: 1.11.1.9) was determined by in non-denaturing method as described earlier (41). Tissue extract containing 40 μg proteins was loaded in each lane of 10% non-denaturing PAGE, Gels were incubated in specific staining mixture containing Tris-Cl buffer (50 mM, pH 7.9) 3 mM GSH, 0.004% H_2O_2, 1.2 mM NBT, and 1.6 mM PMS. Achromatic bands of GPx activity appeared against the violet blue background. The intensity of bands was quantified by gel densitometry using ImageJ software.

Detection of antioxidant enzymes on the basis of native PAGE, development of enzyme specific bands were confirmed by comparing the results of similarly run the gels stained in the presence and absence of specific substrates. In each PAGE were performed 4-5 times and mean ± SD of densitometry values of the bands as relative density of control lane from all the gel run were presented with gels photograph.

Statistical Analysis

Results expressed as mean ± SD and student t-test was applied for determining the level of significance between controls and treated groups Fisher (42)

4. Results and Discussion

The level of T3, T4 decrease significantly ($p<0.002$, $p<0.01$) after *E.coli* exposure compared with control group and increased significantly ($p<0.01$, $p<0.02$) in the mt-c treated group compared with *E.coli* treated group. However, the TSH level increased significantly ($p>0.001$, $p<0.002$) in both *E.coli* and mt-c treated group compared with the control group but decreased significantly ($p>0.001$) in mt-c treated group compare with *E.coli* treated group (**Histogram 1**)

The results of the effect of curcumin on oxidative toxicity induced in thyroid gland are presented in **histogram 2**. It is clear from the results that *E. coli* administration led to a significant ($p<0.001$) increase in MDA (34.30±0.11 μM) as compared to control. Upon supplementation of mt-c for 7 days to mice, we observed a significant ($p<0.001$) decrease in MDA concentration (24.04±0.07) of the thyroid gland when compared with *E. coli* treated group.

The results clarified inhibition on lipid peroxidation by mt-c. The activity of SOD decreased significantly ($p<0.05$) in thyroid gland when the exposed of mt-c, whereas SOD activity, increased significantly ($p>0.002$) when exposed to *E.coli* through the injection (**Histogram 3**).

The activity of non-enzymatic antioxidant GSH is most abundant thiol that an important major reducing agent against oxidative damage. As shown in (**Histogram 4**), the bacterial treatment caused a significant ($p>0.05$) decrease in GSH level as compared to control. However, treatment of mt-c increased GSH level significantly ($p>0.05$) in the thyroid gland.

The activity of catalase increase significantly ($p<0.02$) when the exposed with *E.coli*, whereas its activity decreased significantly ($p>0.05$) when exposed with mt-c orally (**Histogram 5**).

The first step of neutralization of O_2 is completed by synchronized activity of SOD and GPx in mice cell. As compared to control group of mice, the enzyme activity of SOD was observed to be increased significantly ($p<0.001$) when treated with *E.coli*, whereas its value decreased significantly ($p<0.01$) in the thyroid gland (**Histogram 6, and 7**).

In mammalian tissues, four types of GPx have been reported (43). According to histogram as compared to control lanes all GPx isoform increased slightly ($p<0.001$) when treated with *E.coli*, whereas value of GPx was decreased significantly ($p<0.01$) (**Histogram 8and 9**)

Generation of free radical causes oxidative damage when exceeds the body's natural antioxidant defense systems and damage deoxyribonucleic acid, lipid, and proteins.

Mitochondria are the major source of ROS and thus it may be an easy target to damage a cell by various oxidants and oxidative metabolism (44-47). One of the most prominent mechanisms to counter the oxidative stress is through the use of effective antioxidants as curcumin (48-51). Curcumin has both phenolic and β- diketone functional group shows significant antioxidant and free radical scavenging activities (52,53), and it also enhances the activities of antioxidant enzymes such as SOD, GPx, and catalase (54). In the mitochondria, curcumin reduced ROS and prevent oxidative damage, To enhance the efficacy of curcumin against ROS in mitochondria. We synthesized mt-c by conjugation of curcumin with lipophilic cation TPP and they may be rapidly entered in the mitochondria and reduce oxidative stress and mitochondrial impairment. The mt-c is conjugated anionic compound which passes easily through mitochondrial membrane. The *E.coli* of respiratory chain is an important source for reactive oxygen species (ROS), and mutants in the cytochrome oxidase complex of *E.coli* have increased ROS levels, as well as increased sensitivity to exogenous oxidative stress (55). We consider the possibility that microbial ROS may induce a mitochondrial stress response.

Curcumin interact with ROS by scavenging or neutralizing free radicals, inhibiting LPO and maintain cell membrane integrity and their function (56). The lipid peroxides are presumptive markers of oxidative damage(57), so we tested first the effect of *E.coli* on thyroid mitochondria. Our results showed a significant increase in the LPO by *E.coli* treatment. After the treatment of mt-c, it's a significant decrease. The present study shows the efficacy of mt-c to reduced LPO induced by *E.coli*. Curcumin was also found to ameliorate indomethacin drug induced LPO in mitochondria isolated from the small intestine.

Tha level of reduced glutathione (GSH), a tripeptide (L-γ-glutamyl-L-cysteinyl glycine) responsible for maintaining reducing equivalents under oxidative stress, another critical factor is assessing the level of oxidative stress in mammalian cells(58). Mitochondrial GSH also has an important role in the maintain the mitochondrial integrity(59). The level of GSH under stress condition have been depleted can increase mitochondrial damage and cause a defect in mitochondrial energy conservation (60). In the present study, *E.coli* treatment caused a significance decrease of GSH in the mitochondria. After treatment of mt-c, its significant increase the level.our observation is clearly showing the effectiveness of mt-c defending the mitochondria from lethal effect of oxidative stress.

The excess of superoxide anion (O_2^-), is maximum, produced ROS in mitochondria is converted to H_2O_2 by SOD. Simultaneous H_2O_2 is removed by either catalase/GPx, these are crucial for preventing membrane damage due to oxidative stress. In thyroid gland, SOD-GPx-GR pathway is considered to play a major role of antioxidant activity(61), with the increased level of ROS after the treatment of *E.coli*, is significantly increased GPx, SOD, and catalase and ameliorate with mt-c it's a significant decrease.

The investigation of SOD and GPx by native PAGE its activity significantly increase with the treatment of *E.coli*

and after amelioration with mt-c its significantly activity decrease.

5. Conclusion

In conclusion, the present study professed that the mt-c will be able to reduce oxidative damage in mitochondria of the thyroid gland against bacterial toxicity. This study will provide additional information on the role of free radical and biochemical changes in the mt-c treated mice.

6. Acknowledgment

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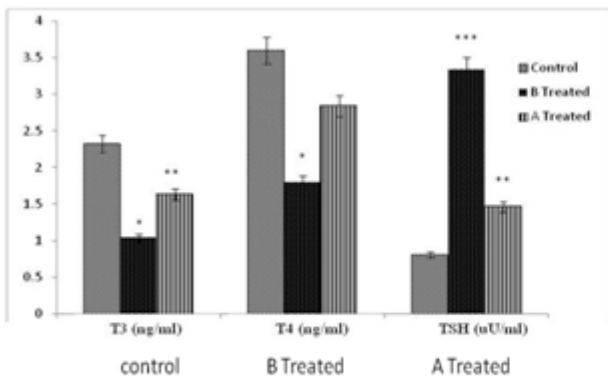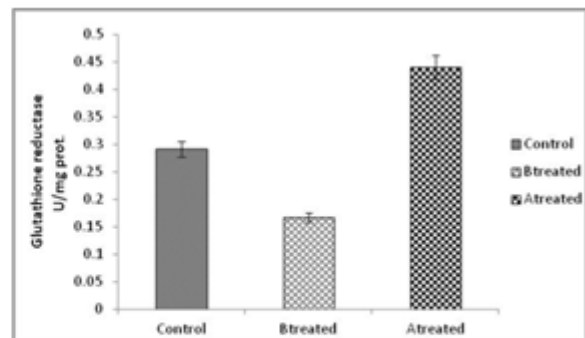
Histogram 1. Effect of *E.coli* and mt-c on mice serum, hormone levels in the group studied. T3, T4, TSH (Thyroid stimulating hormone). B treated- *E.coli* treated, Atreated- m-tc treated.
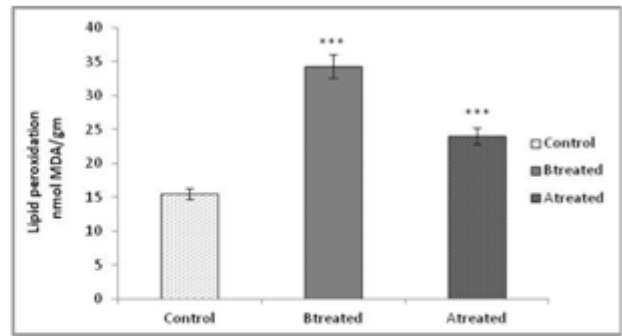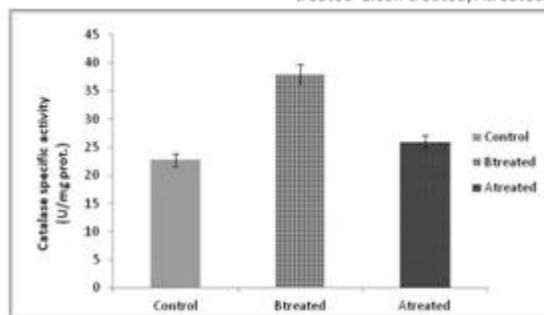

Histogram 2. Effects of *E.coli* and m-tc, LPO in the thyroid gland. Data in panel represents mean± SD from 4-5 observation. ***p<0.001(control vs treated group).Btreated- *E.coli* treated, Atreated- m-tc treated.
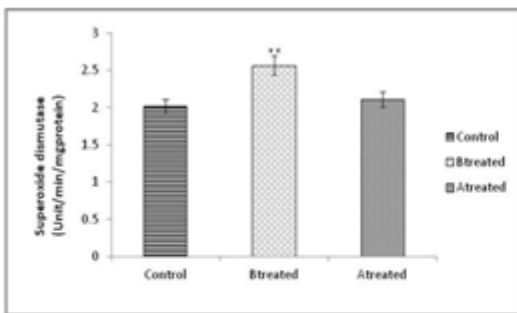

Histogram 3 Effect of *E.coli* and mt-c on the specific activity of superoxide dismutase on the thyroid gland of mice. data panel represents mean± SD from 3-4 obserservation. p<0.002, p>0.05 (Control vs. treated group). Btreated- *E.coli* treated, Atreated- m-tc treated.


Histogram 4 Effect of *E.coli* and mt-c on specific activity of glutathione reductase on the thyroid gland of mice. data panel represents mean± SD from 3-4 observation. p<0.05, p<0.05 (Control vs treated group). B treated- *E.coli* treated, Atreated- m-tc treated.

Histogram 5. Effect of *E.coli* and mt-c on the specific activity of catalase in the thyroid gland. data in panel represents mean± SD. from 3- 4 observation. p<0.02, p>0.05 (Control vs treated group). Btreated- *E.coli* treated, Atreated- m-tc treated.
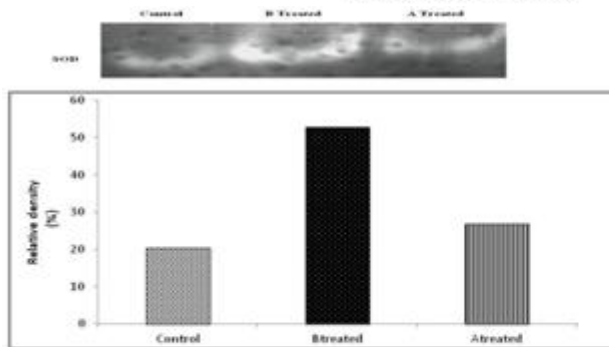
Histogram 6 and 7. Effects of *E.coli* and m-tc, superoxide dismutase in thyroid gland. Represents relative intensity of SOD band taking three lanes.Btreated-*E.coli* treated, Atreated- m-tc treated.
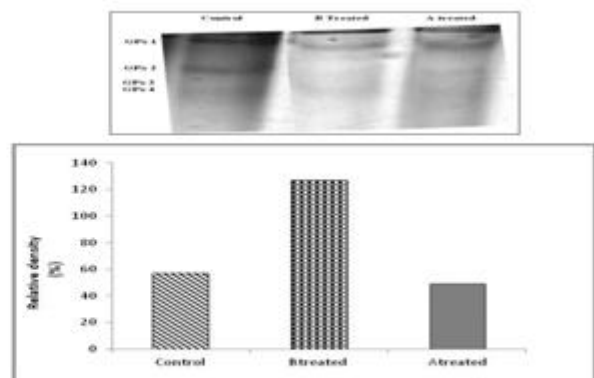
Histogram 8 and 9. Effects of *E.coli* and m-tc, a glutathione peroxidase in thyroid gland. Represents relative intensity of GPx band taking three lanes. Btreated- *E.coli* treated, Atreated- m-tc treated.

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